Stress and Recurrent Miscarriage

Thesis submitted for the degree of doctor of medicine

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This thesis is dedicated to my family…

To my mother who is a brave women with open mind,

To my father (deceased), who was intelligent and inspired me,

To my two elder sisters and their husbands who are loving and giving,

To my elder brother and his wife who are always available for moral support,

To my beloved and bright nephew MR Hongsheng Liu and my beautiful niece Miss Xueqing Li, who are angels of the family,

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Abstract

This thesis investigated the role of stress in RM using both psychometric and biochemical measures. The majority of previous studies on the impact of stress on RM only addressed the psychological aspect without concurrent biochemical stress measurements. In this thesis the stress status in women with RM was evaluated by using a validated questionnaire package and biochemical measurements of stress markers including cortisol, natural killer cells and prolactin.

The first part of this thesis compared the psychological stress status in a cohort of women with unexplained RM with that of fertile health women. The results showed that women with RM had higher levels of psychological stress than fertile women. RM women with higher perceived stress and being less optimistic appeared to have an increased likelihood of live birth. Based on the findings in the present study we speculate that stress coping strategies adopted by individuals may have a prognostic value on subsequent pregnancy outcomes more than stress levels in women with RM.

The next part of this thesis investigated the biochemical stress markers including natural killer cells, cortisol and prolactin in the same cohort of women with unexplained RM. The increases in pNK CDdim cells were found to be associated with a higher risk of a subsequent miscarriage in women with RM, suggesting a prognostic value of measuring pNK subset of CDdim cells in RM. In addition, no correlation between the measurements of pNK cells and those of uNK cells was found in women with RM, suggesting that pNK measurements do not reflect uNK measurements in RM.

Next, we conducted a study to investigate the relationship between prolactin and RM. We found that within normal physiological range low plasma prolactin concentrations were associated with an increased risk of a subsequent miscarriage in women with RM. No significant difference in the measurements of endometrial prolactin receptor was found between women with RM and fertile

women. There was also no association between the expression of endometrial prolactin receptor and subsequent pregnancy outcomes in women with RM.

The following part of this thesis examined the activation of the hypothalamo-pituitary-adrenal (HPA) axis in response to stress in women with RM with cortisol measurements. No non-suppression of cortisol following Dexamethasone Suppression Test (DST) was found in women with RM. There was also no association between cortisol measurements and pregnancy outcomes in women with RM. Based on the findings in this study we speculate that chronic stress of RM has no effect on feedback dysregulation of the HPA axis.

The final part of this thesis correlated the results of various stress measurements. High levels of fertility stress were associated with a decrease in the numbers of pNK CDbright cells. High basal salivary cortisol concentrations were associated with an increase in uNK cell measurements. The results of serum cortisol suppression following DST had an inverse correlation with the values of pNK CDdim cells whereas the results of salivary cortisol suppression following DST had a positive association with uNK cell measurements. The exact physiological mechanism of this observation is not known.

In summary, the studies presented in this thesis showed that stress was associated with RM. There was some evidence that stress affected subsequent pregnancy outcomes in women with RM.

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List of abbreviations

ACU: Assistant Conception Unit

ABC: Avidin-Biotin Complex

ACTH: Adrenocorticotrophic Hormone

AVP: Arginine Vasopressin

APS: Antiphospholipid Syndrome

AS: Acrosomal Status

BV: Bacterial Vaginosis

β-HCG: β Human Chorionic Gonadotropin

CG: Chorionic Gonadotropin

CD: Cluster Differentiation

COSHH: Control of Substances Hazardous to Health

CBG: Corticosteroid-Binding Globulin

CRH: Corticotrophin Releasing Hormone

CV: coefficient of variation

CBG: Corticosteroid-binding Globulin

DAB: Diaminobenzidine

DST: Dexamethasone Suppression Test

$E_{2:}$ Oestradiol

FPI: Fertility Problem Inventory

FSH: Follicular Stimulating Hormone

FSC: Forward Scatter

GnRH: Gonadotropin Releasing Hormone

GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor

HSV: Herpes simplex virus

HLA: Human Leukocyte Antigen

HMG: Human Menopausal Gonadotropin

HPA: Hypothalamo-pituitary-adrenal

HPG: Hypothalamo-pituitary-gonadal axis

HOS: hypo-osmotic swelling

11β-HSD2: 11β-hydroxysteriod dehydrogenase type2

IFN-γ: Interferon γ

IgG: Immunoglobulin G

IHC: Immunohistochemistry

IVF: *In Vitro* Fertilization

KIR: Killer Inhibitory Receptor

LC-MS/MS: Liquid Chromatography-tandem Mass Spectrometry

LPD: Luteal Phase Deficiency

LH: Luteinizing Hormone

MHC: Major Histocompatibility Complex

MTHFR: Methylenetetrahydrofolate Reductase (MTHFR)

NK cells: Natural Killer Cells

dNK: Decidual Natural Killer

pNK cells: Peripheral Natural Killer Cells

uNK cells: Uterine Natural Killer Cells

NCD: Nuclear Chromatin Decondensation

PVN: Parvocellular Neurons

PSS: Perceived Stress Scale

PBS: Phosphate Buffered Saline

PCOS: Polycystic Ovarian Syndrome

PANAS: Positive Affect and Negative Affect Schedule

PGD: Preimplantation Genetic Diagnosis

PGS: Preimplantation Genetic Screening

RCT: Randomized Controlled Trail

RIF: Recurrent Implantation Failure

RM: Recurrent Miscarriage

RPL: Repeated Pregnancy Loss

SSC: Side Scatter

SNPs: Single-nucleotide Polymorphisms

SPSS: Statistical Package for the Social Sciences

TNF-α: Tumor Necrosis Factor α

Chapter I: Literature review

I. Definition of Recurrent Miscarriage

Recurrent miscarriage (RM), which is also referred to as repeated pregnancy loss (RPL) and habitual abortion, is defined as three or more consecutive spontaneous miscarriages (ESHRE, 2006). The experience of repeated pregnancy loss is physically and emotionally traumatic to women who are trying to have children. The overall frequency of RM was estimated from 1% to 3% (ESHRE, 2006, Christiansen et al., 2008). The exact prevalence of RM depends on its definition. To date there is no consensus on the definition of RM with regard to the numbers of previous miscarriages and the gestational age of RM. The American Society for Reproductive Medicine defines the numbers of previous miscarriages in RM as two or more whereas Europe Society of Reproduction and Embryology defines it as three or more (ASRM, 2008, ESHRE, 2006). In some studies only pregnancy losses in the first trimester (≤14weeks) were included whereas in other studies pregnancy losses in the second trimester (≤24weeks) were also investigated (Franssen et al., 2007, Raffaelli et al., 2009, Tempfer et al., 2006, Garrisi et al., 2009, Jaslow et al., 2009). In some studies the gestational age of repeated miscarriages was not clarified (Zolghadri et al., 2008). Therefore, it is possible that different study populations were examined in current studies. Thus the incidence of RM derived from these studies may not be comparable.

II. Aetiology of Recurrent Miscarriage

RM is a heterogeneous condition and it is unlikely that only a single pathological factor is attributed to RM. Current literature suggests that the cause of RM is only identifiable in up to 40%-50% of cases. The remaining RM cases are classified as idiopathic. Hence, this merits further research to seek other possible underlying causes of RM. To date the identifiable causes of RM have been categorized as parental, fetal, environmental and psychological factors.

1 Parental Factors

1.1 Chromosomal Abnormality

Parental balanced structural chromosomal rearrangement accounts for 2%-4% of RM (Ford and Schust, 2009). The most common chromosomal rearrangement is balanced reciprocal or Robertsonian translocation which may lead to unbalanced gene translocations in the fetus, resulting in miscarriage (Fortuny et al., 1988, Suzumori and Sugiura-Ogasawara, 2010). Other chromosomal anomalies associated with RM include chromosomal inversion, insertions and mosaicism (De la Fuente-Cortes et al., 2009). Couples with chromosome defects may benefit from pre-implantation genetic diagnosis (PGD) in conjunction with in vitro fertilization (IVF).

1.2 Maternal Factors

i. Age

It is well recognized that female fertility declines with advancing age, which manifests in increases in miscarriage and trisomy 21 and monosomy X of the fetus. RM as part of a range of reproductive failures shares common risk factors. Studies have shown that in women with RM maternal age is positively associated with the numbers of repeated miscarriages (Garrisi et al., 2009) and also is an important factor predicting the occurrence of miscarriage (Metwally et al., 2010). In IVF treatment the pregnancy rate of women with RM declined with advancing maternal age (Zhang et al., 2008). The value of preimplantation genetic screening (PGS) on the reduction of pregnancy loss in RM women with advanced age is not yet clear. Some studies have shown that PGS significantly reduces the rate of pregnancy loss following IVF treatment in RM patients older than 35 years (Munne et al., 2005). However, a recent randomized controlled trail (RCT) shows that PGS did not improve the pregnancy rate or the live birth rate in RM women with advanced age (>37 years) (Staessen et al., 2004). Another multicenter RCT study also found that in IVF women aged between 35 years to 41 years and who had PGS had significantly lower ongoing pregnancy rates and live birth rates compared with those who did not undergo PGS (Mastenbroek et al., 2007), suggesting that PGS may reduce the rates of ongoing pregnancies and live births in IVF in women with advanced age and PGS has no beneficial effect on screening for embryonic aneuploidy in women with advanced maternal age (Twisk et al., 2008). Adoption or the use of donor gametes may be recommended to the older couples if IVF has failed.

ii. Anatomic Factors

Anatomic abnormalities account for 16 - 18% of RM cases (Jaslow et al., 2009). The common anatomic abnormalities include congenital uterine anomalies, uterine adhesions, uterine fibroids and polyps. These abnormalities may cause inadequate vascularity of the

endometrium where the embryos implant, resulting in placental abruption and consequently miscarriage (Propst and Hill, 2000). Among these anatomic abnormalities, congenital uterine abnormalities such as arcuate, septate or bicornuate uterus may be associated with second trimester miscarriages more than early pregnancy losses (Saravelos et al., 2010). Women with anatomic anomalies may benefit from uterine sonography and HSG in the initial diagnosis. A definitive diagnosis can be obtained by using combined laparoscopy and hysteroscopy as well as 3D sonography (Saravelos et al., 2008). Surgical resection of the uterine septum and adhesions, removal of submuscous fibroids and polyps may improve subsequent pregnancy outcomes in these women (Propst and Hill, 2000).

iii. Endocrinological Factors

Endocrinological disorders account for approximately 17% to 20% of RM cases (Stephenson, 1996, Li et al., 2000). Luteal phase deficiency (LPD), polycystic ovarian syndrome (PCOS) and hyperprolactinaemia are the commonest endocrinological abnormalities in RM. LPD is defined as the retardation of the endometrium development in the histological dating more than 2 days behind the corresponding chronological dating (Olive, 1991). The inadequate secretion of progesterone, premature regression of the luteal corpus and a suboptimal response of the endometrium to progesterone may be present in LPD. The measurements for LPD include LH timed endometrial biopsy characterized by the retardation of the histological development of endometrium, a single serum progesterone concentration < 31.8 nmol/L in mid luteal phase or three random serum progesterone concentrations < 95.4 nmol/L (Jordan et al., 1994, Li and Cooke, 1991). LPD has been associated with RM and substitution treatments with progesterone or human menopausal gonadotropin (HMG) have been reported

to reduce the risk of miscarriage in RM patients with LPD (Daya et al., 1988, Vanrell and Balasch, 1986, Li et al., 2001).

PCOS induced increase in luteinizing hormone and androgen levels may cause premature ageing of oocytes and/or incoordinated development of endometrium, subsequently resulting in spontaneous miscarriages. Case studies have reported that successful live births had been achieved in RM patients with PCOS following metformin and ovarian drilling treatments (Palomba et al., 2006, Ballian et al., 2006), suggesting that PCOS is associated with RM. In a prospective study with a large cohort of 2199 women with RM, Rai et al found that the presence of PCO morphology in RM women was significantly higher than that in general population but the morphological PCO of RM patients was not linked to an increased risk of miscarriage in a subsequent pregnancy (Rai et al., 2000). The presence of raised LH and testosterone has also been found to be higher in women with RM but is not associated with an increased risk of miscarriage (Nardo et al., 2002, Rai et al., 2000, Clifford et al., 1994, Watson et al., 1993). A study suggested that free androgen index (FAI) may be a predictive factor of subsequent miscarriage. Raised FAI has been found to be associated with a high risk of subsequent miscarriage in women with RM (Cocksedge et al., 2008).

The role of hyperprolactinaemia in RM remains controversial. Earlier studies have reported on the high prevalence of hyperprolactinaemia in the RM population and the improvement in pregnancy outcome after bromocriptine treatment (Bussen et al., 1999, Csemiczky et al., 2000, Hirahara et al., 1998), suggesting that hyperprolactinaemia may be a contributory factor of RM. However recent studies found that hyperprolactinaemia occurs rarely in women with RM (Li et al., 2000). The relationship between prolactin and RM will be further discussed in the relevant chapter of this thesis as appropriate.

iv. Prothrombotic States

A successful pregnancy requires a well developed placenta and sufficient placental function to sustain an adequate fetomaternal micro-circulation. This system may be compromised by prothrombotic disorders leading to subsequent miscarriages (Larciprete et al., 2007). However, recent RCTs have shown that thromboprophylaxis using aspirin combined with heparin, aspirin alone or placebo does not improve pregnancy outcomes in women with unexplained RM (Kaandorp et al., 2010, Clark et al., 2010), suggesting that it is inappropriate to extend thromboprophylaxis to RM women without recognized thrombophilia conditions.

It is estimated that prothrombotic states may account for 12.5% of RM (Carp et al., 2004). This disorder is categorized as inherited and acquired conditions.

a. Inherited thrombophilia

The most common inherited thrombophilic disorders are deficiencies of antithrombin III, protein C and protein S, Factor V Leiden mutation and prothrombin gene mutation (G20210A).

a.1 Antithrombin III deficiencies

Antithrombin III is the most important inhibitor of thrombin, factor Xa, IXa and XII a. Antithrombin III deficiency results from the decrease in the concentration or the function of antithrombin III (Patnaik and Moll, 2008). It is an autosomal disorder with a high risk of thromboembolism in life-long time. Women with antithrombin deficiency have been found to

have a high risk of miscarriage. A prospective multicenter study investigated 843 European women with thrombophilia disorders and 541 control women without thrombophilia. They found that antithrombin deficiency was associated with a high risk of recurrent late pregnancy losses (Preston et al., 1996).

a.2 Protein C and Protein S deficiencies

Protein C inactivates factor Va and VIIIa involved in the anticoagulant process and this function is enhanced in the presence of protein S. Protein C deficiency results from a decrease in protein C antigen or the activity of protein C. Protein S deficiency results from a decrease in the concentration or the function of protein S (ten Kate and van der Meer, 2008). Protein C and S deficiencies have been linked to an increased risk of miscarriage. For example, a study examined the effect of thromboprophylaxis on the reduction of pregnancy losses. They found that women who received low-molecular-weight heparin treatment for deficiency of Protein C and S had a significantly lower miscarriage rate than those who did not receive the treatment (Folkeringa et al., 2007), suggesting that Protein C and Protein S deficiencies may be a contributory factor of miscarriage.

a.3 Factor V Leiden mutation

The mutation of Factor V Leiden causes acquired protein C resistance, resulting in thrombophilia both in veins and spiral arteries of the placenta. This may lead to placenta abruption and consequently result in miscarriage. Factor V Leiden has been found to be associated with RM. Yenicesu *et al.* conducted a study among 272 homogenous Caucasian women with RM and 56 fertile controls to investigate the relationship between12

thrombophilic gene mutations and RM. They found that Factor V Leiden mutation along with other six gene mutations was positively associated with RM, suggesting that Factor V Leiden may be a causative factor of RM (Yenicesu et al., 2010). Jivraj et al also found that the couples with one or more thrombophilic allele of Factor V Leiden, prothrombin G20210A or methylenetetrahydrofolate reductase (MTHFR) C677T had an increased risk of miscarriage in a subsequent untreated pregnancy (Jivraj et al., 2006). However, in a recent study they compared the pregnancy outcomes between women with RM who were Factor V Leiden carriers and those with unexplained RM but did not find an association between the occurrence of Factor V Leiden and the increased risk of miscarriage (Jivraj et al., 2009). It has been suggested that the increased presence of single-nucleotide polymorphisms (SNPs) on the Factor V gene may predispose women to acquired activated protein C resistance and therefore is associated with repeated pregnancy losses (Dawood et al., 2007).

a.4 Prothrombin Gene mutation (G20210 mutation)

Prothrombin gene (G20210A) mutation is associated with an increased risk of thrombosis and it is the most identifiable risk factor for venous thrombosis (Zoller et al., 1999). More recent studies have shown that G20210A mutation is associated with RM (Yenicesu et al., 2010, Goodman et al., 2006). However several studies found that the incidence of G20210A mutation was rare in women with RM (Agorastos et al., 2002, Pickering et al., 2001, Altintas et al., 2007, Behjati et al., 2006). Therefore, the role of G20210A mutation in RM still needs to be elucidated.

b. Acquired thrombophilia

The most common acquired thrombophilia associated with RM is the antiphospholipid syndrome (APS). Antiphospholipid antibodies are autoantibodies against negatively charged phospholipids. APS is categorized as primary (where it occurs in isolation) and secondary (where it occurs concurrently with other diseases, such as systemic lupus erythematosus). APS is diagnosed if at least one of the following clinical criteria and one of the following laboratory criteria are met (George and Erkan, 2009).

Clinical criteria:

1. Vascular thrombosis

One or more clinical episodes of arterial, venous, or small vessel thrombosis in any tissue or organ

2. Pregnancy morbidity

(1) One or more unexplained deaths of a morphologically normal fetus at or beyond 10 weeks of gestation, or

(2) One or more premature births of a morphologically normal neonate before 34 weeks of gestation because of eclampsia, severe preeclampsia, or recognized features of placental insufficiency, or

(3) Three or more consecutive spontaneous miscarriages before 10 weeks of gestation, excluded maternal anatomic and endocrinological abnormality and parental chromosomal anomaly.

Laboratory criteria:

1. Lupus anticoagulant presents in plasma on 2 or more occasions at least 12 weeks apart,

2. IgG and/or IgM isotypes of anticardiolipin antibody in plasma or serum present in medium or high titer on 2 or more occasions at least 12 weeks apart,
3. IgG and/or IgM isotypes of anti-β_2-glycoprotein I antibody in serum or plasma present on 2 or more occasions at least 12 weeks apart.

APS has been recognized as a cause of RM. Rai *et al* examined the prevalence of APS in a cohort of 500 consecutive women with RM and found that approximately 15% of them had APS (Rai et al., 1995b). Studies have reported the achievement of live birth in women with RM by treating APS (Farquharson et al., 2002, Sugiura-Ogasawara et al., 2008), suggesting that APS is a causative factor of repeated pregnancy loss. A prospective study compared the pregnancy outcome between women with APS and those with unexplained RM. They found that patients with APS were more likely to have miscarriages again in their subsequent pregnancies compared with those with unexplained RM (Rai et al., 1995a), confirming that women with APS have a high risk of repeated pregnancy loss.

v. Immunological Factors

In utero survival of the semiallogenic embryo depends on the successful adaptation of the maternal immune system to the fetal antigens. Dysregulation of this immunological adaptive process may lead to pregnancy loss. To date there is no systematic diagnostic workup or therapy for RM due to immunological abnormalities.

a. Thyroid antibody

The association between thyroid antibody and RM has been highlighted in several studies. Iravani *et al* conducted a study in 641 patients with RM and 269 fertile controls. They found that the presence of thyroid antibodies including thyroglobulin and thyroid peroxide antibody were associated with RM, suggesting that the dysfunction of thyroid autoimmunity may contribute to RM (Iravani et al., 2008). Dendrinos *et al* examined the occurrence of antithyroid antibody in women with RM and found that women with RM had a significantly higher frequency of antithyroid antibody than those without RM (37% versus 13%, $\rho<0.05$) (Dendrinos et al., 2000), suggesting that the presence of antithyroid antibody is associated with RM.

b. Natural killer cells

In the past decade, considerable effort has been made to identify the relationship between cytokines and RM. Natural killer (NK) cells have been most extensively studied. NK cells are an important part of innate immunity and constitute the predominant leukocyte population of the endometrium at the time of implantation and in early pregnancy. NK cells play an immunoregulatory role in the maternal adaptive immunity to the semiallograft embryo through secreting cytokines, chemokines and angiogenic factors. An increasing number of studies have shown that the increase of NK cells both in peripheral blood and endometrium tissue is associated with RM (Tuckerman et al., 2007, Larciprete et al., 2007, Clifford et al., 1999, King et al., 2010, Perricone et al., 2006, Quenby et al., 2005). Empirical treatment with prednisolone has been shown to have the effect of reducing the number of endometrial NK cells and improving subsequent pregnancy outcomes in women with RM (Quenby et al., 2005, Quenby et al., 2003). It has been proposed that the imbalanced increase in

CD56+/CD16+ phenotype of endometrial NK cells may contribute to RM rather than the increase in the total number of NK cells (Lachapelle et al., 1996).

vi. Cervical Incompetence

Cervical incompetence, also termed as cervical insufficiency, refers to the dilatation and shortening of the cervix in the absence of contractions of labour due to the defect of the cervical sphincter (Romero et al., 2006). Cervical incompetence has been recognized as a cause of repeated mid-trimester spontaneous miscarriage. The clinical presentation is the consequent membrane protruding eventually leading to membrane rupture. Several methods have been proposed for its diagnosis, including the reduction in cervical resistance index in the nonpregnant state and the reduction in sonographic cervical length to less than 25mm with funnel sign in mid-trimester (Anthony et al., 2007, Feingold et al., 1984). Women with cervical incompetence may benefit from cervical cerclage to prevent miscarriages.

vii. Infections

The role of infection in RM is not clear. A number of micro organisms have been suggested to be associated with spontaneous miscarriage, including Chlamydia trachomatis, Listeria monocytogenes, Toxoplasma gondii, rubella, herpes simplex virus (HSV). Bacterial vaginosis (BV) seems to be associated with premature rupture of membranes resulting in mid trimester loss and preterm labour more than early pregnancy losses (Nelson et al., 2007). The association between chlamydia trachomatis and RM had been highlighted in one study (Kishore et al., 2003) but later this was refuted. A study examined 504 women with RM and 154 controls without RM and found that the occurrence of Chlamydia in RM patients was not

significantly different to that of controls (Sugiura-Ogasawara et al., 2005). Therefore, the role of Chlamydia in RM is still not clear.

1.3 Paternal Factors

The association between the morphology and function of sperm and RM is not certain yet due to the paucity of studies in this area. Some preliminary studies suggest that abnormal integrity of sperm DNA may increase the risk of miscarriage (Check et al., 2005, Carrell et al., 2003). However these studies focus more on sporadic miscarriages rather than RM. A study assessed sperm functional parameters, such as hypo-osmotic swelling (HOS), acrosomal status (AS), and nuclear chromatin decondensation (NCD) in vitro between men of partners with unexplained RM and men who had recently conceived a child. They found a significant reduction in sperm function in the case group compared with the control group (Saxena et al., 2008), suggesting that impaired sperm function may play a role in RM. Carrell et al also found a significant increase of sperm chromosome aneuploidy, apoptosis, and abnormal sperm morphology in men with partners having a history of RM (Carrell et al., 2003).

2 Fetal Factors

Chromosomal anomaly is the commonest fetal cause of RM. Aneuploidy is the most prevalent chromosomal abnormalities of abortuses in RM (Carp et al., 2001). Evidence from preimplantation genetic diagnosis (PGD) has shown that women with RM had a higher incidence of chromosomally abnormal embryos after aneuploidy screening than those without RM (Rubio et al., 2005, Rubio et al., 2003).This finding is consistent with those from other previous studies that couples with RM had significantly higher rates of chromosomally

abnormal embryos in IVF treatment (Pellicer et al., 1999, Simon et al., 1998), suggesting that fetal chromosomal anomaly may be the cause of RM. However, some studies have described contradictory findings that fetal aneuploidy occurred less in women with RM and with the increase in the numbers of repeated miscarriages the occurrence of normal chromosomal abortuses increased in women with RM (Sullivan et al., 2004, Ogasawara et al., 2000). Therefore, more research is needed to clarify this issue. Interestingly, Carp *et al* found that patients with karyotypically abnormal abortuses had better pregnancy outcomes in their subsequent pregnancies than those with karyotypically normal miscarried fetuses (Carp et al., 2001). Ogasawara et al also found that RM women with a previous karyotypically normal abortus were more likely to miscarry in the subsequent pregnancies than those with an karyotypically abnormal abortus (Ogasawara et al., 2000) The exact explanation for this observation is not known yet.

3 Environmental Factors

Caffeine consumption and smoking have been implicated in increasing the risk of miscarriage. Recent studies have shown that women who are homozygous for CYP1A2*1F alleles (an enzyme responsible for caffeine metabolism) had a high risk of RM with a dose dependent effect of daily caffeine intake (Sata et al., 2005). Other studies have shown that women exposed to environmental tobacco smoke had a high risk of spontaneous miscarriage in combination with caffeine and alcohol consumption (Windham et al., 1999).

4 Psychological Factors

4.1 The impact of stress on reproduction

Recently, the impact of psychological stress on RM has received more attention. Studies have suggested that stress may play a role in RM through maternal neuro-endocrine-immune network response (Andalib et al., 2006). Stress, namely pressure or tension, has been defined as a form of psychological, physiological and behavioural transaction between people and environment, ie. person-environment fit. In this definition the external environmental stimulus is regarded as a stressor and the psychological response to the external stimulus is termed as stress (Ogden, 2007). Two types of stress are differentiated including acute stress and chronic stress. Acute stress is a response to an immediate threat, such as an exam or a public presentation. Chronic stress is a response to a prolonged or frequent stimulus, such as job stress or poverty (McGonagle and Kessler, 1990). It suggests that a good person environment fit may cause no or a low level of stress whereas a poor fit may result in a high level of stress. The effect of stress may be beneficial to enable fight or flight adaptive response to escape from the harmful situation and the homeostasis may still be resumed. However, effect of stress may be detrimental by mediating a series of physiological responses resulting in adverse somatic consequences, including impaired cognition, abnormal metabolism, impaired immune function and impaired reproduction.

There are an increasing number of studies that reported on the adverse impact of stress on reproductive performances, causing premenstrual pain, loss of libido, sexual dysfunction, functional hypothalamic amenorrhea, anovulation, infertility, miscarriage, still birth, low weight birth and postpartum morbidity (Donadio et al., 2007, Lechner et al., 2007, Botros et al., 2006, Diego et al., 2006, Lopez-Gatius et al., 2005, Xiao et al., 2002). Pregnancy is a

special physiological and psychological transaction in women with increased sensitivity and anxiety. This transaction could be a form of stress causing a negative impact on a woman's wellbeing with a consequent deleterious effect on pregnancy. An adverse pregnancy outcome could also be a form of stressor, mediating negative psychological responses such as grief, mourning, anxiety, depression, coping, loss of self-esteem and frustration (Kersting et al., 2004, Nikcevic et al., 1998). Recently the link between negative prenatal psychological stress and adverse pregnancy outcome has been highlighted. For example, in IVF treatment it has been found that high stress levels are associated with poor biological responses to treatment and low pregnancy rates (Boivin and Schmidt, 2005). In a prospective study in women who planned their first pregnancies, during a period of 6-month follow-up it was observed that women with higher distress scores had lower pregnancy rates and higher early miscarriage rates than those with lower distress scores (Hjollund et al., 1999). Studies have also shown that both in rat models and in humans stress induces repeated pregnancy loss by up-regulating Th1 family cytokines (Knackstedt et al., 2003). In women with RM it has been found that a high stress level is associated with an increased risk of subsequent pregnancy loss (Sugiura-Ogasawara et al., 2002, Andalib et al., 2006).

4.2 The mechanism of stress effect on RM

Stress stimulation can trigger a series of physiological adaptive responses. The systems that respond to stress are the hypothalamo-pituitary-adrenal (HPA) axis and the sympathoadrenal system. Stress can affect the secretion of the parvocellular neurons (PVN) in the hypothalamus and the release of neuropeptides, corticotrophin releasing hormone (CRH) and arginine vasopression (AVP). The interaction of these mediators affects the secretion of adrenocorticotrophic hormone (ACTH) in the anterior pituitary gland, which in turn increases

the synthesis of glucocorticoids in the adrenal cortex. Simultaneously glucocorticoids have a negative feedback on the hypothalamo-pituitary axis, regulating the secretion of CRH and ACTH. It is also known that stress can trigger the release of catecholamines and noradrenaline via the sympathetic nervous system. Therefore, these increased neurohormones and sympathetic factors can activate a series of physiological responses, disrupting homeostasis and causing a range of adverse somatic changes, consequently damaging pregnancy (Tilbrook and Clarke, 2006, Tilbrook et al., 2006, Ferin, 1999, Chorusos et al., 1998).

It has been suggested that stress may play a role in pregnancy loss via the interaction between psycho-neuroendocrine-immune networks. Overt or latent stress may alter steroidgenesis by activating the HPA axis and neuropeptides through the sympathoadrenal system. The interaction of these stress-related mediators, such as glucocorticoids, CRH, ACTH, AVP, PVN, endogenous opioid peptides and catecholamines, may modify the synthesis and secretion of gonadotropin releasing hormone (GnRH) from the hypothalamus and gonadotropins from the pituitary. The regulation of GnRH on gonadotropins and the negative feedback of gonadal steroids to GnRH could also be modulated by stress-related mediators (Tilbrook and Clarke, 2006, Ferin, 1999, Parker and Douglas, 2010). Therefore, stress may interfere with the gonadal axis, resulting in adverse pregnancy via neuro-endocrine pathways. In addition, it is possible that stress may also interfere with reproductive immunity. Stress may alter cytokine profiles which are responsible for the maternal immunity adaption to tolerate the semi-allograft embryo during pregnancy, thus adversely affecting implantation and trophoblastic invasion, consequently resulting in early pregnancy loss (Glaser, 2005, Glaser and Kiecolt-Glaser, 2005, Parker and Douglas, 2010).

RM as a part of pregnancy failure shares the common mechanism with pregnancy loss resulting from stress. Recently, there are an increasing number of studies exploring the relationship between stress and RM. Because many of the psychological concepts are subjective and difficult to define precisely, individuals may be uncertain as to how to classify themselves into the response ranking categories. In addition, the perception of stress may vary between individuals. Some events which are stressful to some individuals may be not to others. Therefore, stress scores derived from individual's self-reports may not reflect the real stress status.

Recently, the correlation between psychological stress and some biochemical stress markers, including endocrine and immune factors, has been emphasized. Some hormones and immune cells responsive to stress, such as cortisol and NK cells, have been examined in studies on the relationship between stress and adverse pregnancy outcome (Diego et al., 2006, Andalib et al., 2006). Therefore, investigation of biochemical stress markers may provide an objective physiological pathway to evaluate the role of stress in RM. However, most of the current studies look at the effect of a single stress mediator on RM. So far there is no comprehensive study examining the role of stress with regard to the interaction between stress mediators. From current literature the possible mechanism of the effect of stress mediators on RM is detailed below.

i. Cortisol

Cortisol is widely recognized as a stress hormone due to its sensitivity and responsiveness to stress stimulation. Studies have shown that peripheral cortisol levels are elevated under stressful circumstances (Schlotz et al., 2004, Aardal-Eriksson et al., 1999) and cortisol

elevation is positively correlated with stress scores (Diego et al., 2006). In pregnant women it has been found that prenatal psychosocial stress may increase cortisol secretion (Diego et al., 2006, Wadhwa et al., 1996). High levels of cortisol have been found to have a negative effect on pregnancy. In IVF treatment it has been observed that women who failed in IVF had higher cortisol concentrations than those who had successful pregnancies (Demyttenaere et al., 1992). In a prospective study high levels of cortisol have been found to be associated with an increased risk of miscarriage, suggesting that cortisol elevation may contribute to miscarriage (Nepomnaschy et al., 2006).

It is unclear how high cortisol levels affect pregnancy. It has been suggested that high levels of cortisol may interfere with gonadal steroid production. In the follicular phase cortisol concentrations have been found to be negatively correlated with the concentrations of FSH, progesterone and β-HCG (Demyttenaere et al., 1992). Cortisol increase also has the effect of suppressing progestin secretion (Nepomnaschy et al., 2004). These inhibitory effects of cortisol elevation on gonadal-steroid production may consequently affect pregnancy, resulting in miscarriage. However, studies have shown that only sustained elevation of cortisol may have this effect. Repeated transient elevation of cortisol does not appear to have a similar effect (Turner et al., 1999a, Turner et al., 1999b). It is therefore possible that chronic stress, which is more likely to induce sustained increase of cortisol, has a more profound deleterious effect on pregnancy than acute stress.

ii. Prolactin

Stress has been found to have the effect of increasing plasma prolactin secretion. These stressful circumstances include war, negative life events and academic examinations (Sonino

et al., 2004, Sabioncello et al., 2000, Armario et al., 1996). Studies also found that women who underwent IVF treatments had higher anxiety levels and plasma prolactin concentrations than fertile controls, suggesting that anxiety may increase plasma prolactin concentrations through the HPA activation (Csemiczky et al., 2000). The increase of prolactin in response to stress often occurs in parallel with cortisol elevation. Yet to date the exact physiological pathway of how stress may increase prolactin secretion is not understood.

Plasma prolactin elevation has been previously related to infertility and RM. For example, Csemiczky et al. (2000) have found that infertile women had significantly higher concentrations of plasma prolactin throughout the menstrual cycle than fertile women (Csemiczky et al., 2000). Some previous studies have reported on the high prevalence of hyperprolactinaemia in women with RM (Hirahara et al., 1998, Bussen et al., 1999) and improved pregnancy outcomes after treatment of hyperprolactinaemia with bromocriptine (Hirahara et al., 1998), suggesting that hyperprolactinaemia may be a possible aetiological factor of RM. However, contrary to the above findings, our earlier study showed that hyperprolactinaemia rarely occurs in women with RM (Li et al., 2000). Recent studies have also shown high levels of plasma prolactin may have a beneficial role in pregnancy and lack of expression of prolactin and its receptors in endometrium is associated with RM (Gåfvels et al., 1992, Gonen and Casper, 1989, Ozaki et al., 2001). Therefore, it is still unclear whether high prolactin levels are associated with miscarriage. Further discussion on the relationship between prolactin and RM will be further discussed in the relevant chapter of this thesis as appropriate.

iii. Natural Killer Cells

It has been found that natural killer (NK) cells may be altered in numbers and activities under stress stimulations. In women with RM pre-conceptional stress has been found to have the effect of altering peripheral NK (pNK) cells (Hori et al., 2000, Andalib et al., 2006). Of the various types of stress, depression appears to have the greatest effect on increasing pNK cell cytotoxicity (Hori et al., 2000, Andalib et al., 2006, Shakhar et al., 2006). The alteration of pNK cells has been found to be associated with RM (Fukui et al., 2008, Emmer et al., 2000, King et al., 2010). Uterine NK (uNK) cells have also been found to be associated with RM. Some studies have shown that uNK is elevated in women with RM compared with fertile women (Tuckerman et al., 2007, Clifford et al., 1999, Quenby et al., 2005) and the increase of uNK cells is positively correlated with the number of previous miscarriages of RM (Tuckerman et al., 2004), suggesting that uNK cells play a role in RM. The relationship between NK cells and RM will be elaborated in the relevant chapter of this thesis as appropriate.

Chapter II: Hypotheses to be explored in this thesis

Hypotheses

1. Women with RM have higher stress levels than fertile women without miscarriage

2. ‘Tender loving care’ in the form of clinical consultation, explanation and reassurance from the medical staff at the dedicated clinic may reduce stress levels of women with RM

3. Stress may have a prognostic value on subsequent pregnancy outcomes in women with RM

Chapter III: Materials and methods

As several studies are presented in this thesis, in this section only materials and methods which are common to all studies will be described. Details of the specific materials and methods of each individual study will be described in the relevant sections as appropriate.

I. Health and Safety

All biological specimens, materials and reagents were handled according to the health and safety regulations. Laboratory coats and gloves were worn during specimen or reagent handling. Any biological specimens or chemical reagents producing toxic fumes were handled in safety cabinets. All recommendations as required by COSHH (Control of Substances Hazardous to Health), or in correspondence to the safety data sheets of each reagent, were followed.

II. Ethics

Ethical approval for the recruitment of participants was obtained from the South Sheffield Research Ethics Committee (08/H1308/80) in October 2008 (Appendix I). R & D approval was granted in July 2009 (Appendix II). Each participant was provided with an information sheet describing the nature of the study (Appendix III). All the patients who took part in the study were seen by the researcher for consent and to further explain details about the study. Instructions with regard to the time and methods of sample collections were delivered to the participants once written consent forms were obtained.

Data regarding clinical profiles of the patients were collected from the medical notes. The collected data was input in a pre-designed chart for research purposes (Appendix IV). The chart sheets were kept in a locked cabinet in a secure office where was only accessible to the authorized persons. The data was processed anonymously into SPSS software by the researcher for the subsequent analyses. The computer where the data was input was secured and password protected.

III. Study Setting

This study was conducted at the Recurrent Miscarriage Clinic in Jessop Wing for Women, Royal Hallamshire Hospital, Sheffield Teaching Hospital. The laboratory work was conducted in the biomedical laboratories on level 4 in the Academic Unit of Reproductive and Developmental Medicine in the Jossop Wing hospital for Women and the flow cytometry department on floor D in the Medical School of the University of Sheffield.

IV. Materials

1 Cases

Women with a history of recurrent miscarriages, who were referred to the Recurrent Miscarriage Clinic, were recruited at their first clinical attendance. Recruitment began in November 2008. Due to the long term nature of the study period for each participant according to the study protocol, recruitment was ended in September 2009 so that the follow-up of the participants could be completed within the time frame. We adhered strictly to the inclusion criteria during recruitment and therefore excluded a significant number of patients

who attended the RM clinic during the study period. There were a total of 52 patients eligible to the study during the recruitment period and 45 of them agreed to participate in the study.

Among these 45 recruits, two patients were diagnosed with depression during the study period and were referred to psychiatric treatment by their GPs; one patient declined treatment at the RM clinic after consent; two patients conceived promptly after consent; four patients withdrew from the research due to the distance reason. Therefore, they were excluded from the study. The remaining 36 patients completed the research investigations on two occasions in accordance with the study protocol.

1.1 Inclusion Criteria

Women with unexplained RM, who were fertile and ovulatory, were eligible for this study. The definition of RM was defined as three or more consecutive spontaneous miscarriages. RM without the presence of previous live births was categorized as primary RM and RM with the presence of previous live births was categorized as secondary RM. All the patients underwent investigations in accordance to an established protocol (Li, 1998), including parental chromosomal analysis, thrombotic study, antiphospholipid antibody screen, thyroid function and thyroid antibody test, FSH, LH, estradiol, prolactin, free androgen index, transvaginal ultrasonography and hysterosalpingography. Hysteroscopy was only performed if a uterine abnormality was suspected on pelvic ultrasound or hysterosalpingography. Unexplained RM fulfilled the criteria of normal parental chromosomal analysis, absence of endocrinological abnormalities, tested negative for antiphospholipid antibody screens, normal coagulation studies, pelvis ultrasonography and hysterosalpingography which shows no evidence of structural uterine anomaly.

1.2 Exclusion Criteria

Women who had pituitary disorders, psychiatric illnesses and who had depression on anti-depressants were excluded from this study.

2 Controls

Thirty female volunteers aged between 20 to 40 years, who had no history of miscarriage, infertility, pituitary diseases, psychiatric disorders and depression, were recruited from the Gynaecology Outpatient Unit and the University Department. Parity was not taken into account as an inclusion criterion for controls as only the history of miscarriage or infertility was considered as the factor which may affect the level of fertility-related stress.

V. Methods

1 Study Design

1.1 Case study

Women with RM, who were referred to the Recurrent Miscarriage Clinic, were approached on their first visits at the clinic. There were approximately two new referrals in each clinic per week. New referrals were interviewed by the specialist nurses before the clinical consultation with regard to the history of their previous miscarriages. Eligible candidates were identified after the interview and the investigator was introduced to the potential recruits by the specialist nurses. The patients were then seen by the investigator and the information

sheets about the purpose of the study were delivered to them. Women who were interested in the study were further explained the details of the study, which included the number of the visits, the types of samples required from them and the invasive procedures involved for these investigations. They were reassured regarding the anonymity and confidentiality of their data and their right of withdrawal from the study at any time. The written consent forms were obtained after patients fully understood the entire study.

i. Procedure

1 Baseline assessment:

1.1 First visit:

At the first visit in the study, patients were asked to complete questionnaires to identify baseline stress levels. The pre-designed flow chart (Appendix V) outlining subsequent research visits was provided to the patient. Patients were required to use barrier contraception during the study period until all of the baseline tests were completed.

1.2 Dexamethasone Suppression Test (DST) visit:

The participant was required to contact Dr Wei Li on day 1 of her next menstrual cycle. The appointment was arranged for the serum cortisol measurement at 8:30 am on day 3 to5 of the menstrual cycle. On the day prior to the blood test a saliva sample was collected by the patient at 11pm at home. A dexamethasone tablet at the dose of 1mg was administrated orally after the collection of the saliva sample. At 8:30am the following morning a 5ml blood sample was obtained for the serum cortisol estimation. A saliva sample was collected simultaneously for salivary cortisol estimation.

1.3 Biopsy visit:

Patients were asked to do daily urine ovulation test with the ovulation test sticks (Clearblue, UK) from day 10 of the cycle onwards. They were required to contact Dr Wei Li once positive results for ovulation were identified. Their appointments for the LH timed endometrium biopsy for uterine nature killer (uNK) cell measurements were arranged on day 7 to 9 after LH surge. The blood samples for peripheral natural killer (pNK) cell measurements were obtained simultaneously with the endometrial biopsies. The sequence of the biopsy visit and the DST visit may vary depending on the day they fell in the cycle on their first visits.

2. Follow-up assessment

According to the established protocol of clinical investigations, approximately 3 months afterwards patients were offered appointments to discuss the results of their investigations. Patients who participated in the research were seen by the investigator again. They were required to complete the same questionnaires in order to compare the change of their stress levels during this period. Simultaneously a blood sample was obtained from them for pNK cell measurements.

Participants were then followed up across the study period until they conceived again after the recruitment. The data regarding their pregnancy outcomes and the information of their clinical profiles were collected from the medical records for the analysis. Participants who did not conceive within a six-month period after the second assessment were asked to

complete the study. They were categorized into the non-pregnant group in the analysis and the data regarding their clinical information was also collected from the medical records.

1.2 Control study

Female volunteers were approached in the Gynaecology Outpatient Unit and the University Department in the Jessop Wing. They were informed about the purpose of the study with the poster. After giving written consent, they were asked to do daily urine dipstick tests (Clearblue, UK) from day 10 of the cycle onwards with a view to detecting the LH surge. On day 7 to10 after the LH surge a blood sample was obtained for pNK cell measurements. They were asked to complete the same questionnaires to identify their stress levels.

1.3 Outcome measures

1.3.1 Clinical measures

The conception outcome was defined as the outcome of the first conception within a six-month period after the second assessment. The pregnancy outcome was measured as the outcome of the first pregnancy during the study period. Miscarriage was defined as the spontaneous loss of pregnancy following a pregnancy test, including biochemical pregnancies, pregnancies with the presence of a sac on ultrasound scan and pregnancies with the presence of fetal cardiac activity in the first trimester.

First trimester spontaneous miscarriages occur in 15% of all pregnancies and are most frequently due to abnormal embryos or fetuses, particularly aneuploidies like trisomy 21, 18, or 13 (Donoso et al., 2007). Spontaneous miscarriage can be classified as threatened, delayed,

inevitable, complete or incomplete. In threatened miscarriage, the embryo or fetus is viable but there is vaginal bleeding with a closed cervical os. In delayed miscarriage, the fetus with a minimum crown-rump length of 6 mm is either non-viable or an empty gestation sac measures more than 20mm in average diameter. In delayed miscarriage, there may or may not be associated vaginal bleeding. Inevitable miscarriage is often characterised by a dilating cervical os and abdominal pain. In this thesis we did not take into account threatened miscarriage in the study analysis.

No treatment was given to the study subjects apart from tender loving care. 'Tender loving care' was defined as clinical consultation, explanation and reassurance by a team of dedicated staff in the RM clinic. RM women who were referred to the Recurrent Miscarriage Clinic were interviewed by the two specialist staff nurses regarding the past history of repeated pregnancy losses and the relevant medical history. A series of investigations were then performed according to an established protocol (Li, 1998) in the clinic as described in the preceding context. The questions asked by the patients regarding the causes of RM and available treatments were explained explicitly and patiently as appropriate by the dedicated clinicians at the clinic. The patients were reassured of a likely profound improvement in pregnancy outcomes after treating the identified causative factor of RM and the likelihood of a subsequent successful pregnancy in women with unexplained RM condition regardless of the past unsuccessful pregnancies. They were offered a fast track to access to appointment for their cycle timed investigations by direct telephone contact with the two specialist nurses at the clinic.

1.3.2 Laboratory measures

Psychometric measures

The psychological stress status was defined as the scores of the validated questionnaires predesigned for this thesis.

Biochemical measures

The biochemical stress status was defined as the results of biochemical stress markers measured in both peripheral blood samples and endometrial or salivary samples, including natural killer cells, prolactin and cortisol.

2 Laboratory Equipments

2.1 Heating equipment

Heating blocks and waterbath were from Techne (Duxford, Cambridge, UK). A Panasonic microwave oven was also used for the heating antigen retrieval procedure in immunohistochemistry.

2.2 Light microscope

A standard light microscope (Zeiss, Germany) was used for microscopic observation of immunostaining. A haemometre was used for microscopic calculation of cells isolated from the blood samples for flow cytometry.

2.3 Measuring equipment

All amounts were weighed by using a Mettler PM 4000 balance (Mettler-Toledo Ltd, Leicester, UK). PH was measured with a Jenway pH metre3020 (Thermo orion, Beverly,

USA). Fluid volumes were dispensed with Eppendorfs pipettes with tips of P10, P100 and P1000 as appropriate.

2.4 Moist chamber

A plastic chamber with suitable platform inside was used to incubate sections with reagents for immunohistochemistry at the appropriate incubation temperature.

2.5 Storage equipment

A laboratory cold refrigerator at +4℃ (Lec) and a -20℃ freezer (Sparkfree Lab Freezer) were used for storage of agents and for overnight incubation of sections with antibodies for immunohistochemistry.

2.6 Centrifuge equipment

A Beckman GS-6R centrifuge machine was used for the centrifugation of samples.

2.7 Flow cytometry facility

A four-colour fluorescence capable flowcytometer (BD FACSCalibur^{TM} system, UK) was employed for the flow cytometry experiment.

Chapter IV: The impact of psychological stress on RM

I. Introduction

Definition of stress

Stress, namely pressure or tension, describes experiencing events which can produce psychological consequences (Smith et al., 2003, Atkinson and Hilgard, 1996). Stress occurs when the threats are beyond an individual's ability to cope. There are two types of stress, namely eustress and distress. Eustress which is positive and motivating enables individuals to fight or flee. For example, in a racing competition eustress exerts excitement and mobilizes the muscles to accomplish the competition. Distress which is negative and harmful alters emotional or physical homeostasis due to failure in coping with the threats (Selye, 1975, Carstens and Moberg, 2000). The events which can cause stress are referred to as stressors (Atkinson and Hilgard, 2000). There are two types of stressors, namely acute stressors which are short term events such as hungry and a car accident; and chronic stressors which are long term events such as poverty or physical health (Ogden, 2007).

The reactions triggered by stress are termed as stress responses (Atkinson and Hilgard, 1996). The psychological responses to stress include anxiety, anger and aggression, apathy and depression and cognitive impairment. The physiological reactions to stress can include increased metabolic rate, increased heart rate, dilations of pupils, high blood pressure, tensing of muscles, increased secretion of neural and endocrine hormones and elevated blood sugar (Atkinson and Hilgard, 2000). However, not only the stressful events determine the individual's stress responses but also the way how an individual perceives stress affects stress responses. So far several stress response models have been proposed by researchers. An

essential model of stress responses is the ‘fight-flight’ which was first described in 1932 by Walter Cannon. It describes the arousal and mobility of the body in response to stress stimulus via activation of the sympathetic nervous system and the endocrine system. This arousal may be protective by facilitating confrontation or flight from the threat. On the other hand, it may be harmful because it causes psychosomatic symptoms over time (Cannon, 1932).

Another widely recognized stress response model is ‘general adaptation syndrome’ which describes a series of physiological changes in stress responses. It consists of three phases. In the first phase of alarm, the body expends resources and lowers resistance to confront a threat by activating sympathetic nervous system. In the second phase of resistance, the body increases its resistance and actively confronts the threat by fleeing or fighting it. In the third phase of ‘exhaustion’ the body depletes its physical resources resulting in decompensation, leading to illness (Selye, 1975).

Lazarus and colleagues (1984) have proposed an ‘interaction’ theory which emphasizes the interaction between the individual and environment in stress responses. In this theory an individual’s internal factors such as attitudes, personality and lifestyle, and environmental factors interact with stressors, reducing or increasing a person’s susceptibility to stressors. Stress responses are associated with the coping methods adopted by the person and past experiences with the stressors (Lazarus and Folkman, 1984) . Therefore, stress is the consequence of a person’s appraisal process of the stressful events. Individuals may feel less stress when they feel their resources adequate to deal with the stressors whereas an individual may feel more stress when they feel resources inadequate (Ogden, 2007).

Difficulties in measuring stress

The cause of stress varies between individuals. Some events which are stressful and overwhelming to some individuals may be not to others. Individuals may deal with stressors differently based on their own experiences and resources. Therefore, stress measurements have been inherently difficult. Several confounding factors, such as gender, age, social status and education, can also affect stress measurements (Fortuny et al., 1988). To date a wide variety of methods have been employed to measure stress, including self-report questionnaires; behavioral measures, such as task performances under stress; physiological measures, such as heart rates and blood pressure; and biochemical measures, such as cortisol and catecholamines (Lovallo, 1997). However, each type of measurement has its own bias. For example, cortisol secretion is affected and interacts with a variety of neuro-endocrine factors other than those enhanced by stress (Tilbrook and Clarke, 2006). Self-report measures are subject to the variation of stress perception and responses between individuals (Suzumori and Sugiura-Ogasawara, 2010). Behavioral measurements are subject to multiple explanations. Performance decline may be due to decline in motivation, fatigue or cognitive strain (Pilcher et al., 2007). Therefore, bias may be produced in stress measurements by using a single stress measure. The possibility of obtaining a good measurement of stress may be increased by combining several measurements. In this thesis we combined both self-report questionnaire measurements and biochemical measurements to comprehensively assess stress in women with RM.

Stress and miscarriage

A long term arousal of the sympathetic or the neuro-immune-endocrine systems exposed to stress stimulation can increase a person's susceptibility to disease or illness (Ogden, 2007). This overarousal has a negative effect on arteries, immune and endocrine systems causing

health problems (Smith et al., 2003). It has been widely recognized that stress may play a role in some disease causation, such as asthma, stomach ulcer and cardiac diseases. Recently the relationship between stress and miscarriage has been paid more attention.

Stress following miscarriages

For many couples pregnancy and childbirth are desired outcomes of their relationship. However, approximately 15% to 20% of pregnancies result in spontaneous miscarriages (Darroch et al., 1999, Henshaw, 1998). Couples who suffer miscarriages often describe the experience as stressful. The symptoms of the psychological distress caused by miscarriages are often referred to as grief, mourning, guilt, anxiety, depression, coping, loss of self-esteem and frustration (Andalib et al., 2006, Kersting et al., 2004, Nikcevic et al., 1998, Nishikawa et al., 1991, Dingle et al., 2008). Although there is an increasing body of studies examining the occurrence of psychological distress following miscarriages, the incidence of psychological morbidity after miscarriages varies between studies (see table IV-1). The reason for this variation may be due to the lack of consensus on stress measurements used in current studies. Although questionnaires are the most widely used instruments to measure stress, there is no consensus on the psychometric measures used to assess fertility-related stress, which should be sought in the questionnaire based study. Different authors employed different questionnaires measuring different psycho-parameters in their studies. For example, some studies measured anxiety and depression as the principal symptoms of the fertility-related stress whereas others examined generic personality disorder and health concerns (Sham et al., 2010, Stallman et al., 2010, Lok et al., 2010, Obi et al., 2009, Dingle et al., 2008, Cumming et al., 2007). Therefore, the results obtained from different questionnaires varied significantly. Table IV-1 illustrates the variation in the instruments used to measure the impact of miscarriage on women's psychological wellbeing.

Table IV 1. Studies which have assessed psychological morbidity in women who have experienced miscarriages

Author	Assessment time	Sample size	Instruments	Items	Psychological morbidity
Sham et al (2010)	3 months after miscarriage	161	General Health Questionnaire (GHQ) The Structured Clinical Interview for DSM-IV Axis Disorder	12 ?	10% of depressive disorder 1.2% of anxiety disorder 0.6% of obsessive compulsive disorder 0.6% of posttraumatic disorder
Stallman et al (2010)	3 months post-miscarriage	117	The Kessler 10	10	81.2% of distress
Lok et al (2010)	Immediately after miscarriage 3 months after miscarriage 6 months after miscarriage 12 months after miscarriage	280	General Health Questionnaire (GHQ) Beck Depression Inventory (BDI)	12 21	55% scored high on GHQ and 26.8% high on BDI 25% high on GHQ and 18.4% on BDI 17.8% high on GHD and 16.4% high on BDI 10.8% high on GHD and 9.3% high on BDI
Obi et al (2009)	1~3 months after miscarriage	202	Zung Self-Rating Depression Scale	20	74.3% had minimal depressive symptoms 3% had moderate depressive symptoms 13.9% had sever depressive symptoms
Dingle et al (2008)	Previous miscarriages in lifetime	82 women at age 21	Composite International Diagnostic Interview computerised version 2.1	?	53.7% of anxiety and 43.2% for affective
Cumming et al (2007)	1 month after miscarriage 6months after miscarriage 13 months after miscarriage	400 women in the 1st assessment 317 women in 2nd assessment 275 women in 3rd assessment	The Hospital Anxiety and Depression Scales	14	28.3% for anxiety and 10% for depression in the first assessment 20% for anxiety and 4% for depression in the 2nd assessment 17% for anxiety and 2% for depression in the 3rd aseessment
Nishikawa et al., 1991	Within a period of 13 months of a miscarriage at 10 to 14 weeks of gestation	204 women	The Hospital Anxiety and Depression Scales The Expanded Texas Inventory of Grief	14 17	45% of anxiety and 15% of depression

? authors did not describe the numbers of items containing in the instruments

Stress causing miscarriages

Recently, there are an increasing number of studies exploring the effect of stress on miscarriage. Stress may activate the HPA axis affecting the establishment of pregnancy through the interaction of the psycho-neuro-endocrine-immune network. The physiological mechanism of stress effect on pregnancy has been discussed in chapter I.

In animal studies it has been shown that sound stress induces miscarriages in mice by up-regulating inflammatory factors, such as TNF-α (Knackstedt et al., 2003). In humans it has been reported that stress reduces the conception probability and high levels of stress are associated with an increased risk of miscarriage (Buck Louis et al., 2010, Ebbesen et al., 2009, Sugiura-Ogasawara et al., 2002, Andalib et al., 2006, Arck et al., 2001) . For example, Boivin et al (2005) conducted a prospective epidemiological study with a large cohort of 818 couples who underwent fertility treatments. They found that both men and women with higher levels of fertility stress were less likely to conceive than those with lower levels of stress. In addition, they found that this effect was more significantly pronounced in women than in men (Anthony et al., 2007). Arck et al (2008) also examined a large cohort of 1098 women and found that high stress levels were associated with an increased risk of subsequent miscarriages in the first trimester of pregnancy (Arck et al., 2008). Another large epidemiological study based on UK population investigated 603 women with a recent miscarriage in the first trimester compared with 6116 women without a recent miscarriage. They found that stress is a risk factor of miscarriage (Maconochie et al., 2007), confirming that stress plays a role in miscarriage.

However, in contrast to the above studies, there are some studies which found a negative association between stress and miscarriage. For example, a prospective study investigating 783 women found that anxiety and depression had no impact on pregnancy rates in IVF treatment (Lintsen et al., 2009). Studies also found that psychological stress had no effect of increasing the risk of miscarriage in a subsequent pregnancy (Nelson et al., 2003b, Nelson et al., 2003a). Furthermore, one study showed that high stress levels were associated with a better pregnancy outcome rather than miscarriage (Cooper et al., 2007).

The reason for the above conflicting findings may be due to the variation in the psychometric measures used to assess stress. To address this issue, we searched for the relevant articles on the association between stress and miscarriage in Pubmed, using a combination of the following search terms: stress and miscarriage, stress and pregnancy loss, emotion and miscarriage/pregnancy loss and psychology and miscarriage/pregnancy loss. Table IV-2 shows the psychological instruments employed in current studies to examine the relationship between stress and miscarriage.

Table IV 2. Studies on the association between stress and miscarriage

Authors	Study population	Controls	Psycho-parameters measured	Findings
Cooper et al., 2009	783 women in their first IVF or ICSI	No	Dutch version of the State Anxiety Inventory the Beck Depression Index for primary care Dutch version of The State–Trait–Anxiety Inventory	Neither anxiety nor depression affected the ongoing pregnancy rates.
Arck et al., 2008	55 women with a subsequent miscarriage between 4 to 12 weeks of gestation	809 women with a progressing pregnancies beyond 20 weeks of gestation	PSQ short version The Quality of Life The Short Form 12 Depressive symptoms Social Support (SOZU)	Women who subsequently miscarried perceived higher levels of stress than those who had successful pregnancies.
Maconochie et al., 2007	603 women with a recent miscarriage in the first trimester	6116 women with a pregnancy beyond 12 weeks	Interviews on social demographic factors and behavioural factors	Feeling stressed is associated with a high risk of miscarriage
Cooper et al., 2007	129 couples undergoing their first IVF	No	Fertility Problem Inventory	Women who had a live birth (defined as >20 weeks) scored significantly higher in the negative view of a child-free lifestyle scale and the need for parenthood scale than those who miscarried. High stress is associated with a better pregnancy outcome rather than miscarriage.
Nelson et al., 2003	98 women with a spontaneous miscarriage	228 pregnant women	Perceived Stress Scale Prenatal Social Environment Inventory Index of Spousal Abuse	High levels of psychological stress are not associated with risks of miscarriage. The influence of stress on miscarriage is not clear.
Nelson et al., 2003	174 women experienced a spontaneous miscarriage	798 women with ongoing pregnancies >22 weeks	using the Center for Epidemiologic Studies Depression Scale the Life Oriented Test-Revised	There was no association between depression and optimism and miscarriage.
Arck et al., 2001	94 women with a miscarriage in the first trimester	No	German version of Perceived Stress Questionnaire WHO-Quality of Life Social Support (SOZU K22)	Women with miscarriage who had higher stress scores were associated with higher numbers of immunocompetent cells such as MCT+, CD8+ T cells and TNF α cells. Stress may modulate immunological factors which cause miscarriage

Stress and RM

Recurrent miscarriage (RM) is a particularly frustrating condition, causing pronounced psychological distress in couples who desire to have children. Studies have shown that apart from having grief, depression and low self-esteem symptoms, couples also reported changes in sexuality and communication difficulties after RM (Serrano and Lima, 2006). It has been found that approximately 32% of women also develop depression and anxiety following RM (Klock et al., 1997).

Although there are an increasing number of studies reporting on the impact of stress on miscarriage, few studies have been published examining the association between stress and RM. We searched on Pubmed for all the relevant articles using a combination of the following search terms: stress and recurrent miscarriage, stress and repeated pregnancy loss, emotion and recurrent miscarriage, and stress and habitual miscarriage. The articles related to the searched papers were also searched to avoid missing any relevant reference. Only six relevant studies were identified in Pubmed. However, among these six papers, two of them were written in German and one of them was written in French. Therefore, the remaining three studies were obtained. Table IV-3 illustrates the psychological measurements used in these three studies to assess stress in women with RM. Among these three studies, one study examined both the psychological and physiological risk factors of RM and found that psychological factors were less important than pathological abnormalities in the cause of RM. In their study pathological abnormalities showed a more significant association with miscarriages than psychological factors (Bergant et al., 1997). Another study critically examined the role of stress in RM combining both psychological measurements and biochemical measurements. They found that women with RM had higher stress scores than fertile women and high stress scores were associated with an elevated activity of peripheral

natural killer cells, suggesting that psychological stress may contribute to RM through the effect of modulating immunological functions (Andalib et al., 2006). This finding was supported by a prospective study which showed that depression was associated with a high risk of miscarriage in a subsequent pregnancy in women with RM, suggesting that stress is a contributory factor of RM (Sugiura-Ogasawara et al., 2002). However, due to the paucity of studies in this area, whether or not stress is a cause of RM is still not clear.

Table IV 3. The psychological measurements used in studies on the relationship between stress and RM

Authors	Study population	Control population	Psychological instruments	Findings
Andalib et al., 2006	45 women with three or more previous miscarriages	45 multi-parous women without a history of miscarriage	Life Change Units the Beck Depression Inventory	Women with RM had significant higher scores in depression scale than controls.
Bergant et al., 1997	36 women with more than two previous consecutive miscarriages	36 controls without RM and matched for age and occupation	The State–Trait–Anxiety Inventory The 'Giessener Beschwerdebogen' The Life Satisfaction Questionnaire The Beck Depression Inventory	No significant difference in the stress measurements was found between study population and controls. Psychological factors is less importance as a cause for RM.
Sugiura-Ogasawara et al., 2002	60 couples with more than two previous consecutive miscarriages	No	Symptom Checklist-90 Revised The NEO Five Factor Index Semi-structured Interviews	High scores in depression is associated with an increased risk of a subsequent miscarriage.

Although researchers have endeavored to study the relationship between stress and miscarriage, there have been few efforts engaged on developing a specific questionnaire to measure miscarriage-related stress. To date most studies examined miscarriage-related stress with a variety of standard psychological instruments which measured personality, anxiety, depression and health status, etc (see table IV-1). There was no standardized psychological instrument with the advantage of reliability, validity and sensitivity in measuring specific stress related with miscarriage.

Concerning this issue, a few studies have been conducted with the aim of developing a valid, sensitive and reliable questionnaire to measure stress related to fertility problems (Newton et al., 1999, Lok et al., 2004, Lee et al., 1997). Newton et al (1999) conducted a prospective study sampling 1,153 women and 1,149 men with infertility problems in order to develop a reliable instrument to assess infertility-related stress. They found that the Fertility Problem Inventory (FPI) was a reliable and sensitive self-report instrument which could identify an individual's global level of infertility-related stress (Newton et al., 1999).

46 items were selected to construct the FPI with good reliability and responsiveness. These questions typically addressed marital relationships, social relationships, sexual relationships, parenthood, childfree view, general quality of life, anxiety and depression, etc. This multidomain questionnaire comprised five relevant independent scales, measuring five psychometric domains of social concern, sexual concern, relationship concern, the need for parenthood and rejection of a childfree lifestyle. Furthermore, a comprehensive score summing across all five scales was used as a global measure of the level of perceived infertility-related stress. All five scales and the composite total scale showed high internal consistency (Social concern=0.87, Sexual concern=0.77, Relationship concern=0.82,

Rejection of childfree lifestyle=0.80, Need for parenthood=0.84 and Global stress=0.93), suggesting that each of the scales was composed of a relatively homogeneous set of items which yield producible and consistent results. The Test-retest reliability of FPI was 0.83 for women and 0.84 for men, indicating the greater reliability of responsiveness over time. The discriminant validity of FPI was 0.45 and the convergent validity of FPI was significant ($\rho<0.01$), suggesting that the questionnaire was measuring separate but related dimensions of fertility-related stress. Currently, the FPI inventory is widely used as a reliable instrument in the assessment of fertility-related stress. In the present study we employed FPI as the principal psychometric measure to assess the stress status in women with unexplained RM.

Although there is an increasing body of studies on the relationship between stress and miscarriage, few efforts have been made to examine the association between stress and RM. Due to the paucity of studies in this area it is still not clear whether stress is a consequence of RM or a cause of RM. Therefore, we conducted this prospective study in a cohort of women with unexplained RM. The aim of the present study was to i) examine the role of stress in RM by comparing the stress levels between women with unexplained RM and fertile controls; ii) examine the effect of Tender Loving Care on stress reduction by comparing the change of stress levels before and after clinical care; iii) examine the prognostic value of stress on the subsequent conception and pregnancy outcome.

II. Materials and Methods

1 Subjects

A total of 36 women with unexplained RM (case group) and 30 female fertile volunteers (control group) were included in this study. They all met the inclusion criteria of the study. The case group was assessed with a questionnaire package on two occasions during the study period in accordance with the study protocol. The first assessment was measured at the time of initial recruitment (baseline assessment). After the clinical consultation, explanation and reassurance by a team of dedicated staff, the participants were assessed again with the identical questionnaire package at a follow-up visit (follow-up assessment) prior to conception. The control group was assessed with the same questionnaire package only once at the time of recruitment.

2 Methods

A comprehensive questionnaire package (Appendix VI) was used in this study to measure the psychological stress levels in the case group and control group. To prevent data invalidation associated with missing data, the investigator double checked the questionnaire answers on obtaining it from the participants to ensure that each of the questions was answered and the answer was interpretable. The pre-designed questionnaire package consisted of three validated individual instruments, including the Fertility Problem Inventory (FPI), the Perceived Stress Scale (PSS) and the Positive and Negative Affect Schedule (PANAS). The questions in these questionnaires were brief, clear, easily understood and easy to response. The validity, reliability and sensitivity of these instruments have been testified through a large body of research (Newton et al., 1999, Cohen et al., 1983, Watson, 1988). The copyright permissions were obtained from the authors and the authorized organizations (Appendix VII).

- **The Fertility Problem Inventory (FPI)**

FPI was developed by Newton et al to evaluate fertility problem stress. It had 46 items and comprised five constructs including Social Concern, Sexual Concern, Relationship Concern, Need for Parenthood, and Rejection of Childfree Lifestyle. Each of the five scales was composed of a relatively homogeneous set of items measuring the intended psychometric domain. A 6-point ordinal scale was referred in the scoring of FPI, categorizing the response from ‘1= strongly disagree’ to ‘6 = strongly agree’. The scale score was obtained from reversing the scores on some items and summing up each raw score of the multiple items in each scale. The composite measure of the global stress of FPI was also obtained by summing across all five scales. High scores in the psychometric domain of Social Concern were

interpreted as more sensitivity to reminders, comments and questions about infertility, feelings of alienation or isolation from peers and difficult in social activities. High scores in the domain of Sexual Concern were interpreted as loss of sexual enjoyment, feeling pressure to schedule sex and loss of sexual self-esteem. High scores in the scale of Relationship Concern were interpreted as having communication difficulties about infertility problems and future relationship concerns. High scores in the domain of Rejection of Childfree Lifestyle were interpreted as having negative view of childfree lifestyle or status. High scores in the scale of Need for Parenthood reflected a close identification with the role of parent. High scores of global stress reflected high levels of perceived infertility-related stress. The FPI has demonstrated a good reliability, sensitivity and validity (Newton et al., 1999).

- **The Perceived Stress Scale (PSS)**

PSS was developed by Cohen et al to evaluate the stressfulness of the situations in the past month of individuals' lives. It is an empirically established index of general stress appraisal and is a most widely used psychological instrument for measuring the perception of stress. It was a 10-item self report questionnaire and relatively free of content to any population group. The items were designed to reflect how unpredictable, uncontrollable and overloaded that the respondents found their lives (Cohen et al., 1983). A 4-point scale rating from '0= never' to '4= very often' was referred to rank the successive degrees of each item. The scale score was obtained from reversing the scores on some items and then summing the scores across all 10 items. High scores of PSS indicated a high level of stress.

- **The Positive and Negative Affect Schedule (PANAS)**

PANAS was developed by Watson et al to briefly measure independent mood factors of the positive and negative affects which related to personality states and traits. It consisted of 10

positive affects (interested, excited, strong, enthusiastic, proud, alert, inspired, determined, attentive and active) and 10 negative affects (distressed, upset, guilty, scared, hostile, irritable, ashamed, nervous, jittery and afraid) (Watson, 1988). PANAS measured the extent to which the respondents had experienced these different emotions and feelings within the past week. The reliability of PANAS instrument was 0.89, suggesting that it was composed of a relatively homogeneous set of items (Crawford and Henry, 2004). A 5-point scale was referred in the scoring of PANAS, rating from 1 to 5 increasingly. PANAS was scored by simply summing up the raw scores of each scale. Respondents with a sum-score above 25 in the Positive Affects scale were suggested to be more optimistic and those with scores above 25 in the Negative Affects scale were suggested to be more pessimistic.

III. Statistical Analysis

Statistical analyses were performed using the SPSS (Statistical Package for the Social Sciences) version 16. Differences in the scores of the stress measurements intergroup and intragroup were analyzed by independent *t* tests. The comparison of the scores of stress measurements between the two assessments were analyzed by paired *t* tests. The correlation between the scores of psychometric measurements and plasma prolactin concentrations was analyzed by Pearson correlation coefficient test. Statistical significance was set at *p* value <0.05.

V. Results

1. The demographic characteristics of the study population

A total of 36 women with unexplained RM were included in the study population. They all completed two questionnaires at the time of baseline assessment and the follow-up assessment respectively. The demographic characteristics of these 36 women are shown in table IV-4. No significant correlation between the psychometric measurements and the age or the number of previous miscarriages was found in these 36 cases ($\rho>0.05$) (table VI-5). There was also no significant association between plasma prolactin measurements and the results of psychometric parameters ($\rho>0.05$).

Table IV 4. Demographic characteristics of the 36 women with unexplained RM

Women with unexplained RM n = 36		
Age (years)		35.9 ± 4.6
No. of previous miscarriages (n) (median, range)		4 (3-8)
BMI (kg/cm^2)		25.8 ± 4.7
FSH (IU/l)		6.5 ± 2.3
LH (IU/l)		5.1 ± 2.2
Oestradiol (pmol/l)		174.3 ± 58.3
Progesterone (nmol/l)		40.6 ± 15.3
FAI		2.8 ± 2.0
Prolactin (mIU/l)		191.9 ± 121.7
Type of RM (n, %)	Primary RM	24 (66.67%)
	Secondary RM	12 (33.33%)
Family history of RM (n, %)	yes	8 (22.2%)
	no	28 (77.8%)

All values are shown in the form of mean ± SD unless otherwise specified.

Table IV 5. The correlation between psychometric measures and the demographics of the 36 cases

		Age (years)	No. of previous miscarriages
Infertility Problem Inventory global scores		$\rho = 0.95$	$\rho = 0.13$
Subscales	Relationship Concern	$\rho = 0.72$	$\rho = 0.07$
	Sexual Concern	$\rho = 0.65$	$\rho = 0.11$
	Need for Parenthood	$\rho = 0.71$	$\rho = 0.19$
	Rejection of Childfree Lifestyle	$\rho = 0.72$	$\rho = 0.69$
	Social Concern	$\rho = 0.85$	$\rho = 0.68$
Perceived Stress Scale		$\rho = 0.43$	$\rho = 0.47$
PANAS Positive Affect		$\rho = 0.61$	$\rho = 0.25$
PANAS Negative Affect		$\rho = 0.61$	$\rho = 0.25$

2. The differences in stress measurements between cases and controls

The scores of stress measurements of the 36 cases and 30 controls are shown in table IV-6. Cases had significantly higher global scores of the Fertility Problem Inventory than controls ($p<0.05$). The same pattern was shown in its subscales of Sexual Concern, Need for Parenthood, Rejection of Childfree Lifestyle and Social Concern ($p<0.05$). Cases also had significantly higher scores in the Perceived Stress Scale and the PANAS Negative Affect scale, and significantly lower scores in the PANAS Positive Affect scale than controls ($p<0.05$).

Table IV 6. Differences in the scores of psychometric measurements between cases and controls

		case group N=36	Control group N=30	P value
Age (years)		35.9 ± 4.6	34.0 ± 6.1	ρ >0.05
Infertility Problem Inventory global scores		162.0 ± 35.2	137.2 ± 35.2	ρ <0.05*
Subscales	Relationship Concern	28.4 ± 10.6	27.6 ± 9.1	ρ >0.05
	Sexual Concern	24.5 ± 8.8	19.0 ± 9.5	ρ <0.05*
	Need for Parenthood	43.6 ± 9.4	36.7 ± 7.5	ρ <0.05*
	Rejection of Childfree Lifestyle	31.1 ± 8.7	26.4 ± 7.1	ρ <0.05*
	Social Concern	34.5 ± 11.7	27.4 ± 10.3	ρ <0.05*
Perceived Stress Scale		21.2 ± 7.5	16.56 ± 4.2	ρ <0.05*
PANAS Positive Affect		29.4 ± 9.9	36.8 ± 5.3	ρ <0.05*
PANAS Negative Affect		25.1 ± 9.7	18.4 ± 5.0	ρ <0.05*

All the values are in the form of mean ± SD.

* significant

3. The association between stress and conception outcomes

Of the 36 cases, 26 of them subsequently conceived during the study period and 10 of them did not conceive. Table IV-7 shows the results of stress measurements of these two subgroups in the two assessments. There was no significant difference in the stress scores between the pregnant group and the non-pregnant group in the baseline assessment or in the follow-up assessment ($p>0.05$). No significant difference in the numbers of previous miscarriages was found between the pregnant group and non-pregnant group ($p>0.05$). Age was the only significant factor associated with conception outcomes. Women who conceived again were younger than those who did not, as we expected ($p<0.05$).

Table IV 7. Differences in the demographics and the scores of psychometric measurements between cases with different conception outcomes

		Pregnant group N=26	Non-pregnant group N=10	P value
Age (years) (mean ± SD, range)		34.9 ± 4.7 (34-42)	38.6 ± 2.9 (25-42)	ρ <0.05*
No. of previous miscarriages (median, range)		4.0 (3-6)	4.0 (3-8)	ρ >0.05
Baseline assessment				
Infertility Problem Inventory global scores		164.5 ± 37.6	155.4 ± 28.5	ρ >0.05
Subscales	Relationship Concern	29.0 ± 11.7	26.6 ± 7.2	ρ>0.05
	Sexual Concern	24.5 ± 9.2	24.4 ± 8.2	ρ >0.05
	Need for Parenthood	43.3 ± 8.4	44.1 ± 12.1	ρ >0.05
	Rejection of Childfree Lifestyle	31.3 ± 8.9	30.4 ± 8.5	ρ >0.05
	Social Concern	36.3 ± 10.5	29.9 ± 13.8	ρ >0.05
Perceived Stress Scale		21.8 ± 7.6	19.5 ± 7.2	ρ >0.05
PANAS Positive Affect		28.4 ± 9.8	31.8 ± 10.4	ρ >0.05
PANAS Negative Affect		25.8 ± 8.4	23.3 ± 12.9	ρ >0.05
Follow-up assessment				
Infertility Problem Inventory global scores		162.6 ± 18.0	161.5 ± 33.6	ρ >0.05
Subscales	Relationship Concern	30.4 ± 7.1	31.6 ± 5.6	ρ >0.05
	Sexual Concern	22.7 ± 5.1	26.0 ± 9.7	ρ >0.05
	Need for Parenthood	43.4 ± 5.8	41.9 ± 10.2	ρ >0.05
	Rejection of Childfree Lifestyle	31.4 ± 5.9	31.9 ± 7.3	ρ >0.05
	Social Concern	34.7 ± 5.6	30.1 ± 11.8	ρ >0.05
Perceived Stress Scale		19.8 ± 4.0	17.5 ± 5.1	ρ >0.05
PANAS Positive Affect		30.9 ± 5.8	32.2 ± 5.6	ρ >0.05
PANAS Negative Affect		24.8 ± 6.3	22.1 ± 9.5	ρ >0.05

All the values are shown in the form of mean ± SD unless otherwise specified.

*significant

4. The association between stress measurements and pregnancy outcomes

Among the 26 cases who subsequently conceived, 22 of them conceived spontaneously and 4 of them conceived through the assisted conception techniques. Of these 26 pregnancies, 18 (69.2%) of them were live births and 8 (30.8%) of them resulted in miscarriage again. The results of stress measurements of these two subgroups are shown in table IV-8. There was no significant difference in the age and the number of previous miscarriages between the two subgroups. The significant factors associated with pregnancy outcomes were the scores of the PANAS Positive Affect scale and the Perceived Stress Scale. In the baseline assessment the live birth group had significantly lower scores in the PANAS Positive Affect Scale and significantly higher scores in the Perceived Stress Scale than the miscarriage group ($p<0.05$). In the follow-up assessment the live birth group also had significantly lower scores in the PANAS Positive Affect Scale than the miscarriage group ($p<0.05$). No significant difference in the scores of the Fertility Problem Inventory was found between the two subgroups in the two assessments.

Table IV 8. A comparison of the results of stress measurements of 26 cases with different pregnancy outcomes

		Live birth group N=18	Miscarriage group N=8	P value
Age (years)		35.0 ± 4.6	34.5 ± 5.3	ρ > 0.05
No. of previous miscarriages (n) (median, range)		4.0 (3-6)	4.0 (3-8)	ρ > 0.05
Baseline assessment				
Infertility Problem Inventory global scores		166.6 ± 30.6	159.9 ± 52.4	ρ > 0.05
Subscales	Relationship concern	29.0 ± 10.6	29.1 ± 14.6	ρ > 0.05
	Sexual concern	24.3 ± 7.9	25.0 ± 12.3	ρ > 0.05
	Need for parenthood	44.1 ± 7.5	41.8 ± 10.6	ρ > 0.05
	Rejection of childfree lifestyle	31.5 ± 8.5	31.0 ± 10.5	ρ > 0.05
	Social concern	37.8 ± 8.0	33.0 ± 14.8	ρ > 0.05
Perceived Stress Scale		23.8 ± 6.1	17.5 ± 9.3	ρ < 0.05*
PANAS Positive Affect		25.0 ± 7.1	36.1 ± 11.1	ρ < 0.05*
PANAS Negative Affect		26.7 ± 7.2	24.0 ± 10.9	ρ > 0.05
Follow-up assessment				
Infertility Problem Inventory global scores		164.1 ± 16.6	159.2 ± 21.8	ρ > 0.05
subscales	Relationship concern	30.2 ± 7.3	30.9 ± 7.1	ρ > 0.05
	Sexual concern	24.3 ± 4.7	19.0 ± 4.4	ρ > 0.05
	Need for parenthood	43.5 ± 5.3	43.0 ± 7.0	ρ > 0.05
	Rejection of childfree life style	31.9 ± 6.2	30.5 ± 5.4	ρ > 0.05
	Social concern	34.1 ± 4.7	35.9 ± 7.5	ρ > 0.05
Perceived Stress Scale		19.5 ± 4.1	20.5 ± 3.8	ρ > 0.05
PANAS Positive Affect		29.0 ± 4.5	35.1 ± 6.6	ρ < 0.05*
PANAS Negative Affect		24.5 ± 6.4	25.5 ± 6.4	ρ > 0.05

All the values are shown in the form of mean ± SD unless otherwise specified.

*significant

5. The stress status of the 36 cases before and after 'tender loving care'

The results of the stress measurements of the 36 cases in the baseline assessment and in the follow-up assessment are shown in table IV-9. No significant difference in the stress scores of each psychometric parameter was found between the two assessments in this group of women. Table IV-10 shows the results of stress measurements at the baseline assessment and the follow-up assessment of the 36 cases with different conception outcomes. There was no significant difference in stress measurements of the pregnant group and the non-pregnant group between the two assessments.

Table IV-11 shows the results of stress measurements of the 26 cases with different pregnancy outcomes at the two assessments. In the live birth subgroup of 18 cases, there were significant differences in the scores of Perceived Stress scale and Social Concern scale ($p<0.05$) between the two assessments. The levels of perceived stress and social relationship concerns in live birth subgroup reduced significantly after receiving 'tender loving care' whereas no stress reduction was found in the miscarriage subgroup after receiving 'tender loving care'.

Table IV 9. The results of psychometric measurements of the 36 cases at the baseline assessment and the follow-up assessment

		Baseline stress assessment N=36	Follow-up stress assessment N=36	P value
Infertility Problem Inventory global scores		162.0 ± 35.1	162.3 ± 22.9	ρ >0.05
Subscales	Relationship concern	28.4 ± 10.6	30.8 ± 6.6	ρ >0.05
	Sexual concern	24.5 ± 8.8	23.6 ± 6.7	ρ >0.05
	Need for parenthood	43.6 ± 9.4	43.0 ± 7.1	ρ >0.05
	Rejection of childfree lifestyle	31.1 ± 8.7	31.5 ± 6.2	ρ >0.05
	Social concern	34.5 ± 11.7	33.4 ± 7.9	ρ >0.05
Perceived Stress Scale		21.2 ± 7.5	19.2 ± 4.4	ρ >0.05
PANAS Positive Affects		29.4 ± 10.0	31.2 ± 5.7	ρ >0.05
PANAS Negative Affects		25.1 ± 9.7	24.0 ± 7.2	ρ >0.05

All the values are shown in the form of mean ± SD.

Table IV 10. The results of stress measurements of the 36 cases with different conception outcomes at the baseline assessment and the follow-up assessment

		Pregnant group (n=26)			Non-pregnant group (n=10)		
		Baseline assessment	Follow-up assessment	P value	Baseline assessment	Follow-up assessment	P value
Infertility Problem Inventory global scores		164.5 ± 37.6	162.6 ± 18.0	ρ > 0.05	155.4 ± 28.5	161.5 ± 33.6	ρ > 0.05
Subscales	Relationship concern	29.0 ± 11.7	30.4 ± 7.1	ρ > 0.05	26.6 ± 7.2	31.6 ± 5.6	ρ > 0.05
	Sexual concern	24.5 ± 9.2	22.7 ± 5.1	ρ > 0.05	24.4 ± 8.2	26.0 ± 9.7	ρ > 0.05
	Need for parenthood	43.3 ± 8.4	43.4 ± 5.8	ρ > 0.05	44.1 ± 12.1	41.9 ± 10.2	ρ > 0.05
	Rejection of childfree lifestyle	31.3 ± 8.9	31.4 ± 5.9	ρ > 0.05	30.4 ± 8.5	31.9 ± 7.3	ρ > 0.05
	Social concern	36.3 ± 10.5	34.7 ± 5.6	ρ > 0.05	29.9 ± 13.8	30.1 ± 11.8	ρ > 0.05
Perceived Stress Scale		21.8 ± 7.6	19.8 ± 4.0	ρ > 0.05	19.5 ± 7.2	17.5 ± 5.1	ρ > 0.05
PANAS Positive Affect		28.4 ± 9.8	30.9 ± 5.8	ρ > 0.05	31.8 ± 10.4	32.2 ± 5.6	ρ > 0.05
PANAS Negative Affect		25.8 ± 8.4	24.8 ± 6.3	ρ > 0.05	23.3 ± 12.9	22.1 ± 9.5	ρ > 0.05

All the values are shown in the form of mean ± SD.

Table IV 11. The results of stress measurements of the 26 cases with different pregnancy outcomes at the baseline assessment and the follow-up assessment

		Live birth group (n=18)			Miscarriage group (n=8)		
		Baseline assessment	Follow-up assessment	P value	Baseline assessment	Follow-up assessment	P value
Infertility Problem Inventory global scores		166.6 ± 30.6	164.1 ± 16.6	ρ > 0.05	159.9 ± 52.4	159.2 ± 21.8	ρ > 0.05
Subscales	Relationship concern	29.0 ± 10.6	30.2 ± 7.3	ρ > 0.05	29.1± 14.6	30.9 ± 7.1	ρ > 0.05
	Sexual concern	24.3 ± 7.9	24.3 ± 4.7	ρ > 0.05	25.0 ± 12.3	19.0 ± 4.4	ρ > 0.05
	Need for parenthood	44.1 ± 7.5	43.5 ± 5.3	ρ > 0.05	41.8 ± 10.6	43.0 ± 7.0	ρ > 0.05
	Rejection of childfree lifestyle	31.5 ± 8.5	31.9 ± 6.2	ρ > 0.05	31.0 ± 10.5	30.5 ± 5.4	ρ > 0.05
	Social concern	37.8 ± 8.0	34.1 ± 4.7	ρ < 0.05*	33.0 ± 14.8	35.9 ± 7.5	ρ > 0.05
Perceived Stress Scale		23.8 ± 6.1	19.5 ± 4.1	ρ < 0.05*	17.5 ± 9.3	20.5 ± 3.8	ρ > 0.05
PANAS Positive Affect		25.0 ± 7.1	29.0 ± 4.5	ρ > 0.05	36.1 ± 11.1	35.1 ± 6.6	ρ > 0.05
PANAS Negative Affect		26.7 ± 7.2	24.5 ± 6.4	ρ > 0.05	24.0 ± 10.9	25.5 ± 6.4	ρ > 0.05

All the values are shown in the form of mean ± SD.

VI. Discussion

The relationship between stress and RM

Whether stress is a cause or a consequence of miscarriage has always been debated. Some studies have found evidence that stress levels in women with miscarriages are higher than women without miscarriages. In addition, high stress levels are found to be associated with a poor chance of conception and an increased risk of miscarriage, suggesting that stress is a causative factor of miscarriage (Buck Louis et al., 2010, Ebbesen et al., 2009). In this study we examined the stress status in women with unexplained RM in which other identifiable causes of RM had been excluded to allow stress as the factor affecting RM. We found that women with unexplained RM had higher levels of overall fertility-related stress and perceived stress and were more pessimistic than fertile women, supporting the notion that stress may be a contributory factor of RM.

The association between stress and conception rate

Although in this study we found high levels of stress in women with unexplained RM, it is still not clear whether high stress levels are a consequence of the previous miscarriages or are the causative factor of future adverse pregnancies. Therefore, we further examined the impact of stress on subsequent conception rates and pregnancy outcomes in this group of women. In this study we found no significant difference in stress levels between pregnant cases and non-pregnant cases, suggesting that stress has no impact on subsequent conception rates.

Our finding is in contrast with previous studies in which stress has been found to be associated with a poor chance of conception (Buck Louis et al., 2010, Klonoff-Cohen et al.,

2001, Ebbesen et al., 2009). However, there are also some studies which have found a negative association between stress and conception chances. For example, one study examined a large cohort of 783 women undergoing IVF treatment using the State Anxiety Inventory and the Beck Depression Inventory. No significant difference in stress levels were found between women who conceived following IVF and those who did not, suggesting that psychological stress had no impact on pregnancy (Lintsen et al., 2009). Another study also found no effect of stress on subsequent conception performance in IVF treatment (Anderheim et al., 2005). Our finding is consistent with the findings from the above studies, supporting the notion that stress has no influence on conception rates. In our study only age was found to be the significant factor associated with conception rates. Younger women with RM had a better conception chance than older women, as we expected. Studies have also shown the rapid decline in the oocyte quality in IVF treatment and the decrease in conception rates in women older than 35 years (Ledger, 2009, Zhang et al., 2008), suggesting that fertility declines with advancing age.

The association between stress and pregnancy outcome

In the subgroup analysis on the association between stress and pregnancy outcomes, we found that cases with subsequent live births had higher stress scores in the Perceived Stress Scale and the Social Relationship Scale of the FPI than fertile women. Cases with live births also had significantly lower scores in the PANAS Positive scale than controls, suggesting that women with unexplained RM who are comparatively more stressed and less optimistic are more likely to have a better pregnancy outcome.

This finding was unexpected and is contradictory to previous research where a positive association between stress and miscarriage was found (Klonoff-Cohen et al., 2001).

However, there are some studies which found a reverse association between stress and pregnancy outcomes. For example, one study investigated 129 couples in IVF treatment using the Fertility Problem Inventory (FPI). They found that both young and old couples with higher stress scores in Social Concern and Need for Parenthood scales subsequently had a higher pregnancy rate than those with lower stress scores. With regard to the rate of ongoing pregnancy (defined as a live birth at > 20 weeks of gestation), the same pattern was shown that couples with higher scores in the Fertility Problem Inventory had significantly higher ongoing pregnancy rates (Cooper et al., 2007), suggesting that moderate stress may be beneficial to successful pregnancy establishment according to the Yerkes-Dondson theory of moderate stress resulting in optimal performance (Yerkes and Dodson, 1908).

In this study we also found that high stress levels were more likely to be associated with a better pregnancy outcome in women with RM. The discrepancies of the current findings on the impact of stress on pregnancy outcomes may be attributed to several factors. Firstly, apart from lack of consensus on the psychological instruments used to measure stress, the type of stress assessed is an important confounding factor in stress measurements in women with RM. The nonspecific questionnaires used to predict pregnancy outcomes in current studies may cause bias. Some studies measured anxiety, relationship concerns or depression to examine the impact of stress on pregnancy whereas other studies measured personality, mood, generic health status or perceived stress. It is argued that the questionnaires used in the current studies may be inadequate to capture specific fertility stress. Studies have shown that general psychological characteristics, such as active and passive coping, personality characteristics, dependency, self-criticism and intrusiveness, are more important in measuring the variability of psychological distress in women with fertility problems than infertility specific concerns (Van den Broeck et al., 2010). It has been proposed that measuring

pregnancy specific anxiety of worries or fears about pregnancy, childbirth, the health of the infant and future parenting, are more predictive on pregnancy outcomes than measuring general anxiety or depression (Huizink et al., 2004). It has also been suggested that stress coping strategies adapted by individuals may be a relevant factor in predicting pregnancy outcomes rather than stress measurements (Panagopoulou et al., 2006). The fact that a significant reduction in stress levels prior to the conception in the subgroup of cases with subsequent live births suggests that the coping strategy may have an effect on pregnancy outcomes.

Secondly, a limitation of this study is that stress was measured at the time of initial recruitment and the follow-up time point before the subsequent pregnancies. The interval between the last stress assessment and a subsequent pregnancy varies from 1 month to 6 months between participants. The stress status of these participants may change during this interval and thus may not truly reflect the pre-pregnant stress status. Therefore, stress measurements of non-conceptional cycle may be of limited value on predicting subsequent pregnancy outcomes. However, it is hardly practical to measure stress in each reproductive cycle in a clinical research in order to identify the exact pre-pregnant stress.

Thirdly, the short-term nature of the study follow-up may prevent us from identifying the significance of the adverse effect of stress on pregnancy outcomes. In this study only pregnancies achieved in a short period of 6 months were taken into account in the study analysis. The pregnancies achieved beyond this period were not followed therefore pregnancies with poor or good prognosis may be over presented in this study. In a future study this bias should be avoided by following a longer period of fertility outcome of at least 12 months.

The effect of ‘tender loving care’ on stress reduction

In this study we hypothesized that ‘tender loving care’ in the form of clinical consultation, explanation and reassurance by the dedicated staff may help to reduce stress levels. However, we did not find an obvious effect of this type of tender loving care on stress reduction in women with RM. There was no significant stress reduction before and after the tender loving care in the whole study population of 36 women with RM. However, in the subgroup of 18 cases with subsequent live births there was a small but significant stress reduction in the perceived stress scale and social concern scale, suggesting that this subgroup of women may respond to tender loving care better, which leads to their live births. This finding supports the notion that the stress coping strategies adapted by individuals are an important factor in predicting pregnancy outcomes rather than stress evaluation.

VII. Conclusion

In this study, we found that women with RM had higher stress levels than fertile women, suggesting that high stress levels are associated with RM. The intensity of stress has no predictive value on subsequent conception rates. However, RM women who conceived again and who have relatively higher stress levels are less likely to miscarry. It is possible that RM women with more stress and concerns may adopt coping strategies or reduce their activities, leading to a better pregnancy outcome.

Chapter V: The relationship between natural killer cells and RM

I. Introduction

1 Morphology and phenotype of natural killer cells

Natural killer (NK) cells are a subset of non-B, non-T peripheral lymphocytes regulating the innate immune response (Moretta et al., 1994). NK cells which comprise up to 15% of peripheral lymphocytes are the large granular lymphocytes expressing antigen receptors capable of recognizing and binding target cells. NK cells can mediate cell contact-dependent cytolysis of target cells which can express foreign major histocompatibility complex (MHC) molecules. This action can be exerted without prior sensitization and restriction by human leukocyte antigen (HLA) (Robertson and Ritz, 1990). The unique cell surface antigens of NK cells are CD56 and CD16. CD56 is expressed essentially on all NK cells and a small portion of cytotoxic T lymphocytes; CD16 is the receptor responsible for NK-mediated antibody-dependent cellular cytotoxicity. There are two subsets of NK cells based on the intensity of CD56 expression. They are $CD56^{dim}$ with low expression of CD56 and high expression of CD16 (CD56+16+); and $CD56^{bright}$ with high expression of CD56 and low or no expression of CD16 (CD56+16-) (Cooper et al., 2001b).

2 The role of peripheral NK cells in RM

In peripheral blood, $CD56^{dim}$ NK cells are predominant and more cytotoxic whereas $CD56^{bright}$ cells which compose a small portion of total peripheral NK (pNK) cells are the main source of NK released immunoregulatory cytokines (Cooper et al., 2001a, Nagler et al., 1989). Studies have shown that the pre-pregnant stress levels may elevate the cytotoxity of

peripheral NK cells in women with RM (Andalib et al., 2006). It has also been found that increases of pNK cells in numbers and activities are associated with RM. Table V-1 illustrates the current studies on the relationship between the NK cell alteration and RM. RM patients with high pNK activities have been found to have a significantly higher miscarriage rate in their subsequent pregnancies (Matsubayashi et al., 2001, Aoki et al., 1995). It has been demonstrated that women with RM and implantation failures have a significantly higher ratio of $CD56^{bright}$ pNK cells expressing TNFα (tumour necrosis factor-α)/GM-CSF (granulocyte-macrophage colony-stimulating factor) than fertile controls (Fukui et al., 2008). A longitudinal study comparing the pre- and post-conceptional profile of NK cells between women with RM and fertile controls, suggested that in preconception the number and cytotoxicity of peripheral CD56+/CD16+ NK cells were similar between women with RM and fertile controls. However, during early pregnancy, women with RM expressed a higher number of CD56+/CD16+ NK cells compared to fertile controls, paralleled with an increase in cytotoxicity of NK cells (Emmer et al., 2000), suggesting that the increase in the subset of CDdim pNK cells may be the cause of RM.

However, to date it is disputed whether pNK alteration in RM subjects is due to the effect of RM or merely a sympathetic response to venipuncture stress. Significant increases in pNK number and activity of women with RM have been found immediately after peripheral venous cannulation, compared with fertile controls. However, 20 minutes after cannulation there was no difference in the pNK measurement between the two groups (Shakhar et al., 2006), suggesting that high sympathetic responsiveness to venipuncture stress in RM subjects may be responsible to the pNK alteration.

Table V 1. Studies on the relationship between pNK cells and RM

Authors	RM Criteria	Study Group	Control Group	Diagnostic Definition	Results	Findings
King et al., 2010	3 or more consecutive previous miscarriage	104 non-pregnant RM subjects	33 fertile women	1. NK cells and the CD56dim subset were expressed as a percentage of lymphocytes as well as absolute number. 2. The activated CD69+CD56dim subset was expressed as a percentage of CD56dim cells as well as an absolute number	RM subjects had significantly higher NK percentage and lower CD56bright/CDdim ratio than controls.	The percentage of pNK increased in RM women, especially in women with unexplained RM.
Fukui et al., 2008	2 or more previous spontaneous miscarriages	25 women with RM	20 women with implantation failures 15 fertile healthy women	The expression of type-1 cytokines of TNFα, IFNγ, GM-CSF and IL-4,5,10 and 13 were measured to determine the cytokine shift in NK cells	NK cells with TNFα/GM-CFS expression or IFNγ expression were significantly higher in women with RM or implantation failures than fertile controls.	Increases in type 1 cytokine expression on NK cells are associated with RM.
Perricone et al., 2006	3 or more consecutive previous miscarriages	394 non-pregnant RM subjects; 187 pregnant RM subjects	42 fertile non-pregnant women	Did not mention	1. Non-pregnant RM subjects had higher absolute numbers of NK cells than non-pregnant fertile women 2. Pregnant RM subjects had significantly higher numbers of NK cells at first trimester than fertile pregnant women	1. Increased NK cells are associated with pregnancy loss. 2. NK levels should always be evaluated in women with RM
Emmer et al., 2000	2 or more previous unexplained miscarriages	43 women with RM	37 fertile women	The percentage of NK cell cytotoxicity was determined as: % lysis= (sample value – spontaneous release)/ (maximum release-spontaneous release)	1. Before pregnancy, levels of NK cytotoxicity and numbers of both single CD56+ and CD56+/CD16+ were similar between RM women and controls 2. Women with RM had higher number of CD56+CD16+ paralleled with the increases in cytotoxicity during early pregnancy than controls. However, single CD56+ numbers decreased in pregnant women with RM. 3. Between RM group, women with NK cells <12% had a positive association with a subsequent live birth.	1. Analysis of pNK cell profile is of diagnostic value in RM. 2. NK cell cytotoxity appears promising in RM diagnosis.
Michou et al., 2003	2 or more consecutive previous miscarriages	25 women with RM; 30 women with sporadic previous miscarriages; 33 infertile women	11 fertile women	1. Percentage of CD56+/CD16- in the total peripheral blood NK cell is given by the measurement of the CD3- cells (CD3- are estimated by gating on the CD56PE/CD16FITC primary lymphocyte analysis distribution). 2. Percentage of uNK cells was counted as a fraction of the total NK cell population	1. Study group had significantly higher CD56+/CD16- fraction than fertile controls. 2. Study group had significantly higher concentration of CD56+/CD16- cells than fertile controls. 3. Women with sporadic miscarriage and infertile women had significantly higher NK cell number than fertile controls.	The fraction of the concentration of CD56+/CD16- cells in total NK cells could be used as an indicator of subsequent successful pregnancy.
Yamada et al., 2003	2 or more previous miscarriages	85 women with RM	no	Did not mention	CD56+ NK cell activities and numbers in RM subjects following a subsequent miscarriage (n=26) were higher than those with live birth (n=59).	High activity and number of pre-conceptional CD56+ NK cells were predictive of spontaneous miscarriage
Aoki et al., 1995	2 previous unexplained miscarriages	68 women with RM	47 fertile women	High level of NK activity was defined as the mean + 1SD of NK activity in controls	1. Women with RM had higher NK activity (39.4%) than controls (29.0%) 2. Women with high level of NK activity had a much higher miscarriage rate (71%) than those with normal NK activity (20%)	Raised NK cell activity may be the cause of miscarriage and may be used as a predictor of pregnancy loss.

3 **The role of uterine NK cells in RM**

Recently, the importance of the uterine in situ environment in RM has been paid more attention. Endometrial leukocytes constitute about 10% of stromal cells in the proliferative phase and increase to 20% in the secretory phase; in early pregnancy, they accumulate consistently to 30-40% in decidua and implantation sites (King et al., 1991). NK cells dominate the endometrial leukocyte population and are referred to as uterine NK (uNK) cells (Bulmer et al., 1991). The number of uNK cells changes during the menstrual cycle. They are few in the proliferative phase and increase significantly in the secretory phase (Trundley and Moffett, 2004). In the first trimester of normal pregnancy up to 70% of decidual lymphocytes are NK cell, namely decidual NK (dNK) cells (Moffett-King, 2002). uNK cells accumulate around spiral arteries and endometrial glands in the late secretory phase of endometrium and early pregnancy decidua. The time and the site of uNK expression suggest that uNK may play an important role in immunoregulation and angiogenesis in early pregnancy (Quenby et al., 2009). Yet to date the precise mechanism of uNK function is not clear.

The CD56+ population constitutes mainly uNK and dNK cells(Moffett et al., 2004). It is suggested that uNK may derive from blood $CD56^{dim}$ population, formed from stem cell precursors in bone marrow and migrate to endometrium where they proliferate and differentiate in situ (Chantakru et al., 2002). Table V-2 shows the phenotypic differences between pNK and uNK cells (Dosiou and Giudice, 2005).

Table V 2. Expression of surface antigens on NK cells

Antigen	Peripheral NK ($CD56^{dim}$)	uNK ($CD56^{bright}$)
CD2 (early T cell marker)	+	+
CD7 (early T cell marker)	+	+
CD18 (integrin)	+	+
CD16 (NK marker)	+	-
CD45 (hematopoietic cell)	+	+
CD56 (NK marker)	+	+
CD57 (NK marker)	+	-
CD62L (adhesion molecule)	+/-	-
CD69 (activation marker)	-	+
KIR (NK marker)	+	+
c-kit (cytokine receptor)	-	+
IL-2Rβ (cytokine receptor)	+	+

This table was adapted from ref. (Dosiou and Giudice, 2005)

The majority of uNK cells are $CD56^{bright}$ CD16- NK cells with high affinity of IL-2 receptors (Rai et al., 2005, Nishikawa et al., 1991). uNK cells also express CD38 and CD69 phenotypically, but lack expression of CD3, CD4, CD8 and CD57. Among these uNK phenotypes, CD56+ and CD38+ cells increase from nonpregnant state of endometrium up to 75% in the first trimester decidua (Moffett-King, 2002, Bulmer et al., 1991). dNK cells also express higher intensities of CD56 markers (Koopman et al., 2003, Hill et al., 1995). Double immunohistochemical labelling for decidual CD56+ and CD38+ completely overlaps (Bulmer et al., 1991), suggesting that CD56+ is the predominant NK cell subset of decidua.

3.1 uNK cells in normal pregnancy

The role of uNK in normal pregnancy is uncertain. A review concluded that there are several possible functions of uNK, including the regulation of placental and trophoblast growth, implantation, decidual vascularisation via mediating cytokines (GM-CSF, CSF-1) and the involvement of trophoblast invasion (Dosiou and Giudice, 2005). It has been demonstrated that in nonpregnant endometrium, uNK cells are closely located to stromal cells around glands and blood vessels (Quenby et al., 2008). During pregnancy, they are in close proximity to the implantation site, close to the infiltrating extravillous trophoblast (Slukvin et al., 2004). The accumulation of uNK cells around implantation site during early normal pregnancy suggests that they contribute to the establishment of pregnancy (Cooper et al., 2001a). However the exact mechanism by which semi-allogenic trophoblasts can escape lysis from accumulating uNK cells in implantation site is uncertain. It has been suggested that uNK cells may participate in local immuno-suppression at the maternal-fetal interface through the productin of immunomodulatory cytokines during implantation (Koopman et al., 2003). uNK CD56 cells may also produce interferonγ in early pregnancy remodelling

decidual transformation and spiral artery modification, thus affect pregnancy in this way (Ashkar and Croy, 2001). The angiogenesis effect of uNK has been donmenstrated in a study which found that uNK immunostaining density was positively correlated with the formation of vessels, spiral arteriole smooth muscle differentiation and endometrial oedema; and uNK cell measurements were associated with a reduced uterine artery resistance to blood flow in women with RM or recurrent implantation failure (RIF), suggesting that uNK may contribute to miscarriage due to increased angiogenesis and blood flow during implantation leading to excessive oxidative stress (Quenby et al., 2009).

3.2 uNK cells in RM

An increasing number of studies have shown that uNK cells increase in women with RM compared with fertile women (Tuckerman et al., 2007, Clifford et al., 1999) and the number of previous miscarriages of RM is associated with uNK increases (Tuckerman et al., 2004), suggesting a contributory role of uNK in RM. It has been proposed that the imbalanced expression of uNK phenotype with a decrease of CD56+CD16- and an increase of CD56+CD16+ may be responsible for RM (Lachapelle et al., 1996). A study investigated the immunophenotypic profile of uNK cells in women with RM comparing with fertile women using a panel of 14 monoclonal antibodies to immunostain the luteal phase endometrium. They found that women with RM had significantly higher expression of CD56+, CD16+, CD4+ and CD14+ phenotypes than fertile women. Further comparison of the difference of uNK phenotype between RM subjects with different subsequent pregnancy outcomes showed that RM women with subsequent miscarriages had higher expression of CD56+, CD16+ CD4+, CD8+ and CD14+ than those with subsequent live births (Quenby et al., 1999). This suggests that high expression of CD56+CD16+ uNK subset may play an important role in RM.

However, in contrast to the above findings, there are some studies which have not found an increase of uNK in RM subjects (Shimada et al., 2004, Michimata et al., 2002). There are several possible reasons for these conflicting results. Firstly, the sampling of study population may be not consistent between studies. Some researchers have strictly included women with three or more miscarriages into the study group (Tuckerman et al., 2007, Clifford et al., 1999, Quenby et al., 1999) whereas others have also included women with two previous miscarriages (see table V3). Secondly, there is no consensus on uNK counting or the normal

reference range of uNK in current studies. Some authors have defined the mean number of uNK cells as a percentage of CD45+ cells (Michimata et al., 2002, Clifford et al., 1999) whereas others have counted the number of uNK cells as a percentage of total stromal cells (Tuckerman et al., 2007, Quenby et al., 1999) (see table V3). Finally, the normal variation of the number of uNK cells is unclear due to the paucity of epidemiological studies on the presence of uNK cell in normal fertile women. So far there is no consensus on the reference range of uNK used between centers. Therefore, the results from different studies yield conflicting findings.

The aim of this study was i) to examine the role of NK cells in RM by measuring both peripheral and endometrial NK cells in women with unexplained RM; ii) to examine the prognostic value of NK cell measurements on a subsequent pregnancy outcome in women with RM by correlating NK cell measurements with pregnancy outcomes; iii) to examine if NK cell measurements in peripheral blood can reflect NK cell changes in endometrium by comparing the result of pNK cell measurements with that of uNK cell measurements

Table V 3. A summary of studies on the relationship between uNK and RM

uthors	RM criteria	Study Group	Control Group	Method	uNk Counting	Results	Findings
Lachapelle et al., 1996	≥3 previous miscarriages	20 women with RM	15 fertile women	Flow cytometry	CD56+ cells was counted as the percentage of total CD45+ cells	1.uNK CD16-CD56+ subset decreased in RM subjects compared with controls 2.uNK CD16+CD56+ increased in RM subjects compared with controls	uNK cell subset of CDdim plays an immunological role in RM
Clifford et at., 1999	≥3 previous miscarriages	29 women with RM	10 fertile women	IHC	The number of positively stained cells in 10 non-overlapping high-powered fields was counted	Women with RM had significantly higher uNK CD56+ than controls	Increases in uNK cells is the possible pathogenesis of RM
Quenby et at., 1999	≥3 previous miscarriages	22 women with RM	9 women for laparoscopic sterilization	IHC	uNKCD56+,CD16,CD14,CD 8,CD4 were counted as a percentage of total stromal cells	1.uNK CD56CD16CD14CD4 was significantly higher in women with RM than controls 2.RM women who subsequently miscarried had significantly higher uNK CD56CD16CD8CD4 than those had live birth or controls	Women with RM have a different population of leucocyte in the pre-implantation endometrium compared with fertile controls
Quack et al., 2001	≥3 previous miscarriages	38 women with RM	20 women with elective termination of pregnancy	IHC	CD56+ cells were counted in the ratio of CD56+ cells to CD45+ cells	1. CD56+ NK cells decreased in women with RM compared with controls 2. Unexplained RM subjects with normal male karyotype had significantly more activated leukocytes than those unexplained RM subjects with a trisomy 16 (n=21) and controls	Cellular immunity with increased number of leukocytes may be involved in RM
Michimata et al., 2002	≥2 previous miscarriages	17 women with RM	15 women with solely male infertility	IHC	uNK CD56+ , CD16+, CD3+, CD8+ cells were counted as a percentage of CD45+ cells	1. No significant difference of uNK CD56, CD16, CD45 and CD3, CD8 was found between women with RM and controls. 2. No significant difference in numbers or ratios of lymphocyte subsets were found between cases with subsequent miscarriage and those with live birth	uNK cells have no predictive value for subsequent pregnancy outcomes in women with RM
Quenby et al., 2005	≥3 previous miscarriages	85 women with unexplained RM	18 women for sterilization	IHC	uNK CD56+ cells were counted as a percentage of total stromal cells	1. Women with RM had significantly more uNK cells than controls 2. Prednisolone significantly reduced the number of uNK cells from 14% to 9%.	1. The presence of uNK cells is high in peri-implantation endometriumof women with RM. 2. Prednisolone has a therapeutic effect in reducing high uNK cells in RM
Tuckerman et al., 2007	≥3 previous miscarriages	87 women with RM	10 women with 7 of them with proven fertility	IHC	uNK CD56+ cells were counted as a percentage of total stromal cells	1. The number of uNK cells was significantly higher than that in controls. 2. No significant difference in uNK numbers was found between RM women who subsequently miscarried and those who had live births. 3. No correlation was found between the presence of uNK cells and the numbers of previous miscarriages.	uNK has the diagnostic value in RM but has no prognostic value on a subsequent pregnancy outcome

II. Materials and Methods

1 Subjects

Cases

A total of 36 women with unexplained RM were recruited in this study. Of these 36 recruits, two patients declined endometrial biopsy procedures on their consent; we failed to obtain sufficient endometrial tissue in one patient and later she withdrew from the study following her referral to the assistant conception unit (ACU) for further fertility treatment; two patients neglected to use contraception in the biopsy cycle and later declined the study due to time pressure; two patients were not accessible to any contact means during the biopsy cycle and later were withdrawn from this study.

The remaining 29 patients underwent an endometrial biopsy for uterine NK cell measurements and two blood tests for peripheral NK cell measurements on two occasions in correspondence with the study protocol. Among these 29 patients, the blood sample of one patient was incorrectly stored in a tube without heparin and another blood sample was spoiled due to unexpected disruption of the electricity supply during the centrifugation procedure for cell isolation. These two patients were withdrawn from the peripheral NK cell measurements. Therefore, a total of 29 patients were included in uNK cell measurements and 27 of them were included in pNK cell measurements

Controls

A total of 30 fertile female volunteers were recruited in this study. Among them, 21 of them agreed to give blood samples for pNK cell measurements in the mid-luteal phase of the cycle. Therefore, these 21 fertile women were used as controls for pNK cell measurements in this study.

2 Methods

2.1 Procedure

a Endometrial biopsy

A LH-timed endometrial biopsy during the peri-implantation window was conducted to measure natural killer cells in endometrium. Participants were required to test ovulation with dip sticks from an ovulation test kit (Clearblue, UK) provided by the researcher from day 7 of the menstrual cycle onwards to precisely indentify LH surge. They were required to contact Dr Wei Li once positive ovulation test was detected. An appointment for LH-timed biopsy was then arranged on Ward G1 in the mid-luteal phase between day 7 and day 9 after ovulation. An endometrium biopsy was performed as an outpatient procedure without prior dilatation of the cervix or any form of analgesia. The procedure was operated with a pipelle sampler (Prodimed, France) induced through the cervical canal and the endometrium tissue was obtained by creating a negative suction pressure. The samples were then fixed in formalin and wax embedded in the Histology Department for future immunohistochemistry. Slides were cut for research and sent to the laboratory in the Academic Unit of Reproductive and Developmental Medicine.

b. Peripheral blood tests

The first blood test for pNK cell measurements was conducted concurrently with the endometrial biopsy in accordance with the research protocol. Another blood test for pNK cell measurements was conducted at a follow-up clinical attendance prior to conception. A 5ml blood sample was obtained in a green top tube with heparin (EDTA tube) from the patients in each assessment. The samples were conveyed immediately to the laboratory in the Academic Unit of Reproductive and

Developmental Medicine. All samples were processed within 4 hours after sampling for the flow cytometry experiment.

2.2 Flow cytometry for pNK cell measurements

a. The principle of flow cytometry

Standard flow cytometry techniques were used to determine the expression of pNK cells within plasma samples from women with unexplained RM and fertile controls. The protocol was optimized to determine the best conditions for maximal fluorescence labelling with the target cells. The phenotypes of CD56 and CD16 were detected as the surface markers to peripheral NK cells in flow cytometry with four-colour fluorescence capable FACSCalibur^{TM} system (BD, Oxford, UK). Mouse anti-human CD56 and CD16 antibodies were used for fluorescent labelling NK cells. Because T cells and platelets, which have the same density with NK cells, could not be eliminated from the isolation solution of NK cells from the plasma, mouse anti-human CD3 antibody which specifically conjugates with T cells; and CD45 antibody which conjugates with leucocytes but not with platelets were used to exclude these two types of cells. Only cells with positive of CD45+CD56+CD16+/- expression and negative of CD3 expression were identified as NK cells in this study. The results of pNK cell measurements of controls were used as the reference for determination.

Flow cytometry measures and then analyzes the property of each individual cell from 0.2 to 150 micrometres as they flow in a fluid steam through a beam of laser light of a single wavelength. The single cells either scatter or absorb and then re-emit (fluorescence) the light when they pass through the light beam one in a line. The light signals emitted by the particles are detected by a number of detectors: one in line with the light beam (Forward Scatter or FSC) and several perpendicular to it (Side Scatter or SSC). FSC correlates with the cell volume and SSC correlates

with the inner complexity of the particle (i.e., shape of the nucleus, the amount and type of cytoplasmic granules or the membrane roughness). This combination of scattered and fluorescent light is collected by the detectors and various types of information about the properties of each individual cell are generated. The properties of the cells measured include the size of the particles, relative granularity or internal complexity, and relative fluorescent intensity.

b. Flow cytometry technique

A 5ml blood sample was obtained in a tube with heparin (EDTA tube) and was diluted with equal volume of PBS. 10ml of the diluted sample was then layered over 5ml of Lymphoprep (1.077) gently and centrifuged at 600g for 30minutes without brake. After centrifugation, the mononuclear cells which banded at the interface were removed by pipette. Mononuclear cells were then washed in 10ml of PBS and centrifuge at 500-600g for 5 minutes with brake setting up at rate 3. The suspension was decanted and the cells were re-suspended with 10ml PBS and centrifuged for 5 minutes again twice. Then the suspension was decanted and the cells were re-suspended with 1ml PBS. Cells were counted on a haemocytometer and $1X10^6$ cells were transferred into each microtube. 20ul of appropriate antibodies or isotypes were added into each tube as appropriate. The agents applied were as follows:

Tube-a: sample only (unstained control)

Tube-b: sample + CD56 antibody (APC Mouse Anti-Human CD56, BD Pharmingen^{TM})

Tube-c: sample + CD 16 antibody (PE Mouse Anti-Human CD16, BD Pharmingen^{TM})

Tube-d: sample + CD 45 antibody (FITC Mouse Anti-Human CD45, BD Pharmingen^{TM})

Tube-e: sample + CD 3 antibody (PreCP Mouse Anti-Human CD3, BD Pharmingen^{TM})

Tube-f: sample + all antibodies (CD56 antibody, CD16 antibody, CD45 antibody and CD3 antibody, BD Pharmingen^{TM})

Tube-g: sample + all isotype controls (CD56 IgG1 k, CD16 IgG1 k, CD3 IgG1 k and CD45 IgG1 k, BD Pharmingen™)

After applying reagents, the samples were incubated on ice in the dark for 30 minutes. After incubation 0.5ml of flow buffer was added into each tube and the cells were washed twice by centrifugation for 5 minutes at 500g. Then the cells were re-suspended in 0.5ml flow buffer and fixed with cellfix solution (100ul/tube) (BD, UK). The samples were processed by the flow cytometer after connecting the password protected computer to the FACSCalibur™ machine (BD, UK). The voltage and compensation was adjusted for each sample to achieve the maximal detection of the target cells. The computer then converted light analogue signals into digital data and generated the results for the future analysis.

2.3 Immunohistochemistry for uNK cell determination

a. The principle of immunohistochemistry

Standard immunohistochemistry techniques were used to measure uNK cells within endometrium tissue from women with unexplained RM. The protocol was optimized to determine the best conditions for maximal immunostaining with minimal background staining. Phenotype CD56 was detected as the surface marker of natural killer cells in the immunohistochemistry measurement. Monoclonal mouse anti Human CD 56 at a dilution of 1:50 (Serotec, UK) was used as the primary antibody within this study.

An indirect staining method where a biotin labelled secondary antibody raised against the IgG of the primary antibody was used. As avidin has four binding sites for biotin, the signal of end products was further amplified through the biotinylated secondary antibody reacted with the pre-

formed avidin-biotin complexes on the primary antibody. This tertiary signal amplification increased the sensitivity of staining techniques and helped to reduce unwanted background staining. To visualize the areas of antibody-antigen binding, enzyme-substrate reactions was employed to convert colourless chromagens into coloured end products. Chromagen solution (Diaminobenzidine, DAB) was used to produce brown staining to visualize the target antigen.

The key technique of the immunohistochemistry was antigen retrieval method. When aldehyde-based fixatives (eg. formalin) were used during tissue preparation, inter- and intra-molecular cross links were produced masking certain structural proteins of tissue antigens. This adverse effect was caused by the formation of methylene bridges between reactive sites on tissue proteins. The degree of masking of the antigenic sites depended upon the length of fixation time, temperature, concentration of fixatives and the availability of other nearby proteins able to undergo cross-linkage. In order to expose these hidden antigenic sites, heat mediated antigen retrieval technique was employed in the current study. The heat mediated antigen retrieval techniques included microwave oven irradiation, combined microwave oven irradiation and proteolytic enzyme digestion, pressure cooker heating, autoclave heating, waterbath heating, steam heating and high temperature incubation. We used microwave oven irradiation for heating mediated antigen retrieval due to the advantages of having a digital timer for precise time adjustment, a turntable platform for uniform heating of the retrieval solution and an ideal working power ranged from 700W to 1000W.

b. The technique of immunohistochemistry

Immunohistochemistry was performed on de-waxed and re-hydrated 5 mm tissue sections. The majority of procedures were carried out at room temperature. The exceptions were antigen unmasking which was carried out in a microwave oven and incubation with the primary antibody

which was performed overnight at +4°C. All sections were quenched in 0.3% hydrogen peroxide in methanol for 20 min prior to antigen unmasking. Unmasking was performed in an 800 W microwave oven in 10 nmol/l boiling citrate buffer at pH 6.0. The slides were incubated for 12 minutes at medium heat and then left to cool in the buffer for 20 minutes. Then slides were blocked for 1 hour in the blocking buffer (Universal Elite ABC kit, Vector Laboratories, UK) which contained avidin and then incubated overnight with primary antibody (Monoclonal Mouse anti Human CD 56 1:50; Serotec, UK). The next day slides were incubated with the secondary antibody (Universal Elite ABC kit, Vector Laboratories, UK) for 30 minutes at room temperature. Then slides were incubated with peroxidase substrate DAB (Diaminobenzidine) for 8 minutes (Vector Laboratories, UK). Slides were then washed in distilled water for 5 minutes and stained with 10% haematoxylin for 15 minutes. After slides were differentiated, dehydrated, cleared and mounted, the number of endometrial CD56+ NK cells was counted as the percentage of CD56+ cells over total endometrial stromal cells in 10 random selected microscopic fields at magnification of X400. Only cells positive for CD56 staining in stroma were counted as uNK cells. Glandular epithelial cells were not counted as stroma cells in this thesis. Only cells with clear nuclei in the clumps were counted as effective cells. The inter and intra observer errors were 1. 5(±1.2)% and 2.6(±2.2)% respectively. The normal reference of the expression of natural killer cells in endometrium was defined from 2.2% to 13.9% (Tuckerman et al., 2007).

III. Statistical Analysis

Statistical analyses were performed using the SPSS (Statistical Package for the Social Sciences) version 16. Differences in the results of natural killer cell measurements intergroup and intragroup were analyzed with independent T tests. The comparison of the results of peripheral natural killer cell measurements between the baseline assessment and the follow-up assessment were analyzed with paired T tests. The correlation between NK cell measurements and psychometric measurements, and pNK cell measurements and uNK cell measurements were analyzed with Pearson correlation coefficient tests. Statistical significance was set at p value <0.05.

IV. Results

1. Peripheral NK (pNK) cell measurements

A total of 27 cases and 21 controls had pNK cell measurements with flow cytometry. Figure V-1 (a, b) shows the representative density plot graphs of pNK cell measurements by flow cytometry. The demographic characteristics of the 27 cases are shown in table V-4. The mean (± SE) age of the 27 cases was 36.0 ± 0.9 years. The mean (± SE) age of the 21 controls was 34.1 ± 1.2 years. There was no significant difference in the mean age between cases and controls ($p>0.05$). No significant difference in the results of pNK cell measurements was found between cases and controls ($p>0.05$) (table V-5).

Figure V-2 (a, b) shows the comparison of pNK cell measurements of the 27 cases between the baseline assessment and the follow-up assessment. No significant differences in the measurements of the two subsets of pNK CDdim and pNK CDbright were found between the two assessments.

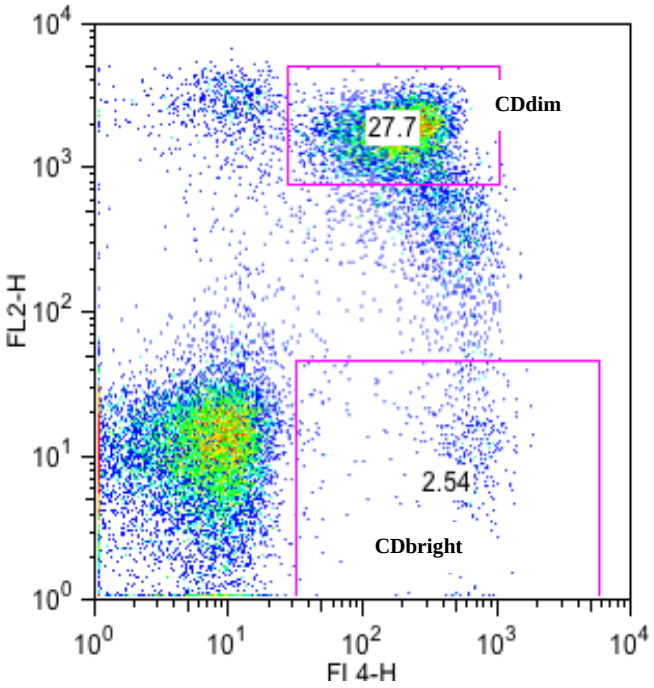
Figure V-3(a, b) shows the comparison of pNK cell measurements at the baseline assessment and the follow-up assessment between the 27 cases with different conception outcomes. There were no significant differences in pNK cell measurements of the two assessments between cases who subsequently conceived and those who did not ($p>0.05$).

Figure V-4 (a,b) shows the comparison of pNK cell measurements at the two assessments between 18 cases with different pregnancy outcomes. In the baseline assessment no significant difference in the pNK cell measurements was found between cases who had subsequent live births and those who miscarried again ($p>0.05$). However, in the follow-up assessment a significant difference in the results of pNK CDdim cells was found between the live birth group and the miscarriage group

($p<0.05$). Cases who miscarried again had significantly higher pNK CDdim cells than those who had live births. No significant difference in pNK CDbright cell measurements was found between the live birth group and miscarriage group in the follow-up assessment ($p>0.05$).

Figure V 1 (a, b). Representative density plot graphs of peripheral NK cell measurements with flow cytometry. FL4 axis represents CD56 expression and FL2 axis represents CD16 expression.

(a) A representative density plot graph of pNK measurements of cases



(b) A representative density plot graph of peripheral NK cell measurements of controls

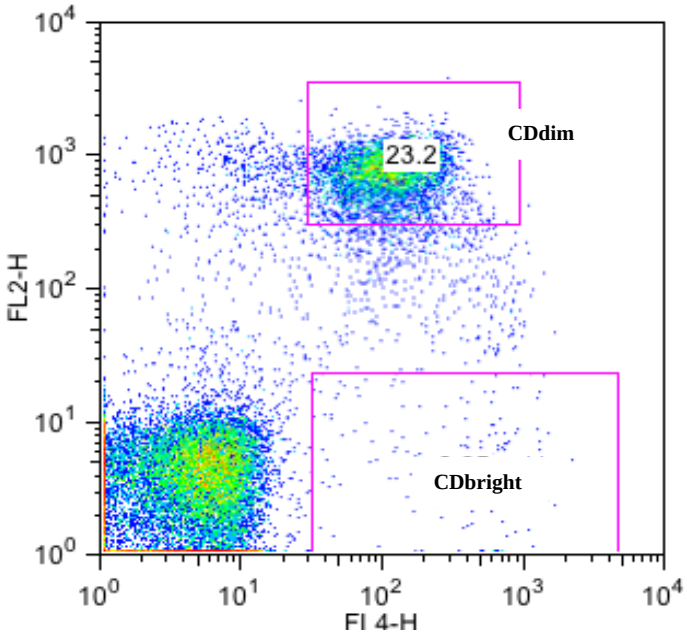


Table V 4. Demographics of 27 cases with peripheral NK cell measurements

Age (years)	pNK CDdim %	pNK CDbright %	No. of previous miscarriages (median, range)	type of RM	
				primary RM (n, %)	secondary RM (n, %)
36.0 ± 0.9	23.4 ± 3.0	1.8 ± 0.1	4 (3-8)	19 (70.4%)	8 (29.6%)

All the values are shown in the form of mean ± SE unless otherwise specified.

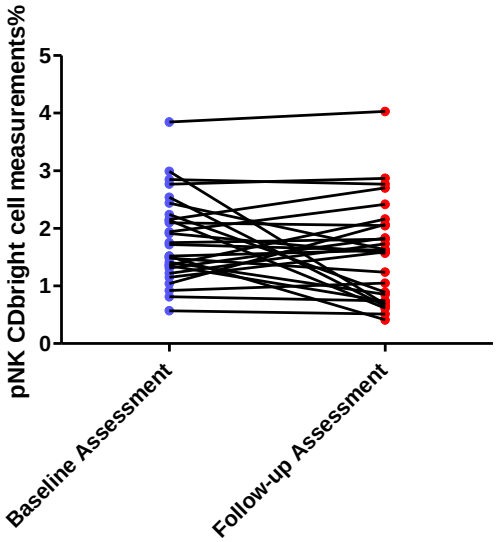
Table V 5. A comparison of peripheral NK cell measurements between cases and controls

	Cases N=27	Controls N=21	ρ value
Age (yrs)	36.0 ± 0.9	34.1 ± 1.2	ρ =0.19
pNK CDdim%	23.4 ± 3.0	21.9 ± 3.0	ρ =0.72
pNK CDbright%	1.8 ± 0.1	1.5 ± 0.3	ρ =0.29

All the values are shown in the form of mean ± SE.

Figure V 2 (a, b). The comparison of peripheral NK cell measurements of the 27 cases between the baseline assessment and the follow-up assessment.

(a) The comparison of pNK CDbright cell measurements of the 27 cases between the two assessments



(b) The comparison of pNK CDdim cell measurements of the 27 cases between the two assessments

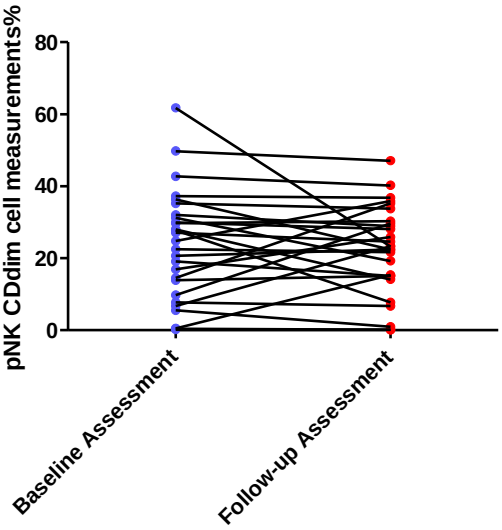
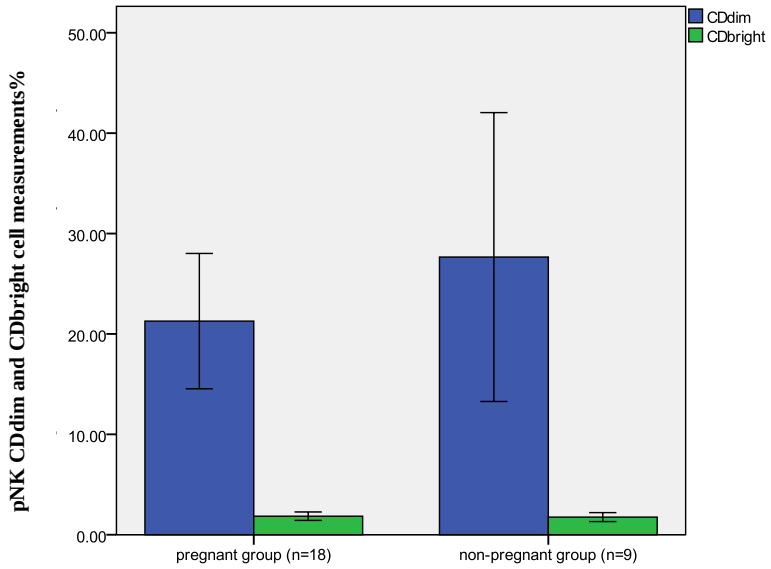


Figure V 3 (a, b). The comparison of peripheral NK cell measurements of 27 cases with different conception outcomes in the baseline assessment and the follow-up assessment

(a) A comparison of pNK cell measurements of the 27 cases with different conception outcomes at the baseline assessment



(b) A comparison of pNK cell measurements of the 27 cases with different conception outcomes at the follow-up assessment

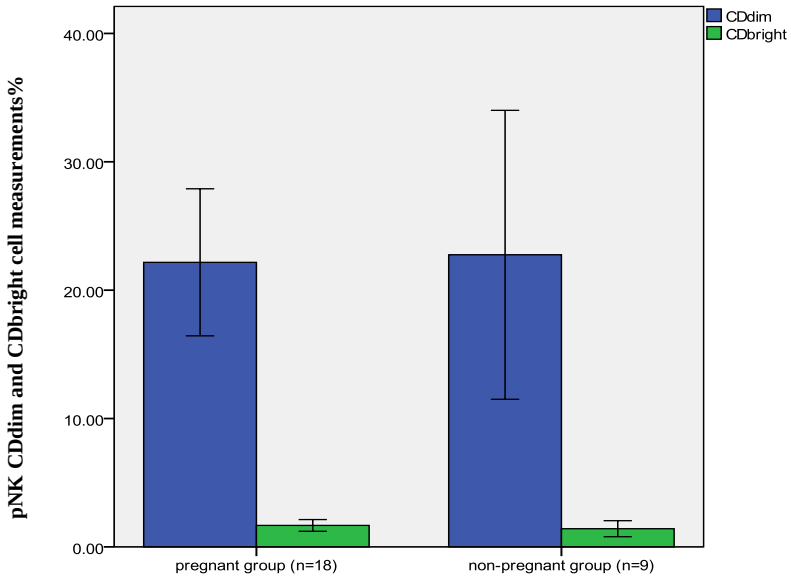
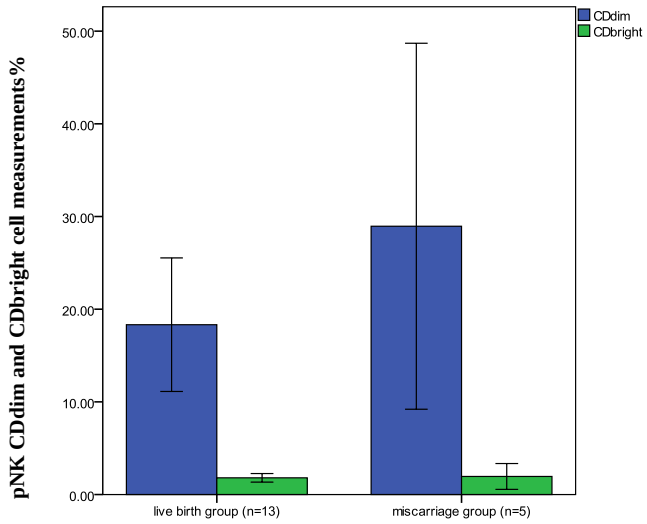
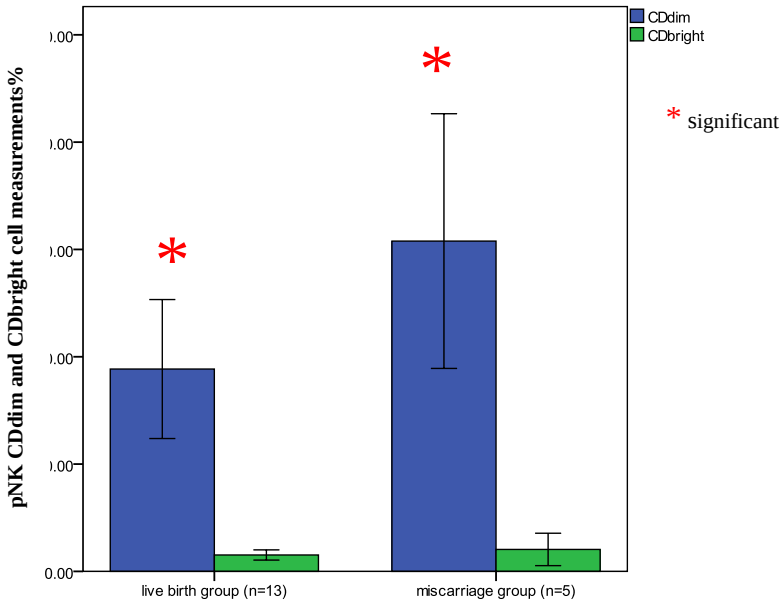


Figure V 4 (a, b). The comparison of peripheral NK cell measurements of cases with different pregnancy outcomes in the baseline assessment and the follow-up assessment

(a) A comparison of pNK cell measurements of the 18 cases with different pregnancy outcomes at the baseline assessment



(b) A comparison of pNK cell measurements of the 18 cases with different pregnancy outcomes at the follow-up assessment

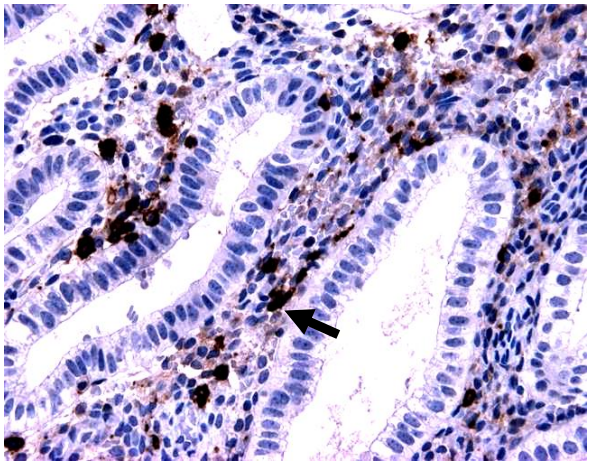


2. Uterine NK (uNK) cell measurements

A total of 29 women with unexplained RM had uNK cell measurements with the LH timed endometrial biopsies. Figure V-5 shows the immunohistochemical staining for CD56+ NK cells in the endometrium of the mid-luteal phase. uNK CD56+ cells were present as clumps of cells around the glands or vessels in stroma. The demographic characteristics of these 29 cases are shown in table V-6. Of these 29 endometrial biopsies, one had a high result of uNK cells of 20.2% (figure V-6). The results of uNK cell measurements for the rest 28 samples were all within the normal range (defined as the range from 2.2% to 13.9%) (Tuckerman et al., 2007). There was no significant difference in the results of uNK cell measurements between cases with different conception outcomes or different pregnancy outcomes ($p>0.05$). Figure V-7 and 8 show the results of uNK measurements of cases with different conception outcomes and pregnancy outcomes.

Figure V 5 (a, b). Immunohistochemical staining of CD56 cells in endometrium tissue of women with unexplained RM. Magnification X400.

(a) CD56+ cells were present as clumps of cells in stroma around the glands.



(b) CD56+ cells were present as clumps of cells in stroma around the vessels.

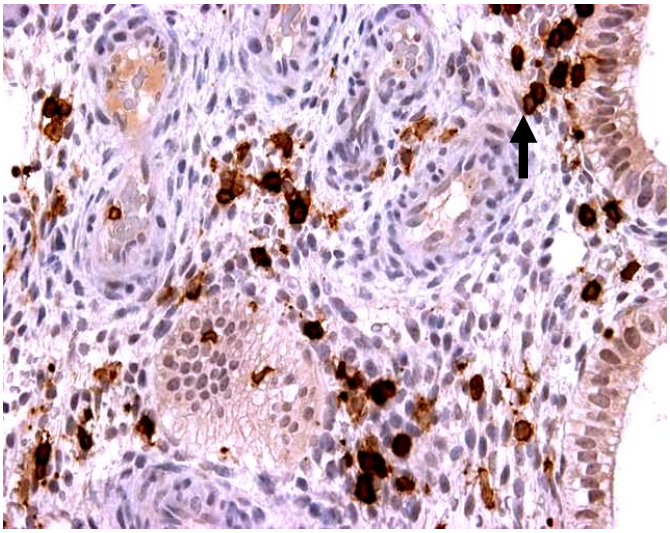


Figure V 6. Image of immunohistochemical staining of the endometrium tissue of women with unexplained RM with a high value of uNK CD56 cell measurement. Magnification X400.

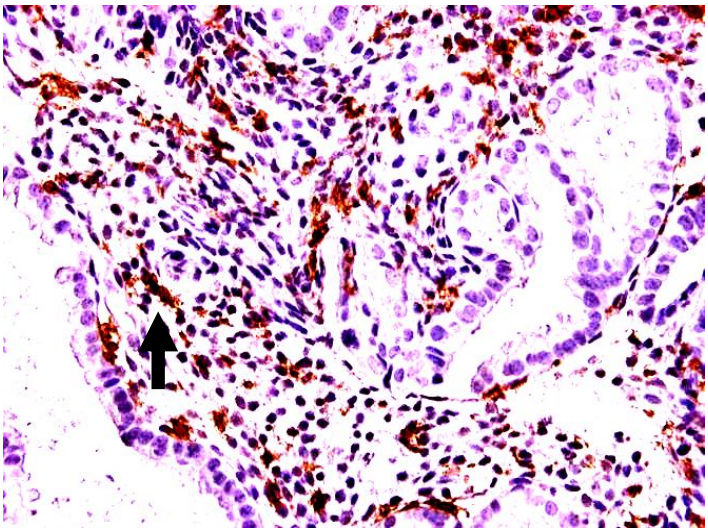


Table V 6. Demographic characteristics of 29 cases with uNK cell measurements

cases	age	uNK	No. of previous miscarriages (median, range)	type of RM	
				primary RM (n, %)	secondary RM (n, %)
n= 29	35.9 ± 0.8 yrs	7.1 ± 0.73	4 (3-8)	21 (72.4%)	8 (27.6%)

All the values are shown in the form of mean ± SE unless otherwise specified.

Figure V 7. Results of uNK CD56 cell measurements of 29 cases with different conception outcomes

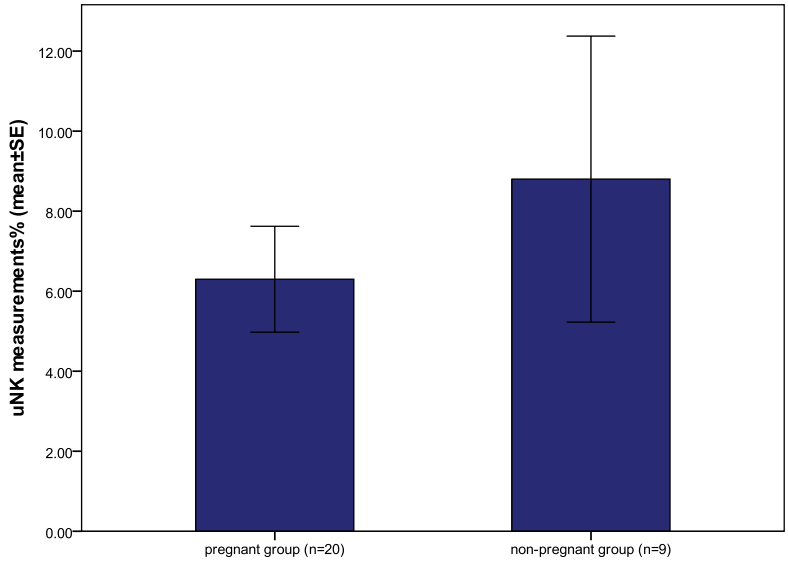
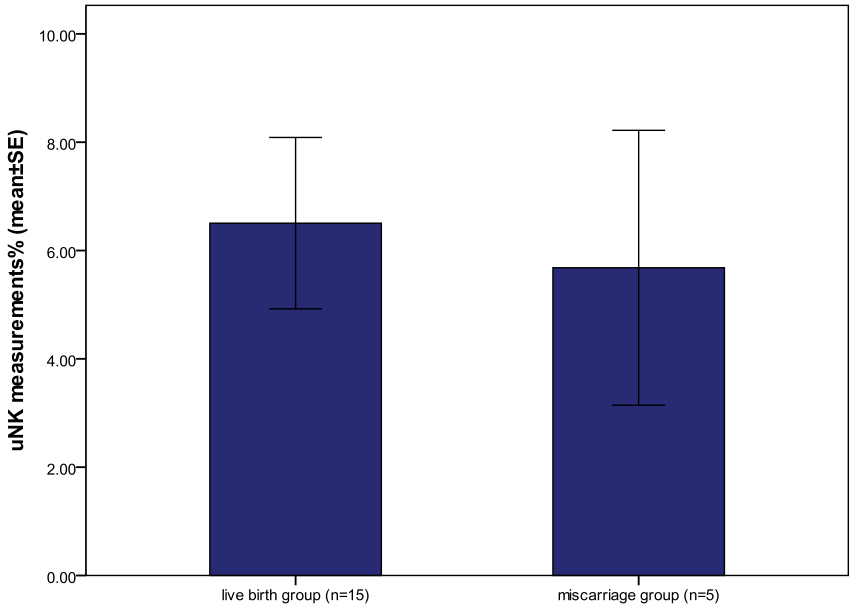


Figure V 8. Results of uNK CD56 cell measurements of 20 cases with different pregnancy outcomes



3. Correlation between pNK cell measurements and uNK cell measurements

A total of 27 matched blood and endometrial samples were obtained from the same individual and at the same time in the mid-luteal phase for the uNK cell and pNK cell measurements. No significant correlation was found between the results of uNK cell measurements and the results of pNK CDdim or CDbright measurements ($\rho>0.05$). Figure V-9 shows the association between the measurements of uNK cells and pNK CDdim cells in the 27 matched samples. Figure V-10 shows the association between the measurements of uNK cells and pNK CDbright cells in the 27 matched samples. There was also no association between the peripheral and uterine NK cell measurements and the demographics (table V-7).

Figure V 9. No correlation between uNK cell measurements and pNK CDdim cell measurements

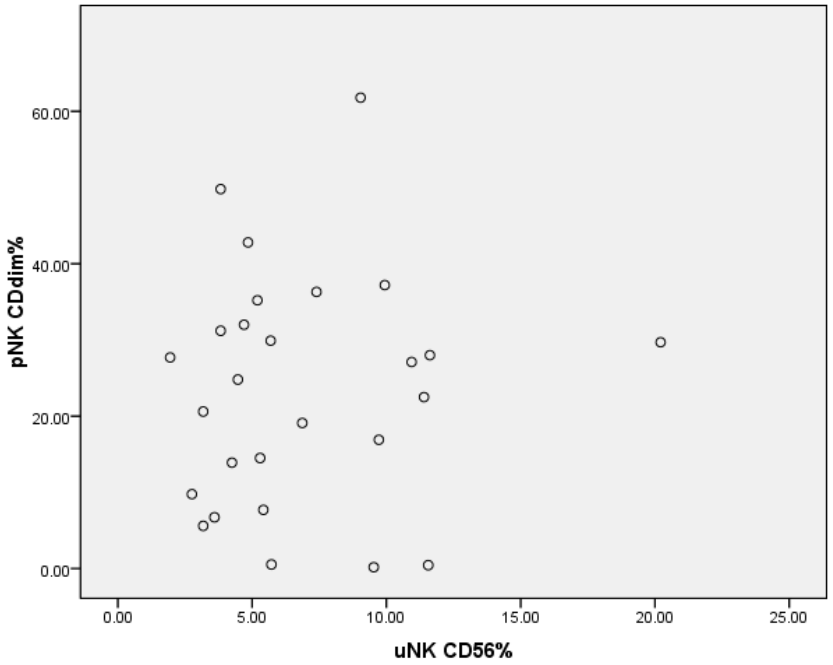


Figure V 10. No correlation between uNK cell measurements and pNK CDbright cell measurements

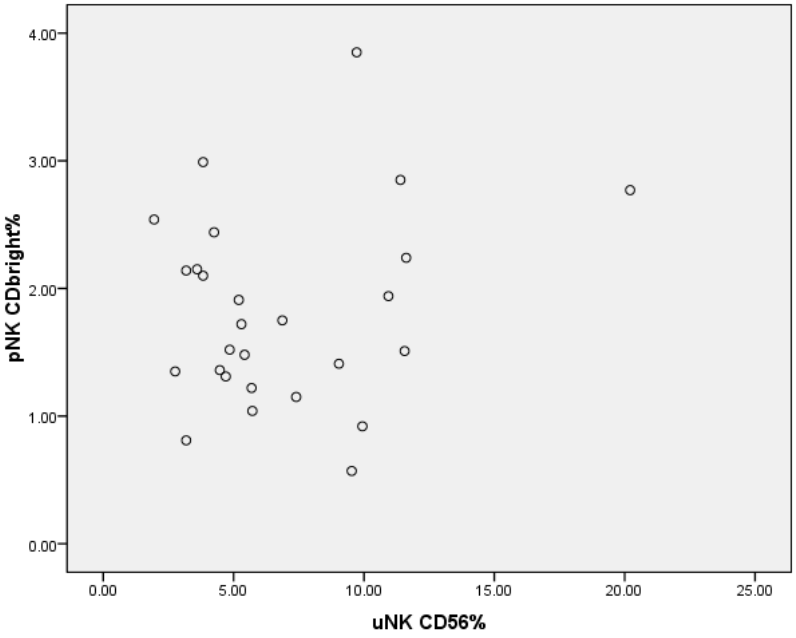


Table V 7. The correlation between peripheral and uterine NK cell measurements and the demographics

	Age	No. of previous miscarriages
uNK measurements n=29	ρ =0.08	ρ =0.93
pNK CD dim measurements n=27	ρ =0.65	ρ =0.07
pNK CD bright measurements n=27	ρ =0.30	ρ =0.38

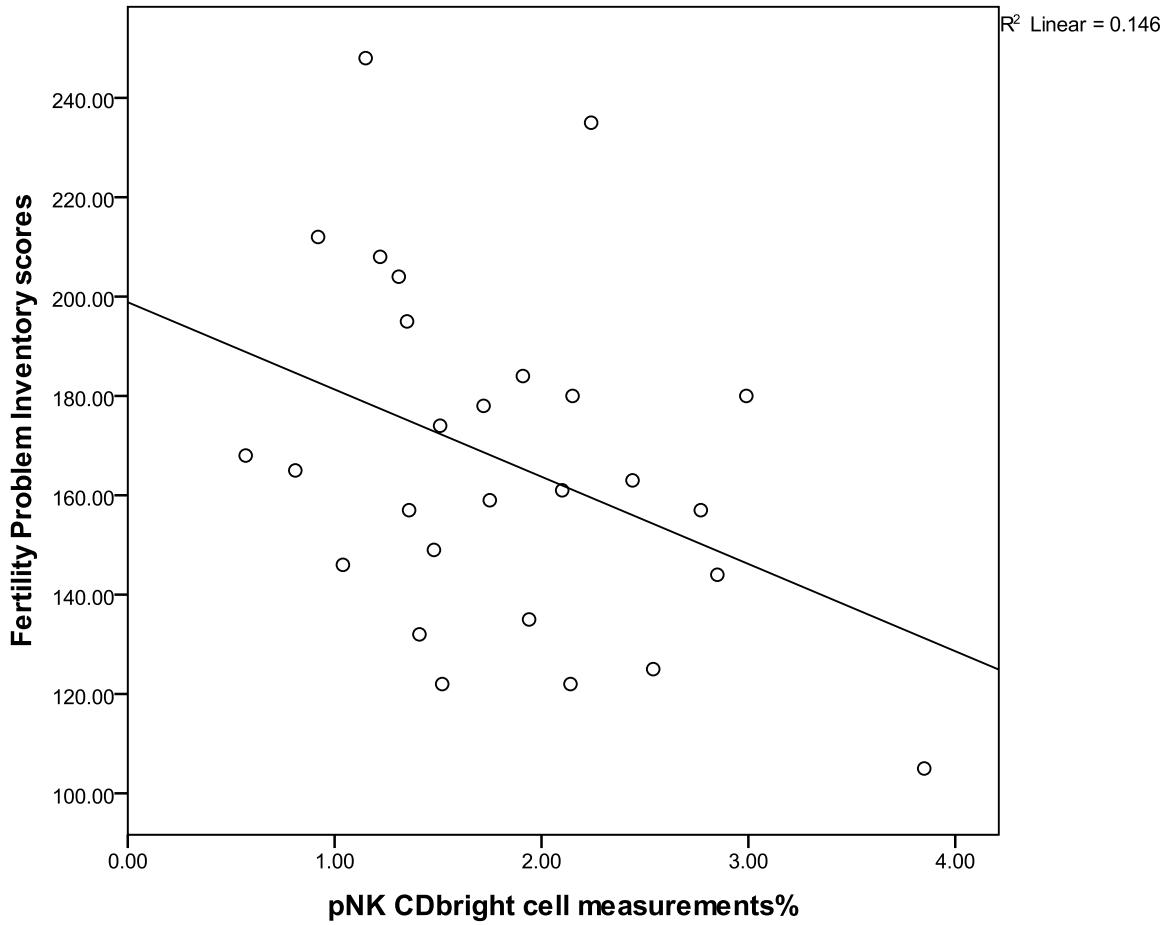
4. Correlation between NK cell measurements and psychometric measurements

Table V-8 shows the correlation between the NK cell measurements and psychometric measurements. There was a significant inverse correlation between the results of pNK CDbright cells and the scores of fertility problem stress. Women with higher levels of fertility problem stress had significantly lower expression of pNK CDbright cells ($\rho < 0.05$) (figure V-11).

Table V 8. The relationship between NK cell measurements and psychometric measurements in women with unexplained RM

	NK cell measurements		
	pNK CDdim% n=27	pNK CDbright% n=27	uNK% n=29
Fertility Problem Inventory	ρ =0.64	ρ =0.04*	ρ =0.94
Perceived Stress Scales	ρ =0.37	ρ =0.09	ρ =0.49
PANAS Positive Affect	ρ =0.96	ρ =0.37	ρ =0.91
PANAS Negative Affect	ρ =0.42	ρ =0.13	ρ =0.24

Figure V 11. An inverse correlation between the results of pNK CDbright cell measurements and FPI scores



V. Discussion

Natural killer cells, which are part of innate immune system, have been found in both peripheral blood and endometrium. Recently, there has been an increasing body of studies reporting on the increases in the number of peripheral NK cells or endometrial NK cells in women with RM, suggesting that NK cell elevation either in peripheral blood or in endometrium may be a causative factor of RM. However, the lack of clarity of the relationship between pNK and uNK measurements makes interpretation of these results difficult. Measurements of peripheral NK cells by flow cytometry are considerably less invasive compared to uNK measurements with an endometrial biopsy. Flow cytometry can also precisely detect subsets of pNK cells such as CDdim and CDbright separately, which are postulated to play different roles in reproductive processes (Kwak-Kim and Gilman-Sachs, 2008, Laird et al., 2003). Therefore, measurements of pNK cells may act as a surrogate for uNK cell measurements in studies on RM if pNK cell measurements reflect the results of uNK cell measurements. We conducted this study measuring NK cells in both peripheral blood and endometrium in matched samples obtained at the same time from the same individual in a group of women with unexplained RM in order to examine the role of NK cells in RM and the relationship between pNK cell measurements and uNK cell measurements in RM.

The relationship between pNK measurements and RM

Current studies on the relationship between pNK cells and RM have yield conflicting results. Some studies have found the evidence of the increases of pNK cells in both numbers and activities in women with RM or RIF (recurrent implantation failure) (King et al., 2010, Prado-Drayer et al., 2008, Yamada et al., 2003) and the achievement of live births in women with RM or RIF by treating high pNK cells with prednisolone or immunoglobulin G (Stricker et al., 2000, van den Heuvel et al., 2007, Coulam and Goodman, 2000), suggesting that increased pNK cells may be an

immunological factor of RM. However, there are also studies which found a negative association between pNK cell measurements and RM. For example, a study comparing 85 RM women with 27 fertile controls found that there was no significant difference in pNK cell numbers between women with RM and controls, suggesting that pNK cell measurements have no diagnostic value on RM (Wang et al., 2008). Another study also suggests that enumeration of pNK cells including the counting of total number of pNK CD56+ and the two subsets of CDdim and CDbright cells has no predictive value on pregnancy outcomes (Thum et al., 2005). In our study we found no differences in the numbers of pNK cell measurements between women with RM and fertile controls.

There are several factors which may contribute to the current conflicting findings. Firstly, there is no consensus on the antibodies used to measure pNK cells with flow cytometry. Some studies labeled pNK cells with CD56, CD16 and CD69 antibodies (Thum et al., 2004) whereas others labeled pNK cells only with CD56 antibody (Wang et al., 2008). It is well known that T cells which contaminate in the NK cell isolation from blood samples also express CD56 antigen apart from its unique antigen of CD3. Therefore, it is likely that different subsets of monocytes are measured in studies with different methods of antibody labeling, which lead to the conflicting results. In our study we employed four antibodies of CD56, CD16, CD3 and CD45 to define pNK cells. Only cells with expression of CD56+CD16+/- CD45+CD3- were defined as pNK cells.

Secondly, the setting of the gate and the compensation of the fluorescent chromosone spectrum on the flow cytometer is quite arbitrary and need to be adjusted from sample to sample in each experiment. Therefore, the results derived from different experiments and operated by different researchers may vary and lead to different conclusions.

Thirdly, it is possible that the increase of pNK cells found in RM studies is the result of sympathetic responses to the stressful venipuncture. A study has observed the change in the numbers of pNK cell measurements in women with RM between the first blood samples obtained by venipuncture and the second blood samples obtained 20minutes after cannulation, suggesting that high sympathetic responsiveness to venipuncture stress in RM subjects may be responsible to the pNK cell increase (Shakhar et al., 2006).

The association between pNK cell measurements and subsequent pregnancy outcomes

Although we did not find a significant difference in pNK cell measurements between women with RM and fertile controls, we found that RM women who had miscarriages in a subsequent pregnancy had higher numbers of pNK CDdim subset prior to conception than those who had live births. This finding suggests that the elevation of pNK CDdim cells prior to conception is a risk factor of subsequent miscarriage in women with RM. The exact mechanism of how pNK CDdim adversely affects pregnancy is not known. It will be of interest to examine the cytotoxity of pNK CDdim cells in women with RM in future studies. However, due to the limitation of the numbers of women with pNK measurements, this finding needs to be verified in a future study.

The effect of 'tender loving care' on pNK cell measurements in women with RM

In this study we hypothesized that 'tender loving care' in the form of clinical consultation, explanation and reassurance by a team of dedicated staff may help to reduce the stress level in women with RM. No significant change in the measurements of pNK cells was found after' tender loving care', suggesting that this kind of 'tender loving care' has no effect on reducing the results of stress marker measurements. This finding is consistent with the result from the psychometric measurements where no evidence of the effect of 'tender loving care' on psychological measurements was found.

However, interestingly we found a small but significant inverse association between the results of pNK measurements and psychometric measurements. High fertility stress was associated with a decrease in pNK CDbright cells in women with RM. The exact mechanism of how fertility specific stress affects the pNK CDbright cells is yet not known. Previous studies have reported on stress increasing the cytotoxicity of peripheral NK cells in women with RM (Andalib et al., 2006) and found a negative association between stress levels and the number of peripheral NK cells (Arck et al., 2001). Due to the paucity of studies in this area, it is still not clear how stress may affect the number of pNK cells.

The relationship between uNK cell measurements and RM

uNK cells, which are the most predominant leucocyte in the endometrium, are present adjacently to the trophoblast cells in implantation site. uNK cell expression is maximal in the mid-to late-luteal phase of the cycle and increases in early pregnancy. The site and the time of uNK cell expression suggest that uNK cells are involved in implantation. Recently, the role of this endometrial factor in RM has been a focus of research interest. Previous studies have found the positive association between raised uNK cells and RM, and the reduction in uNK cell numbers achieved by prednisolone treatment in women with RM (Clifford et al., 1999, Tuckerman et al., 2007, Larciprete et al., 2007, Quenby et al., 2005), suggesting that the increase of uNK cells is a contributory factor of RM. In this prospective study, we did not find an obvious association between uNK cell measurements and RM or pregnancy outcomes in women with RM. Due to the small number of cases examined in this study, the relationship between uNK cells and RM still need to be investigated in future studies.

The correlation between peripheral NK cell measurements and endometrial NK cell measurements

Peripheral and endometrial NK cells are in contact with trophoblasts in different areas. The peripheral NK cells bath the trophoblast cells through the circulation whereas endometrial NK cells are in contact with the trophoblast cells in the implantation sites. Peripheral blood NK cells are part of the innate immune system and are involved in the maternal immunological regulation to adapt the semi-allograft embryo in the early pregnancy. The precise role of uNK cells in the implantation process is still not very clear. Research has proposed several functions of uNK including direct interaction with MHC molecules on the invading trophoblast, production of chemokines and cytokines, angiogenesis and spiral artery remodeling (Bulmer et al. 2010; Bulmer and Lash 2005; Quenby et al. 2009).

The majority of pNK cells are CDdim (CD56+16+) whereas the majority of uNK cells are CDbright (CD56+16-). Studies have found considerable differences in the cell surface markers between pNK and uNK, suggesting they are different phenotypically and functionally (Schallhammer et al., 1997). This raises a question whether the measurement of pNK reflects uNK measurements in studies on RM. Up to date only one published study has examined the association between endometrial NK cells and peripheral NK cells in women with RM (Park et al., 2010). In the study of Park et al, they examined numbers of NK cells in decidual tissue and peripheral blood in matched samples obtained at the same time from 21 women with more than 2 previous miscarriages. They found that there was a positive correlation between the numbers of NK cells measured in the decidual tissue and those measured in the peripheral blood. Therefore, they concluded that peripheral NK cell changes reflected endometrial NK cell alteration in women with RM and pNK measurements can be a surrogate for uNK cell measurements in the diagnosis of RM with the advantage of less invasive procedure.

However, we should note that this study investigated the presence of NK cells in decidual tissue of early pregnancy comparing with the results of peripheral NK cells. The presence of endometrial NK cells varies significantly in different phases of the menstrual cycle and during pregnancy. They increase from the proliferative phase reaching maximum in the secretory phase (King et al., 1991). In the first trimester of pregnancy, endometrial NK cells dominantly form70% of leucocytes in the decidua (Moffett-King, 2002). However, the number of pNK cells increases in the luteal phase (Lee et al., 2010) and does not change throughout the pregnancy (Southcombe et al., 2010). In addition, studies revealed a decrease in the total number of lymphocytes in the third trimester associated with a suppressed regulatory effect on maternal immunity to tolerate the semi-allograft fetus (Mahmoud et al., 2001). Therefore, the dynamic change of NK cells in decidua of the early pregnancy may be not synchronized with the change of NK cells in circulation and thus pNK measurements in early pregnancy may not reflect the measurements of NK cells in decidua. In addition studies have shown that peripheral NK cells can be artificially increased by the stress of venipuncture (Shakhar et al., 2006). Therefore, the significance picked up in the above study may be a type II error.

In our prospective study we investigated the numbers of NK cells in the peripheral blood and endometrium in the matched samples obtained at the same time from the same individual in a group of women with unexplained RM. In this study we found no correlation between pNK measurements and uNK measurements in RM women in the pre-conceptional state. Our study is the first study to investigate the association between uNK and pNK measurements in pre-conceptional state in women with RM. The limitation of the study is that it was carried out in women with RM. A control study in fertile women would also be of interest to further examine

the association between pNK and uNK measurements with simultaneously obtained blood and endometrial samples.

The negative association between pNK cell results and uNK cell results found in this study raises the question about the role of pNK cell measurements in RM. The finding that the pNK cell measurements are not related to uNK cell measurements suggests that it may be inappropriate to extrapolate results from peripheral blood to interpret changes in the endometrium. Therefore, the role of peripheral NK cell measurements in RM remains to be ascertained.

VI. Conclusion

In the present study we found that the increase in numbers of pNK CDdim cells is associated with an increased risk of a subsequent miscarriage in women with RM. The exact pathogenesis of how increased pNK CDdim cells adversely affect pregnancy is not clear. In this study we also find no correlation between the measurements of NK cells in peripheral blood and those in endometrium in women with RM, suggesting that pNK cell measurements do not reflect uNK cell measurements.

Chapter VI: The relationship between plasma prolactin concentrations and RM

I. Introduction

Recurrent miscarriage (RM) is a heterogeneous condition in which 50% of the cases remain unexplained. To date several aetiological factors of RM have been identified, including parental chromosomal anomalies, uterine pathology, haematological abnormalities, immunological factors and endocrinological disorders. However, most previous studies in relation to the endocrine abnormalities of RM have focused on the luteal phase deficiency (LPD) and PCOS (polycystic ovarian syndrome) (Cocksedge et al., 2009, Palomba et al., 2006, Nardo et al., 2002). Less attention has been paid to the role of prolactin in RM.

Prolactin is a polypeptide hormone synthesized by the lactotroph cell in the anterior pituitary gland and in extrapituitary sites such as mammary gland, placenta and uterus (Goffin et al., 2002, Eyal et al., 2007, Reis et al., 2002). Apart from the lactation and endocrine effects, prolactin may have the luteotrophic function by potentiating the effect of luteinizing hormone (LH) on steroidgenesis in granulosa-luteal cells and inhibiting the 20α-hydroxysteroid dehydrogenase enzyme which inactivates progesterone (Freeman et al., 2000, Kanuka et al., 1997, Richards and Williams, 1976). Therefore, prolactin plays an important role in maintaining corpus luteum function and progesterone secretion, with potential impact on establishment of pregnancy. The exact mechanism of the luteotrophic effects of prolactin is still unclear.

Recently the role of prolactin in RM has attracted more attention. Several studies have reported a high prevalence of hyperprolactinaemia in the follicular phase in women with RM and described

the suppression of prolactin secretion with bromocriptine to improve pregnancy outcome (Hirahara et al., 1998, Csemiczky et al., 2000, Bussen et al., 1999), suggesting that high plasma prolactin levels may be a possible aetiological factor of RM. However, our earlier study showed that hyperprolactinaemia rarely occurs in women with RM (2.5%, 3/122) (Li et al., 2000). In some studies it has been suggested that an adequate level of plasma prolactin is essential to maintain corpus luteum function (Gåfvels et al., 1992) and establish a successful pregnancy (Oda et al., 1991). In IVF treatment it has been observed that transient hyperprolactinaemia is associated with a better chance of successful pregnancy outcomes (Ozaki et al., 2001). The inadequate expression of prolactin and its receptor in endometrium during the implantation window has been found to cause premature luteolysis and subsequent infertility or recurrent miscarriages (Garzia et al., 2004). Therefore, this raises the question of whether or not a high plasma prolactin level is associated with an increased risk of recurrent miscarriage. Due to the paucity of the studies in this area, the relationship between the plasma prolactin level and RM is still unclear. The aim of this retrospective study was to investigate the relationship between plasma prolactin concentrations and the pregnancy outcomes in a large cohort of women with unexplained RM.

II. Materials and Methods

Subjects

A series of 174 consecutive women with unexplained RM, who had plasma prolactin concentrations measured in the early follicular phase from January 1999 to September 2009, were identified from the clinical database and were included in this study. All the patients who conceived again after the referral were offered telephone pregnancy counselling service as part of 'tender loving care' throughout their subsequent pregnancies. No other treatment was given to this group of women.

Outcome measures

Pregnancy outcome was defined as the outcome of the first pregnancy after referral. In patients who conceived more than once during the study period, only the first pregnancy was considered.

Prolactin measurement

Blood samples for plasma prolactin measurements were taken in the early follicular phase. Prolactin assay was performed by automated microparticle enzyme-immunoassay (Abbott Axsym Analyser, Abbott Diagnostics) at the Clinical Chemistry Department, Royal Hallamshire Hospital. The intra-and inter-assay coefficient of variation for prolactin measurement was 3.7% and 11.5%. The presence of hyperprolactinaemia was defined as plasma prolactin concentration > 660 mIU/l.

III. Statistical Analysis

Results were expressed as numbers, mean ±SE, range and percentages as appropriate. Normal distribution of prolactin value was tested with *Kolmogorov-Smirnov* test. Differences in plasma prolactin concentrations between women who subsequently miscarried and those who had a live birth were analysed with T test. The difference in the miscarriage rate between the two subgroups was analysed with *Pearson* x^2 test. Statistical significance was set at *p* value <0.05 and analysis were performed using SPSS (Statistical Package for the Social Sciences) version 16.

IV. Results

A total of 174 women with unexplained RM had their plasma prolactin measured. The mean age (±SE) was 32.2 (± 0.4) years; the median number of previous miscarriages was 4 (ranged from 3 to14). The mean (±SE) prolactin concentration was 226 (±9.3) mIU/l. Among those 174 women with unexplained RM, 40 of them did not conceive and 9 of them were lost to follow-up. The remaining 125 women with unexplained RM conceived again after the referral. The differences in clinical profile between women who conceived (n=125) and those who did not conceive (n=40) are shown in table VI-1. As expected, women who did not conceive were older than those who conceived ($P< 0.05$). Women who conceived again had higher mean prolactin concentrations than those who did not conceive. However, the difference was not significant.

Among those 125 women with unexplained RM who conceived again, 2 women had ectopic pregnancies, 3 women had mid-term miscarriages due to cervical incompetence, 3 women had mid-term terminations of pregnancies due to fetal deformation and 8 women lost follow-up of their pregnancy outcomes. Therefore, they were excluded from the study analysis. A total of 109 women with subsequent pregnancies were included in the study analysis. The mean age was 31.9 (±0.6) years. The median of the numbers of previous miscarriages was 3 (ranged from 3 to 7). The mean (±SE) concentration of prolactin was 278 (±13.1)mIU/L.

In the 109 subsequent pregnancies, 49 (45%) pregnancies resulted in the first trimester loss again whereas 60 (55%) pregnancies resulted in live birth. Women with a subsequent live birth had significantly ($\rho=0.01$) higher prolactin concentrations (308± 12.2mIU/l) than those with a subsequent miscarriage (240±16.8mIU/l) (Figure VI-1).

These 109 women were further divided into two subgroups according to the mean value of the prolactin measurements. 45 women with prolactin concentrations above the mean level were allocated into high normal prolactin group and 64 women with those below the mean level were allocated into low normal prolactin group (Table VI 2). The miscarriage rate in women with high normal prolactin (15/45, 33.3%) was significantly lower ($p<0.05$) than that in women with low normal results (34/64, 53.1%).

Table VI 1. The differences in demographics between 125 women with unexplained RM who conceived again and 40 women with unexplained RM who did not conceive

	Women who conceived (n=125)	Women who did not conceive (n=40)	ρ value
Age (years)	31.0 ± 0.6	32.6 ± 0.5	ρ = 0.02*
No. of previous miscarriages (median, range)	4 (3-10)	4 (3-14)	ρ = 0.47
Prolactin concentrations (mIU/l)	234.5 ± 12.6	196.3 ± 9.9	ρ = 0.26

All the values are shown in the form of mean ± SE unless otherwise specified.

* Significant

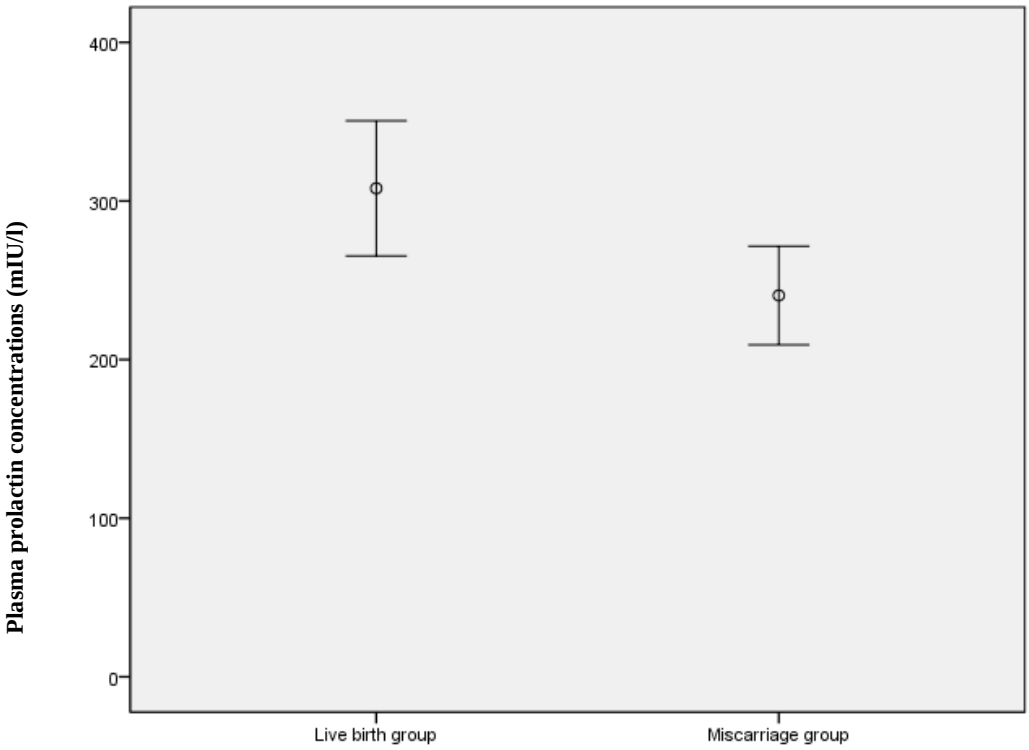


Figure VI 1. Prolactin concentrations of women with unexplained RM who had a subsequent live birth and those who miscarried again

Table VI 2. The differences in the miscarriage rates between the two subgroups

	High normal prolactin subgroup (prolactin≥278mIU/l) n=45	Low normal prolactin subgroup (prolactin<278mIU/l) n=64	p value
Age (years)	31.5 ± 0.8	32.1 ± 0.7	ρ >0.05
No. of previous miscarriages (median, range)	3 (3-6)	3 (3-7)	ρ >0.05
No. of miscarriages (n, %)	15 (33.3%)	34(53.1%)	ρ <0.05*
Prolactin concentration (mIU/l)	415 ± 17.9	181 ± 7.0	ρ <0.05*

All the values are shown in the form of mean ± SE unless otherwise specified.

*significant

V. Discussion

The role of prolactin in RM is controversial. Some studies have reported a high occurrence of hyperprolactinaemia in women with RM and a positive association between hyperprolactinaemia and adverse pregnancy outcomes (Bussen et al., 1999, Csemiczky et al., 2000, Hirahara et al., 1998). However, evidence from observational studies in IVF shows that women with high prolactin concentrations have a better pregnancy rate and live birth rate, indicating high prolactin may benefit pregnancy (Ozaki et al., 2001). This conclusion is supported by other studies in which a lack of endometrial expression of prolactin was found to be associated with an increased risk of miscarriage (Garzia et al., 2004). These contradictory findings failed to resolve the question as to whether prolactin is detrimental or beneficial in pregnancy. Therefore, we conducted a study to examine the role of plasma prolactin in RM.

In this study the role prolactin in RM was examined in a large cohort of 174 women with unexplained RM using patient data taken from the clinical database. We only included women with unexplained RM in whom other possible causes of RM have been excluded to enable prolactin concentration to be the only factor affecting pregnancy. In this study we found that women with plasma prolactin concentrations in the lower end of the physiological range had an increased risk of miscarriage, suggesting that adequate concentrations of plasma prolactin participate in the maintenance of pregnancy.

The adverse effect of low prolactin concentration on pregnancy outcome found in this study is consistent with some previous studies where the relationship between the plasma prolactin concentration and the outcome of assisted conception treatment were examined. Ozaki *et al* (2001) compared plasma prolactin concentrations between women who had subsequent live births

following IVF treatment and those who had early pregnancy losses. They found that women with successful pregnancies had significantly higher prolactin levels than those with early losses (Ozaki et al., 2001). In a study by Gonen and Casper (1989) transient hyperprolactinaemia following ovarian stimulation was found to be associated with a better chance of pregnancy following IVF treatment. Before HCG administration, there was no significant difference in the basal prolactin level between women who subsequently conceived and those who did not conceive (11.1±0.6ug/l vs 10.1±0.4ug/l). After HCG administration, the prolactin level in the pregnant group was significantly higher than that in nonpregnant group (20.8±1.6ug/l vs 16.0±0.9ug/l) (Gonen and Casper, 1989). It is not yet understood why in assisted conception women with high prolactin levels had better pregnancy outcomes. It has been observed that women with higher prolactin concentrations had significantly higher fertilization rates and cleavage rates of oocytes in IVF treatment than those with low prolactin concentrations (Oda et al., 1991).

While some studies reported that high prolactin levels had an inhibitory effect on GnRH release and HCG-induced ovulation, and a negative regulatory function on endometrial decidualization in pregnancy (Milenković et al., 1994, Liu et al., 1997, Eyal et al., 2007), other studies have reported a beneficial effect of prolactin on pregnancy. Plasma prolactin was found to have a beneficial effect on maintaining corpus luteum in early pregnancy via modulation of luteinizing hormone/chorionic gonadotropin (LH/CG) receptors (Gåfvels et al., 1992). LH/CG receptors are expressed in corpus luteum tissue and chorionic villous macrophages after implantation (Sonoda et al., 2005, Saint-Dizier et al., 2004). In a rat model it has been demonstrated that inhibition of plasma prolactin secretion with bromocriptine during early pregnancy deceased LH/CG receptors and led to premature luteolysis, subsequently resulting in miscarriage. This effect can be reversed by prolactin supplementation after implantation (Gåfvels et al., 1992, Chan et al., 1980), suggesting that adequate prolactin secretion is of protective to pregnancy.

The relationship between the prolactin concentration and the outcome of spontaneous pregnancy, especially in women with a history of recurrent pregnancy loss has not been previously studied. In our study, we found women who subsequently miscarried had a lower plasma prolactin concentration than those who had a live birth. We also found that plasma prolactin level appears to have a prognostic value on the outcome of pregnancy in women with RM, with low physiological levels having a worse outcome than high levels. Thus our data supports the notion that low prolactin level is associated with an adverse pregnancy outcome.

VI. Conclusion

In conclusion, we found that in women with unexplained RM a low prolactin level is associated with an increased risk of miscarriage. The exact reason for the observation is unclear but it seems likely that an adequate level of prolactin concentration is needed for the maintenance of successful pregnancies. It will be of interest to further examine the expression prolactin and its receptor in the endometrium to ascertain the mechanism of RM in association with low prolactin concentrations.

Chapter VII: The expression of endometrial prolactin receptor in RM

I. Introduction

The success of pregnancy relies on two aspects, namely uterine receptivity and embryo development. It is crucial that the endometrium undergoes a series of morphological changes and tissue remodeling in the decidualization process of early pregnancy to facilitate the invasion and implantation of the proliferating trophoblast cells. It is well recognized that a number of growth factors, cytokines and protein hormones are involved in the initiation and development of decidualization.

Prolactin is a polypeptide hormone and is synthesized and secreted by lactotroph cells in the anterior pituitary gland (Freeman et al., 2000). Prolactin is a reproductive hormone and regulates lactation. The extra-pituitary expression of prolactin and its receptors in the glandular epithelial cells of secretory endometrium and in the decidua of early pregnancy has been identified (Jones et al., 1999). Endometrial prolactin increases steadily after implantation, reaching a peak at about 20-25 weeks of pregnancy and declines towards term (Wu et al., 1995). Endometrial prolactin RNA transcript is longer than pituitary prolactin. Endometrial prolactin has been considered to be distinct from pituitary prolactin structurally, chemically, immunologically and biologically (Gellersen et al., 1989, Gellersen et al., 1994). Endometrial prolactin may have a role in decidualization and embryo implantation through modifying the expression of adhesion and proteolytic molecules which facilitate trophoblast cell invasion following extracellular matrix degradation of the endometrium (Starzyk et al., 1999, Ormandy et al., 1997, Horseman et al., 1997).

The biological functions of prolactin are mediated by interaction with its receptors of long or short isoforms (Binart et al., 2010, Bole-Feysot et al., 1998). Studies have found prolactin induced up-regulation of prolactin receptor expression in the corpus luteum during pregnancy (Telleria et al., 1997). Prolactin receptors are transmembrane cytokine receptors that initiate kinase signaling pathways such as JAK/STAT (Bachelot and Binart, 2007, Jabbour et al., 1998). The expression of endometrial prolactin receptor has been found to be upregulated during the secretory phase of the menstrual cycle and influence glandular epithelial and stromal proliferation and gene transcription of the mitogenic and differentiative effect of hormones (Jabbour et al., 1998). The target hormones activated by prolactin receptor in endometrium is still not clear. The time and site of its expression in endometrial tissue suggest a crucial role for prolactin and its receptor in establishment and maintenance of early pregnancy. In human endometrium prolactin receptor has been found to regulate the decidualization via autocrine pathway (Eyal et al., 2007).

Prolactin receptors have also been identified in uterine natural killer (NK) cells (Gubbay et al., 2002). The distinctive presence of uterine $CD56^{bright}$ NK cells around trophoblast cells during implantation suggests that they may play an important role in angiogenesis and immunoregulation between the fetal-maternal interface in early pregnancy (Quenby et al., 2008). Therefore, prolactin may also affect uterine NK cells function or differentiation with mitogenic effect through ERK phosphorylation pathway (Gubbay et al., 2002) and thus affect implantation in this way.

The aim of this study was to examine the relationship between endometrial prolactin receptor and RM by comparing the expression of prolactin receptor in endometrium between women with

unexplained RM and fertile women; and correlating the expression of prolactin receptor with the subsequent pregnancy outcome in women with unexplained RM.

II. Materials and Methods

1 Subjects

1.1 Cases

A total of 22 endometrial biopsies from cases with unexplained RM were used for prolactin receptor measurements. The LH timed endometrial tissues were obtained with a pipelle sampler (Prodimed, France) on LH day 7 to 9. The LH surge was detected with daily urine dipsticks (Clearblue, UK) from day 10 of the cycle onwards until LH surge was identified. The details of the endometrial biopsy collection procedure has been described in Chapter V. Samples were fixed in formalin and wax embedded in the Histology Department for immunohistochemistry. Slides were cut from these biopsy samples and sent to the laboratory in the Academic Unit of Reproductive and Developmental Medicine. The mean (±SE) age of these cases is 35.7 (±1.0) years.

1.2 Controls

Nine endometrial samples from healthy women of proven fertility were retrieved from the Pathology Department of the Royal Hallamshire Hospital. The endometrial biopsies were collected in the mid luteal phase from LH day 7 to 9. The endometrial tissue was fixed in formalin and wax embedded in the Histology Department. Slides were cut from these biopsy samples and sent to the laboratory in the Academic Unit of Reproductive and Developmental Medicine. The mean (±SE) age of these controls is 34.3 (±0.8) years.

2 Methods

Technical optimization of the immunohistochemical procedure

Standard immunohistochemistry techniques were used in this study to identify the expression of prolactin receptor in endometrium from women with unexplained RM and healthy fertile women. The principle of immunohistochemistry has been discussed in the previous relevant chapter. The protocol was optimized to determine the best conditions for the best immunostaining with minimal background staining in the laboratory in the Academic Unit of Reproductive and Developmental Medicine. The monoclonal mouse anti human prolactin receptor antibody (AbCam, UK) was used as the primary antibody to determine the expression of prolactin receptor in this study. The antibody was tested with the positive control tissue of breast carcinoma to verify the specificity of the antibody. A series of dilutions of the prolactin receptor antibodies were prepared at the concentrations of 1:250, 1:500, 1:750, 1:1000, 1:1250 and 1:1500 respectively with PBS solution as the diluent. The optimal dilution of prolactin receptor antibodies was determined when a clear product colour with a minimal background tissue staining was achieved at the end of the immunohistochemistry procedure. The optimal concentration of antibody dilution was confirmed at the ratio of 1: 500 after several pilot experiments. The preferred antigen retrieval method for prolactin receptor immunostaining was established with heat induced enzymatic procedure where sections were unmasked with 0.5% pepsin solution for 30mins in a waterbath under the temperature of 37°C. The detail of the procedure was illustrated as below.

The paraffin embedded endometrial sections were dewaxed in the xylene twice and then rehydrated in the alcohol solution in descending concentrations three times. The sections were then washed in the tap water and quenched in the fresh made 3% of hydrogen peroxidise in methanol for 20 minutes to eliminate the endogenous nonspecific antigens. The sections were then washed again and digested with the 0.5% pepsin in hydrogen chloride in a moisture chamber for

30 minutes in a waterbath at 37°C. After antigen retrieval, the sections were blocked with the horse serum for 30 minutes at room temperature and then incubated with the prolactin receptor antibody at the dilution of 1:500 for 1 hour at room temperature. The biotinylated secondary antibody was applied for 30 minutes at room temperature after the sections were washed with the TBS buffer. Vectorstain was performed for 30 minutes followed by DAB application to colour the end products for 10 minutes. The sections were then counter stained with haematoxylin for 30 seconds and dehydrated in the alcohol solution and rewaxed in the xylene solution respectively. The slides were then mounted and observed under the light microscopy.

Quantification of immunohistochemical staining

The immunostaining intensity in the tissue sections was graded and scored using H Scoring system (Lessey et al., 1996a, Lessey et al., 1996b) following the equation:

H-score = $\sum P_i (i + 1)$, where I = staining intensity (1= weak, 2 = moderate, 3 = strong) and Pi = percentage of cells stained at each intensity (0-100%). The expression of prolactin receptor was assessed separately in the luminal and glandular epithelium and in the stroma in mircroscopic fields at X10 magnification. The numbers of stromal cells with positive prolactin receptor expression were counted and expressed as the average of the cell numbers in ten fields at X400 magnification.

The scoring was conducted by two independent observers (SML and WL) both blinded to the status of the slides. In the case of a large discrepancy in the results between the two observers, the slides were reanalyzed in the presence of both observers until the final agreement in the scoring was reached. The results were determined by taking the average of the results from each individual observer. The staining procedure for prolactin receptor was performed and scored twice

to verify the reproducibility and the reliability of the results. The final results for the expression of prolactin receptor were obtained by using the average of each individual study.

III. Statistical Analysis

Statistical analyses were performed using the SPSS (Statistical Package for the Social Sciences) version 16. As the data of the H-scores of prolactin receptor was non-parametric, Mann Whitney U test was chosen for the statistical analysis to compare the differences in prolactin receptor measurements between groups. The correlation between prolactin receptor measurements and demographic features or psychometric measurements was analyzed with Spearman's correlation coefficient test. Statistical significance was set at p value <0.05.

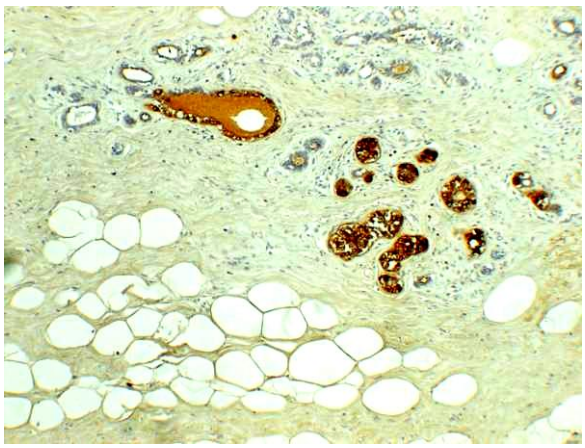
IV. Results

1. The results of endometrial prolactin receptor measurements

A total of 22 endometrial biopsies from women with unexplained RM and 9 endometrial biopsies from the fertile controls were used for prolactin receptor measurements. The immunohistochemical staining of prolactin receptor was identified in glands of breast carcinoma tissue from the positive control slides (figure VII-1). Positive immunostaining of prolactin receptor was identified in the luminal epithelium, glandular epithelium and stroma both in the endometrial tissue from women with RM and that from fertile controls. The pattern of the prolactin receptor expression in the endometrium of fertile women was seen with the strongest intensity of staining in the luminal epithelium, moderate to weak intensity in the glandular epithelium and very weak or no staining in the stoma (figure VII-2a). Women with unexplained RM were found to have a similar pattern of endometrial prolactin receptor expression (figure VII-2b).

Figure VII 1 (a, b). Immunohistochemical staining for prolactin receptor in positive control slides (breast carcinoma tissue). Magnification X100

(a)



(b)

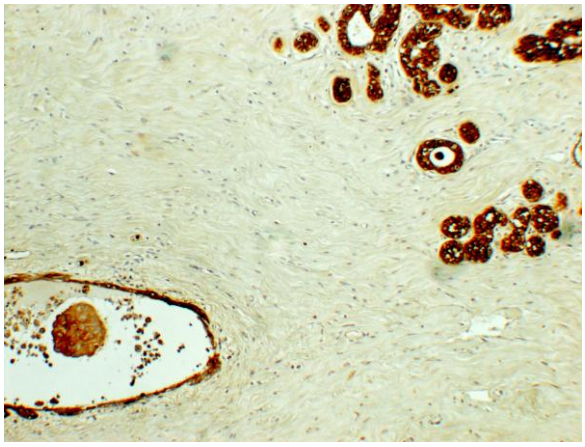
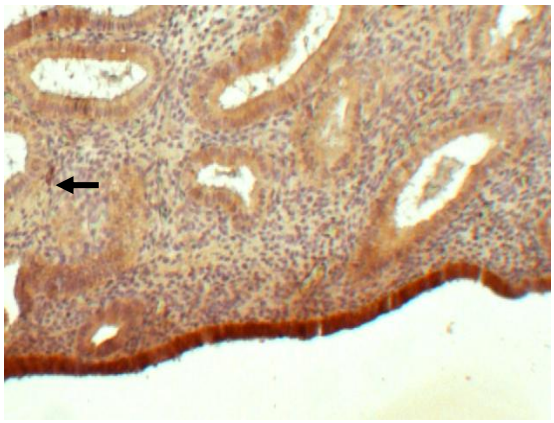
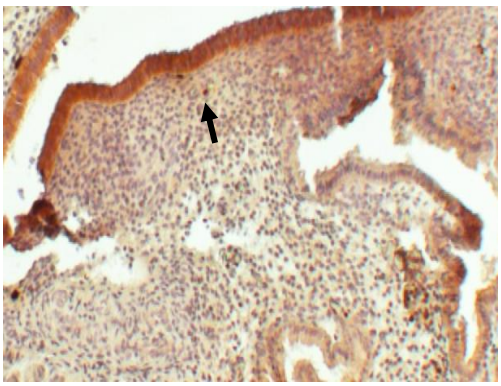


Figure VII 2 (a, b). Immunohistochemical staining for prolactin receptor in the endometrium of fertile women and women with unexplained RM. Magnification X100. Prolactin receptor was stained strongly in the luminal epithelium, moderately in glandular epithelium and weakly in stroma. The black arrow points to the positive staining cells for prolactin receptor.

(a). The expression of prolactin receptor in the endometrium of fertile women in the mid luteal phase



(b). The expression of prolactin receptor in the endometrium of women with unexplained RM in the mid luteal phase



2. The comparison of the scores of endometrial prolactin receptor between cases and controls

Table VII-1 shows the results of prolactin receptor H-scores in each compartment of the endometrium of the 22 cases and 9 controls and the positive cell counting in stroma between the two groups. No significant difference was found in the endometrial prolactin receptor expression between cases and controls ($p>0.05$). When comparing the difference in prolactin receptor expression between cases with different conception outcomes or different pregnancy outcomes, no significant difference was seen between the subgroups with different conception outcomes or pregnancy outcomes ($p>0.05$) (table VII-2, 3).

Table VII 1. A comparison of the H scores of the immunohistochemical staining of prolactin receptor in each compartment of the endometrial tissue between cases and controls

	Women with unexplained RM N=22	Fertile women N=9	ρ value
Luminal epithelium	223.8 (55-300)	220 (135-312.5)	ρ = 0.54
Glandular epithelium	165.0 (0-255)	200 (50-300)	ρ = 0.19
Stroma	20.0 (0-80)	25 (0-60)	ρ = 0.71
Cell counting	1.0 (0.35-1.85)	1.1 (0.7-2.1)	ρ = 0.59

All the values are shown in the form of median and range.

Table VII 2. A comparison of the H scores of the immunohistochemical staining for prolactin receptor in each compartment of endometrium between 22 cases with different conception outcomes

	Pregnant group N=16	Non-pregnant group N=6	ρ value
Luminal epithelium	240 (55-300)	228.8 (140-292.5)	ρ =0.77
Glandular epithelium	160 (0-255)	180 (80-225)	ρ =0.44
Stroma	16.3 (0-70)	32.5 (0-80)	ρ =0.32

Table VII 3. A comparison of the H scores of immunohistochemical staining for prolactin receptor in each compartment of endometrium tissue between 16 cases with different pregnancy outcomes

	Live birth group N=7	Miscarriage group N=9	ρ value
Luminal epithelium	205 (55-262.5)	240 (100-300)	ρ =0.61
Glandular epithelium	170 (0-255)	150 (40-245)	ρ =0.53
Stroma	5 (0-70)	35 (0-52.5)	ρ =0.23

3. The correlation between the endometrial prolactin receptor measurements and the demographics, plasma prolactin measurements and the psychometric measurements

Spearman's correlation analysis shows that there was a significant correlation between prolactin receptor expression in the luminal epithelium and the numbers of previous miscarriages ($p < 0.05$) (figure VII-4). Women with unexplained RM who had more previous miscarriages had higher expression of prolactin receptor in endometrium. No significant correlation was found between plasma prolactin measurements and the prolactin receptor measurements in various compartments of the endometrium tissue ($p > 0.05$) (table VII-4). Table VII-5 shows the correlation between endometrial prolactin receptor expression and the results of psychological measurements in the 22 cases. No significant correlation was found between endometrial prolactin measurements and psychometric measurements.

Table VII 4. The correlation between the H scores of endometrial prolactin receptor and the demographics and the plasma prolactin concentrations

	prolactin receptor expression in luminal epithelium	prolactin receptor expression in glandular epithelium	prolactin receptor expression in stroma
Age (years)	ρ =0.24	ρ =0.43	ρ =0.65
No. of previous miscarriages	ρ =0.03*	ρ =0.12	ρ =0.91
Plasma prolactin concentrations	ρ =0.11	ρ =0.18	ρ =0.19

Figure VII 3. A positive correlation between the H scores of prolactin receptor in luminal epithelium and the numbers of previous miscarriages

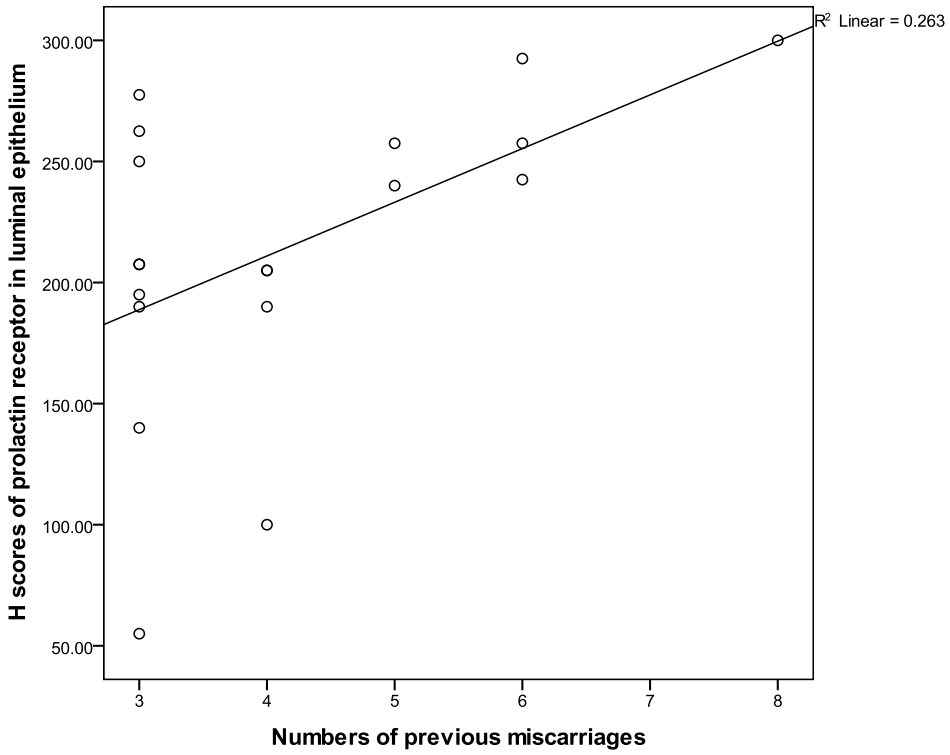


Table VII 5. Correlation between psychometric measurements and the expression of prolactin receptor in each compartment of endometrium in women with unexplained RM

	FPI	PSS	PANAS Positive Affect	PANAS Negative Affect
prolactin receptor in luminal epithelium n=22	ρ =0.16	ρ =0.32	ρ =0.62	ρ =0.65
prolactin receptor in glandular epithelium n=22	ρ =0.46	ρ =0.23	ρ =0.84	ρ =0.91
prolactin receptor in stroma n=22	ρ =0.22	ρ =0.06	ρ =0.14	ρ =0.20

V. Discussion

The expression of endometrial prolactin receptor in RM

In the preceding chapter we demonstrated a reverse association between plasma prolactin and pregnancy outcomes in women with unexplained RM. In this chapter we extended our study to the endometrial level to examine the relationship between the endometrial prolactin receptor and RM. In this present study we examined the expression of prolactin receptor in each endometrial compartment including luminal epithelium, glandular epithelium and stroma. Immunohistochemical localization revealed that endometrial prolactin receptor is strongly expressed in the epithelium of lumina and glands and weakly expressed by the stroma. This finding is consistent with previous studies which showed strong expression of endometrial prolactin receptor in epithelium layers of lumina and glands either in the mid to late luteal phase of endometrium or in deciduas (Jones et al., 1998, Garzia et al., 2004). In addition, we also found some individual cells which expressed prolactin receptor in the stroma of the mid-luteal phase of endometrium. In previous studies double immunofluorescence using antibodies against both prolactin receptor and CD56 showed that the prolactin receptor positive cells in stroma were also positive for CD56, suggesting that prolactin receptor was expressed in the uNK CD56 cell population in endometrial stroma (Gubbay et al., 2002). Therefore, the individual brown stained cells found in this study may be a subpopulation of uNK cells.

In this study we did not find any significant difference in the expression of the endometrial prolactin receptor between women with RM and fertile women. However, we found that the numbers of previous miscarriages was correlated with the expression of prolactin receptor in luminal epithelium.

Our finding is in contrast with a previous study in which a significant reduction of prolactin receptor expression in the endometrium was observed in women with RM (Garzia et al., 2004). In the study of Garzia et al, the quantity of the expression of endometrial prolactin receptor was examined by RT-PCR method and the localization of its expression in endometrium was examined by immunohistochemistry method. In our study we immunolocalized the pattern and site of endometrial prolactin receptor expression in women with RM and employed H-score to quantify the significance. H-score is a semi-quantitative calculation method which takes into the consideration of the staining intensity in conjunction with the percentage of positive stained cells. The results of H-score are subject to a variety of subjective factors, such as observers and quality of the staining. Therefore, different observers may produce different results based on their own judgment upon the extent of the staining. In the present study we also found discrepancy in the results of H-score of several slides between the two observers who conducted the scoring. We overcame this problem by identifying the disputed slides and double checking the results with the presence of both observers until the agreement was reached. Therefore, the finding of no significant differences in the expression of endometrial prolactin receptor in RM in this study with H-score method needs to be verified in a future study. In the future it will be of interest to combine both qualitative PCR and quantitative immunohistochemistry methods to further identify the expression of prolactin receptor in women with RM.

The relationship between endometrial prolactin receptor and pregnancy outcomes in women with RM

We examined the impact of endometrial prolactin receptor expression on pregnancy outcome in women with RM. So far this is the first study examining the relationship between endometrial prolactin receptor and pregnancy outcomes. In this study we did not find a significant association between prolactin receptor expression and pregnancy outcomes. Apart from the limitation of H-

score method we used in this study, it worth to note that the small numbers of cases included in this part of study may limit the power to detect a significant difference. Therefore, a future prospectively designed study with a larger sample size may be necessary to verify whether or not there is an association between prolactin receptor expression and pregnancy outcome in women with RM.

VI. Conclusion

In this study we identified the pattern and site of the expression of the prolactin receptor in the endometrium tissue in women with RM. We did not find a significant difference in the expression of the endometrial prolactin receptor expression between women with RM and fertile controls. Instead we found RM women with more previous miscarriages had significantly higher expression of prolactin receptor in the luminal epithelium. The exact mechanism for this observation is not clear. It will be of interest to employ both qualitative and quantitative methods in the future study to further examine the relationship between the expression of endometrial prolactin receptor and RM.

Chapter VIII: Measurement of cortisol in the evaluation of the relationship between stress and RM

I. Introduction

Cortisol is synthesized from cholesterol in the adrenal cortex and is the end product of the HPA (Hypothalamic-pituitary-adrenal) axis activation. Recently the measurement of cortisol as a biomarker in the evaluation of the HPA response to the psychological stress and related mental health disorders has been paid more attention. One of the prominent physiological changes in response to stress stimulation is the activation of the HPA system and the subsequent increased release of cortisol from the adrenal cortex into peripheral blood. Excessive secretion of glucocorticoids under stress exerts multiple metabolic effects and subsequently compromises homeostasis, thereby impairing pregnancy.

There is increasing evidence showing that psychosocial stress in the antenatal period may cause serum cortisol elevation (Diego et al., 2006, Demyttenaere et al., 1992, Wadhwa et al., 1996). For example, one study examined 54 women prior to 28 weeks gestation and found that the stress scores in these women were positively correlated with the serum cortisol elevation (Wadhwa et al., 1996). It has been suggested that high cortisol levels may have a negative effect on the establishment of pregnancy. For example, a study examined 40 women undergoing IVF treatment and found that women with higher concentrations of cortisol had poorer pregnancy rates following IVF than those with lower concentrations of cortisol (Demyttenaere et al., 1992). In a prospective study Nepomnaschy and colleagues measured peripheral serum cortisol concentrations in 22 pregnant women during the first three weeks after conception and found that women whose pregnancies subsequently miscarried (n=13) had significantly higher mean serum cortisol

concentrations than those whose pregnancies resulted in live births (n=9) (Nepomnaschy et al., 2006). In the same study population, they also found that women with high serum cortisol levels (n=12) were 2.7 times more likely to miscarry in their subsequent pregnancies than those with normal cortisol levels (n=10), suggesting that high levels of cortisol may contribute to miscarriage. However, the exact physiological mechanism of the adverse effect of high cortisol on pregnancy outcome is yet unknown.

The measurement of cortisol in the evaluation of the HPA function may be affected by various factors. Cortisol secretion has a circadian rhythm, usually peaking between 07:00 and 11:00 hours and declining across the day such that lower levels are present in the circulation between 21:00 and 00:00 hours (Nomura et al., 1997). In most studies the level of serum cortisol in the morning has been measured to evaluate the pituitary and adrenal function at the time of maximal secretion (Dickerson and Kemeny, 2004). Serum cortisol (hydrocortisone; 11β, 17α, 21-trihydroxy-4-pregnane-3, 20-dione) may be free or bound to corticosteroid-binding globulin (CBG) and albumin in the circulation (figure IV-1). Approximately 75~80% of cortisol in circulation is bound to CBG and 15% of cortisol is bound to albumin (Brien, 1981). Free cortisol which is the unbound portion is biologically active and measured as an index of cortisol concentration (Gozansky et al., 2005, Morineau et al., 1997).

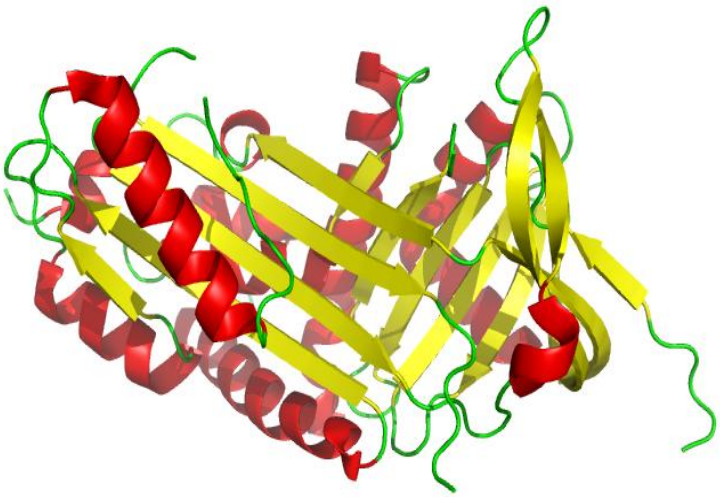


Figure VIII 1. Structure of corticosteroid-binding globulin in complex with cortisol. This work has been released into the public by its author I, Lijealso. This applies worldwide.

Recently, studies have reported the measurement of serum cortisol in the evaluation of HPA dysregulation and the escape of cortisol suppression in the HPA function test in women with psycho-psychiatric disorders (Fountoulakis et al., 2004, Wingenfeld et al., 2007, Gaab et al., 2003). The Dexamethasone Suppression Test (DST) has been widely used to assess pituitary and adrenal function. In normal subjects dexamethasone suppresses ACTH (Adrenocorticotrophic Hormone) and therefore cortisol secretion. Significantly impaired adrenocortical responsiveness following the DST was found in subjects with depression or borderline personality disorder, compared with healthy subjects (Wingenfeld et al., 2007, Fountoulakis et al., 2004). Half of the patients with a depressive syndrome were found to be the non-DST suppressors and DST results were shown to be a useful measure of depression rather than symptomatology (Fountoulakis et al., 2004). It was suggested that DST non-suppression in women with distress was due to the development of a reversible glucocorticoid resistance with compromise of the inhibitory feedback of exogenous cortisol on the HPA axis after the DST (Wingenfeld et al., 2007, Stokes and Sikes, 1988, Herman and Cullinan, 1997). In addition, significant menstrual cycle changes in cortisol suppression after dexamethasone administration have been found. Tandon et al conducted a study in 25 hospitalized women with major depression who had weekly DST measurements. They found that cortisol suppression were lowest at 3.84 μg/dl during menstrual period, rose to 4.94 μg/dl in the follicular phase, reached a peak at 7.61 μg/dl in the periovulation period and declined in the luteal phase to 5.00 μg/dl (Tandon et al., 1991). This finding suggests that the phase of the menstrual cycle is an important confounding variable in the interpretation of the result of the DST in women. There is likely to be less suppression with DST in women in the luteal phase than in the follicular phase. Therefore, cortisol suppression in women is usually assessed in the follicular phase to precisely reflect the pituitary-adrenal function. Other factors that may cause false positive responses in the DST test include malabsorption of dexamethasone, drugs that increase the hepatic clearance of dexamethasone and oestrogen therapy or pregnancy (Newell-Price, 2008).

The alteration of HPA function in response to stress is modulated by several other factors. The sampling of serum cortisol involves venipuncture which can act as an acute stressor to increase HPA activity and therefore potentially giving a false positive result of HPA activation. It is not practical to perform a series of blood collections throughout normal daily activity to assess the HPA function in routine clinical practice. Recently studies have provided evidence that urinary and salivary cortisol can be used as surrogates for plasma cortisol with the advantage of non-invasive sample collection and ease of repetition (Gozansky et al., 2005, Nakamura et al., 2008). It has been found that 24-hour urinary cortisol value has a positive and significant correlation with plasma cortisol (Nelson et al., 2002). Urinary cortisol increased in response to stress and its increases were associated with the intensity of the stress (Smitha and French, 1997).

Recently the advantage of the measurement of salivary cortisol as a surrogate to serum cortisol in the study of the HPA axis has received more attention (Perogamvros et al., 2010b, Perogamvros et al., 2009). Salivary cortisol at 23:00 hours has been proposed to replace serum midnight cortisol due to its sensitivity and specificity in diagnosis for the presence of subtle cortisol excess (Putignano et al., 2001, Gozansky et al., 2005, Perogamvros et al., 2010b, Perogamvros et al., 2009). Salivary cortisol reflects the bioactive fraction of serum free cortisol and is not affected by changes in cortisol-binding globulin (Perogamvros et al., 2010b). Salivary cortisol as a practical surrogate to serum free cortisol has been found to be increased under stress stimulation (Federenko et al., 2004) and its elevation is positively correlated with the stress scores (Ng et al., 2003). Studies compared the utility of salivary cortisol and serum cortisol in the measurement of psychological dysfunction of the HPA axis and concluded that saliva is a sensitive and reliable medium for cortisol measurement (Deutschbein et al., 2009, Baghai et al., 2002). In studies comparing salivary cortisol with simultaneously obtained serum cortisol in assessing the dynamic

HPA axis activity, salivary cortisol demonstrated a higher sensitivity and specificity in the HPA function test than serum cortisol (Gozansky et al., 2005, Perogamvros et al., 2010a), suggesting that measurement of salivary cortisol may replace serum cortisol with the advantage of non-invasive sample collection and free of cortisol-binding globulin influence.

The immunoassay for the measurement of salivary cortisol is affected by variable cortisol interferences. The enzyme 11β-hydroxysteriod dehydrogenase type2 (11β-HSD2) expressed in the parotid glands is capable of converting active cortisol into inactive cortisone, which causes the cross-reactivity of cortisone in the determination of cortisol concentration with immunoassays (Smith et al., 1996). To address this limitation, Perogamvros et al conducted a series of studies to develop a novel, robust liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay for the combined measurement of salivary cortisol and salivary cortisone (Perogamvros et al., 2009). They compared the value of salivary cortisol measurement of LC-MS/MS assay with serum free cortisol measurement in the HPA function test and found that salivary cortisol measurement is superior to serum cortisol measurement with a higher sensitivity and specificity (Perogamvros et al., 2010b). In the present study we employed this accurate assay to precisely measure salivary cortisol in the stress assessment of women with unexplained RM.

The aim of this study was to determine if RM was associated with the feedback dysregulation of the HPA axis by measuring cortisol suppression in serum and saliva following the Dexamethasone Suppression Test and basal midnight salivary cortisol.

II. Materials and Methods

1 Subjects

Among 36 participants who were recruited in the stress and RM project, six of them declined the Dexamethasone Suppression Test (DST) due to concerns about the side effects of steroid administration; four of them canceled the DST after they were prescribed the dexamethasone due to time pressure; one of them could not attend the scheduled appointment for the blood test after the dexamethasone administration due to heavy snow.

The remaining 25 participants had blood samples taken for the morning serum cortisol measurement following the DST. 19 of them had primary RM and 6 of them had secondary RM. They all met the inclusion criteria of unexplained RM and none of them had the pituitary disorders, psychiatric illnesses or depression. They were informed of the nature of the study with a patient information leaflet and gave the written consent before the commencement of the cortisol measurement. The study was approved by the South Sheffield Research Ethics Committee (08/H1308/80).

Among these 25 participants who underwent serum cortisol measurements following DST, three of them reported omitting saliva collection and thus did not collect saliva samples; three of them forgot to collect saliva samples with DST. Therefore, a total of 19 participants collected saliva samples on two occasions in correspondence to the study protocol for the salivary cortisol measurements.

2 Procedure

2.1 Serum Cortisol Measurement

All the eligible participants were given an instruction leaflet on the Low Dose Overnight Dexamethasone Suppression Test (DST) for serum cortisol measurement at the RM clinic. The DST was explained in detail by the investigator. They were reassured that no side effect would occur with the administration of a single low dose of exogenous glucocorticoid. They were required to take a tablet of Dexamethasone at the dose of 1mg at 23:00 hours on day 3, 4 or 5 of the menstrual cycle. At 9:00 hours the next morning a blood sample was obtained for serum cortisol measurement by the specialist nurse on the ward G1 in the Royal Hallamshire Hospital. The samples were analyzed in the Clinical Chemistry department of the Royal Hallamshire Hospital using the serum cortisol assay.

2.2 Salivary Cortisol Measurement

Salivary cortisol was measured during the DST. Participants were given sample collectors (Salivette, SARSTEDT, UK) to collect the midnight saliva samples at 23:00 hours at home prior to dexamethasone administration. Patients were instructed to refrain from brushing their teeth, smoking, eating or drinking anything but water for at least 60 minutes prior to sampling. The saliva sample was obtained by chewing the cotton wool swab which contained citrate acid to simulate the saliva flow. A sufficient volume of saliva was achieved when the swab was fully wet. The participants were required to store the samples in the freezer at home and return them back to the investigator the next morning on their visit for the blood tests. A second saliva sample was collected simultaneously with the collection of a blood test for the morning serum cortisol measurement as part of the dexamethasone suppression test. The saliva fluid was recovered from

the swab by spinning the container at 1000g for 5 minutes in the centrifuge and was stored at -20 °C in the freezer for later analysis in batches in the University of Manchester.

3 Methods

3.1 Serum Cortisol Measurement

Serum cortisol was measured by the Clinical Chemistry Department in the Royal Hallamshire Hospital with Bayer Advia Centaur automated immunoassay (Bayer, Newbury, UK). The interassay coefficient of variation (CV) was 7% at 200nmol/l and 8% at 1050nmol/l. The intraassay CV was 4% at 28ng/l. The laboratory reference of the 1mg Dexamethasone Suppression Test is set at the suppression of the morning serum cortisol to less than 50nmol/l.

3.2 Salivary Cortisol Measurement

Salivary cortisol was measured with a liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay in the Department of Biochemistry in the University of Manchester with a lower limit of quantitation at 0.39nmol. The intra-assay CVs was 9.3% and inter-assay CVs was 9.7% at 1.8-52.2 nmol/L of salivary free cortisol. The cut-off value for the midnight salivary cortisol estimation was recommended as 4nmol/l in LC-MS/MS assay with no blood contamination in the samples (Newell-Price, 2009).

III. Statistical Analysis

Statistical analyses were performed using the SPSS (Statistical Package for the Social Sciences) version 16. Differences in the concentration of cortisol measurements between cases with different conception outcomes or different pregnancy outcomes were analyzed with independent T test. The correlation between cortisol measurements and case demographics was analyzed with Pearson Correlation coefficient test. The correlation between cortisol measurements and psychometric measurements was analyzed by Spearman's rank correlation coefficient test. Statistical significance was set at p value <0.05.

IV. Results

3.1 Serum cortisol measurements

Among 25 patients who underwent the morning serum cortisol measurements following DST, 20 of them had primary RM and 5 of them had secondary RM. The demographics of these 25 patients are shown in table VIII-1. There was no significant difference in cortisol suppression between cases who subsequently conceived and those who did not, or between cases who had subsequent live births and those who had miscarriages again ($\rho>0.05$) (figure VIII-2, 3).

Table VIII 1. Demographics of the 25 cases with serum cortisol measurements

Cases with serum cortisol measurements N=25	
Age (years)	35.7 ± 0.9
No. of previous miscarriages median (range)	4 (3-8)
Serum cortisol suppression (nmol/l)	23.5 ± 4.3

All the values are shown in the form of mean ± SE unless otherwise specified.

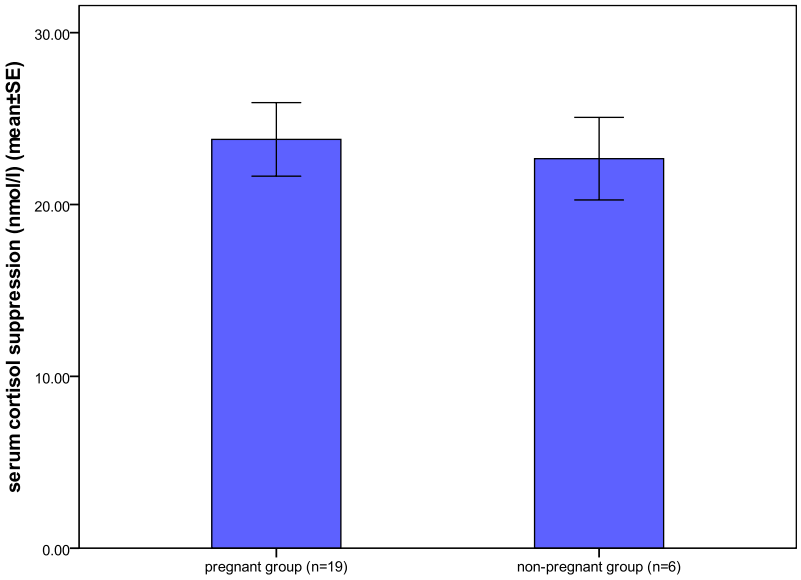


Figure VIII 2. A comparison of the resluts of serum cortisol suppression between 25 cases with different conception outcomes

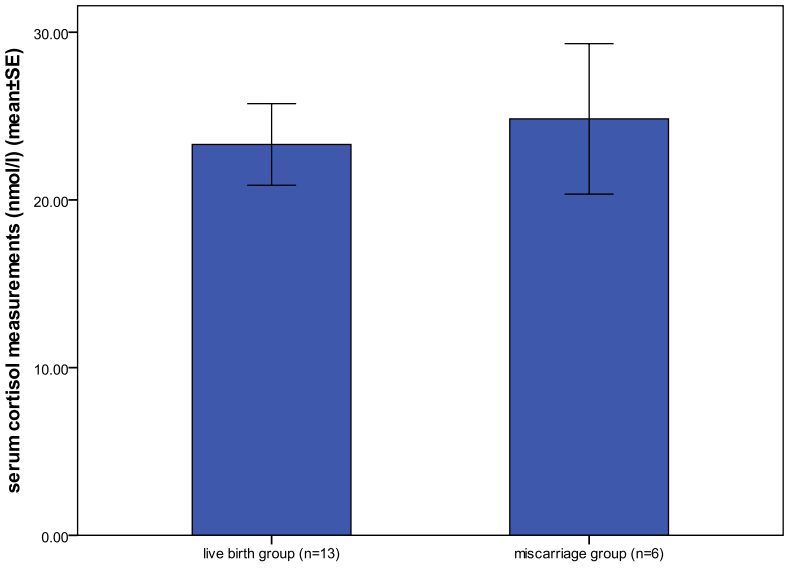


Figure VIII 3. A comparison of the results of serum cortisol suppression between 18 cases with different pregnancy outcomes

3.2 Salivary cortisol measurements

Among the 19 cases with salivary cortisol measurements, 14 of them had primary RM and 5 of them had secondary RM. Table VII-2 shows the demographics of these 19 cases. There was no significant difference in the mid-night basal salivary cortisol measurements between cases with different conception outcomes or different pregnancy outcomes ($p>0.05$). The same pattern was seen in the morning salivary cortisol suppression where no significant difference was seen between cases with different conception outcomes or different pregnancy outcomes ($p>0.05$) (Table VII-3,4).

Table VIII 2. Demographic characteristics of 19 cases with salivary cortisol measurements

Cases with salivary cortisol measurements N=19	
Age (years)	36 ± 1
No. of previous miscarriages median (range)	4 (3-8)
Mid-night salivary cortisol (nmol/l)	2.5 ± 0.9
Morning salivary cortisol (nmol/l)	1.9 ± 0.7

All the values are shown in the form of mean ± SE unless otherwise specified.

Table VIII 3. The comparison of mid-night salivary cortisol concentrations and morning salivary cortisol suppression between 19 cases with different pregnancy outcomes

	Pregnant group N=13	Non-pregnant group N=6	ρ value
Mid-night salivary cortisol (nmol/l)	1.2 ± 0.4	5.2 ± 2.9	> 0.05
Morning salivary cortisol (nmol/l)	1.7 ± 0.9	2.3 ±1.5	> 0.05

All the values are shown in the form of mean ± SE.

Table VIII 4. The comparison of the mid-night salivary cortisol concentrations and morning salivary cortisol suppression between 13 cases with different pregnancy outcomes

	Live birth group N=7	Miscarriage group N=6	ρ value
Mid-night salivary cortisol (nmol/l)	0.8 ± 0.4	1.7 ± 1.0	> 0.05
Morning salivary cortisol (nmol/l)	2.4 ± 1.6	0.8 ± 0.1	> 0.05

All the values are shown in the form of mean ± SE.

3.3 The correlation between serum cortisol and salivary cortisol

There was a significant positive correlation between the morning serum cortisol suppression following DST and morning salivary cortisol suppression ($p=0.05$) (figure VII-5). No significant association was seen between serum cortisol suppression and the basal midnight cortisol measurements ($p=0.35$). There was also no significant correlation shown between the morning serum cortisol suppression and the mid-night salivary cortisol measurements ($p=0.38$). Cortisol measurements in both serum and saliva were found to have no association with the demographics of age and the numbers of previous miscarriages ($p>0.05$) (table VII-5).

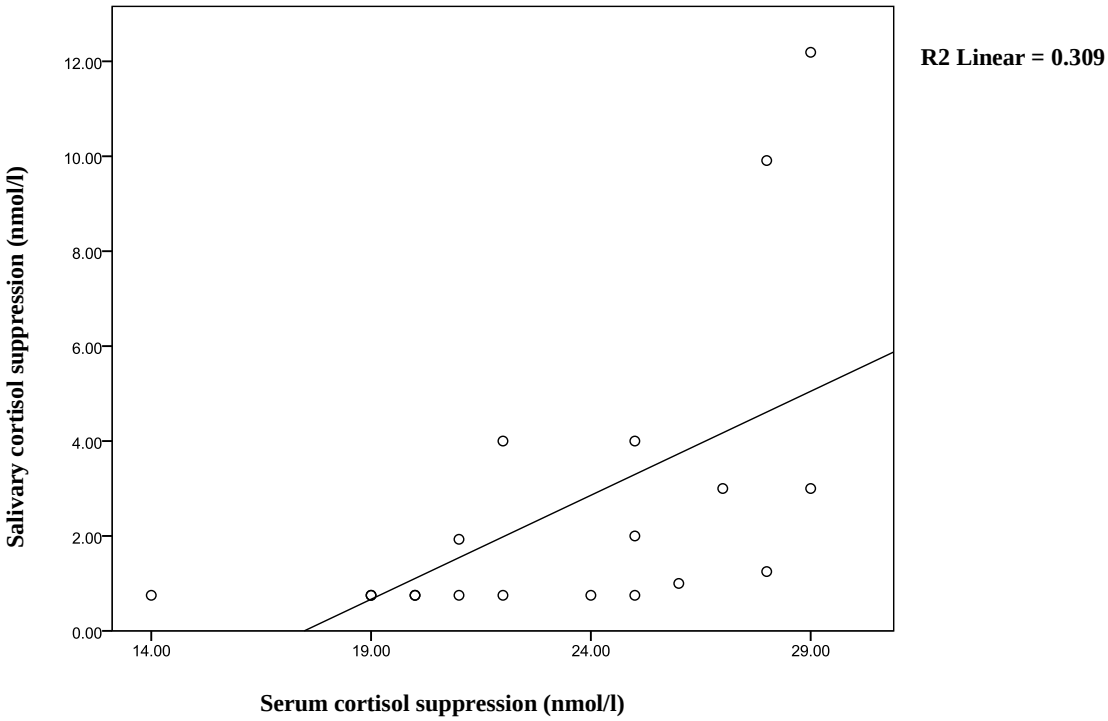


Figure VIII 4. The correlation between the results of serum cortisol and salivary cortisol following 1mg Dexamethasone Suppression Test

Table VIII 5. The correlation between serum cortisol measurements and salivary cortisol measurements and the demographics

	Serum cortisol suppression	Morning salivary cortisol suppression	Midnight basal salivary cortisol
Age	ρ= 0.45	ρ= 0.62	ρ= 0.06
No. of previous miscarriages	ρ= 0.54	ρ= 0.49	ρ= 0.33

3.4 The association between psychometric measures and cortisol measurements

No significant correlation was found between cortisol measurements in serum and saliva and the each individual psychometric measures ($\rho>0.05$) (Table VII-6).

Table VIII 6. The correlation between the scores of psychometric measurements and results of cortisol measurements in the 19 cases

	Serum cortisol	Salivary cortisol	
		Midnight salivary cortisol	Morning salivary cortisol
FPI	$\rho= 0.11$	$\rho= 0.63$	$\rho= 0.88$
PSS	$\rho= 0.81$	$\rho= 0.41$	$\rho= 0.66$
PANAS Positive Affect	$\rho= 0.82$	$\rho= 0.16$	$\rho= 0.99$
PANAS Negative Affect	$\rho= 0.23$	$\rho= 0.32$	$\rho= 0.62$

V. Discussion

The relationship between stress and cortisol alteration

There is an increasing body of studies using cortisol as a biochemical marker in research on stress. In animal models, it has been demonstrated that assessment of the sensitivity of the HPA glucocorticoid feedback system is a better diagnostic tool for stress than the basal cortisol measurements (Kalin et al., 1981). In the human non-suppression following the Dexamethasone Suppression Test (DST) has been found to be associated with stress symptoms such as depression and stressful live events in which 35% patients were non-suppressors compared with only 6% of non-suppressors in healthy controls (Faravelli et al., 2010), suggesting that the DST is an useful screen test in stress assessment.

In this study we hypothesized that chronic stress caused by RM impaired the feedback regulation of the HPA system. Therefore, we employed the usual low dose 1mg DST to measure the HPA response to the stress from RM. Apart from measuring the serum cortisol suppression, the novelty of the present study lies in employing concurrent salivary cortisol measurements during the DST to more precisely identify the HPA response to RM stress. To overcome interference of the cross-reactivity of cortisone in the determination of cortisol concentration with immunoassays, the salivary cortisol assay was performed in collaboration with the clinical laboratory of the Manchester University using a novel robust liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay. In this study we found that the morning serum cortisol suppression is correlated with the suppression in the morning salivary cortisol, supporting the notion that salivary cortisol measurement is consistent with serum cortisol measurements and salivary cortisol is a sensitive surrogate to serum cortisol in the HPA function test for stress assessment.

In the present study we did not find any case of non-suppression in the morning serum cortisol following DST in women with stress from RM. With regard to the effect of stress on cortisol alteration, no correlation was shown between the intensity of stress in women with RM and the cortisol measurements both in serum and saliva. When using the cut-off value of late night salivary cortisol at 4 nmol/l (Newell-Price, 2009), only 3 out of 19 participants had high midnight salivary cortisol measurements.

Despite of increasing interests in using cortisol as a biochemical marker to assess stress, empirical studies assessing the association between cortisol alteration and stress have yielded conflicting findings. In contrast to early studies where positive correlation between cortisol non-suppression in DST and stress were demonstrated (Quenby et al., 2005, Wingenfeld et al., 2007), other studies did not find the association between the feedback dysfunction of the HPA system and stress (Lazarus and Folkman, 1984, Lange et al., 2005). As an example, Lange et al found no increase in the incidence of cortisol non-suppression following DST in subjects with personality disorders compared with healthy controls (Lange et al., 2005). In another study examining the association between stress and the cortisol alteration in saliva in 78 nurses who experienced examination stress, they found no significant change in salivary cortisol concentration before and after stress (Takatsuji et al., 2008).

It has been proposed that the type of stress may be an important factor in determining the DST response. For example, in a study assessing the effect of childhood trauma on the feedback dysregulation of the HPA system using DST as the method, they found that there was no significant difference in scores of psychometric measurements between suppressors and non-suppressors. However, non-suppressors had a significantly higher occurence of some types of stress symptoms than suppressors, suggesting that certain types of stress cause activation of the

HPA dysfunction, rather than the severity of stress (Quenby et al., 2005). In addition, another study found an enhanced suppression of salivary cortisol following DST in women with stress from chronic pelvic pain, indicating that in contrast to the current assumption of non-suppression of cortisol in women with stress, enhanced suppression may
exist in a stressful condition (Kwak-Kim and Gilman-Sachs, 2008) . In the current literature, the use of DST as a diagnostic method has been extended to a number of psychiatric disorders without consensus on the type of stress measured, the psychometric measures adapted and the dose of dexamethasone administered in DST. As a result of these methodological issues, it is not surprising that conflicting findings are presented between studies.

Our study is the first study employing DST in the assessment of stress in women with RM. Assessment of stress using DST is an imprecise measure of the HPA axis function. Failure of cortisol suppression in response to dexamethasone represents an abnormal physiological state which can be the result of several separate dysfunctions involving activation and interaction of neuronal and endocrine pathways involved in the HPA systems. Failure of cortisol suppression in response to dexamethasone has been used as a screening test in several studies in psychiatry, but has not been used to assess stress responses in patients without psychiatric disorders but who are placed in abnormal stress situations, such as women experiencing recurrent miscarriages. Evaluation of the precise neuroendocrine changes in the HPA system in stress condition is beyond the scope of this study. Based on the findings presented in the current study, it is likely that subclinical stress experienced by women with RM does not impair the feedback regulation of the HPA system.

The relationship between cortisol concentrations and pregnancy

Previous studies have found high serum cortisol concentrations are associated with a poor conception rate and an increased risk of miscarriage (Nepomnaschy et al., 2006, Demyttenaere et al., 1992). However, other studies failed to confirm an association between serum cortisol concentrations and the risk of miscarriage (Nelson et al., 2003a). The lack of consensus on the methods used to measure cortisol concentrations between studies may account for this variation. For example, some studies measured cortisol concentrations with serum samples 4 times a day in the first trimester (Seng et al., 2005) whereas others measured cortisol with first-morning urinary samples every other day three times a week (Nepomnaschy 2006). Therefore, the results from studies with different methodologies are not comparable and may lead to conflicting findings.

In this prospective study we compared cortisol suppression following DST in both serum and saliva, and the basal midnight salivary cortisol concentration between unexplained RM women with different conception outcomes and pregnancy outcomes. No association was found between cortisol concentrations and the conception probability or pregnancy outcomes in women with unexplained RM. However, we should note that the finding from the present study was obtained from a group of women with cortisol suppression within normal physiological range. None of the cases in this study had non-suppression of cortisol. It will be of interest in the future to examine the impact of cortisol concentrations on pregnancy outcomes with a more diverse study population stratified by different degrees of cortisol suppression. In addition, the limitations of the number of patients with cortisol measurements and the insufficient numbers in each subgroup in the analysis comparing different pregnancy outcomes may be inadequate to pick up the true difference. In the future study these issues should be addressed by using a larger sample size.

VI. Conclusion

In this study we found that women with unexplained RM had normal cortisol suppression in the low dose of 1mg Dexamethasone Suppression Test. Cortisol concentrations in women with RM have no predictive value on conception rates and pregnancy outcomes. Based on the results derived from this study, we speculate that chronic RM-related stress has no effect on HPA feedback dysregulation. The findings in the present study need to be verified in future studies.

Chapter IX: The correlation of various biochemical stress markers in women with unexplained RM

Results

A total of 19 women with unexplained RM had the measurements of all the stress markers, including NK cell measurements both in peripheral blood and endometrium, cortisol measurements both in serum and saliva, and the measurements of prolactin in plasma and its receptor in endometrium. Table IX-1 shows the correlation between NK cell measurements and cortisol measurements and prolactin measurements in the 19 women with unexplained RM. Table IX-2 shows the correlation between cortisol measurements and prolactin measurements in the 19 women with unexplained RM.

Cortisol measurements were found to be significantly associated with NK cell measurements. Serum cortisol suppression was found to be inversely correlated with pNK CDbright expression ($\rho<0.05$) (figure IX-1). A lesser degree of serum cortisol suppression is associated with an increased expression of pNK CDbright subset. Salivary cortisol measurements were found to have a significant association with uNK cell measurements ($\rho<0.05$) (table IX-2). Increased uNK cell measurements were associated with an increase in the basal mid-night salivary cortisol levels and a decrease in the morning salivary cortisol suppression (figure VIII-2,3)

Table IX 1. The correlation between NK cell measurements and the measurements of cortisol and prolactin (n=19)

		NK cell measurements		
		pNK CDdim%	pNK CDbright%	uNK%
Cortisol measurements	Serum cortisol suppression (nmol/l)	ρ = 0.58	ρ = 0.02*	ρ = 0.85
	Moring salivary cortisol suppression (nmol/l)	ρ = 0.19	ρ = 0.59	ρ = 0.03*
	Midnight salivary cortisol(nmol/l)	ρ = 0.14	ρ = 0.81	ρ = 0.02*
Prolactin measurements	Plasma prolactin (mIU/l)	ρ = 0.12	ρ = 0.31	ρ = 0.87
	Prolactin receptor in luminal epithelium	ρ = 0.39	ρ = 0.47	ρ = 0.48
	Prolactin receptor in glandular epithelium	ρ = 0.73	ρ = 0.62	ρ = 0.32
	Prolactin receptor in stroma	ρ = 0.79	ρ = 0.97	ρ = 0.82

Table IX 2. The correlation between the results of cortisol measurements and the plasma prolactin concentrations and the H scores of endometrial prolactin receptor (n=19)

		Cortisol measurements (nmol/l)		
		Serum cortisol suppression	Salivary cortisol suppression	Midnight salivary cortisol
Prolactin measurements	Plasma prolactin (mIU/l)	$\rho = 0.60$	$\rho = 0.51$	$\rho = 0.51$
	prolactin receptor in luminal epithelium	$\rho = 0.76$	$\rho = 0.84$	$\rho = 0.83$
	Prolactin receptor in glandular epithelium	$\rho = 0.76$	$\rho = 0.10$	$\rho = 0.75$
	Prolactin receptor in stroma	$\rho = 0.51$	$\rho = 0.98$	$\rho = 0.75$

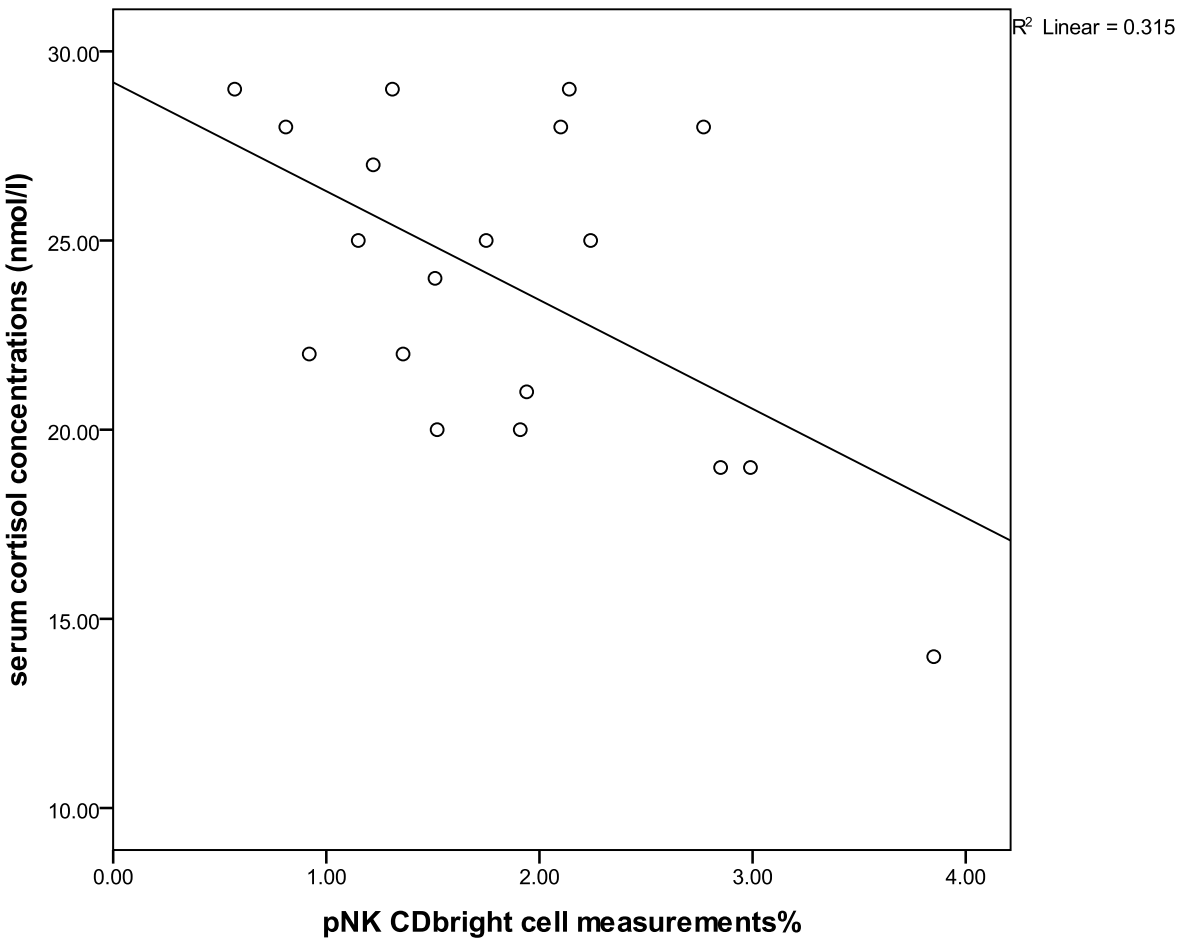


Figure IX 1. The correlation between the results of peripheral NK CDbright cells and the concentrations of serum cortisol suppression following the Dexamethasone Suppression Test

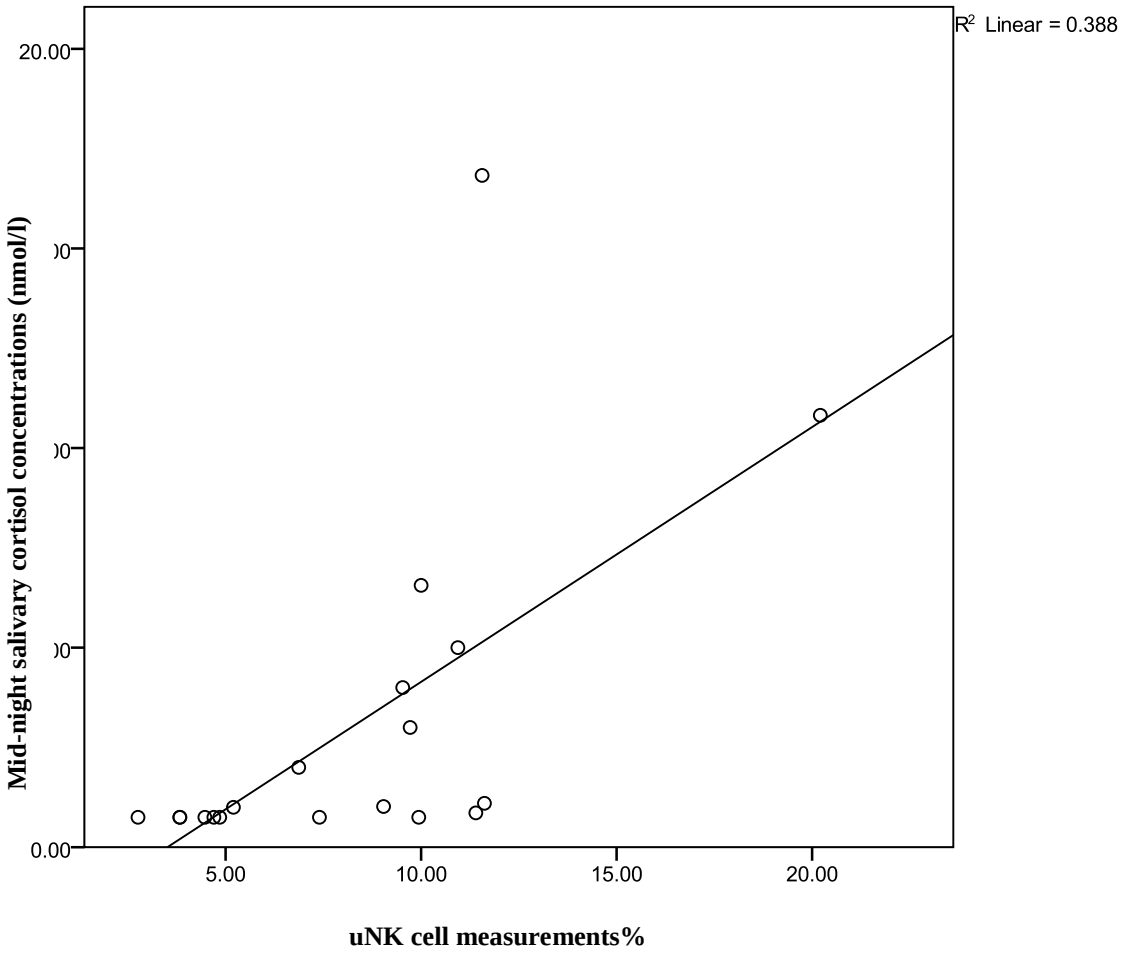


Figure IX 2. The correlation between uNK cell measurements and midnight salivary cortisol measurements

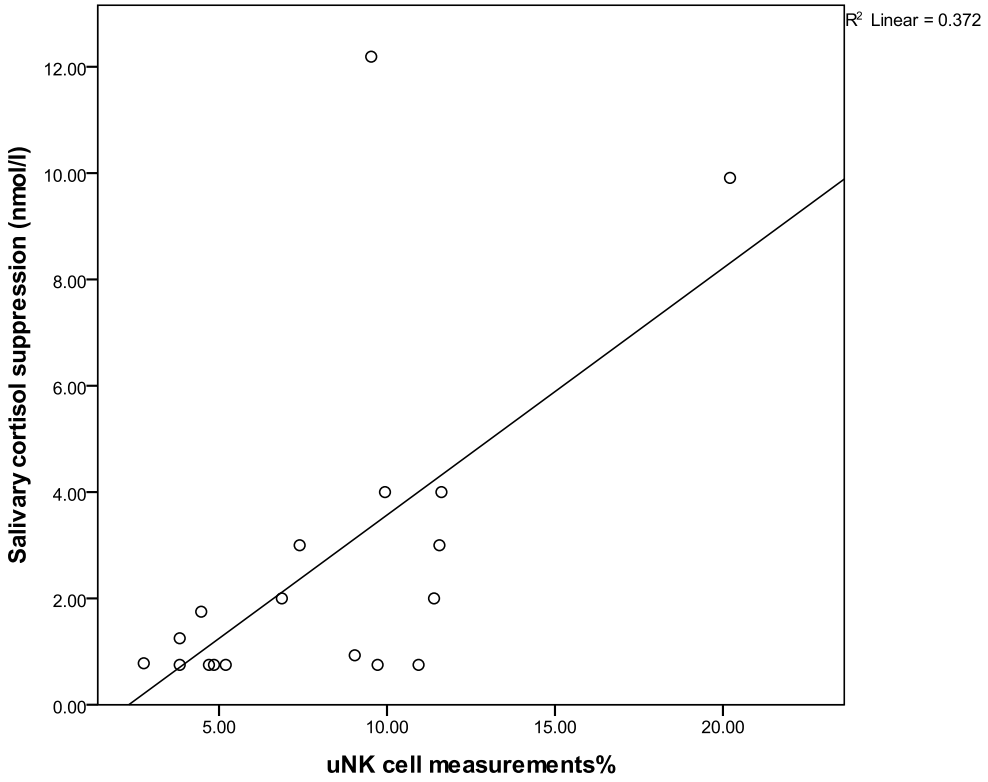


Figure IX 3. The correlation between uNK cell measurements and the measurements of morning salivary cortisol suppression following the Dexamethasone Suppression Test

Discussion

In the previous chapters of this thesis we have examined the relationship between psychometric measurements and the measurements of each biochemical stress markers. In this chapter we correlated the results of various biochemical stress markers with each other to further investigate whether there are interactions among these stress markers in women with RM.

Cortisol measurements have been found to be significantly associated with NK cell measurements. An inverse correlation between serum cortisol suppression following DST and pNK CDbright cells has been found. Morning salivary cortisol suppression following DST has a positive correlation with uNK cell measurements. Basal mid-night salivary cortisol concentrations are positively associated with the increase in uNK cell measurements. Therefore, it seems that the cortisol concentrations can affect the numbers of pNK or uNK cells in women with RM or vice versa. The underlying reason for such an observation is unclear. Some previous studies have showed the effect of cortisol on inhibiting peripheral natural killer cells (van Ierssel et al., 1996) and uNK cell expression (Quenby et al., 2005). Future research needs to be conducted to clarify this issue.

Chapter X: Overall Conclusion

In summary, in this thesis we found that women with RM had higher levels of stress than fertile women. Higher fertility stress levels are associated with a better pregnancy outcome in women with RM, suggesting that coping strategies may be of more prognostic value on subsequent pregnancy outcomes than measuring stress levels in women with RM. We also found that the increase in the pNK subset of CDdim cells was associated with a higher risk of a subsequent miscarriage in women with RM, suggesting the role of the increase of pNK CDdim subset in RM. No correlation was found between pNK cell measurements and uNK cell measurements, suggesting that pNK cell measurements do not reflect uNK cell measurements and therefore it is inappropriate to extrapolate the findings of NK cell measurements in peripheral blood to the endometrium. In this thesis low plasma prolactin concentrations within normal physiological range were associated with an increased risk of miscarriage in women with RM, suggesting that adequate plasma prolactin concentrations may benefit pregnancy. However, no significant difference in the expression of endometrial prolactin receptor was found between RM women and fertile women. In this thesis we did not find the effect of stress caused by RM on the feedback dysregualtion of the HPA axis. Cortisol measurements have been found to have no association with subsequent conception rates or pregnancy outcomes in women with RM.

With regard to the interaction between various stress measurements, pNK CDbrigtht cells have been found to be inversely associated with the levels of fertility stress, suggesting a role of pNK CDbright cells as a sensitive biochemical marker in stress

assessment in women with RM. Cortisol measurements were found to be associated with NK cell measurements in women with RM or vice versa. The increase in serum cortisol concentrations after DST was associated with a decrease in pNK CDdim cells whereas the increase in salivary cortisol concentrations after DST was associated with an increase in uNK cells. In addition, the increase in the basal mid-night salivary cortisol concentrations was associated with an increase in uNK cells. The exact mechanism of how cortisol affects NK cells in women with RM is not clear.

Strength and weakness

Strength

The novelty of the work presenting in this thesis lies in the comprehensiveness of the measurements used in examining the role of stress in RM. This study employed a multifaceted approach including psychometric, endocrinological, immunological and histological measurements. This is the first study which thoroughly examined stress in women with RM from both psychological and physiological aspects.

In the present study we only included a cohort of women with unexplained RM as the study population. It was clearly important to study a homogenous group of unexplained RM patients to enable us to exclude other causative factors of RM. This allowed stress to be studied as the only identifiable confounding factor affecting pregnancy in the present study analysis.

At present there is no consensus on the definition of RM with regard to the numbers of previous miscarriages and previous live births. In this thesis we followed the

definition of RM consistent with European criteria proposed by ESHRE in 2006, i.e three or more consecutive miscarriages. Participants who had previous live births were also included in this study, as it has been shown that a preceding live birth has no impact on a subsequent pregnancy (van den Boogaard et al., 2010).

Weakness

One possible criticism of this study is the relatively small number of subjects presenting in the analyses. The limited number of the eligible referrals attending the RM clinic each week restricted us to recruit more participants. In the future a multicenter approach should be involved to overcome this issue.

Other factors to be considered in the stress study are the ethnic, educational and occupational backgrounds. Among 36 women with unexplained RM presenting in the study, two of them were of Asian Indian origin and two of them were of African origin. The rest of the 32 participants were white Caucasian. The small number of minority ethnic group members did not allow us to perform a subgroup analysis to examine the difference in stress status stratified by ethnicity. Further information about educational and occupational background was not collected during the information collection from participants due to ethical concerns. The patients many have become anxious that their education or occupation was related to the cause of their miscarriages if these questions were asked by the investigators. This could potentially cause additional psychological burden and affect their stress status. In the future it will be of interest to include a more diverse population stratified by ethnic, educational and occupational backgrounds to compare the stress levels between these women.

Another concern in the present studies is that all the stress measurements were based on samples collected in the non-conception cycle. These results may not be extrapolated into changes in conception cycle where pregnancies are established. The psycho-neuroendocrine-immune network is altered during pregnancy, thus the results from nonconception cycles may be of limited predictive value for pregnancy. However, it is difficult to conduct a clinical research obtaining samples from ongoing pregnancy to precisely reflect the stress status of pregnant women with RM.

In addition, another weakness of this thesis lies in the confounding effect of anxiety before venipuncture on random prolactin assays. Some patients may become very anxious or stressed before venipuncture. Like plasma cortisol, the anxiety of venepuncture can cause a falsely elevated prolactin level. As a result, our hospital, on its intranet site, has a Cannulated Prolactin Protocol to re-assess basal prolactin levels for any hyperprolactinaemia after random levels. In our retrospective study on the relationship between plasma prolactin concentrations and pregnancy outcomes we obtained the data of the 174 consecutive women with RM, who had plasma prolactin measured between 1999 and 2009, from the existing clinical database. It is not clear whether this protocol was always followed in the clinic.

Proposal for future studies

Proposed prolactin receptor study

Owing to the small numbers of biopsies in this study we could not determine if endometrial prolactin receptor measurements has a prognostic value on the outcome of a subsequent pregnancy in women with recurrent miscarriage. It is possible to

examine achieved endometrial specimens collected over the last 10 years from the RM clinic at the Jessop Wing Hospital, Sheffield to further investigate the relationship between the expression of prolactin receptor and pregnancy outcomes in women with RM. It is estimated that almost at least 100 biopsies could be retrieved for analysis.

Proposed stress study

The results of this thesis suggest that coping strategies may be of predictive value on subsequent pregnancy outcomes, more than pre-pregnant stress levels. Therefore, in the future it will be of interest to conduct a study assessing the relationship between stress coping strategies and pregnancy outcomes in women with RM. The pregnancy outcome of women with RM may be improved by using effective coping intervention tools such as the positive reappraisal intervention card (Lancastle and Boivin, 2008), instruments designed for improving quality of life for women experiencing fertility treatments (www. fertiqol.org) or information and support for women who suffer miscarriages available on the websites of miscarriage association (www.miscarraigeassociation.org.uk).

The results of this research have implications for future clinical consultations where there is a need to help decrease the stress experienced by women with RM. Patients should be encouraged to adopt effective coping strategies and/or seek emotional support to optimize their pregnancy outcomes in conjunction with clinical treatments, although more research is needed in this area. It is anticipated that women with RM who adapt effective stress coping strategies may respond to stress

well with no activation of the HPA system, and no alteration in the measurements of natural killer cells, prolactin and cortisol.

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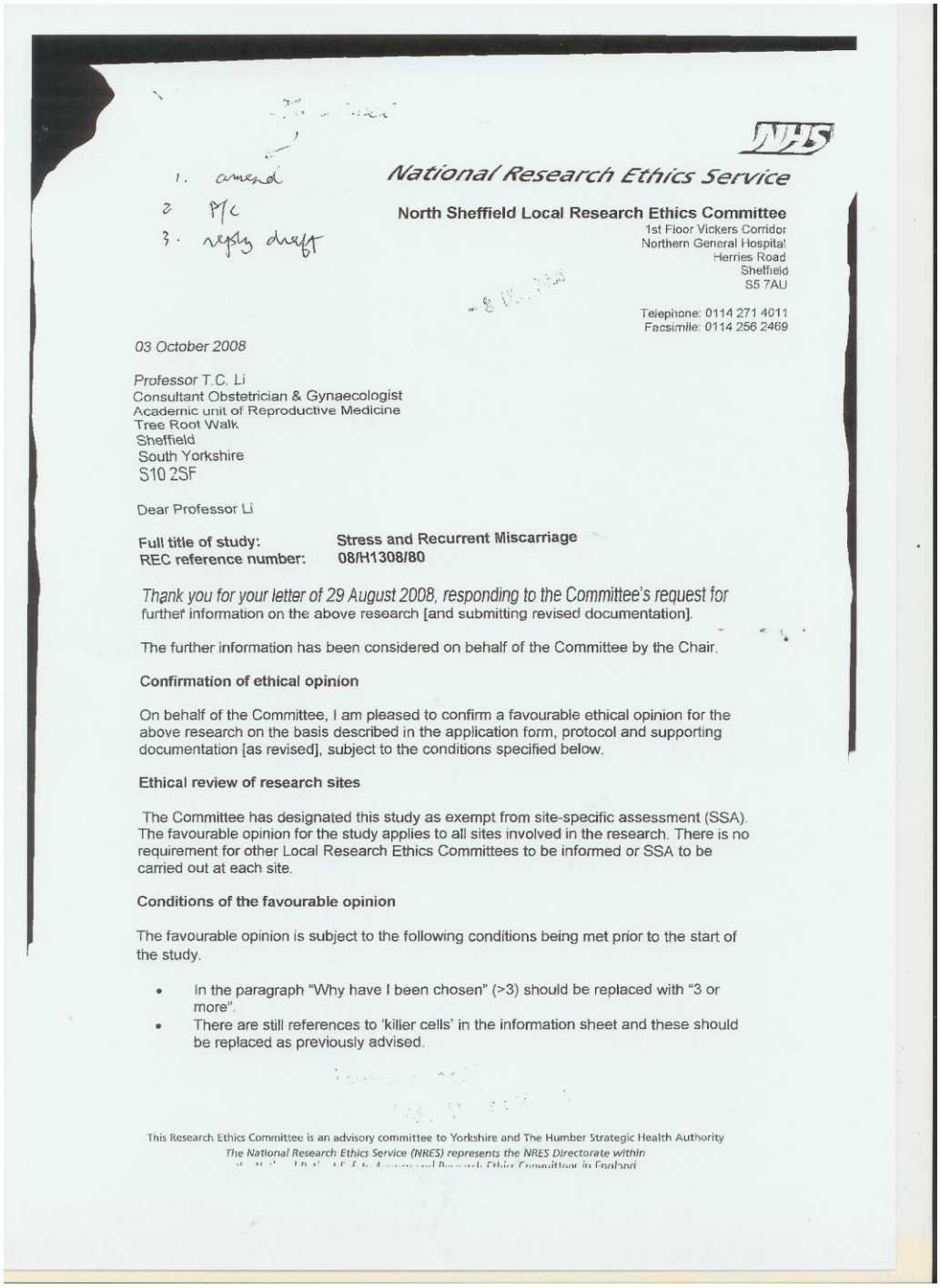
Appendix I. Ethics Approval



1. amend
2 P/C
3. reply draft

National Research Ethics Service

North Sheffield Local Research Ethics Committee
1st Floor Vickers Corridor
Northern General Hospital
Herries Road
Sheffield
S5 7AU

Telephone: 0114 271 4011
Facsimile: 0114 256 2469

03 October 2008

Professor T.C. Li
Consultant Obstetrician & Gynaecologist
Academic unit of Reproductive Medicine
Tree Root Walk
Sheffield
South Yorkshire
S10 2SF

Dear Professor Li

Full title of study: **Stress and Recurrent Miscarriage**
REC reference number: **08/H1308/80**

Thank you for your letter of 29 August 2008, responding to the Committee's request for further information on the above research [and submitting revised documentation].

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation [as revised], subject to the conditions specified below.

Ethical review of research sites

The Committee has designated this study as exempt from site-specific assessment (SSA). The favourable opinion for the study applies to all sites involved in the research. There is no requirement for other Local Research Ethics Committees to be informed or SSA to be carried out at each site.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

- In the paragraph "Why have I been chosen" (>3) should be replaced with "3 or more".
- There are still references to 'killer cells' in the information sheet and these should be replaced as previously advised.

This Research Ethics Committee is an advisory committee to Yorkshire and The Humber Strategic Health Authority
The National Research Ethics Service (NRES) represents the NRES Directorate within

- In the paragraph "What will I have to do" The sentence "The endometrial biopsy will be performed as an outpatient procedure and immediately drawn out" should be revised, as the meaning of "immediately drawn out" is unclear.
- The Committee reviewed version 5.6 of the information sheet and consent form, your revised information sheet and consent form cannot, therefore, be version 2. These documents have been re-referenced as version 5.7 in the office and your copies should be re-referenced the same. Any revised version will become version 5.8.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission at NHS sites ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Information for dexamethasone suppression test	2	24 July 2008
Leaflet for 24 hour urine collection	2	24 July 2008
Participant Consent Form	5.7	24 July 2008
Participant Information Sheet	5.7	24 July 2008
Laboratory Protocol	5.6	28 May 2008
Questionnaire	5.6	28 May 2008
Peer Review	Evidence of peer review	30 May 2008
Summary/Synopsis	Flow chart	28 May 2008
Protocol	5.6	28 May 2008
Investigator CV		
Application		25 May 2008
Response to Request for Further Information		29 August 2008

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

08/H1308/80	Please quote this number on all correspondence

With the Committee's best wishes for the success of this project

Yours sincerely

Dr C A Moore
Chair

Email: april.dagnall@sth.nhs.uk

Enclosures: "After ethical review – guidance for researchers" *[SL-AR1 for CTIMPs, SL- AR2 for other studies]*

Copy to: *Prof Simon Heller, STH R & D Department*

Appendix II. R&D Approval

Ref: STH15026/AD

Sheffield Teaching Hospitals NHS
NHS Foundation Trust

16 June 2009

Professor T C Li
Consultant Obstetrician and Gynaecologist
Academic Unit of Reproductive Medicine
4th Floor
Jessop Wing
Tree Root Walk

Dear Professor Li

Acknowledgement of Project Continuation

STH ref:	STH15026
Study title:	The role of stress and recurrent miscarriage
Chief Investigator:	Professor T C Li, STH
Principal Investigator:	Professor T C Li, STH
Sponsor:	Sheffield Teaching Hospitals NHS Foundation Trust
Funder:	NA

The Research Department has received the required documentation for the study as listed below:

1.	**Sponsorship IMP studies (non-commercial)** **Sponsorship responsibilities between institutions** **Responsibilities of investigators** **Monitoring Arrangements**	NA NA NA NA
2.	**STH registration document: completed and signed**	STH Finance Form, Professor T C Li, 13 May 09
3.	**Evidence of favourable scientific review**	Sheffield Hospitals Charitable Trust, 14 February 08
4.	**Protocol – final version**	V5.6, 28 May 08
5.	**Participant Information sheet – final version**	V5.9, 09 April 09
6.	**Consent form – final version**	V5.8, 24 October 08
7.	**Signed letters of indemnity**	NA
8.	**ARSAC / IRMER certificate**	NA
9.	**Evidence of hosting approval from STH directorate**	STH Finance Form, Mr A Farkas, 13 May 09

Ref: STH15026/AD

10. Evidence of approval from STH Data Protection Officer	STH Finance Form, Mr P Wilson, 13 May 09
11. Letter of approval from REC	North Sheffield REC, 08/H1308/80, 03 October 08 Confirmation of updated documents, Sheffield REC, 08/H1308/80, 30 April 09 Acknowledgement of minor amendment 1, Sheffield REC, 08/H1308/80, 30 April 09
12. Proof of locality approval	NA
13. Clinical Trial Authorisation from MHRA	NA
14. Honorary Contract	NA
15. Associated documents	
- **Laboratory Protocol**	V5.6, 28 May 08
- **Questionnaires** - **Leaflet for the collection of a 24-hour urine specimen**	V2, 24 July 08
16. Signed financial agreement/contract	STH Finance Form, Professor S Heller, 09 June 09

The project has been reviewed by the Research Department and authorised by the Director of R&D on behalf of STH NHS Foundation Trust to begin.

Yours sincerely

Professor S Heller
Director of R&D, Sheffield Teaching Hospitals NHS Foundation Trust
Telephone +44 (0) 114 2265934
Fax +44 (0) 114 2265937

cc. Sheila Duffy

Appendix III. Patient Information Sheet

Sheffield Teaching Hospitals NHS
NHS Foundation Trust

Jessop Wing Hospital

Tree Root Walk Road

S10 2SF

Sheffield

Stress and Recurrent Miscarriage Study (STH 15026)

Patients Information Sheet

An invitation to take part in medical research

We would like to invite you to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. If you would like further information please contact: Dr Wei Li on 07502255073 or Prof T.C. Li on 0114 226 1064. The research study will form the basis of an MD thesis.

What is the purpose of the study?

In 50% of women with recurrent miscarriage (RM) the exact cause is unknown, one hypothesis is that stress plays a significant role on recurrent miscarriage via its effect on the brain which in turn affects the hormone and immune system. In this study we aim to assess the level of stress in women attending the RM Clinic and relate the measurements to reproductive hormone and the immune cells.

Validated Questionnaires, blood samples, a saliva sample and biopsy of the lining of the uterus will be collected to measure stress markers, hormone and immune cells. The result of this study will provide new data on stress in women with RM and the predictive value of stress on a subsequent pregnancy.

Why have I been chosen?

You have been chosen because you have experienced recurrent miscarriages (3 or more). We hope to find out if stress may be a contributory factor for your repeated losses. With your participation, we can understand more about stress and RM.

With your permission, we will inform your GP that you are taking part in this study.

Do I have to take part?

No. Taking part is entirely voluntary. If you prefer not to take part, you don't have to give a reason. Your clinical care will not be affected in any way. If you agree to take part, but later change your mind, you may withdraw at any time without affecting your care in any way.

If you withdraw from the study later, the indentifiable data and tissue already collected with consent would be retained and used in the study unless you request them to be destroyed.

What will I have to do if I take part?

If you agree to take part in this study

(1) You will be asked to complete the questionnaires, have a blood sample (about one teaspoon) and a salivary sample taken. These will be taken to measure your stress levels at your first visit in the clinic.

(2) On your second visit which is usually on day 21 of your menstrual cycle (or 7 days after ovulation), a blood sample (about two teaspoon volume) will be obtained to measure progesterone as part of the routine investigation for your condition; a portion of the blood sample obtained will be used to measure an immune cell (eg. NK cell). In addition **a biopsy of the lining of the uterus** will be obtained to measure immune cells and stress receptors. The endometrial biopsy will be performed as an outpatient procedure. The biopsy procedure itself will take about 10 seconds.

(3) At your third visit which is usually on day 2-5 of the cycle, a blood sample (about 3 teaspoon volume) will be obtained for hormone tests as part of the routine investigation for your condition. Prior to this visit you will be asked to take a tablet the night before your visit (Dexamethasone Suppression Test). Dexamethasone is a tablet which usually suppresses stress hormone, and during your visit you will have a blood sample (about one teaspoon volume) and salivary sample collected to measure stress markers. For the instruction of Dexamethasone Suppression Test please see the attached leaflet.

(4) As part of your routine investigation, you will be asked to reattend the clinic at about 2 months later to discuss the results of your investigations. At this visit you will be asked to complete the questionnaires for the second time and have your blood sample (one teaspoon volume) and salivary sample taken to measure stress markers.

(5) If you conceive again, we will see you when you are 6-8 weeks pregnant as part of your routine care. This will be for an ultrasound examination to confirm that the pregnancy is progressing normally. At this visit you will be asked to complete the questionnaires again and have your blood and salivary sample collected as before.

(6) If your pregnancy reaches 14 weeks successfully or if you miscarry again, and or if you have not conceived 4 months after the initial visit, you will be seen again in the clinic when you will be asked to complete the questionnaires for the final time as well as have your blood and salivary sample taken.

Confidentiality

All reasonable steps will be taken to ensure confidentiality. Any information collected in the 4 validated questionnaires is anonymous and can only be used with your permission by the clinicians involved in your care. All the information that is collected about you during the course of the research will

be kept strictly confidential. When the results are published, no names will be used, and it will not possible to identify anyone who has taken part.

Will my taking part involve any extra visit?

No, it will not involve extra visits and the additional investigations have all been planned during your routine visits.

What are the possible risks of taking part?

We don't anticipate any health risks as a result of your taking part in this study.

The **endometrial biopsy** is a very commonly performed outpatient investigative procedure in gynaecology. It may be uncomfortable and sometimes painful, but the procedure usually lasts only a few seconds. Infection is a very rare complication of the procedure. It is not necessary to do a pregnancy test prior the biopsy, but if you are concerned that you may be pregnant, we would advise you not to proceed with the biopsy.

Questionnaires: There are 4 **questionnaires** for you to complete on 3-4 occasion. It may take 30-40 minutes on each occasion. You will be asked about how you feel about the recurrent miscarriages and its impact on your relationship. You may find some of the questionnaires of a sensitive nature. However, you don't have to complete every questionnaire if you don't feel comfortable about doing so.

What will happen to the results of the research study?

The results of this research will help guide future management of patients. The results will be written up and published in medical journals.

Storage of samples: The endometrial sample will be used to measure the presence of various stress markers. Part of the specimens will be frozen and stored up to for 3 years. We will only keep the specimen for more than 3 years if you consent to it. The specimen will be stored in line with the Human Tissue Act.

Who is organizing the research?

The research is being conducted by a team of clinicians and scientists who work in the Royal Hallamshire Hospital and University of Sheffield. The team is led by Professor TC Li who has been running the recurrent miscarriage clinic in Sheffield for 15 years.

What if I wish to complain about the way in which this study has been conducted?

If you have any cause to complain about any aspect of the way in which you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms are available to you and are not compromised in any way because you have taken part in a research study. You can use the normal Sheffield Teaching Hospital complaints

procedure and contact: Miss Alison Neild, Head of Patient Services, B Floor, Royal Hallamshire Hospital 0114-226-1766.

What do I do now?

Now you have read the information sheet, if you are happy to participate, please complete the consent form and return it to the informing clinician, then the detail of your appointment and further care will be arranged.

Appendix IV. Data Collection Sheet

Case No.		Date of birth				Family history of RM	Yes No	Height		Weight	
Smoke		coffee		Alcohol		: menstruation regularity		Yes			
Amount		Amount		Units				No			
Occupation						Ethnicity					
Past Pregnancy history	Date of pregnancy					Pregnancy outcome					
Hormonal profile	Items.	Date	Result								
	LH										
	FSH										
	Estrodiol										
	Progesterone										
	Testosterone										
	FAI										
	Prolactin										
Pregnancy outcome											
Subsequent pregnancy	Yes	LMP:									
	No	Reason:									
Pregnancy Outcome	Live birth	Miscarriage		Still Birth		Ectopic preg.		TOP			
		Gestation Week		Gestation Week							

Appendix V. Flow Chart of Sample Collection

How Do You Collect Your Sample

At your first appointment Bring your written consent with you to your appointment

⇩

On day1 of next cycle On the first day you start to bleed, telephone the Pregnancy Support Service (PSS) on 0114 2268589 to arrange your Dexamethasone test

⇩

On day2-5 of cycle Attend PSS on Ward G1 at 8:15 to 8:30 in the morning for your blood test. Please don't forget to take Dexamethasone tablet the night before you attend

⇩

On day7 of your cycle Start to time the day of your ovulation with Ovulation Test kits, on the day of your ovulation contact PSS for your biopsy arrangement

⇩

On 7-10 days after ovulation Attend G1 for the biopsy of the line of your womb and blood test. Please make sure you use condoms or we will be unable to take the biopsy

⇩

2 months after your first visit Return the clinic discussing the results of your tests with doctors

We will continue caring you after this point, if you get pregnant subsequently or repeat miscarriage, please contact us. We are here for you.

YOU WILL HAVE BEEN ASKED TO USE A BARRIER METHOD OF CONTRACEPTION UNTIL YOUR RESULTS HAVE BEEN

Appendix VI. Study Questionnaires

Stress and Recurrent Miscarriage Study

Infertility-related Stress Questionnaires

Name: _____

Date: _____

Please answer every question. Some questions may look like others, but each one is different. Please take the time to read and answer each question carefully by ticking the box that best represents your response. There are no right or wrong answers, don't spend too much time on any one statement but give the answer which seems to describe your present situation best.

Thank you for taking the time to complete these questionnaires

Fertility Problem Inventory

Instruction for completing the questionnaire

The following statements express different opinions about a fertility problem. Please place a number on the line to the left of each statement to show how much you agree or disagree with it.

Please answer every item. Use the following response categories:

6 = strongly agree
5 = moderately agree
4 = slightly agree
3 = slightly disagree
2 = moderately disagree
1 = strongly disagree

1. ___ Couples without a child are just as happy as those with children.
2. ___ Pregnancy and childbirth are the two most important events in a couple's relationship.
3. ___ I find I've lost my enjoyment of sex because of the fertility problem.
4. ___ I feel just as attractive to my partner as before.
5. ___ For me, being a parent is a more important goal than having a satisfying career.
6. ___ My marriage needs a child (or another child).
7. ___ I don't feel any different from other members of my sex.
8. ___ It's hard to feel like a true adult until you have a child.
9. ___ It doesn't bother me when I'm asked questions about children.
10. ___ A future without a child (or another child) would frighten me.
11. ___ I can't show my partner how I feel because it will make him/her feel upset.
12. ___ Family don't seem to treat us any differently.
13. ___ I feel like I've failed at sex.
14. ___ The holidays are especially difficult for me.

6 = strongly agree
5 = moderately agree
4 = slightly agree
3 = slightly disagree
2 = moderately disagree
1 = strongly disagree

15. ___ I could see a number of advantages if we didn't have a child (or another child).

16. ___ My partner doesn't understand the way the fertility problem affects me.

17. ___ During sex, all I can think about is wanting a child (or another child).

18. ___ My partner and I work well together handling questions about our infertility.

19. ___ I feel empty because of our fertility problem.

20. ___ I could visualize a happy life together, without a child (or another child).

21. ___ It bothers me that my partner reacts differently to the problem.

22. ___ Having sex is difficult because I don't want another disappointment.

23. ___ Having a child (or another child) is not the major focus of my life.

24. ___ My partner is quite disappointed with me.

25. ___ At times, I seriously wonder if I want a child (or another child).

26. ___ My partner and I could talk more openly with each other about our fertility problem.

27. ___ Family get-togethers are especially difficult for me.

28. ___ Not having a child (or another child) would allow me time to do other satisfying things.

29. ___ I have often felt that I was born to be a parent.

30. ___ I can't help comparing myself with friends who have children.

31. ___ Having a child (or another child) is not necessary for my happiness.

32. ___ If we miss a critical day to have sex, I can feel quite angry.

6 = strongly agree
5 = moderately agree
4 = slightly agree
3 = slightly disagree
2 = moderately disagree
1 = strongly disagree

33. ___ I couldn't imagine us ever separating because of this.

34. ___ As long as I can remember, I've wanted to be a parent.

35. ___ I still have lots in common with friends who have children.

36. ___ When we try to talk about our fertility problem, it seems to lead to an argument.

37. ___ Sometimes I feel so much pressure, that having sex becomes difficult.

38. ___ We could have a long, happy relationship without a child (or another child).

39. ___ I find it hard to spend time with friends who have young children.

40. ___ When I see families with children I feel left out.

41. ___ There is a certain freedom without children that appeals to me.

42. ___ I will do just about anything to have a child (or another child).

43. ___ I feel like friends or family are leaving us behind.

44. ___ It doesn't bother me when others talk about their children.

45. ___ Because of infertility, I worry that my partner and I are drifting apart.

46. ___ When we talk about our fertility problem, my partner seems comforted by my comments.

Perceived Stress Scale

Instructions: The questions in this scale ask you about your feelings and thoughts during the last month. In each case, please circle how often you felt or thought a certain way.

1. In the last month, how often have you been upset because of something that happened unexpectedly?

0=never 1=almost never 2=sometimes 3=fairly often 4=very often

2. In the last month, how often have you felt that you were unable to control the important things in your life?

0=never 1=almost never 2=sometimes 3=fairly often 4=very often

3. In the last month, how often have you felt nervous and "stressed"?

0=never 1=almost never 2=sometimes 3=fairly often 4=very often

4. In the last month, how often have you felt confident about your ability to handle your personal problems?

0=never 1=almost never 2=sometimes 3=fairly often 4=very often

5. In the last month, how often have you felt that things were going your way?

0=never 1=almost never 2=sometimes 3=fairly often 4=very often

6. In the last month, how often have you found that you could not cope with all the things that you had to do?

0=never 1=almost never 2=sometimes 3=fairly often 4=very often

7. In the last month, how often have you been able to control irritations in your life?

0=never 1=almost never 2=sometimes 3=fairly often 4=very often

8. In the last month, how often have you felt that you were on top of things?

0=never 1=almost never 2=sometimes 3=fairly often 4=very often

9. In the last month, how often have you been angered because of things that were outside of your control?

0=never 1=almost never 2=sometimes 3=fairly often 4=very often

10. In the last month, how often have you felt difficulties were piling up so high that you could not overcome them?

0=never 1=almost never 2=sometimes 3=fairly often 4=very often

PANAS

Directions

This scale consists of a number of words that describe different feelings and emotions. Read each item and then circle the appropriate answer next to that word. Indicate to what extent you have felt this way during the past week.

Use the following scale to record your answers.

(1) = Very slightly or not at all (2) = A little (3) = Moderately (4) = Quite a bit (5) = Extremely

	Very slightly or not at all	A little	Moderately	Quite a bit	Extremely
1. Interested	1	2	3	4	5
2. Distressed	1	2	3	4	5
3. Excited	1	2	3	4	5
4. Upset	1	2	3	4	5
5. Strong	1	2	3	4	5
6. Guilty	1	2	3	4	5
7. Scared	1	2	3	4	5
8. Hostile	1	2	3	4	5
9. Enthusiastic	1	2	3	4	5
10. Proud	1	2	3	4	5
11. Irritable	1	2	3	4	5
12. Alert	1	2	3	4	5
13. Ashamed	1	2	3	4	5
14. Inspired	1	2	3	4	5
15. Nervous	1	2	3	4	5
16. Determined	1	2	3	4	5
17. Attentive	1	2	3	4	5
18. Jittery	1	2	3	4	5
19. Active	1	2	3	4	5
20. Afraid	1	2	3	4	5

Appendix VII. Copy Right Permission Letter

Dear Li Wei,

I appreciate your interest in the PANAS. Please note that to use the =ANAS, you need both our permission and the permission of the American Psyc=ological Association (APA), which is the official copyright holder of the =nstrument. I am pleased to grant you permission to use the PANAS in =our research. Moreover, because I am copying this email to APA, you =o not have to request permission separately from APA; this single e-mail c=nstitutes official approval from both parties.

We make the PANAS available without charge for non-commercial research purp=ses. We do require that all printed versions of the PANAS include a =ull citation and copyright information. Thus, any printed copies sho=ld state:

"From "Development and validation of brief measures of positive a=d negative affect: The PANAS scales," by D. Watson, L. A. Clark= and A. Tellegen, 1988, Journal of Personality and Social Psychology, 54, =063-1070. Copyright © 1988 by the American Psychological Associ=tion. Reproduced with permission."

Finally, as requested, I have attached a sample copy of the PANAS that show= how the scales should be scored.

Good luck with your research.

Sincerely,

David Watson

David Watson, Ph.D.
F. Wendell Miller Professor of Psychology
Editor, Journal of Abnormal Psychology

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Appendix VIII. Works Arising From This Thesis

Publications:
The relationship between plasma prolactin concentration and pregnancy outcome in women with recurrent miscarriage. W. Li, N. Ma, S.M. Laird, T.C. Li, submitted.

Measurements of CD56+ cells in peripheral blood and endometrium by flow cytometry and immunohistochemical staining in situ. S.M. Laird, N. Mariee, W. Li, TC. LI, submitted.

Presentations:
The relationship between plasma prolactin concentrations and pregnancy outcomes in women with recurrent miscarriage. Poster presentation, ESHRE 2010, Rome.

The relationship between plasma prolactin concentrations and pregnancy outcomes in women with recurrent miscarriage. Poster presentation, Annual school research meeting, University of Sheffield, 2010.

The value of cortisol suppression test in the evaluation of the HPA activation in women with recurrent miscarriage. Oral presentation, Second year student presentation, University of Sheffield Medical School, 2009.

The role of stress in recurrent miscarriage. Oral presentation. First year student presentation. University of Sheffield, 2008.

Appendix IV. Copies of Abstract

The Relationship between Plasma Prolactin Concentration and Pregnancy Outcome in Women with Recurrent Miscarriage

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Background: The role of prolactin in pregnancy is controversial. Some early studies have reported on the high prevalence of hyperprolactinaemia in women with recurrent miscarriage (RM) and the possible deleterious effect of high prolactin on pregnancy. However, a few recent studies have shown that prolactin may have a beneficial role in pregnancy through signalling decidualization and embryo implantation. The aim of this study was to evaluate the relationship between the plasma prolactin concentration and pregnancy outcomes in women with RM. **Methods:** Plasma prolactin was measured in 174 women with unexplained RM in the early follicular phase in pre-pregnancy state. Among these women, pregnancy outcomes were correlated with plasma prolactin levels in 109 women with unexplained RM. **Results:** Of the 109 women with unexplained RM and subsequent pregnancies, those who miscarried had significantly ($\rho=0.01$) lower (Mean ± SD) plasma prolactin levels (240+108.3 mIU/l) compared with those who had a live birth (308+164.8 mIU/l). In addition women with prolactin concentrations lower than the mean level had a significantly higher miscarriage rate (34/64, 53.1%) than those with prolactin concentrations higher than the mean level (15/45, 33.3%) ($\rho<0.05$). **Conclusions:** Low plasma prolactin level is associated with an increased risk of miscarriages in a subsequent pregnancy.

Key words: prolactin / hyperprolactinaemia / pregnancy outcome / recurrent miscarriage

Introduction

Recurrent miscarriage (RM) is defined as three or more consecutive spontaneous miscarriages in the first trimester (ESHRE, 2006). It is a heterogeneous condition with a number of possible underlying causes. To date several aetiological factors of RM have been identified, including parental chromosomal anomalies, uterine pathology, haematological abnormalities, endocrinological disorders and immunological factors. However, in 50% of the cases, the cause of RM still remains unexplained. Most previous studies in relation to the endocrine abnormalities of RM have focused on the luteal phase deficiency (LPD) and PCOS (polycystic ovarian syndrome) (Cocksedge et al., 2009, Palomba et al., 2006, Nardo et al., 2002). Little effort has been made on the investigation of the role of prolactin in RM.

Prolactin is a polypeptide hormone synthesized by the lactotroph cell in the anterior pituitary gland and the extrapituitary sites such as mammary gland, placenta and uterus (Goffin et al., 2002, Eyal et al., 2007, Reis et al., 2002). Apart from the lactation and endocrine effects, prolactin may have the luteotrophic function by potentiating the steroidgenic effect of luteinizing hormone (LH) in granulosa-luteal cells and inhibiting the 20α-hydroxysteroid dehydrogenase enzyme which inactivates progesterone. Prolactin may also be luteolytic by inducing programmed cell death in the corpora lutea mediated by CD3 lymphocytes (Freeman et al., 2000, Kanuka et al., 1997, Richards and Williams, 1976). Therefore, prolactin plays an important role in maintaining corpus luteum function and the subsequent steroidgenesis of progesterone secretion, thus affecting pregnancy in this way. The exact mechanism of both the luteotrophic and luteolytic effects of prolactin is still unclear.

Recently the role of prolactin in RM has attracted some attention. Several studies have previously reported on the high prevalence of hyperprolactinaemia in the follicular phase in women with RM and the suppression of prolactin secretion with bromocriptin to improve pregnancy outcome (Hirahara et al., 1998, Csemiczky et al., 2000, Bussen et al., 1999),

suggesting that high prolactin levels may be a possible aetiological factor of RM. However, our earlier study showed that hyperprolactinaemia rarely occurs in women with RM (2.5%, 3/122) (Li et al., 2000). In some studies it has been suggested that an adequate level of serum prolactin is essential to maintain corpus luteum function (Gåfvels et al., 1992) and establish a successful pregnancy(Oda et al., 1991). In IVF treatment it has been observed that transient hyperprolactinaemia is associated with a better chance of successful pregnancy outcomes (Ozaki et al., 2001). The inadequate expression of prolactin and its receptor in endometrium during the implantation window has been found to cause premature luteolysis and subsequent infertility or recurrent miscarriages (Garzia et al., 2004). Therefore, this raises the question of whether or not high prolactin level is associated with an increased risk of recurrent miscarriage. Due to the paucity of the studies in this area, the relationship between the prolactin level and RM is still unclear. The aim of this retrospective study was to investigate the relationship between serum prolactin concentrations and the pregnancy outcomes in a large group of women with unexplained RM.

Materials and Methods

Human subjects

A series of 174 consecutive women with unexplained RM, who had plasma prolactin concentrations measured in the early follicular phase from January 1999 to September 2009, were included in this study. All the patients who conceived again after the referral were offered the telephone pregnancy counselling service as part of tender loving care throughout their subsequent pregnancies. Therefore, their pregnancy outcomes were followed and documented by the pregnancy support team. No treatment was given to this group of women apart from the tender loving care.

Inclusion criteria

All the subjects underwent thorough investigations according to an established protocol (Li, 1998), including maternal and paternal karyotypes, prothrombotic studies, antiphospholipid antibody screen, thyroid function test and thyroid antibodies, FSH, LH, oestradiol, progesterone, androgen profile, prolactin, transvaginal ultrasonography and hysterosalpingography. Hysteroscopy was only performed if an abnormality was suspected on pelvic ultrasound or hysterosalpingography.

Unexplained RM fulfilled the criteria of normal parental chromosomal analysis, absence of endocrinological abnormalities including normal analysis of thyroid function test and normal levels of FSH, LH, oestradiol, progesterone, prolactin and androgen profile, test negative for antiphospholipid antibodies and lupus antibodies, normal coagulation studies and absence of structural uterine abnormalities.

Study Setting

The Recurrent Miscarriage Clinic at Jessop Wing for Women, Royal Hallamshire Hospital, Sheffield, UK

Outcome measures

Pregnancy outcome was measured as the first pregnancy outcome after referral. In patients who conceived more than once during the study period, only the first pregnancy was considered.

Prolactin measurement

Blood samples for plasma prolactin investigations were taken in the early follicular phase. Prolactin assay was performed by automated microparticle enzyme-immunoassay (Abbott Axsym Analyser, Abbott Diagnostics) at the Clinical Chemistry Department, Royal Hallamshire Hospital. The intra-and inter-assay coefficient of variation for prolactin measurement was 3.7% and 11.5%. The presence of hyperprolactinaemia was defined as plasma prolactin concentration > 660 mIU/l.

Data Analysis

Results were expressed as numbers, mean ±SD, range and percentages as appropriate. Normal distribution of prolactin value was tested with *Kolmogorov-Smirnov* test. Differences in plasma prolactin concentrations between women who subsequently miscarried and those who had a live birth were analysed with T test. The difference in the miscarriage rate between the two subgroups was analysed with *Pearson* X^2 test. Statistical significance was set at *p* value <0.05 and analysis were performed using SPSS (Statistical Package for the Social Sciences) version 15.

Results

A total of 174 women with unexplained RM had their plasma prolactin measured during the study period. The mean age (±SD) was 32.2 (± 5.6) years; the median number of previous miscarriages was 4 (ranged from 3 to14). The mean (±SD) prolactin concentration was 226 (±121) mIU/l. Among those 174 women with unexplained RM, 40 of them did not conceive during the study period and 9 of them lost follow-up. The remaining 125 women with unexplained RM conceived again after the referral. The differences of the clinical profile between women who conceived (n=125) and those who did not conceived (n=40) are shown in table 1. As expected, women who did not conceived were significantly older than those who conceived (P< 0.05). Women who conceived again had higher mean prolactin concentrations than those who did not conceive. However, the difference was not significant.

Table I. The differences between 125 women who conceived again and 40 women who did not conceived during the study period

	Women who conceived	Women who did not conceive	p value (<0.05)
N	125	40	
Age	31.0 ± 6.0	32.6 ± 5.5	0.02*
No. of previous miscarriages	4 (3-10)	4 (3-14)	0.47
Prolactin concentrations	234.5 ± 125.8	196.3 ± 98.8	0.26

* Significant

Among those 125 women with unexplained RM who conceived again, 6 women had ectopic pregnancies and 10 women had mid-term terminations of pregnancies due to fetal deformation, such as joint twins and Down's syndrome. Therefore, they were excluded from the study analysis. A total of 109 women with subsequent pregnancies during the study period were included in the study analysis. The mean age was 31.9 (±5.2) years. The median of the numbers of previous miscarriages was 3 (ranged from 3 to 7). The mean concentration of prolactin was 278 (±146) mIU/L.

In the 109 subsequent pregnancies, 49 (45%) pregnancies resulted in the first trimester loss whereas 60 (55%) pregnancies resulted in live birth. Women with a subsequent live birth had significantly (ρ=0.01) higher prolactin concentrations (308±164.8mIU/l) than those with a subsequent miscarriage (240±108.3mIU/l) (figure I).

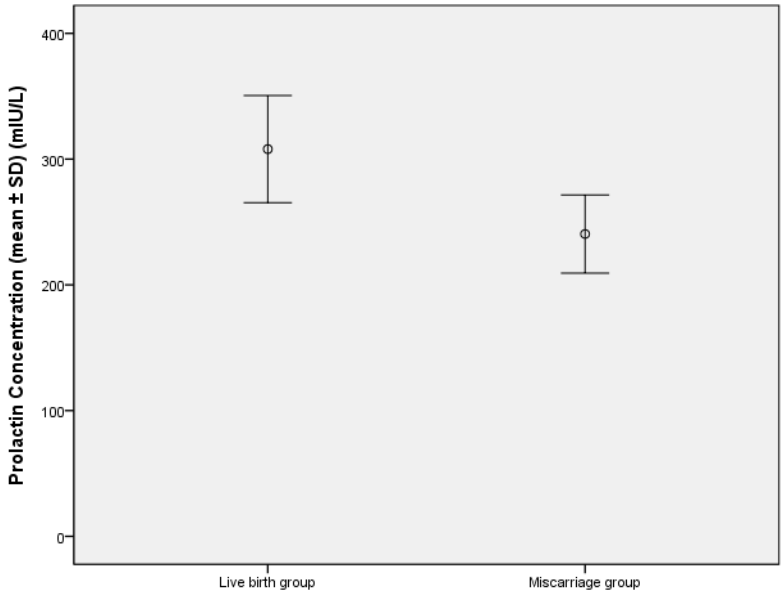

Figure I: Prolactin concentrations in women who subsequently had a live births and those who had a miscarriage

These 109 women were further divided into two subgroups according to the mean value of the prolactin measurements. 45 women with prolactin concentrations above the mean level were allocated into high normal prolactin group and 64 women with those below the mean level were allocated into low normal prolactin group (**Table II**). The miscarriage rate in women with high normal prolactin (15/45, 33.3%) was significantly lower ($p<0.05$) than that in women with low normal results (34/64, 53.1%).

Table II. The difference in miscarriage rates between the two subgroups

	High normal prolactin subgroup (prolactin≥278mIU/l) n=45	Low normal prolactin subgroup (prolactin<278mIU/l) n=64	p value (<0.05)
Age (mean± SD)	31.5 ± 4.5yrs	32.1 ± 5.5yrs	0.66
No. of previous miscarriages (median, range)	3 (3-6)	3 (3-7)	0.47
Prolactin concentration (mean± SD)	397 ± 85 mIU/l	181 ± 56 mIU/l	0.00*
No. of miscarriages (n, %)	15 (33.3%)	34(53.1%)	0.04*

*significant

Discussion

The relationship between the plasma prolactin concentration and pregnancy outcome

In this study we found that low concentrations of plasma prolactin in women with RM are associated with a high risk of miscarriage in a subsequent pregnancy. To examine the relationship between the prolactin level and pregnancy outcome, we only included a cohort of patients with unexplained RM in which other possible causes of RM have been excluded to ensure that prolactin is the only factor affecting pregnancy outcome. The relationship between the plasma prolactin concentration and the outcome of assisted conception treatment has been examined in several studies. Ozaki *et al* (2001) compared the plasma prolactin concentrations between women who had subsequent live births following IVF treatment and those who had early pregnancy losses. They found that women with successful pregnancies had significantly higher prolactin levels than those with early losses (Ozaki et al., 2001). In a study by Gonen and Casper (1989) transient hyperprolactinaemia following ovarian stimulation was found to be associated with a better chance of pregnancy following IVF treatment. Before HCG administration, there was no significant difference in the basal prolactin level between women who subsequently conceived and those who did not conceive (11.1±0.6ug/l vs 10.1±0.4ug/l). After HCG administration, the prolactin level in the pregnant group was significantly higher than that in nonpregnant group (20.8±1.6ug/l vs 16.0±0.9ug/l) (Gonen and Casper, 1989). It is not yet understood why in assisted conception women with high prolactin levels had better pregnancy outcomes. It has been observed that women with higher prolactin concentrations had significantly higher fertilization rates and cleavage rates of oocytes in IVF treatment than those with low prolactin concentrations (Oda et al., 1991).

The relationship between the prolactin concentration and the outcome of spontaneous pregnancy, especially in women with a history of recurrent pregnancy loss has not been previously studied. In our study, we found women who subsequently miscarried had lower plasma prolactin concentrations than those who had a live birth. We also found that plasma prolactin level appears to have a prognostic value on the outcome of pregnancy in women

with RM, with low levels having a worse outcome than high normal levels. Thus our data supports the notion that low prolactin level is associated with an adverse pregnancy outcome.

The mechanism of prolactin action:

Whilst some studies showed that high prolactin levels had an inhibitory effect on GnRH releases and HCG-induced ovulation, and a negative regulatory function on endometrial decidualization (Milenković et al., 1994, Liu et al., 1997, Eyal et al., 2007), other studies have reported a beneficial role of prolactin in pregnancy. Serum prolactin was found to have a beneficial effect on maintaining corpus luteum in early pregnancy via modulating luteinizing hormone/chorionic gonadotrophin (LH/CG) receptors (Gåfvels et al., 1992). LH/CG receptors are expressed in corpus luteum tissue and chorionic villous macrophages after implantation (Sonoda et al., 2005, Saint-Dizier et al., 2004). In a rat model it has been demonstrated that inhibition of serum prolactin secretion with bromocriptine during early pregnancy deceased LH/CG receptors and led to premature luteolysis, subsequently resulting in miscarriage. This effect can be reversed by prolactin supplementation after implantation (Gåfvels et al., 1992, Chan et al., 1980).

Prolactin and its receptor have also been identified in the endometrium (Eyal et al., 2007, Mak et al., 2002, Reis et al., 2002). Lack of expression of the endometrial prolactin during the implantation window have been shown to be associated with repeated pregnancy loss (Garzia et al., 2004). Prolactin and its receptor are expressed in the glandular epithelium and a subset of endometrial stromal cells with maximal expression seen during the mid-late secretory phase of the cycle. Prolactin production by stromal cells increases dramatically during the decidualization process associated with embryo. The time and sites of its expression in endometrium tissue suggests a crucial role for prolactin and its receptor in establishment and maintenance of early pregnancy. Prolactin may affect decidualization through modifying the expression of adhesion and proteolytic molecules, facilitating trophoblast cell invasion following extracellular matrix degradation of the endometrium (Starzyk et al., 1999, Ormandy et al., 1997, Horseman et al., 1997). Prolactin receptors have

also been identified in uterine natural killer (uNK) cells (Gubbay et al., 2002). The distinctive presence of uterine CD56bright NK cells around trophoblast cells during implantation and the positive association between the density of uNK expression and the blood vessel formation suggest that they play an important role in angiogenesis and immunoregulation in the fetal-maternal interface in early pregnancy (Quenby et al., 2008). Therefore, prolactin may also affect uterine NK cells function or differentiation and thus affect implantation in this way.

In conclusion, we found that in women with unexplained RM a low prolactin level is associated with an increased risk of miscarriage. The exact reason for the observation is unclear but it seems likely that an adequate level of prolactin concentration is needed for the maintenance of successful pregnancies. Future studies should be directed to the evaluation of prolactin and its receptor in the endometrium to ascertain the mechanism of reproductive failure in conditions associated with low prolactin concentration.

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Measurements of CD56+ cells in pheripheral blood and endometrium by flow cytometry and immunohistochemical staining in situ.

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Abstract

BACKGROUND: Several studies have suggested that peripheral blood and /or endometrial CD56+ cells may be increased in women with recurrent implantation failure or recurrent miscarriage. However the relationship between numbers of peripheral blood NK cells and uterine NK cells is uncertain and there is also some uncertainty about comparing results of studies of endometrial CD56+ where they have been measured by immunohistochemistry and flow cytometry.

METHOD: Endometrial biopsy samples, together with a blood sample, were obtained from women with recurrent miscarriage (n=25) in the mid-luteal phase of the cycle. Numbers of total CD56+ cells, CD56+ CD16- and CD56+CD16+ cells in peripheral blood were measured by flow cytometry, while the number of CD56+ cells in the equivalent endometrial biopsy sample were determined by immunohistchemistry. In addition endometrial biopsy samples were obtained from normal fertile women (n=20) at different times in the cycle. The biopsy was divided into two parts; one part was used to measure CD56+ and CD45+ cells by flow cytometry, while the other part was used to determine numbers of CD56+ and CD45+ cells by immunostaining.

RESULTS: There was no correlation between the numbers of total CD56+, CD56+CD16- or CD56+CD16+ in peripheral blood and the number of endometrial CD56+ cells in the same women in the recurrent miscarriage group. However in the endometrial biopsy samples from fertile women a significant correlation was found between the measurement of CD56+ cells by flow cytometry and immunohistochemistry both when the cell numbers

were expressed as a percentage of total cells (correlation=0.497, P=0.026) or as a percentage of CD45+ cells (correlation=0.570, P=0.009).

CONCLUSION: The results suggest that measurements of CD56+ cells in peripheral blood do not correlate with endometrial CD56+ cell numbers and therefore it is inappropriate to extrapolate results obtained from peripheral blood to what is happening in the endometrium and decidua. However measurements of endometrial CD56+ cells by flow cytometry and immunostaining seem to correlate well and therefore differences in results from different studies cannot be attributed to differences in methodology.

Introduction

Natural killer (NK) cells, which form part of the innate immune system, are found in peripheral blood and in the endometrium of humans. However the characteristics and possible functions of peripheral blood and endometrial NK cells are very different. The majority (90%) of the peripheral blood NK cells express the characteristic NK cell markers, CD56, CD16 and CD3. In contrast the majority (90%) of NK cells found in the endometrium (uNK cells) express high levels of CD56, but are CD16 and CD3 negative. The remaining 10% of uNK cells resemble peripheral blood NK cells and are CD16+. Expression of NK cell receptors also differs between peripheral blood and uNK cells within any one individual. The numbers of uNK cells in the endometrium increase dramatically during the secretory phase of the menstrual cycle and remain high during the first trimester of pregnancy (Bulmer et al. 1991; Laird et al. 2003).

Although uNK cells and peripheral blood NK cells are clearly separate populations, there is considerable debate about whether the increase in uNK cell numbers during the secretory phase of the cycle is due to the recruitment of immature cells from peripheral blood followed by proliferation and differentiation in the endometrium, or due to proliferation of uNK cells in situ (Bulmer and Lash 2005; Santoni et al. 2008). A number of factors are postulated to control this proliferation and differentiation process, including IL15 (Kitaya et al 2005) and various chemokines (Manaster and Mandelboin. 2008).

There are numerous reports of increased numbers of uNK cells or peripheral blood NK cells in women who suffer recurrent miscarriage or recurrent

implantation failure after IVF (Quenby et al. 1999; Tuckerman et al. 2010; Thum et al. 2004; Coulam et al. 1995: Kwak et al. 1995; Ledee-Bataille et al. 2004; King et al. 2010). However other studies have shown no differences in levels of peripheral blood NK cells or uNK cells in these groups of women (Shimada et al. 2004). In addition, further studies have shown that measurement of uNK in non-pregnant endometrium of women with recurrent miscarriage or measurement of peripheral blood NK cells in women undergoing IVF are not predictive of pregnancy outcome (Tuckerman et al. 2007; Thum et al. 2005).

Measurements of NK cells in blood are traditionally measured by flow cytometry, while those in endometrial tissue may be measured by flow cytometry (Lachapelle et al. 1996) or immunocytochemical staining in situ (Quenby et al. 1999: Tuckerman et al. 2007). It is thought that the differences in results of different studies may be due to the use of these different methods of CD56+ measurement.

Measurements of either peripheral blood NK cells or uNK cells are beginning to be used in clinical practice (Quenby et al. 2005). However the lack of clarity between the different methods used and the relationship between peripheral blood NK cells and uNK cells makes interpretation of the results difficult. There are no studies to-date which have correlated peripheral blood NK cells with uNK cells or that have correlated measurement of endometrial uNK cells by flow cytometry and immunostaining. We have therefore a) measured peripheral blood NK cells by flow cytometry and compared their numbers to those in the endometrium collected from the same women at the

same time and b) measured endometrial uNK cells in the same endometrial biopsy sample by flow cytometry and immunostaining.

Materials and Methods

Human Subjects

Endometrial biopsies were obtained from women attending the Jessop Wing, Sheffield Teaching Hospitals. All samples were collected with informed patients consent and ethical committee approval was obtained for the study. Two groups of women were recruited. The first group (n=25) were women attending the recurrent miscarriage clinic, with unexplained recurrent miscarriage defined according to our established protocol (Li 1998). As part of their on-going investigation a timed endometrial biopsy was taken. Daily measurements of LH in either serum or urine was used to identify the LH surge and the endometrial biopsy was collected with a Pipelle sampler between days LH+7 and LH+9. Biopsies were immediately fixed overnight in formalin and then embedded in wax using an automated process. A 10 ml and a 5 ml blood sample were taken at the same time as the biopsy; the 10 ml sample was used for hormone measurements while the other 5 ml was processed immediately for flow cytometry. The second group of women (n=20) were normal fertile women undergoing surgery for non-endometrial pathology. All these women were aged between 20 and 45 and had menstrual cycles of between 25 and 35 days, had normal uterine anatomy, had no steroid treatment for at least 2 months prior to the study. Samples obtained from the operating theatre were divided into 2 parts; one part was fixed and embedded in wax to detect CD56+ cells by immunohistochemistry, while the other part was used for flow cytometry. Menstrual cycle dating of these biopsies was carried out from the date of the last menstrual period and by the morphological appearance of the immunostained sections.

Hormone assays

Serum was immediately separated from cells by centrifugation and stored at -20ºC until assay. Levels of oestradiol and progesterone were determined by chemiluminesescent assay (Abbott Axstm analyser: Abbott diagnostics). Levels of LH and FSH were measured using the Advia Centaur assay system (Bayer-Siemens plc). The intra and inter assay coefficients of variation were: oestradiol 7.9% and 11.9%; progesterone 6.8% and 12.4%; LH 4.5% and 10.4%; FSH 5.9% and 8.0%.

Immunohistochemistry for CD56 and CD45

5 µm sections were dewaxed in xylene, rehydrated through descending alcohols to Tris buffered saline pH 7.6 (TBS), and quenched in 0.3% hydrogen peroxide in methanol for 20 minutes. A negative control in which the specific antibody was replaced by an equivalent concentration of mouse IgG was included for each marker.

After washing, unmasking was performed in an 800W microwave oven in 10 mmol/l citrate buffer pH 6.0. Buffer was heated in the microwave oven until boiling. Slides were added to the buffer, and left covered at high heat for 3 mins. Slides were further incubated for 12 mins on medium heat and allowed to cool for 20 mins. An ABC kit (Vector Laboratories, UK) was used according to the manufacturer's instructions and the following adaptations. Slides were washed in TBS and blocked in blocking buffer containing 250 µl avidin/ml (Vector Laboratories, UK) for 1 hour at room temperature, and incubated overnight at +4ºC with either a mouse monoclonal primary anti-

CD56 antibody (NCL-CD56-504 Novacastra Laboratories Ltd, UK) diluted 1:50, or a mouse monoclonal anti-CD45 antibody (Dako M0701) diluted 1:50 in antibody buffer containing 250 µl/ml biotin. The addition of avidin in the blocking buffer and biotin with the antibody blocks endogenous activity. Slides were washed in TBS throughout, and after application of secondary antibody and Vectorstain, positive cells were visualised by incubation with peroxidase substrate (3.3'diaminobenzidene terahydrochloride; Vector Laboratories, UK). Slides were washed in distilled water and counterstained with 20% haematoxylin for 30 minutes, differentiated, dehydrated through ascending alcohols, cleared in xylene and mounted in Vectormount (Vector Laboratories, UK).

Flow Cytometry

For comparisons between methods of analysis of CD56+ cells in the same endometrial biopsy the part of the sample used for flow cytometry was collected into 1 ml of RPMI 1640 medium containing 100 IU/ml penicillin, 100 mg/ml streptomycin and 10% heat inactivated pooled human serum. The tissue was chopped into small pieces and strained through a cell strainer (70 µm). The tissue that passed through the strainer was incubated with red blood cell lysing buffer for 5 mins and then the cell suspension was centrifuged at 300 g for 5 mins. The cell pellet was washed in PBS and after a further centrifugation at 300 g was re-suspended in 1 ml cold FACS buffer. The total number of cells was counted using a haemocytometer. Cell suspensions (200 µl) were then incubated with either no antibodies (negative control), or 5 µl anti CD45 fluorescein isothiocyanate, 5 µl anti CD56 allophycocyanin, 5 µl anti CD45 fluorescein isothiocyanate plus anti CD56

allophycocyanin, or isotype controls in the dark, on ice for 30 mins. All antibodies were obtained from BD Biosciences, Oxford, UK. After incubation cold FACs buffer (500 µl) was added to each cell suspension, which were pelleted at 400 g for 4 minutes. The cell pellet was washed again in FACs buffer and resuspended in 300 µl of 1% paraformaldehyde and kept in the dark until analysed using a BD FACS Caliber flow cytometer.

For measurements of CD56+ cells in peripheral blood, leucocytes were prepared from the 5 ml blood sample using Lymphoprep 1.077. The cells at the interface were washed in PBS and re-suspended in PBS prior to counting. $1x10^6$ cells were incubated with either 20 ul of anti CD56 allophycocyanin, 20 ul anti CD45 fluorescein isothiocyanate, 20 ul anti CD16 phycoerythrin, 20 ul of all antibodies, 20 ul of isotype control antibodies or no antibody (negative control). After incubation the cells were fixed and analysed as described above.

Analysis

For immunohistochemistry the numbers of CD56 positive, CD45 positive and total number of negative stromal cells were counted in 10 X 400 magnification microscopic fields for each biopsy. The number of CD56+ cells was expressed as a percentage of either all stromal cells or as a percentage of CD45+ cells.

For flow cytometry the numbers of CD56+ cells were calculated as either a % of total cells in the sample or as a % of CD45+ cells found in the sample.

The relationships between the numbers of endometrial cells measured by flow cytometry and immunohistochemistry in normal control women and the numbers of peripheral blood CD56+ cells measured by flow cytometry and

endometrial CD56+ cells measured by immunohistochemistry in women with recurrent miscarriage was determined using the Pearson correlation test.

Results

The age and peripheral blood levels for LH, FSH, progesterone and oestradiol for the 25 recurrent miscarriage women are shown in Table 1. The concentrations of each hormone are within the normal range.

Figure 1 shows the immunohistochemical staining for CD45+ and CD56+ cells in the proliferative and secretory endometrium from the group of normal fertile women. In proliferative phase endometrium CD45+ and CD56+ cells were present as individual cells scattered throughout the stroma, while secretory phase endometrium contained more CD45+ and CD56+, which were also present as clumps of cells (Figure 1a - d). Figure 1 e and f shows immunohistochemical staining in secretory phase endometrium from women with recurrent miscarriage. It shows CD56+ cells present as individual or groups of cells.

Figure 2 shows the relationship between endometrial CD56+ cells and peripheral blood CD56+ cells in the group of recurrent miscarriage women. There was no significant correlation between numbers of endometrial CD56+ cells and total peripheral blood CD56+, or the CD56+CD16+, CD56+CD16- subsets of CD56+ cells.

There was also no correlation between the numbers of endometrial CD56+ cells or peripheral blood CD56+ cells (or subsets) and the concentration of plasma LH, FSH, oestradiol or progesterone (data not shown).

Figure 3 shows the numbers of CD56+ cells in the endometrium obtained at different times in the menstrual cycle from individual normal fertile women measured by immunohistochemistry. Although there is some variation, the numbers are low during the proliferative and early secretory phase of the

cycle and only start to increase from the mid-late secretory phase. A similar pattern of distribution during the cycle was obtained when the numbers of CD56+ cells were measured by flow cytometry (data not shown).

Figure 4 shows the relationship between numbers of endometrial CD56+ cells measured by flow cytometry and by immunohistochemistry in the same endometrial biopsy samples obtained from normal fertile women throughout the menstrual cycle. When expressed as a percentage of all cells the measurements of CD56+ cells by flow cytometry were lower (0.1-18%) than measurements obtained by immunohistochemistry (3.8-52.8%), whereas when expressed as a percentage of CD45+ cells both methods gave more comparable results (range 3.8-61% for flow cytometry and 10.8-70% for immunohistochemistry).There was a significant correlation between the measurement of cells by flow cytometry and immunohistochemistry both when the cell numbers were expressed as a percentage of total cells (correlation=0.497 P=0.026) or as a percentage of CD45+ cells (correlation=0.570 P=0.009).

Discussion

Previous studies on the possible role of uNK cells in pregnancy outcome have produced contradictory results; some studies have shown that women with reproductive failure such as repeated implantation failure or recurrent miscarriage have increased numbers of uNK cells while others have suggested that there is no difference or reduced numbers (Thum et al. 2004; Moffat et al. 2004; King et al. 2010; Bulmer et al. 2010). It has been suggested that this may be due to measurement of NK cells in different compartments (eg blood, endometrium or decidua) or by different methodologies (eg immunohistochemistry or flow cytometry) (Laird et al. 2003).

In this study we have investigated numbers of NK cells in the endometrium and peripheral blood in samples obtained at the same time from the same individual woman in a group of women undergoing investigation for recurrent miscarriage. We found no correlation between numbers of total CD56+, CD56+CD16- or CD56+CD16+ and endometrial CD56+ cells in these women. A limitation of the study is that it was carried out in women with recurrent miscarriage; a comparable study in control fertile women would also be of interest. However recruitment of large numbers of fertile women to studies which require taking simultaneous blood and biopsy samples is difficult. There are very few studies comparing numbers of CD56+ endometrial and peripheral blood cell numbers even in patients with reproductive disorders attending clinics for diagnosis and treatment, suggesting the difficulties in the logistics of such studies.

The fact that there is no correlation between peripheral blood NK cell number and uNK cell number raises questions about the role of studies and measurements of peripheral blood NK cells in reproductive medicine. Measurements of peripheral blood NK cells by flow cytometry are considerably easier, as they do not require obtaining an endometrial biopsy sample, which is invasive. Flow cytometry can also easily detect sub-populations of CD56+ cells such as CD56+CD16+ and CD56+CD16-, which are postulated to play different roles in pregnancy failure (Laird et al. 2003). However the numbers of peripheral blood NK cells may not relate to those seen in the endometrium and therefore it may be inappropriate to extrapolate results from peripheral blood to what is happening in the endometrium. In addition a recent study has shown that peripheral blood NK cells are increased at times of stress and that the stress of obtaining a blood sample may artificially increase peripheral blood NK cells (Shakhar et al. 2006) again questioning the use of peripheral blood NK cell measurements in the diagnosis and treatment of recurrent miscarriage and other reproductive disorders.

Peripheral blood and endometrial and uNK cells are in contact with different areas of the developing placenta; the peripheral blood baths the syncytiotrophoblasts, while endometrial and decidual cells are in contact with the invading trophoblast. It is thought that the endometrial and decidual NK cells are more likely to be involved in reproductive pathologies because of their proximity to the implantation process. Peripheral blood NK and uNK cells are likely to have different functions. Peripheral blood NK cells are part of the innate immune system and are involved in the protection against

infection. The precise role of uNK cells in the implantation process is unknown, but could include direct interaction with MHC molecules on the invading trophoblast, production of chemokines and cytokines, angiogenesis and spiral artery remodelling or innate immunity (Bulmer et al. 2010; Bulmer and Lash 2005; Quenby et al. 2009).

We also compared the numbers of endometrial uNK cells measured by immunohistochemistry and flow cytometry in endometrial biopsy samples obtained at the same time from individual fertile control women. For endometrial biopsy samples immunohistochemistry is usually used to determine the number of CD56+ cells. The advantage of this technique is that it can be carried out on relatively small samples of tissue and, once fixed, the tissue can be processed at the convenience of the laboratory. Larger endometrial biopsy samples are required to obtain sufficient cells for flow cytometry and the tissue needs to be processed immediately. However the advantage of the flow cytometry method is that it is able to determine sub-populations of CD56+ cells, which can only be carried out by double staining or staining on serial sections by immunohistochemistry.

Our study showed a correlation between the numbers of CD56+cells measured by the 2 different methods. We expressed these measurements as CD56+ cell numbers as a percentage of all cells and CD56+ cells as a percentage of CD45+ cells for both methodologies. When expressed as a percentage of all cells the measurements of CD56+ cells by flow cytometry were lower (0.1-18%) than measurements obtained by immunohistochemistry (3.8-52.8%), whereas when expressed as a percentage of CD45+ cells the methods gave more comparable results

(range 3.8-61% for flow cytometry and 10.8-70% for immunohistochemistry). This means that care should be taken in comparing absolute numbers of uNK cells using the two different methodologies either within the same study or between studies. It also means that before measurement of uNK cells is translated into clinical practice a decision needs to be made about the chosen methodology of measurement.

Although absolute values of CD56+ cells measured in each biopsy differed using the different methods there was a correlation in the cell numbers obtained, meaning that biopsies where there were high numbers of CD56+ cells were at the high end of the range in each method used. This means that in studies comparing numbers of CD56+ cells in two different groups of women or before and after treatments differences in results may not be due to differences in the methods used to detect the cells as long as the same method is used for all groups in the same study.

In a previous study we reported that the numbers of CD56+ cells in endometrium obtained from a small group of control women (n=10) on days LH+7-LH+9 of the menstrual cycle was 2.2-13.9% (Tuckerman et al. 2007). In the current study the numbers ranged from 3.9-52.8%. However in this study samples were taken from throughout the menstrual cycle. A massive increase in CD56+ cells into the endometrium at the end of the cycle just prior to menstruation has been reported (Salamonsen & Lathbury 2000) and very high numbers of CD56+ cells were also only found in the endometrium in samples obtained during the late secretory phase of the cycle in this study. Of the 7 samples obtained in this study in the mid-secretory phase of the

cycle (days 19-23), only 1 was slightly (14.6%) above the 13.9% upper value reported in the previous study.

In conclusion we have shown no correlation between numbers of CD56+ cells in endometrium and peripheral blood in a group of women with recurrent miscarriage, suggesting that it is inappropriate to extrapolate findings in peripheral blood to the endometrium and decidua. In addition we have shown a correlation between measurements of endometrial NK cells by flow cytometry and immunohistochemistry, which suggests that differences in results seen in some studies cannot be attributed to the different methodologies used.

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Legends to Figures

Figure 1. Photomicrograph of immunostaining for a) CD45 in proliferative endometrium from a fertile woman b) CD56 in proliferative endometrium from a fertile woman c) CD45 in secretory endometrium from a fertile woman d) CD56 in secretory endometriun from a fertile woman e) and f) CD56 in secretory endometrium from 2 different women with recurrent miscarriage. (Magnification X400).

Figure 2. Relationship between numbers of endometrial CD56+ cells assessed by immunohistochemistry and a) total peripheral blood CD56+ cells b) CD56+CD16+ peripheral blood cells and c) CC56+CD16- peripheral blood cells in recurrent miscarriage women (n=25).

Figure 3. Variation in numbers of endometrial CD56+ cells assessed by immunohistochemistry in biopsy samples obtained from fertile women throughout the menstrual cycle (n=20).

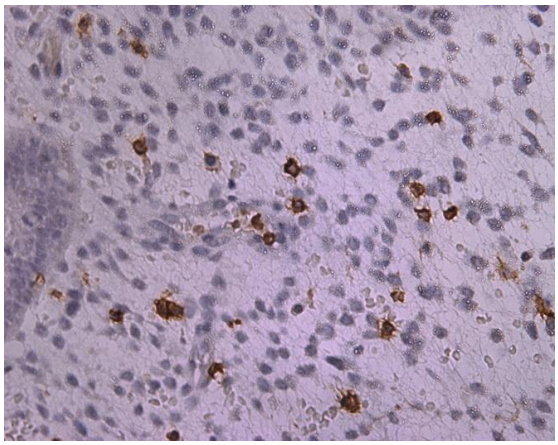
Figure 4. Correlation between measurement of endometrial CD56+ cells measured by immunohistochemistry and flow cytometry expressed as a) % of all cells and b) % CD45+ cells in fertile women (n=20).

Table 1. Age and hormone data for recurrent miscarriage patients

	Median (Range)
Age	36 (25-42) years
LH	5 (2.4-9.2) IU/L
FSH	6.4 (2.9-12.6) IU/L
Progesterone	42.9 (16.8-168) nmol/l
Oestradiol	171 (71-378) pmol/l

Figure 1.

a



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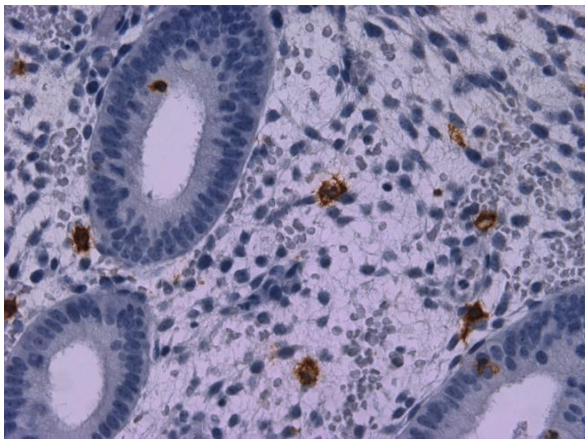

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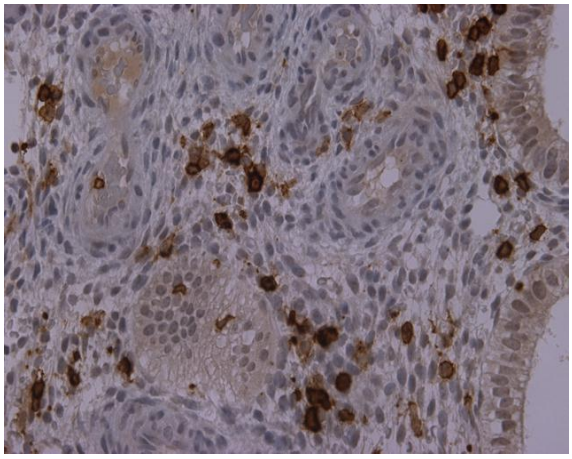

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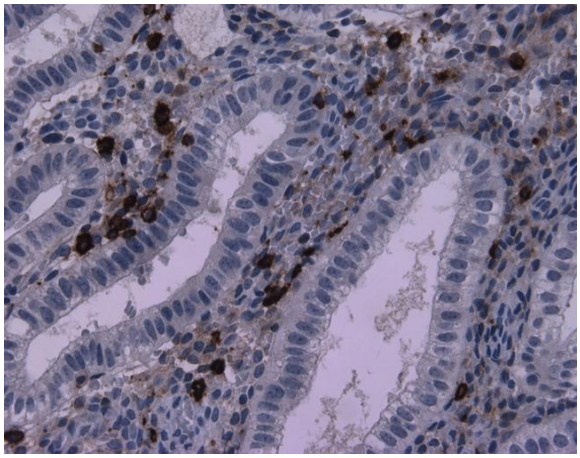

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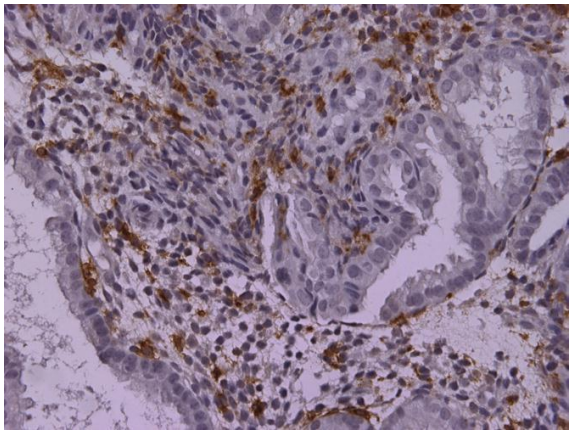

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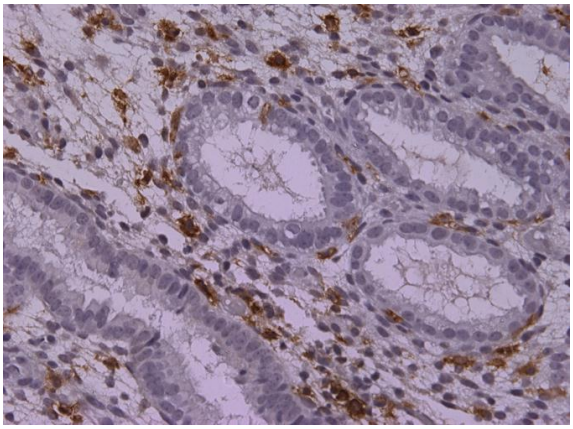
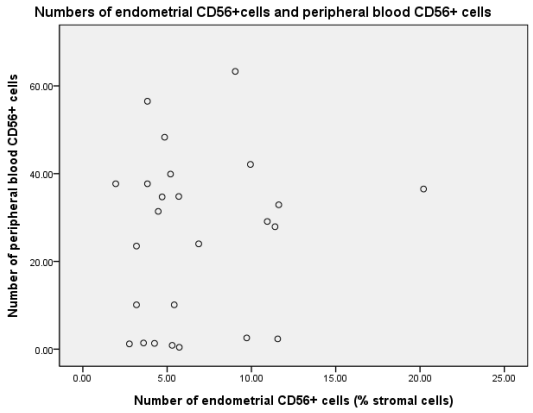
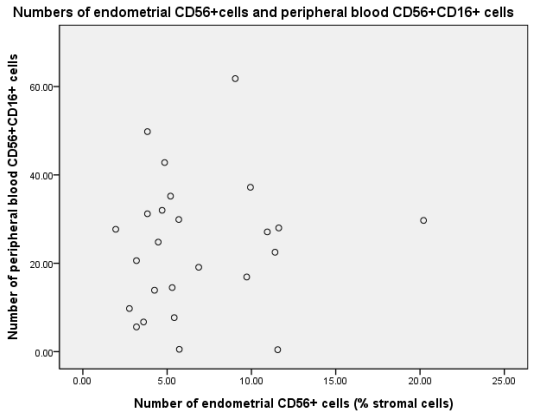


Figure 2

a)


Numbers of endometrial CD56+cells and peripheral blood CD56+ cells
Number of peripheral blood CD56+ cells
60.00
40.00
20.00
0.00
0.00
5.00
10.00
15.00
20.00
25.00
Number of endometrial CD56+ cells (% stromal cells)

b)


Numbers of endometrial CD56+cells and peripheral blood CD56+CD16+ cells
Number of peripheral blood CD56+CD16+ cells
60.00
40.00
20.00
0.00
0.00
5.00
10.00
15.00
20.00
25.00
Number of endometrial CD56+ cells (% stromal cells)

c)

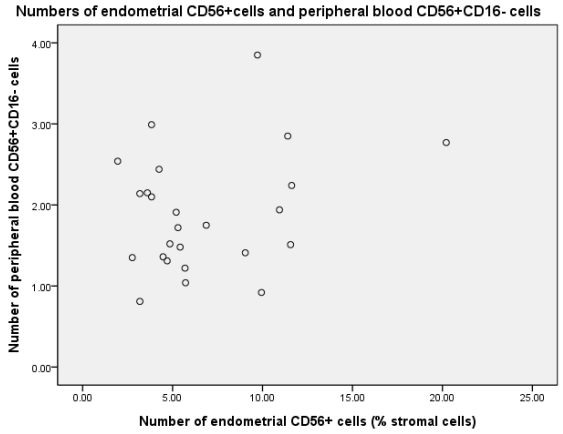

Numbers of endometrial CD56+cells and peripheral blood CD56+CD16- cells
Number of peripheral blood CD56+CD16- cells
4.00
3.00
2.00
1.00
0.00
0.00
5.00
10.00
15.00
20.00
25.00
Number of endometrial CD56+ cells (% stromal cells)

Figure 3

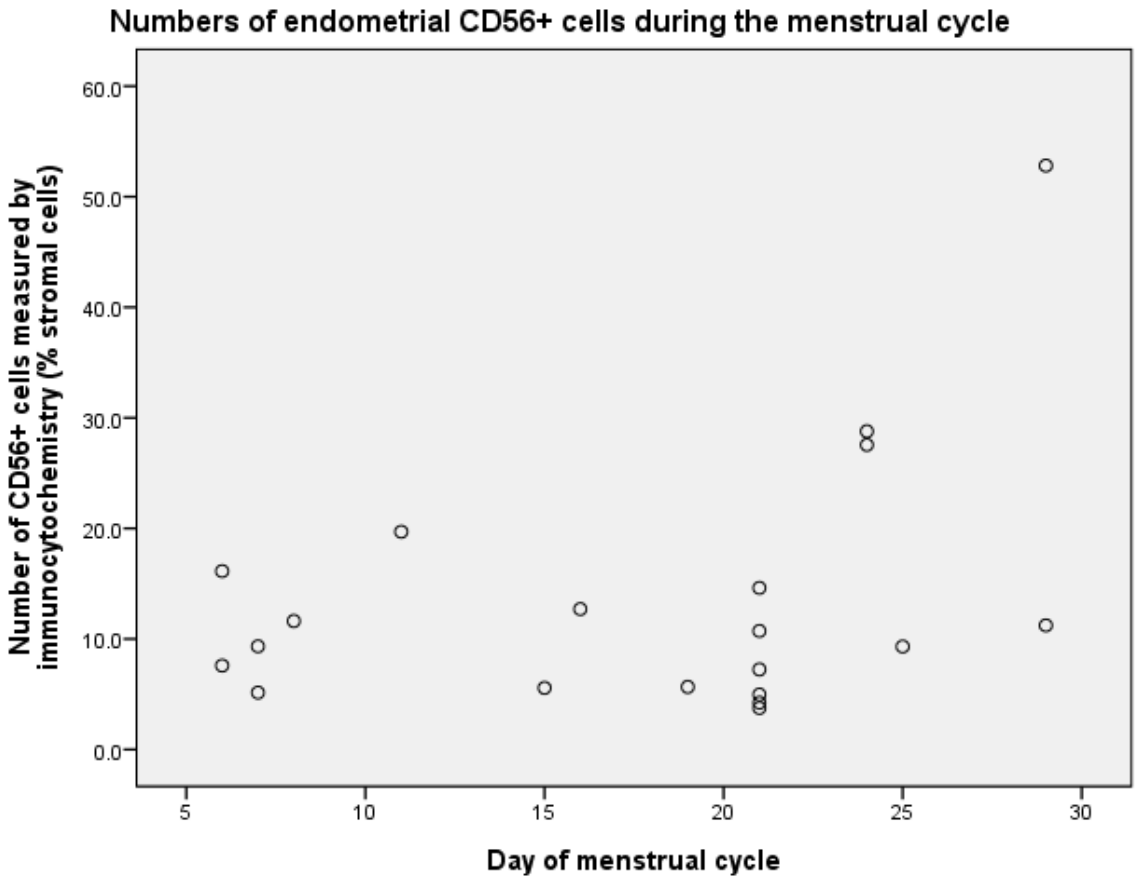
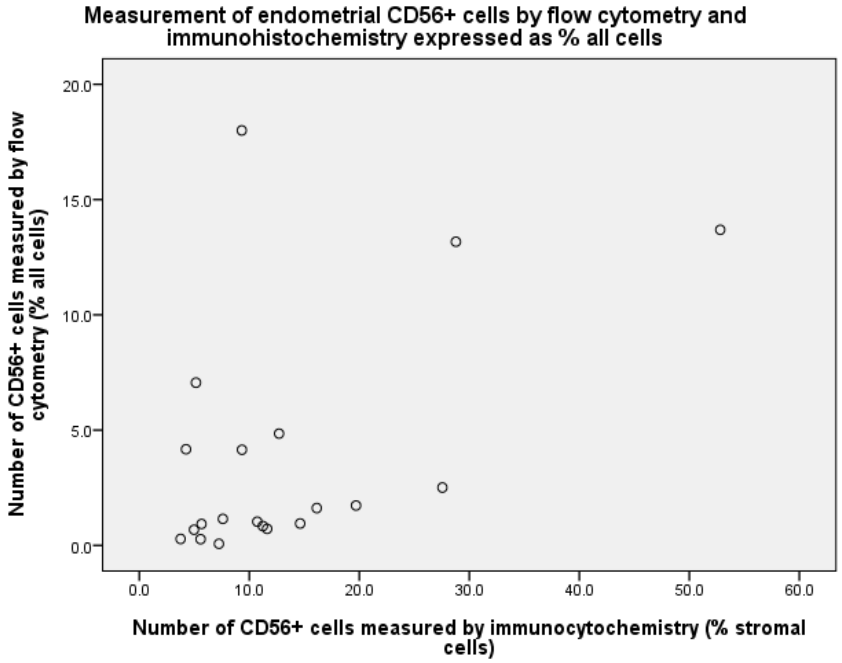
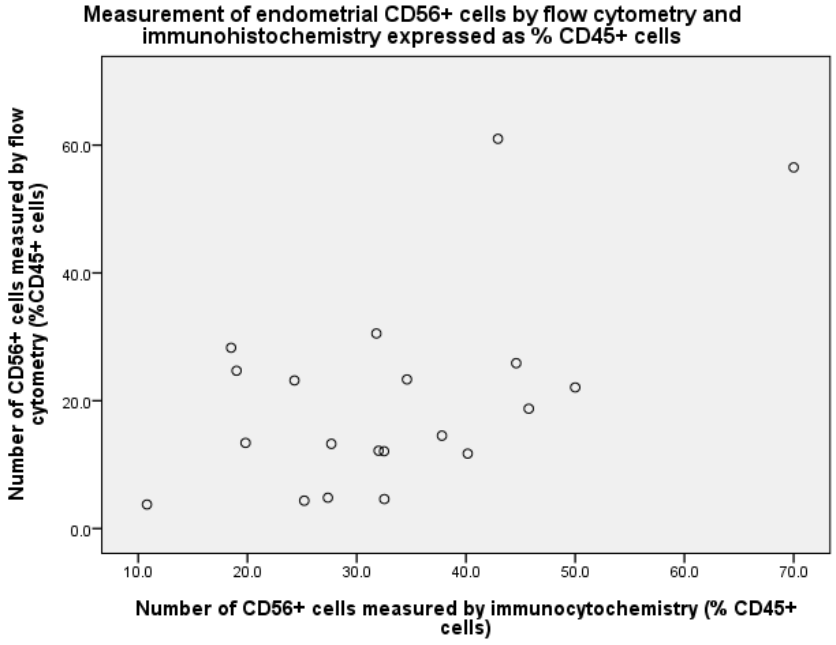

Numbers of endometrial CD56+ cells during the menstrual cycle
Number of CD56+ cells measured by immunocytochemistry (% stromal cells)
60.0
50.0
40.0
30.0
20.0
10.0
0.0
5
10
15
20
25
30
Day of menstrual cycle

Figure 4

a)


Measurement of endometrial CD56+ cells by flow cytometry and immunohistochemistry expressed as % all cells
Number of CD56+ cells measured by flow cytometry (% all cells)
20.0
15.0
10.0
5.0
0.0
0.0
10.0
20.0
30.0
40.0
50.0
60.0
Number of CD56+ cells measured by immunocytochemistry (% stromal cells)

b)


Measurement of endometrial CD56+ cells by flow cytometry and immunohistochemistry expressed as % CD45+ cells
Number of CD56+ cells measured by flow cytometry (%CD45+ cells)
60.0
40.0
20.0
0.0
10.0
20.0
30.0
40.0
50.0
60.0
70.0
Number of CD56+ cells measured by immunocytochemistry (% CD45+ cells)