Markers of Endometrial Receptivity: A Study of Ultrasonographic and Molecular Factors

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One wonders why some people sail through life on fully expanded sails avoiding the treacherous shallows, rough seas and rugged rocks, whereas others remain stuck in the silt or barely manage to keep afloat due to repeated insults to their hull.

I would like to dedicate this thesis to my Father, Marian Polanski, who kept going against all odds and thanks to whom I got to where I am today. May he rest in peace knowing that his efforts have not been in vain.

Tę rozprawę doktorancką, chciałbym zadedykować mojemu ojcu, Marianowi Polańskiemu, który mimo wielu trudności nigdy się nie poddawał i któremu zawdzieczam wszystko co do tej pory osiagnąłem. Był, i nadal jest, on dla mnie modelem człowieczeństwa.

Niech odpoczywa w pokoju, wiedząc że wszelkie jego wysiłki nie poszły na marne.

'(…) now is the time for science and not more marketing. For science is what we should do when we don't know what we are doing. Science and discovery are more important than earnings potential.'

Peter Braude, 2013

Abstract

The endometrium is a complex organ bridging the fetal and maternal environments. Appropriate endometrial function allows for establishment of pregnancy and its successful course. Any deregulation within this organ, can cause various manifestations of reproductive failure. The aim of this thesis was to identify markers that would be predictive of successful pregnancy following assisted reproductive treatment (ART). To accomplish this task, a literature review on the molecular pathways governing endometrial decidualization was carried out. Similarly, an attempt to define recurrent implantation failure (RIF), an iatrogenic ART related condition, was carried out aiming to standardise recruitment to future studies. Subsequent empirical work included a clinical trial of endometrial biopsy prior to ART aiming to answer if the procedure can improve treatment outcomes in an unselected population of infertile women. Contemporaneous sonographic and molecular data has been collected in order to describe a receptive endometrial phenotype favouring pregnancy. The thesis structure plan is presented in Figure 1.

The main study involved 151 women randomised to receiving an endometrial biopsy or no procedure in the mid-luteal phase of the menstrual cycle directly preceding *in vitro* fertilization (IVF), intra-cytoplasmic sperm injection (ICSI) or frozen embryo replacement treatments. The primary outcome of the study was a clinical pregnancy. Of the randomised women, 76 were allocated to the study group and 75 to the control group. There was no significant difference in the clinical pregnancy rates between the biopsy and control groups both on an intention to treat (ITT) basis [53.9% (41/76) versus 44.0% (33/75); risk ratio (RR) 1.23 (95%CI=0.88-1.70); P=0.256] and per embryo transfer [65.1% (41/63) versus 55% (33/60); RR 1.18 (95%CI=0.88-1.58); P=0.254]. Procedure related complications occurred in one woman (1.4%), whom was not able to tolerate the procedure due to pain. No other significant complications were reported.

Sonographic data obtained during the abovementioned study has demonstrated that only triple pattern endometrium was associated with a

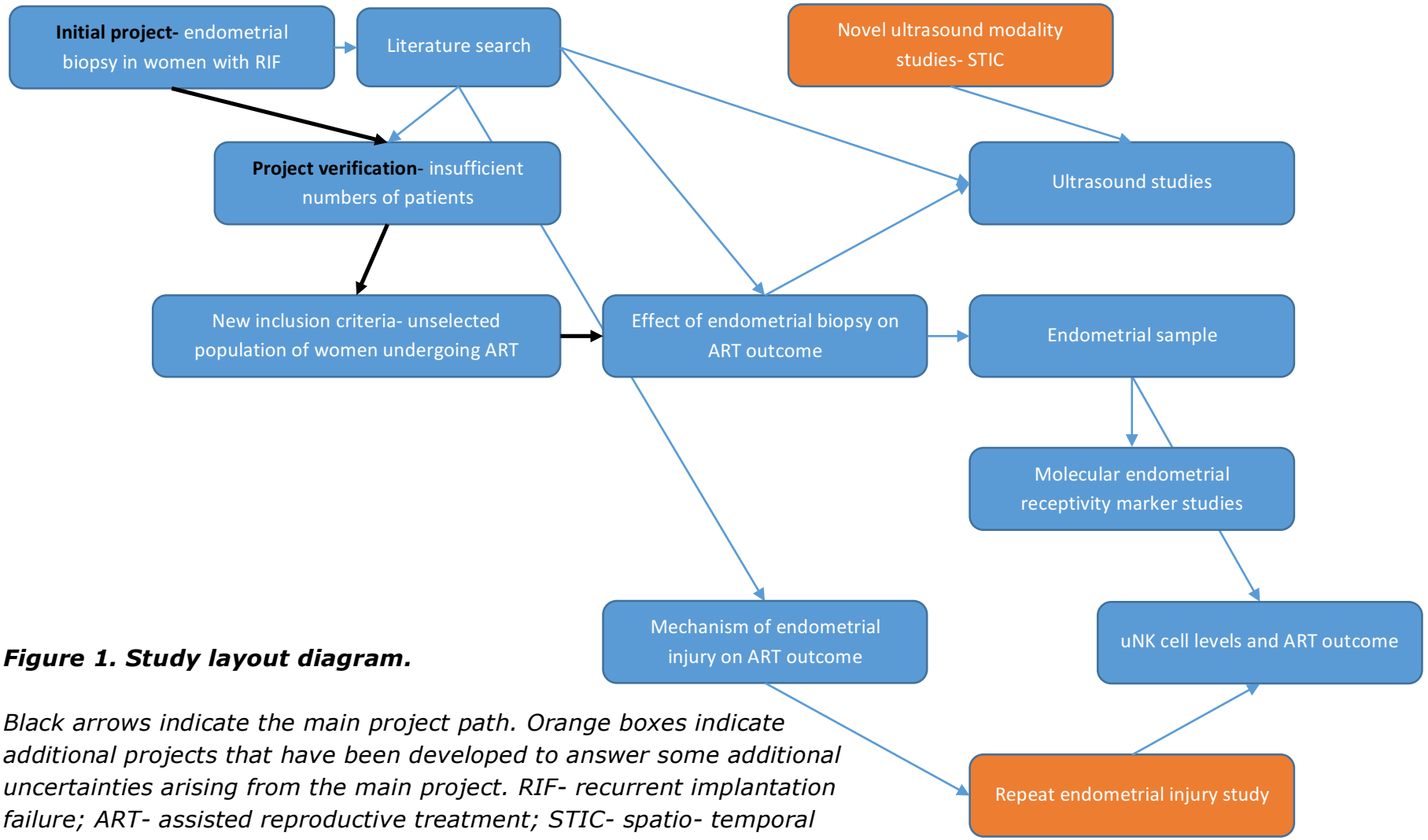

Figure 1. Study layout diagram.

Black arrows indicate the main project path. Orange boxes indicate additional projects that have been developed to answer some additional uncertainties arising from the main project. RIF- recurrent implantation failure; ART- assisted reproductive treatment; STIC- spatio- temporal image correlation.

clinical pregnancy at transvaginal oocyte retrieval (TVOR) (P<0.05). All other sonographic markers were not associated with any pregnancy outcome. There were no significant sonographic differences between the biopsy and control groups in the mid-luteal phase of the cycle, at TVOR and at embryo transfer (ET), though the 3-dimensional power Doppler (3DPD) vascularization index (VI) and vascularization flow index (VFI) appeared to be lower in the biopsy group (P>0.05). When high definition power Doppler (HDPD) changes over time were analysed, the FI differed between the pregnant and non-pregnant population (P=0.032), with the negative group having a lower vascularity at TVOR and at ET. The best predictor of a first trimester miscarriage was mean grey scale (MGS) at TVOR with an area under the curve (AUC) of 0.810.

As part of the study, the feasibility of utilising a novel power Doppler US modality- Spatio-Temporal Image Correlation (STIC), as a non-invasive marker of endometrial receptivity has also been assessed. Initially, optimisation of machine settings has been performed. As STIC allows for analysis of a spherical volume of interest as well as the entire endometrium during a single cardiac cycle, both these methods were assessed and compared. The mean time taken for analysis of one STIC dataset was 1478.86 ± 290.99 s using the manual analysis method and 266.8 ± 39.31 s using spherical sampling (P<0.05). When using manual analysis method, the intraclass correlation coefficients (ICCs) between one and two observers were above 0.8 for VI and VFI, suggesting very good reliability of measures. Spherical sampling revealed a lower ICC >0.5 for FI. The main drawback of the technology has been identified as a significant biological variability of measured vascularity indices between different acquisitions (or cardiac cycles), with the coefficients of variation (CVs) for the systolic, diastolic and average VI, FI and VFI ranging from 8.3% for diastolic FI to 76.3% for diastolic VFI. Nevertheless, STIC vascularity indices as predictive markers of ART outcome have been further evaluated. Manual minimal VI, average VI and minimum VFI STIC vascularity indices prior to TVOR, when considering first trimester pregnancy loss, the were significantly higher compared to the on-going pregnancy group (P<0.05), with minimal manual VI being able to predict

miscarriage with an AUC of 0.8 for a cut-off value ≥0.7 and sensitivity 80.0% and specificity 68.1%. All other indices have proven to be poor predictors of clinical pregnancy.

Collected endometrial samples were processed and analysed in order to assess the levels of $CD56^+$ uterine natural killer (uNK) cells, and correlate these with the ART outcomes. Women recruited to the endometrial biopsy study and sixty-four women with recurrent reproductive failure that attended the host unit for implantation failure screening were recruited. uNK cell levels were compared between the reproductive failure group and women undergoing their first ART cycle. The former population tended to have higher uNK cell levels compared to women undergoing their first IVF treatment cycle [4.25% (1.8-7.6) vs. 2.6% (1.4-7.4) respectively; P>0.05]. No correlation between uNK cell levels and ART outcome was identified. Clinical pregnancy rates of 63.2% (12/19) were achieved in women with uNK cell levels >5% and 69.2% (27/39) in the population of women with uNK cells <5%, respectively (P>0.05). Miscarriage rates were 5.3% (1/19) and 12.8% (5/39), respectively (P>0.05).

Endometrial biopsy and uNK cell level analysis has been carried out in two consecutive menstrual cycles to identify a possible mechanism responsible for the proposed in the literature beneficial effect of endometrial biopsy on pregnancy outcomes. A small cohort of 22 women has been recruited; only 16 repeat samples were available for data comparison. The uNK cell levels in the first biopsy cycle were 2.4% (0.78- 4.8%) with a significant decrease in the second cycle at 0.7% (0.4-0.91%; P<0.01). Contemporaneously acquired sonographic data has shown no difference in endometrial parameters (P>0.05).

Finally, the obtained endometrial stromal cells were processed, cultured and decidualized using medroxyprogesterone acetate (MPA) and 8-Bromoadenosine- cyclic monophosphate (8-Br-cAMP). These cells were then harvested on day 0, 2 and 8 of decidualization and processed using PCR techniques to ascertain the response to the decidualization stimuli and assess if a receptive phenotype can be identified using prolactin

(PRL), insulin-like growth factor binding protein (IGFBP)-1 and 11-β-hydroxysteroid dehydrogenase (HSD) 1. Twenty-four women have been enrolled in this part of the study. Following ART, eleven were found to have a clinical pregnancy, two had a biochemical pregnancy only and eleven had a negative pregnancy test. There was a significant increase in the relative PRL, IGFBP-1 and 11β-HSD1 messenger ribonucleic acid (mRNA) expression between days 0, 2 and 8 of hormone treatment in all analysed samples ($P<0.05$). This difference was not significant between the groups with and without clinical pregnancy. 11β-HSD1 mRNA expression on day 0 of decidualization has demonstrated a positive correlation with uNK cell levels (Pearson r 0.44; $P=0.03$) and day 8 (Pearson r 0.45; $P=0.028$). At TVOR, the IGFBP1 expression on day 2 of decidualization has been found to be negatively correlated with endometrial thickness (Pearson r -0.438, $P=0.047$). None of the assessed markers was able to unequivocally differentiate between the pregnant and non-pregnant populations and, as such, were poor predictors of ART outcome.

In conclusion, in an unselected population of infertile women undergoing ART, routine endometrial scratching in the mid-luteal phase of the cycle directly preceding the treatment has shown no benefit on pregnancy rates. As the study is underpowered, basing clinical practice on the findings reported in this thesis without appropriate validation on a larger sample, should not be undertaken. Women with reproductive failure tend to have elevated uNK cells compared to infertile women undergoing first ART cycles ($P>0.05$). uNK cell levels have been shown to be significantly affected by the endometrial biopsy, which may provide a possible explanation as to why endometrial injury improves the endometrial milieu. Endometrial scratching in women with deregulated endometrial environment might reset the endometrial immune status to a receptive state favouring pregnancy. Analysed sonographic and molecular markers have proven to be poor predictors of ART outcome. The findings reported in this work may be related to the unselected populations studied and require further validation in larger or more selected populations.

Acknowledgements

The research described in this thesis would not be possible without the women that have taken the time and effort to take part in the studies, while undergoing the stressful and demanding infertility treatment. Without their dedication, furthering research in the field of ART would not be possible.

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Other people that require special mention in this thesis, are Mrs Anne Skinner whom has introduced me to the laboratory and held my hand throughout the first experiments; Dr Emma Lucas, Coventry, who introduced me to more complex laboratory analyses; Mr Ruban Peter Durairaj, Coventry, whom took time and effort to culture endometrial cells in order to obtain material for analysis; Miss Kirstyn Sewell, whom helped to retrieve outcome data; and last but not least Dr Amarin Narkwichean, who helped me understand and carry out statistical analysis.

Lastly, I would like to thank all staff of Nurture Fertility, for their help, support and good times spent together.

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Abbreviations

2D	Two-dimensional
3D	Three-dimensional
3DPD	Three-dimensional Power Doppler
8-Br-cAMP	8-BromoAdenosine- cyclic MonoPhosphate
11β HSD	11-β Hydroxysteroid Dehydrogenase
ART	Assisted Reproductive Treatment
AFC	Antral Follicle Count
AMH	Anti-Müllerian Hormone
ANOVA	Analysis of variance
AUC	Area Under the ROC Curve
BMI	Body Mass Index
CI	Confidence Interval
CL	*Corpus luteum*
COH	Controlled Ovarian Hyperstimulation
CV	Coefficient of Variation
DAB	3,3'-Diaminobenzidine
DCC-FCS	Dextran Coated Charcoal treated Fetal Calf Serum
DMSO	Dimethyl Sulfoxide
ET	Embryo Transfer or Endometrial Thickness (depending on context in which used)
FI	Flow Index
FER	Frozen Embryo Replacement
FSH	Follicle Stimulating Hormone

GnRH	Gonadotropin Releasing Hormone
GR	Glucocorticoid Receptor
HCG	Human Chorionic Gonadotropin (beta subunit, unless stated otherwise)
HESC	Human Endometrial Stromal Cells
HLA	Human Leukocyte Antigens
HSG	Hysterosaplingography
ICC	Intra-Class Correlation Coefficient
ICSI	Intra-Cytoplasmic Sperm Injection
IGF	Insulin-like Growth Factor
IGFBP-1	Insulin-like Growth Factor Binding Protein- 1
ITT	Intention To Treat
IQR	Interquartile Range
IUI	Intrauterine Insemination
IVF	*In-vitro* Fertilization
JZ	Junctional Zone
LH	Luteinising Hormone
LOA	Limits of Agreement
LR	Likelihood Ratio
MANOVA	Multivariate analysis of variance
MGS	Mean Grey Scale Value
MHC	Major Histocompatibility Complex
MoM	Multiples of the Mean
MPA	MedroxyPorgesterone Acetate

MR	Mineralocorticoid Receptor
MRI	Magnetic Resonance Imaging
NK	Natural Killer (cells)
NPV	Negative Predicitve Value
OHSS	Ovarian Hyperstimulation Syndrome
OR	Odds Ratio
PCR	Polymerase Chain Reaction
PD	Power Doppler
PDSTIC	Power Doppler Spatio-Temporal Image Correlation
PFA	Paraformaldehyde
PI	Pulsatility Index
PPV	Positive Predictive Value
PRF	Pulse Repetition Frequency
PRL	Prolactin
PSV	Peak Systolic Velocity
PWPD	Pulse Wave Power Doppler
RCT	Randomized Controlled Trial
REC	Research Ethics Committee
RI	Resistance Index
RM	Recurrent Miscarriage
RIF	Recurrent Implantation Failure
RNA	Ribonucleic Acid
ROA	Region Of Interest

ROC	Receiver Operating Characteristics
RR	Risk Ratio (Relative Risk)
SD	Standard Deviation
SonoAVC	Sonography Based Automated Volume Calculation
STIC	Spatio-Temporal Image Correlation
TVOR	Transvaginal Ultrasound Guided Oocyte Retrieval
UA	Uterine Artery
VEGF	Vascular Endothelial Growth Factor
VFI	Vascularisation Flow Index
VI	Vascularisation Index
VOCAL	Virtual Organ Computer-aided AnaLysis ™
WOI	Window Of Implantation

CHAPTER 1. Background and Hypothesis

The stage of human development spanning from fertilization to pregnancy establishment is one of the most fascinating physiological processes that science has yet to understand. Multiple factors are involved in this process, and deregulation of any of these often leads to reproductive failure. This condition encompasses infertility or pregnancy losses at varying stages of gestation, which can affect up to 20% of couples trying to conceive. In this thesis I have focused on the infertile population and women with failed implantation. Failure to achieve a pregnancy and, if such has been achieved, its subsequent loss is a distressing and often life changing event for the couple involved. Hence research into reproductive failure and subsequent pregnancy development is an important one to pursue.

This chapter contains information about reproductive anatomy, physiology, pathophysiology and means of assessing endometrial parameters in the context of endometrial receptivity.

1.1. Female reproductive anatomy

1.1.1. Normal anatomy of the female reproductive tract

The non-pregnant uterus is a female pelvic organ encapsulated in peritoneum with the exception of a small anterior aspect where the uterus comes in direct contact with the bladder. The sigmoid colon and rectum are located posteriorly to the uterus. Broad and round ligaments support the uterine corpus. The non-gravid uterus measures 7.5 x 5 x 2.5 cm and weighs up to 40 gm. The uterus is divided into two parts by the isthmus- the superior body (*corpus*) and the inferiorly located cervix. Within the cavity, the internal cervical *os* corresponds to the externally distinguishable isthmus; part of the uterus lying above tubal *ostia* is termed the fundus. The cavity of the uterus is smaller compared to the overall dimensions of the organ. It is triangular in shape, with the base located between tubal ostia and the apex at the internal cervical os. The uterine cavity communicates with the external environment via the cervical

canal, which terminates at the external cervical os, and with the peritoneal cavity via the Fallopian tubes. The average distance from the fundus to the external cervical os is 6.25 cm (Gray et al. 1918).

The blood supply to the uterus originates mainly from the uterine arteries (branches of anterior division of internal iliac arteries) located on both sides of the organ between the two layers of the broad ligament. Within the broad ligament, the uterine arteries divide into the ascending and descending branches supplying the cervix, uterus and cervix and vagina, respectively. Within the uterus, they give off further branches: the arcuate arteries and spiral arteriolar plexus (Farrer-Brown et al. 1970) giving off radial arteries, which directly feed the endometrium (Chien et al. 2002). A small proportion of the blood supply to the uterus comes from the ovarian arteries, originating from the abdominal aorta and anastomosing with the superior branches of the uterine arteries within the broad ligaments (see Figure 2) (Gray et al. 1918).

The endometrium is composed of two functionally distinct portions- the basal layer (*zona basalis*) and the functional layer (*zona functionalis*) (Heller 1994, Clancy 2009). Rapid endometrial growth of the stroma and glands characterising the follicular phase of the menstrual cycle begins from the *zona basalis*. The endometrial glands at this stage are straight and tubular, and are lined with pseudo-stratified columnar cells. No mucous secretion or vacuolation is present. Around the time of ovulation, the mitotic activity of the endometrium slows down in favour of cellular differentiation. Formation of vacuoles begins initially near the basal membrane and progressively reaches the apical surface of the glandular cells. In the late luteal phase, the endometrial glands are dilated and tortuous. The stromal compartment also undergoes differentiation. On around days 21- 22 of the cycle, spiral arterioles are formed. Extracellular matrix increases and oedema is present. Stromal cells undergo hypertrophy and accumulate cytoplasmic eosinophilia. This process is termed as pre-decidual change and spreads rapidly along the *zona functionalis*. Scattered neutrophils and lymphocytes are

present, which increase in number just before menstruation (Kumar et al. 2005). If no pregnancy occurs, the *zona functionalis* is shed in the form of menstrual bleeding.

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Figure 2. Vascular supply of the uterus and vagina.

Image from Anatomy of the Human Body, Henry Gray; 1918.

1.1.2. **Female reproductive tract abnormalities**

Human sex is determined genetically by the presence of X and Y chromosomes. Up to a certain stage in the development, embryos of both sexes are morphologically similar. Lim1, Pax2, Emx2 and Wnt4 are essential genes responsible for early steps of Müllerian duct development (Yin et al. 2005). Presence of the sex determining region Y gene (*Sry*) on the Y chromosome in males allows for development of male sexual characteristics (Cate et al. 1986, Koopman et al. 1991). Absence of male determining factors (*Sry* and Müllerian inhibiting substance-MIS) allows for uninterrupted proliferation and differentiation of the Müllerian ducts in the female and development of female sexual characteristics (Yin et al. 2005). Uterine development occurs up until the 16th week of fetal life. Two paramesonephric ducts (Müllerian ducts) form the Fallopian tubes, uterine corpus, cervix and upper portion of vagina (Letterie 1998, Braun et al. 2005). Stages of uterine development include

organogenesis of Müllerian ducts, lateral fusion of these structures and absorption of the septum created during Müllerian duct fusion (Saravelos et al. 2008). The Wolffian (or mesonephric) ducts are a precursor and inducer of female reproductive tract development (Hannema et al. 2007). Disruptions in the *in utero* development of the reproductive tract lead to formation of congenital uterine abnormalities, whereas acquired uterine anomalies form during ones' lifetime.

The most recent classification of congenital uterine anomalies is based on anatomical findings and is categorised into classes and sub-classes according to the severity of anatomical deviation (see Figure 3). Cervical and vaginal abnormalities are also included (Grimbizis et al. 2013). This classification differs from the previous classification where uterine anomalies were divided into seven groups based on the degree of Müllerian duct development status (Wold et al. 2006).

Diagnosis of uterine anomalies can be conducted by means of hysterosalpingography (HSG), two- dimensional ultrasound, sonohysterography (SHG), three-dimensional ultrasound (3DUS), hysteroscopy and magnetic resonance imaging (MRI). HSG is a useful screening tool for diagnosis of a normal uterine cavity (Letterie 1998) with a sensitivity and specificity of 78% and 90%, respectively (Saravelos et al. 2008). The external contour of the uterus cannot be assessed using HSG; hence this modality has limited reliability in differentiating between different groups of anomalies (Saravelos et al. 2008). Wide availability of two- and three-dimensional ultrasound makes these tools an easy and non-invasive modality for screening for and diagnosis of uterine anomalies. Three- dimensional ultrasound has been reported to have a 100% sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy in identification and diagnosis of uterine anomalies compared to hysteroscopy (Wu et al. 1997, Radoncic et al. 2000, Makris et al. 2007a, Makris et al. 2007b) making it an effective, minimally invasive, well tolerated,

and inexpensive diagnostic tool. Despite this, some authors still consider the invasive combination of hysteroscopy and laparoscopy as the gold standard for diagnosis of congenital uterine anomalies. Hysteroscopy on its own allows for direct visualization and simultaneous treatment of the uterine cavity, but does not allow for assessment of the outside contour of the uterus, failing to classify different types of anomalies (Saravelos et al. 2008). MRI is reported to be a sensitive tool for diagnosis of uterine anomalies (Pellerito et al. 1992), however the cost and limited availability, limit the use of MRI as a diagnostic tool.

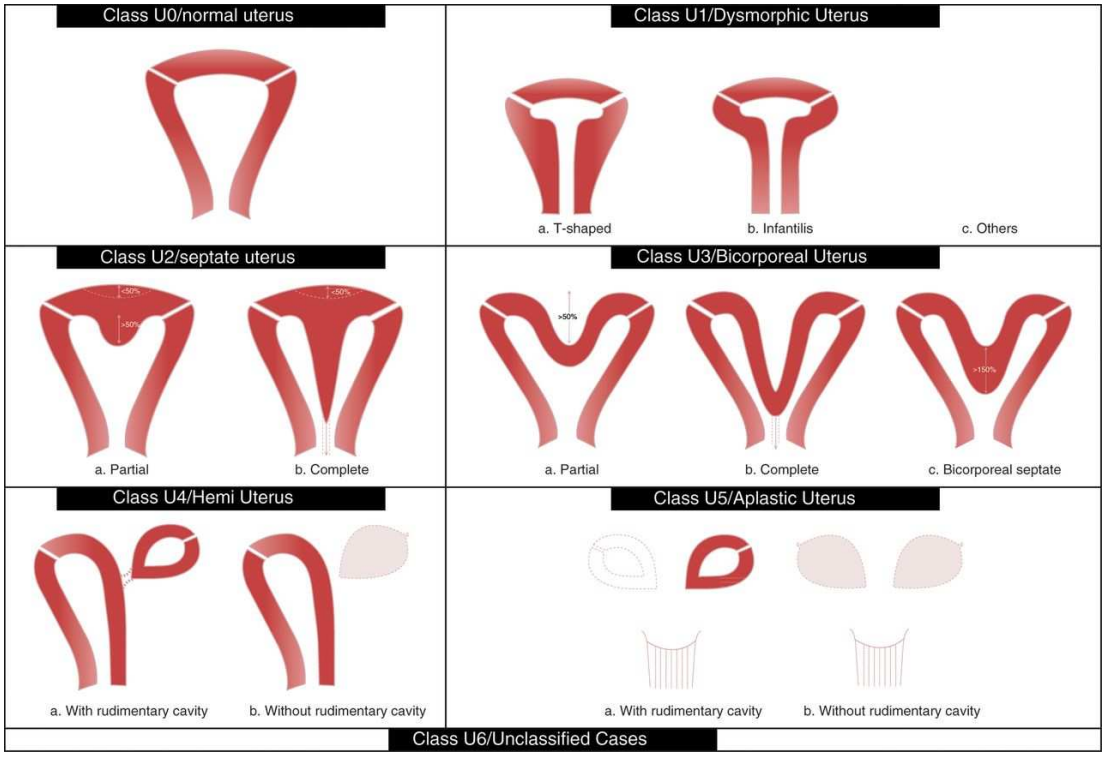


Figure 3. ESHRE/ESGE classification of uterine anomalies: schematic representation.

Class U2: internal indentation >50% of the uterine wall thickness and external contour straight or with indentation <50%, Class U3: external indentation >50% of the uterine wall thickness, Class U3b: width of the fundal indentation at the midline >150% of the uterine wall thickness. Adapted from Grigoris F., et al. (2013).

The prevalence of congenital uterine anomalies in the general population is estimated to be between 2.4% to 6.7%, with arcuate uterus being the most common anomaly (Saravelos et al. 2008). Approximately 7.3% to 10.8% of infertile women have been found to have a congenital uterine anomaly with septate uterus being most common (Saravelos et al. 2008). In women with recurrent miscarriage (RM), congenital uterine anomalies were identified in 13% to 23% of cases suggesting a strong link between RM and structural uterine anomalies (Patton 1994, Kupesic 2001, Salim et al. 2003, Saravelos et al. 2008). Very little evidence exists on the efficacy of treatment of congenital uterine anomalies on pregnancy outcome (Grimbizis et al. 2001). Some authors offer hysteroscopic metroplasty to women with septate and subseptate uteri and history of reproductive problems, however the exact impact of this procedure remains to be assessed fully (Kowalik et al. 2011).

Uterine leyomyomas, endometrial polyps and adenomyosis constitute the majority of acquired uterine anomalies. Uterine leyomyomas are the most common benign tumours of female genital tract and are known to cause various gynaecological symptoms, including subfertility. Approximately 25% of infertile women, possibly even more, are found to have a leyomyoma on baseline scan (Bulletti et al. 1999). Current evidence suggests that submucosal and intramural fibroids have a negative impact on implantation rates (Klatsky et al. 2008, Pritts et al. 2009). Endometrial polyps in women undergoing hysteroscopy for investigation of infertility are present in up to 25% of patients (Kim et al. 2003, Shokeir et al. 2004, Japur De Sa Rosa et al. 2005). Adverse influence of polyps on conception and implantation may possibly be related to formation of a mechanical barrier for gamete migration as well as alteration of receptivity of the endometrium preventing implantation (Rackow et al. 2011). Adenomyosis- the presence of ectopic endometrial glands within the myometrium- has been linked by some authors with infertility (Tremellen et al. 2011), however the frequent co-existence of this condition with

endometriosis, confounds this association. The true prevalence of adenomyosis within the infertile population is thought to be even as high as 50%, however that figure was derived from studies reporting the disruption of the endo-myometrial junctional zone (JZ), a feature of adenomyosis, and not full organ histopathology analysis (de Souza et al. 1995).

Transvaginal ultrasound is highly accurate in detecting intramural and subserosal fibroids, with a sensitivity of 99% and a specificity of 91%, and can be used to categorise the number, size and position of each. Sensitivity and specificity of standard ultrasound for detection of submucosal myomas varies, and has been reported to be as low as 21% and 53% respectively (Farquhar et al. 2003). Colour Doppler sonography detects endometrial polyps with a sensitivity and specificity between 80% and 90% (Hosny et al. 2007, Cil et al. 2010). Presence of myometrial cysts and distorted or irregular endo-myometrial JZ have been reported to be the best diagnostic features of adenomyosis on 2D and 3D sonography with diagnostic accuracy, as compared to histopathology, in the range of 83% and 89% for two- and three-dimensional sonography respectively (Exacoustos et al. 2011).

1.2. The ovary and the menstrual cycle

Uninterrupted cooperation of the female central nervous system and peripheral organs is responsible for physiological cyclical changes leading to menstruation. Menarche, or first period, signifies the transition from puberty in to the reproductive phase of a woman's life. Cessation of menstruation, or menopause, signifies the depletion of ovarian reserve and end of the reproductive years. By consensus, menstrual cycle begins on the first day of menses. The average length of the menstrual cycle is 28 days, ranging from 25 to 32 days (Silberstein et al. 2000). Gonadotropin- releasing hormone (GnRH) secreted by the hypothalamus exerts its action on the pituitary, which in turn produces luteinizing hormone (LH) and follicle stimulating hormone (FSH). The ovaries, in response to the

rising levels of FSH and LH, secrete oestrogen, progesterone, and inhibins. The endometrium being an end organ of oestrogen and progesterone action is the uterine marker of hormonal changes occurring during the menstrual cycle.

Neurons in the pre-optic and arcuate nuclei of the hypothalamus, when stimulated by norepinephrine, serotonin and other neurotransmitters, secrete GnRH into the hypophyseal portal venous system. A pulsatile manner of this production leads to secretion of FSH and LH from the pituitary, whereas continuous presence of GnRH leads to suppression of pituitary secretion of gonadotropins (Dierschke et al. 1970, Conn et al. 1991, Silberstein et al. 2000). In the follicular phase of the menstrual cycle, GnRH pulses occur every 1 to 2 hours (Dierschke et al. 1970, Belchetz et al. 1978, Conn et al. 1991). Progesterone in the luteal phase significantly decreases the pulsatility of GnRH secretion (Backstrom et al. 1982, Filicori et al. 1984, Merriam GR. 1984). Within the ovary, the theca cells of the follicle respond to LH levels and produce progesterone and androgens (Ryan et al. 1966, Ryan et al. 1968, McNatty et al. 1980). The granulosa cells aromatize androgens to oestrogens under the influence of FSH (Silberstein et al. 2000). FSH also promotes the secretion of inhibin, which together with oestrogen, suppresses FSH secretion by the pituitary (negative feedback loop) (Silberstein et al. 2000). The increasing level of oestrogen in the late follicular phase is responsible for a time and dose dependant positive feedback on the LH level (see Figure 4) (Marsh et al. 2011). Threshold levels of oestrogen in cycling women leading to an increase in LH secretion have been estimated to be between 91 pg/ml (334 pmol/L) and 195 pg/ml (716 pmol/L), with higher levels not causing further LH increase (Shaw et al. 2012). Rising levels of LH stimulate follicular progesterone secretion (Silberstein et al. 2000).

Image not available (Copyright)

Figure 4. Hormone level variations throughout the normal menstrual cycle.

FSH- follicle stimulation hormone; LH- luteinizing hormone. Image adapted from Anatomy & Physiology, Connexions Web site. http://cnx.org/content/col11496/1.6/, Jun 19, 2013.

The granulosa cells under the influence of FSH produce plasminogen activator, which converts plasminogen into plasmin. This in turn, converts procollagenase into collagenase, which degrades the follicular basement membrane of the leading follicle (Smith et al. 1996). Following formation of the follicular stigma due to follicular degradation, rupture of the leading follicle and ovulation occur. These processes are prostaglandin dependant (Phi et al. 1977). The rise in LH causes the resumption of meiotic division in the oocyte and causes luteinisation of the granulosa cells (Smith et al. 1996). The ruptured post-ovulatory follicle forms the *corpus luteum (CL)*. Maintenance of the CL is necessary to sustain the early conceptus. In the non- conception cycle, luteolysis occurs leading to loss of structural and functional integrity of the CL. hCG secreted by the pregnancy maintains the progesterone secretion by the CL, minimizes influx of macrophages to the CL in the late luteal phase

(Duncan et al. 1998b) and decreases the activity of matrix metalloproteinase 2 (MMP-2) (Duncan et al. 1998a). Withdrawal of progesterone has been shown to inhibit the secretion of tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) contributing to overall tissue remodelling (Morgan et al. 1994). The exact mechanisms of CL survival and luteolysis still remain unclear, despite extensive research (Maybin et al. 2004).

During the cyclical pituitary- ovarian interplay, increasing levels of oestrogen and progesterone affect the endometrium as the target organ. Oestrogen leads to proliferation, whereas progesterone leads to differentiation of stromal cells into the decidual, secretory phenotype. Withdrawal of progesterone with regression of the CL leads to shedding of the *zona functionalis* and menstruation (Silberstein et al. 2000).

Serum concentrations of oestrogen and progesterone cause sequential changes in the histological appearance of the endometrium and allow for appropriate dating of the specimen, as first described by Noyes et al. in 1950 (Noyes et al. 1950, de Ziegler et al. 1998). A marked alteration in respective levels of these hormones was thought to be reflective of the condition described as luteal phase defect (LPD) first mentioned in 1949 (Jones 1949) causing incomplete secretory transformation of the endometrial stroma. Removal of the CL and resulting inadequate progesterone levels prior to the establishment of placental function, results in a miscarriage (Csapo et al. 1972). When biochemical pregnancy loss occurred, no differences were noted in levels of progesterone compared to on-going pregnancies (Baird et al. 1991), however conception cycles have demonstrated a sharper increase in serum progesterone (Baird et al. 1997) signifying the importance of progesterone on establishment of pregnancy and supporting the supplementation of this medication in the luteal phase (2012). Multiple factors associated with LPD have been described. Infertility (Moszkowski et al. 1962, Blacker et al. 1997), miscarriage (Swyer et al. 1953), stress (Xiao et al. 2002, Kajantie et al. 2006), and PCOS

(Filicori et al. 1991) represent just a few of the conditions. At present, no reliable diagnostic test for LPD exists and in a recent ASRM Practice Committee Guideline, LPD as a condition causing infertility has not been recognised (2012). In spite of the above, supplementation of progesterone in the luteal phase remains still an empirical treatment in some cases of unexplained RM (2012). In the IVF setting, the use of GnRH agonist or antagonist stimulation protocols warrants the supplementation of progesterone and is related to improved outcome (Pritts et al. 2002, Daya et al. 2004, 2008b). It is recommended that its use should be continued until the 10th week of gestation (2012).

Suboptimal rise in the mid-cycle LH levels can cause luteinisation of the lead follicle and initiation of the meiotic division in the oocyte, without follicular rupture or ovulation (Smith et al. 1996). This can be demonstrated laparoscopically (Koninckx et al. 1978, Marik et al. 1978), sonographically (Coulam et al. 1982, Coutts et al. 1982) and by monitoring of the serum progesterone and oestrogen levels. The condition associated with this phenomenon is called luteinized unruptured follicle syndrome (LUFS) and, if occurs repeatedly, can be a cause of infertility (Hamilton et al. 1985). The diagnosis of this condition can be problematic and method dependant. Laparoscopic studies have revealed a prevalence of 9.4% (Donnez et al. 1983) to 46.7% (Vanrell et al. 1982) in infertile women. Sonographic studies show a much lower rate of approximately 4% of women with unexplained infertility (Kugu et al. 1991).

Menstrual irregularity, often linked to polycystic ovarian syndrome (PCOS), is possibly the best-known condition associated with infertility. The original description of PCOS (or Stein- Leventhal syndrome) included obesity, hirsutism, anovulation and infertility associated with enlarged and polycystic ovaries (Stein et al. 1935). In approximately 23% of the general female population without any of the classical PCOS symptoms (Polson et al. 1988), polycystic ovaries can be an incidental finding (Pettigrew et al. 1997). The current definition of PCOS includes the presence of two of the

following factors: oligo- or anovulation, clinical and/or biochemical hyperandrogenism, and polycystic ovaries (Chang et al. 2004, Fauser et al. 2004). The exact aetiology of this condition remains elusive; however a significant body of evidence suggests that a combination of obesity, hyperinsulinaemia and relative insulin resistance in susceptible individuals can lead to the development of this syndrome (Nestler et al. 1989). Menstrual irregularity in obese women in general and in women with PCOS in particular, has been associated with increased levels of circulating oestrogen due to the peripheral conversion of androgens (Kiddy et al. 1990) and a lower level of sex hormone binding globulin (SHBG), when compared to lean women (Insler et al. 1993, Pettigrew et al. 1997). Cycle irregularity and a possible role of increased androgen levels in the follicular microenvironment decrease oocyte quality, and can be responsible for infertility in women with PCOS (Pettigrew et al. 1997). Smoking (Rowland et al. 2002), chronic diseases (Doufas et al. 2000), diabetes (Kjaer et al. 1992, Rowland et al. 2002), treated depression, and stress (Rowland et al. 2002) are additional factors associated with menstrual irregularity and a potential effect on fertility; however their detailed description is beyond the remits of this thesis.

1.3. The endometrium

The *zona basalis* and *zona functionalis* occupy approximately one third and two thirds, respectively, of the entire endometrial thickness (Critchley et al. 2001a). The *zona basalis* is the source of pluripotent stem cells and is neither hormone responsive or hormone dependant (Heller 1994), allowing for regeneration of the *zona functionalis* after menstruation and pregnancy (Chan et al. 2004).

The endometrial stromal compartment demonstrates an increased proliferative and mitotic activity in the first half of the menstrual cycle, which ceases following ovulation. Around day 18 of the cycle,

superficial stromal oedema can be observed, which becomes generalized by day 21.

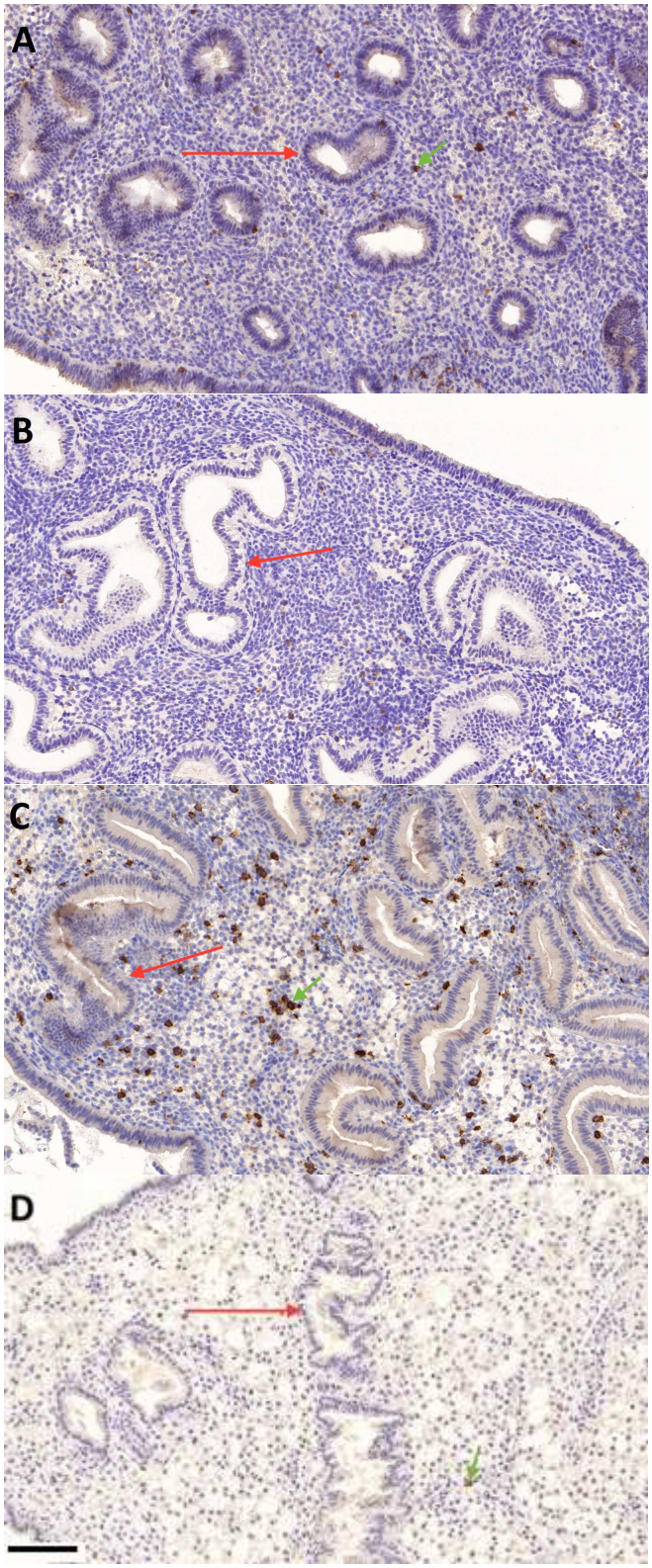


Figure 5. The endometrium at different stages of the menstrual cycle.

***A**. Proliferative phase. Densely packed endometrial stromal cells (ESC) with small straight glands (red arrow).*

***B**. Early luteal phase. Glands increase in tortuosity and spaces appear between ESCs.*

***C**. Mid-luteal phase. Glands are more tortuous (red arrow). Stromal oedema is obvious. Natural killer cells are represented more frequently than earlier in the cycle (green arrow).*

***D**. Late luteal phase. Tortuous glands with significant stromal oedema (red arrow). ESCs have been fully transformed to pale, large decidual cells with abundant cytoplasm.*

Marker represents 100 µm.

A significant increase in the cytoplasm of stromal cells in the proximity of spiral arteries can be seen by day 23. Large stromal cells with pale nuclei form the most superficial part of the endometrium from day 25. By day 27, stromal cells are indistinguishable from decidual cells of pregnancy (see Figure 5). If no pregnancy occurs, massive leukocyte infiltration becomes apparent 2 days prior to menstruation (Rock et al. 1937, Noyes et al. 1950).

1.3.1. **The Endometrial Stroma and Decidualisation**

Two distinct cellular populations within the endometrium are responsible for appropriate pregnancy implantation and development. In the mouse, the attachment of the embryo to the endometrial epithelium, leads to subsequent changes of the endometrial stromal cells- a process termed decidualisation (Mulholland et al. 1991, Ramathal et al. 2010). The term 'decidualisation' is derived from Latin '*decidere'*, meaning to 'fall off' (Gellersen et al. 2003). This process leads to morphological and functional cellular changes. Decidualised endometrial stromal cells (ESCs) become rounded and secrete many proteins, such as prolactin (PRL) and insulin-like growth factor binding protein 1 (IGFBP-1), which are thought to be the universal markers of decidualisation (Lockwood et al. 1993, Gellersen et al. 1994, Oliver et al. 1999, Christian et al. 2001). In the mouse, cells surrounding the site of implantation often are bi-nucleated or polyploid (Ansell et al. 1974) and have a limited life-span, which is thought to allow for placental development and expansion (Das 2009). In the human, decidualisation occurs during the luteal phase of the menstrual cycle irrespective of the presence of an embryo (Ramathal et al. 2010). Decidual changes initially are noted in the stromal cells surrounding the spiral arteries (Bell 1990, Maruyama et al. 2008), spreading throughout the endometrial stroma when pregnancy occurs (Ramathal et al. 2010). All these functional and morphological changes of the endometrial stroma are driven by the cyclical changes of oestradiol and progesterone levels. Insulin, LH, human

chorionic gonadotropin (hCG), oxytocin, and prolactin are involved in the process to a lesser extent (Jabbour et al. 2006). Glucocorticosteroid and androgen receptors have also been identified within the endometrium, with the former almost exclusively expressed in the stromal compartment (Bamberger et al. 2001, Henderson et al. 2003) and playing a role in endometrial cell proliferation (Bigsby et al. 1993), cell death (Jo et al. 1993), cellular remodelling (Salamonsen et al. 1996), and repression of the PRL promoter (Gellersen et al. 1994). Their presence has also been confirmed on uterine NK cells (Henderson et al. 2003). Androgen receptors are also expressed mainly within the stroma (Slayden et al. 2001), however their exact role is not fully known (Jabbour et al. 2006).

Decidualisation of the human endometrial stromal cells (HESCs) begins approximately 10 days following ovulation (Brosens et al. 1999, Cloke et al. 2008), and unlike in other mammalian species, is embryo independent (Finn 1974). As described by Jabbour et al. (Jabbour et al. 2006), decidualisation is a process of cellular differentiation accompanied by a decreased production of growth factors corresponding with decreased mitotic activity within the decidualized endometrium (Jabbour et al. 2006). An increasing pool of evidence suggests that decidualisation of the oestrogen and progesterone primed stromal cells is mediated by increasing levels of cyclic adenosine monophosphate (cAMP) (Telgmann et al. 1997, Brosens et al. 1999, Jones et al. 2006) due to expression of relaxin, corticotropin-releasing hormone and prostaglandin E2, sustained activation of protein kinase A (PKA), as well as contemporaneous down regulation of phophodiesterase enzymes (PDE4) (Bartsch et al. 2004). This stimulation of the HESCs by the increasing levels of cAMP leads to their sensitization and reliance on progesterone (Brosens et al. 1999, Cloke et al. 2008). Glucocorticoid receptors (GR) seem to play a role in the sustained cAMP production by up-regulating the activity of PKA (Kuroda et al. 2013c). cAMP's main intracellular target is PKA, which when activated, phosphorylates

target molecules in the cytoplasm or transcription factors in the nucleus (Skalhegg et al. 2000, Gellersen et al. 2003). HESCs are dependent on persistent cAMP stimulation to achieve and maintain a decidual phenotype (Tanaka et al. 1993, Telgmann et al. 1997). C/EBPβ transcription factor is regulated by cAMP upon decidualisation (Pohnke et al. 1999), and is responsible for transcription of PRL gene (Ramji et al. 2002). Another transcription factor- FoxO1a- is also induced by cAMP in HESCs (Gellersen et al. 2003). It is suggested that the LH surge and FSH peak, might initiate a sequence of events leading to decidualisation through stimulation of endometrial gonadotrophin receptors (Gellersen et al. 2003). LH/hCG receptors and other G-protein subunits, increase the activity of adenylate cyclase (Bergamini et al. 1985, Tanaka et al. 1993, Gellersen et al. 2003). Following ovulation, FSH and LH levels fall, but autocrine and paracrine action of relaxin, corticotrophin-releasing hormone (CRH) and embryo-derived β-hCG, sustain cAMP signalling (Wolkersdorfer et al. 1998, Gravanis et al. 1999, Palejwala et al. 2002). Increasing serum levels of progesterone converge on described below proteins/ transcription factors and sustain a decidualized phenotype of HESCs. It is suggested, that progesterone receptors' transcriptional activity reaches full potential following exposure to increasing levels of cAMP explaining the close correlation of progesterone and cAMP (Gellersen et al. 2003).

The intercellular matrix of the endometrium during decidualisation also undergoes remodelling. Collagen, fibronectin and laminin are produced in abundance (Dunn et al. 2003), accompanied by a decreased activity of matrix metalloproteinases (MMP-1 and MMP-3) by the combined action of progesterone and interleukin (IL) -1 (Schatz et al. 1999, Fazleabas et al. 2004). Increased secretion of IL-15 by decidualized stromal cells promotes growth and differentiation of uterine natural killer (NK) cells (Okada et al. 2000, Verma et al. 2000, Dunn et al. 2002, Jabbour et al. 2006).

Prokineticin I (PROKI)

PROKI (a cytokine promoting endometrial receptivity and induction of LIF in endometrial epithelial cells; or endocrine gland-vascular endothelial growth factor), PRL and IGFBP-1 have been identified by gene profiling studies as possible markers of ESC receptivity (Hochner-Celnikier et al. 1984, Brosens et al. 1999, Cloke et al. 2008, Evans et al. 2009, Salker et al. 2010). In the receptive, normal endometrium, the levels of PRL were significantly higher than in a defective sample (Salker et al. 2010). The local function of PRL on the endometrium seems to comprise of maintenance of oestradiol receptors (Basuray et al. 1983, Frasor et al. 2003) and an immunomodulatory effect by directly effecting uNK cells (Gubbay et al. 2002).

PROKI is a protein with diverse biological function of which endothelial cell proliferation, angiogenesis (LeCouter et al. 2001, Lin et al. 2002) and inflammation (Monnier et al. 2008), are of most importance in terms of successful pregnancy. In the non-pregnant uterus, expression of PROK I is at its peak in the mid-secretory phase (Battersby et al. 2004, Evans et al. 2008), if pregnancy occurs a further increase is noted (Evans et al. 2008). PROKI expression seems to be regulated by progesterone and hCG (Battersby et al. 2004, Ngan et al. 2006, Evans et al. 2009). An association between increased levels of PROKI transcripts and miscarriages was noted (Salker et al. 2010). Decreased levels of PROKI prior to decidualisation have been shown to decrease the levels of PRL (Macdonald et al. 2011).

PROKI exerts its function through G-protein coupled receptors, prokineticin receptor (PROKR)-1 and PROKR2 (Lin et al. 2002, Soga et al. 2002). PROKI and PROKR1 are expressed in the glandular and luminal epithelium, as well as in the stromal compartment (Battersby et al. 2004). The levels of PROKR1 and PROKR2 do not change throughout the menstrual cycle (Evans et al. 2008). Biological effects of PROKI also include increased synthesis of cyclooxygenase (COX) 2 (Evans et al. 2008), leukaemia inhibitory

factor (LIF) (Evans et al. 2009), IL- 8 and -11 (Maldonado-Perez et al. 2009, Cook et al. 2010), and heparin- binding epidermal growth factor (HB-EGF) (Evans et al. 2008), thus establishing the role of PROKI as an important protein responsible for early pregnancy development (Macdonald et al. 2011).

PROKI pathway is also involved in the regulation of the Wnt signalling (Macdonald et al. 2011) promoting differentiation of ESCs (Tulac et al. 2003). The action of PROKI on Wnt pathway is through induction of Dickkopf-related protein 1 (DKK I) expression- an inhibitor of Wnt signalling (Macdonald et al. 2011). Studies by Hou et al. (Hou et al. 2004) and Jeong et al. (Jeong et al. 2009) suggest the Wnt pathway is responsible for endometrial proliferation in the follicular phase, whereas switching the signalling off induces decidual differentiation (Tulac et al. 2003). The deregulation in this fine-tuned system seems to lead to lack of endometrial selectivity and RM (Brosens et al. 2009c, Salker et al. 2010, Teklenburg et al. 2010a).

Prostaglandin E2 (PGE2) plays an important role in transformation of ESCs into the decidual phenotype. PGE2 is synthetized by COX-2, which in turn is controlled by PROKI (Evans et al. 2008). COX-2 mediated prostaglandin synthesis causes an increase in the vascular permeability and oedema at the time of blastocyst implantation (Okada et al. 2001). COX-2- deficient mice have reduced vascular permeability at the site of implantation (Matsumoto et al. 2002a). Significant expression of PROKI by the trophoblast leads to an increase in COX-2 activation and prostaglandin synthesis favouring implantation (Hoffmann et al. 2006). Uterine NK cells also express PROKI, possibly contributing to the vascular remodelling in early pregnancy via the COX-2 and PGE2 pathway (Hoffmann et al. 2006).

Heparin binding epidermal growth factor (HB-EGF)

HB-EGF, a potent mitogen for fibroblasts and smooth muscle cells, is one of a number of growth factors under the control of oestrogen

and progesterone (Thomas 1990, Marikovsky et al. 1993, Lessey et al. 2002b). HB-EGF receptor is stimulated by epidermal growth factor (EGF) and transforming growth factor α (TGF α) (Singh et al. 2011b). *In vitro* studies have demonstrated that in the human endometrium, maximal expression of HB-EGF in the epithelium and HESCs follows exposure to a combination of progesterone and oestrogen (Lessey et al. 2002b); however each hormone on its own induces only minimal HB-EGF expression (Lessey et al. 2002b). All stromal and epithelial endometrial cells seem to express HB-EGF throughout the entire menstrual cycle (Simon et al. 2000), and the expression persists until term within the placental villi (Birdsall et al. 1996). Other studies however, have shown a cycle dependant expression of HB-EGF, with a peak expression at the time of WOI (Das et al. 1994, Tazuke et al. 1996, Lessey et al. 2002b). This growth factor has also been shown to stimulate expression of PRL (Hnasko et al. 2003), IL-11 (Robb et al. 1998, Karpovich et al. 2003), LIF, β3 integrin subunit, and HOXA-10 (Lessey et al. 2002b). Endometrial cell proliferation, inhibition of apoptosis, glandular secretion and decidualisation are also controlled by HB-EGF (Simon et al. 2000, Michalsky et al. 2001).

Blastocyst- sized beads soaked with the growth factor, when introduced into a mouse uterus, caused auto-induction of HB-EGF secretion (Hamatani et al. 2004), as well as localized implantation-like response (increased vascular permeability and decidualisation) (Paria et al. 2001). HB-EGF knock-out mice have been found to be sub-fertile demonstrating the importance of this molecule in pregnancy (Xie et al. 2007). Low levels of HB-EGF have been noted in women with pre-eclampsia suggesting a possible role of this protein in protection against free oxygen radicals and oxidative stress (Leach et al. 2002).

Recently, a correlation has been identified between the sonographic pattern of the endometrium 7 days following ovulation and the levels of HB-EGF (Wang et al. 2012). In the study population, HB-EGF levels were significantly higher in women with a triple layer

endometrial pattern. When the biopsy was repeated in a subsequent menstrual cycle, HB-EGF levels were increased compared to the first biopsy specimen, suggesting a role of this growth factor in improvement of endometrial receptivity following biopsy (Wang et al. 2012). Some authors also postulate a cytoprotective role of the soluble and transmembrane forms of HB-EGF, however further studies are required to substantiate this (Chobotova et al. 2005).

11-β Hydroxysteroid Dehydrogenase (11-β HSD)

11-β HSD in its two isoforms (1 and 2) is responsible for maintenance of tissue levels of glucocorticoids. The bidirectional 11-β HSD1 predominantly converts the inactive cortisone to the bioactive cortisol leading to an increase of active glucocorticoids (Courtney et al. 2008). 11-β HSD2 on the other hand, converts cortisol to cortisone (Ferrari et al. 1996). *In vivo*, 11β HSD1 has been identified on cells also expressing GR, which are the main target for cortisol, 11-β HSD2 has been identified on MR expressing cells (Arriza et al. 1987, Kuroda et al. 2013c).

In the context of reproduction, it is noteworthy that 11-β HSD, GR and MR are all expressed within the endometrium, with expression of GR on HESCs and MR within the stroma and glands (McDonald et al. 2006). Isoform 1 of the enzyme is highly up-regulated during the transition from undifferentiated ESC to decidual cells *in vitro* (Takano et al. 2007). 11-β HSD1 isoform expression is highest in the decidua and menstrual endometrium (McDonald et al. 2006, Michael et al. 2008). Type 2 isoform, is expressed mainly in the glandular epithelium throughout the entire menstrual cycle (Thompson et al. 2002, McDonald et al. 2006). Presence of 11-β HSD1 has also been confirmed on uNK cells suggesting the action of these cells is modulated by the bioavailability and metabolism of glucocorticoids (McDonald et al. 2006). Based on these observations, some authors speculate that the increased activity of the bidirectional isoform of 11-β HSD upon decidualisation leads to modulation of the GR and MR dependant pathways through the increased bioavailability of cortisol (Kuroda et al. 2013c). Formation

of a corticosteroid gradient within the feto-maternal interface might be, at least partially, responsible for the immune tolerance between the maternal immune system and the fetus. Recent evidence seems to suggest that elevated subluminal uNK cells are a marker of relative cortisol deficiency putting these patients at risk of reproductive failure (Kuroda et al. 2013c).

Vascular endothelial growth factor (VEGF)

Cyclical regeneration and decidual transformation of the endometrium is dependent on adequate vascular supply. Vasoconstriction and subsequent ischaemia lead to expression of multiple factors responsible for this regeneration, one of which is VEGF (Kolch et al. 1995, Wenger et al. 1997). VEGF is expressed throughout the menstrual cycle within the stroma and endometrial glands (Charnock-Jones et al. 1993, Shifren et al. 1996) with oestrogen and progesterone being the main regulators of expression (Shifren et al. 1996). Maximal expression of this protein is in the secretory phase (Shifren et al. 1996, Torry et al. 1996). VEGF is a growth factor involved in vascular permeability, vasodilatation, anti-apoptosis (Benjamin et al. 1997, Ferrara et al. 2003), endothelial cell proliferation, migration and differentiation (Ferrara 2004), vascularization of the decidualised endometrium, and placental angiogenesis in early pregnancy (Jackson et al. 1994, Krussel et al. 2000, Zygmunt et al. 2003). HESCs exposed to low oxygen culture environment show elevated expression of this growth factor compared to cells cultured in 20% 0_2 environments (Popovici et al. 1999). Underexpression or VEGF gene mutations leading to low levels of this growth factor can lead to faulty vascularization of the endometrium and subsequent reproductive failure (Watson et al. 2000, Jinno et al. 2001, Boudjenah et al. 2012). Natural cycle levels of VEGF have been shown to correlate to some extent with the sonographic appearance of the endometrium, with homogenous hyperechoic pattern having low expression of this mitogen compared to trilaminar endometrial pattern (Zhu et al. 2012).

1.3.2. **The Endometrial epithelium**

The luminal epithelium is the first point of contact between the embryo and maternal tissues (Cowell 1969, Stewart et al. 1992, Raab et al. 1996). Biochemical signals inducing proliferation and receptivity of the epithelial barrier during implantation are derived from the underlying stromal cells, however the exact factors responsible for this are not fully known (Cooke et al. 1997, Hom et al. 1998, Kurita et al. 1998, Simon et al. 2009, Teklenburg et al. 2010a, Teklenburg et al. 2010b).

Cell surface

Cellular membrane surface projections called pinopodes, have been thought to be a promising marker heralding the window of implantation (Psychoyos et al. 1971, Nikas et al. 1995, Nikas et al. 1999, Lessey 2011). Different studies have failed to prove the reliability of pinopodes as the maker of implantation (Acosta et al. 2000, Usadi et al. 2003, Quinn et al. 2007), hence their role in reproductive failure or success remains unproven (Lessey 2011). Epithelial carbohydrate glycoprotein MUC 1 forms the glycocalyx (Aplin 1999) which in the human endometrial epithelium is consistently expressed throughout the WOI, in contrast to a mouse model, where its expression decreases (Surveyor et al. 1995, DeSouza et al. 1999).

Integrins

In the infertile population, integrin αvβ3 has been identified as a possible marker of endometrial receptivity (Lessey et al. 2002a). It first appears at the beginning of the WOI (day 6-7 following ovulation) (Achache et al. 2006, Casals et al. 2008, Lessey 2011) and persists into pregnancy (Lessey et al. 1992, Lessey et al. 1994a). Expression of αvβ3 integrin is regulated by transcription factor HOXA10 (Daftary et al. 2002, Lessey 2011) and HB-EGF (Lessey 2002). Conditions such as adenomyosis (Fischer et al. 2010), PCOS (Taylor et al. 1999, Cermik et al. 2003), endometriosis (Lessey et al. 1994b, Matsuzaki et al. 2009), and hydrosalpinx

(Daftary et al. 2007) cause suppression of HOXA10 activity, and as a result, αvβ3 integrin levels decrease (Lessey 2011). The role of the mentioned integrin as a sole marker of IVF treatment outcome has not been unequivocally confirmed or refuted (Thomas et al. 2003a, Thomas et al. 2003b, Revel et al. 2005, Casals et al. 2010). Another integrin- α4β1, is only co-expressed with integrin αvβ3 during the WOI, and disappears in the late luteal endometrium (Lessey 2002), indicating that temporal co-expression of multiple surface proteins might herald the receptive phenotype.

Osteopontin

Osteopontin (OPN), an acidic extracellular matrix glycoprotein (Apparao et al. 2001), is a ligand to multiple adhesion molecules, however its primary receptor is integrin αvβ3, and their combined biological effects are related to cell adhesion and migration (Apparao et al. 2001, Lessey 2002). Thought closely related, OPN and integrin αvβ3 are not expressed contemporaneously in the menstrual cycle, with OPN appearing on day 4-5 following ovulation and integrin 2-3 days later (Casals et al. 2008). OPN, integrin αvβ3 and their complex, have been investigated as putative markers of implantation (Lessey 2003, Aplin 2006, Aghajanova et al. 2008b), however the levels of the glycoprotein complex or each protein separately, have not proven useful when assessing endometrial receptivity (Casals et al. 2008, Casals et al. 2010). Induction of glucocorticoid receptors (GR) on the ESCs has been shown to suppress OPN expression suggesting that glucocorticoid exposure around the time of implantation might decrease the chances of a successful pregnancy via this pathway (Altmae et al. 2012, Kuroda et al. 2013c).

Other surface proteins with a possible embryo-binding potential are L-selectin ligand (Lai et al. 2005, Foulk et al. 2007, Wang et al. 2008a, Margarit et al. 2009), trophinin (Fukuda et al. 1995) and HB-EGF (Iwamoto et al. 2000, Kimber 2000, Lessey et al. 2002b, Aghajanova et al. 2008a).

Heparin binding epidermal growth factor

HB-EGF has been shown to stimulate the endometrial epithelial expression of mentioned αvβ3 integrins (the β3 subunit) and HOXA 10 transcription factor, as well as LIF in *in vitro* studies (Lessey et al. 2002b). Some authors have demonstrated a time lag between the maximal expression of HB-EGF (peaking between the early and mid-secretory phase), β3 integrin subunit and HOXA 10 (Lessey et al. 2002b). It is therefore possible, that this growth factor through its cell-bound and soluble form can be a key component of endometrial receptivity (Lessey et al. 2002b).

Leukaemia inhibitory factor (LIF)

Expression of epithelial LIF is mediated by PROKI. LIF is a glycoprotein of the interleukin-6 family, which is essential for implantation in mice (Bhatt et al. 1991, Stewart et al. 1992, Stewart 1994). LIF levels can be detected in small quantities in the proliferative phase of the human menstrual cycle; however these levels increase significantly in the latter half of the cycle- both in the luminal and glandular epithelium (Charnock-Jones et al. 1994, Cullinan et al. 1996). LIF is present in the first trimester decidual samples, and decreases thereafter (Ren et al. 1997, Evans et al. 2009).

Trophoblast cells within LIF-rich environment have an increased ability to adhere to fibronectin and laminin indicating LIFs' possible role in implantation (Evans et al. 2009). LIF has also been shown to modulate trophoblast differentiation towards an anchoring phenotype (Nachtigall et al. 1996, Lass et al. 2001). First trimester abundance of LIF has been shown to increase fibronectin production, decrease gelatinase activity and increased differentiation towards syncytialization (Bischof et al. 1995, Sawai et al. 1995a, Sawai et al. 1995b, Nachtigall et al. 1996, Evans et al. 2009). Some authors also postulate LIF as a mediator between endometrial leukocytes, trophoblast and angiogenesis in the placental villi (Cavagna et al. 2003, Sharkey et al. 2003). In women with unexplained infertility, decreased levels of LIF have been found on days LH+7, LH+10 and

LH+12, suggesting a possible causal relationship between this protein and infertility (Laird et al. 1997).

Serum- and glucocorticoid- inducible kinase (SGK1)

SGK1, a serine-threonine protein kinase, increases in concentration following stimulation with progesterone, initially in the epithelium and then in the endometrial stroma (Jeong et al. 2005, Talbi et al. 2006, Feroze-Zaidi et al. 2007). The main role of SGK1 is regulation of sodium transport through epithelial cell membranes (Lang et al. 2006, Loffing et al. 2006), cellular proliferation and survival (Brunet et al. 2001, Amato et al. 2009).

Murine studies have demonstrated, that increased levels of SGK1 interfere with implantation and alter the histological architecture of the endometrium causing a decrease of the glandular areas (Salker et al. 2011). In SGK1-/- mice, bleeding and immune cell infiltration was noted very frequently at the site of implantation, compared to wild type mice (Salker et al. 2011). Induction of LIF, HOXA10 and HB-EGF has been abolished upon continuous expression of SGK1 (Stewart et al. 1992, Satokata et al. 1995, Wang et al. 2006, Xie et al. 2007).

In humans, SGK1 transcript levels were elevated in infertile women, compared to fertile controls, and decreased in women suffering from RM (Feroze-Zaidi et al. 2007, Salker et al. 2011). A transient loss of SGK1 activity has been noted in the luminal epithelium around the time of implantation (Salker et al. 2011). In primary HESC cultures obtained from fertile women, recurrent pregnancy loss (RPL) and infertile patients, baseline levels of SGK1 were comparable. A steady increase in transcript levels of SGK1 upon decidualisation was noted, but on day 8, cells obtained from RPL patients expressed significantly less SGK1 compared to the other two cell lines (Salker et al. 2011). Decreased levels of SGK1 have also been correlated with decreased levels of oxygen radicals scavenger proteins (i.e. glutathione peroxidase 3, superoxide dismutase 2), thus suggesting

an important role of this protein in prevention of oxidative stress within the feto-maternal interface (Salker et al. 2011).

Levels of LEFTY-A (left right determination factor), a member of the transforming growth factor β family (Sakuma et al. 2002), are also decreased in decidualising stromal cells when SGK1 expression is suppressed (Salker et al. 2011). Higher levels of LEFTY-A have been noted in the infertile population and lower in women with RPL- a manner similar to SGK1 expression, suggesting a correlation between these factors (Salker et al. 2011), hence a decrease of these proteins during the WOI permits establishment of pregnancy (Tabibzadeh et al. 2000, Tang et al. 2005, Salker et al. 2011). Constant expression of SGK1 in the epithelium leads to implantation failure, whereas deregulation of its expression in the stromal compartment leads to poor decidualisation and exposes the placenta to oxidative stress of pregnancy, leading to miscarriage (Jauniaux et al. 2000, Jauniaux et al. 2006b, Leitao et al. 2010, Salker et al. 2011).

Though many factors have been identified with a confirmed role in endometrial transformation and adaptation to pregnancy, more well designed studies with appropriate patient selection are required to identify factors linking the pathways together and identify additional mediators able to predict the ART outcome.

1.3.3. **Oestrogen and Progesterone**

Oestrogen

Oestrogen promotes proliferation of the endometrial tissue and 'primes' the expression of progesterone receptors (Lessey et al. 1988, de Ziegler et al. 1998). It exerts its action through two receptors (ER α and β) (Green et al. 1986, Kuiper et al. 1996, Enmark et al. 1997), which are hormone inducible transcription factors (Tsai et al. 1994). Products of the induced genes result in endometrial tissue proliferation and angiogenesis (Critchley et al. 2001b, Lecce et al. 2001, Jabbour et al. 2006).

ER α and β are present within the nuclei of epithelial and stromal cells, and show a cycle-dependent expression (Critchley et al. 2001a, Jabbour et al. 2006). Oestrogen receptors (ER α) have been reported to decline within the *zona functionalis* in the late secretory phase of the menstrual cycle (Critchley et al. 2001a). Oestrogen receptors are also present on immunocompetent cells, including uNK cells (Henderson et al. 2003). Through the stimulation of ER α, interferon gamma (IF-γ) mRNA production has been increased in murine studies (Fox et al. 1991). Lack of oestrogen stimulation in the follicular phase will not cause a decidual response of the stromal cells, as the expression of progesterone receptors is oestrogen dependant (Jabbour et al. 2006).

Progesterone

The increasing serum levels of progesterone post ovulation, cause differentiation and secretory changes in the endometrial epithelial and stromal cells (Lessey et al. 1988, Kodaman et al. 2004, Clancy et al. 2009). As progesterone is present throughout the menstrual cycle, a threshold level should therefore induce decidual changes within the endometrium and such a level is thought to be >5ng/mL (Usadi et al. 2008, Paulson 2011). Inhibition of cell proliferation is the effect of the antioestrogenic action of progesterone (Jabbour et al. 2006). Progesterone receptors (A and B) are ligand activated transcription factors exhibiting sequence-specific DNA binding ability to regulatory regions of various genes responsible for decidualisation (Gellersen et al. 2003, Jabbour et al. 2006).

Difference in the expression of PR receptors has been noted between the *zona basalis* and *functionalis* with persistence of receptors in the *zona basalis* throughout the cycle (Lessey et al. 1988, Snijders et al. 1992). PRB is significantly down- regulated upon decidualisation (Wang et al. 1998, Brosens et al. 1999, Mote et al. 1999). The expression of ER α as well as PRA and PRB is under the dual control of oestrogen and progesterone. In the follicular (proliferative) phase of the menstrual cycle, these receptors are up-regulated by ovarian oestrogen. A decline in the expression of

progesterone receptors (PRB) within the functional layer of the endometrium (especially within the glands) is well documented upon transition from the proliferative to the secretory phase (Lessey et al. 1988, Chauchereau et al. 1992, Snijders et al. 1992, Jabbour et al. 2006). Endometrial endothelial cells and uNK cells do not express PRs. It is thought that the effect of progesterone on the mentioned cells is indirect, and exerted via perivascular PR rich cells (Perrot-Applanat et al. 1994, Wang et al. 1998, Henderson et al. 2003).

Expression of such factors as Dickkopf-1 protein (Tulac et al. 2003), glycodelin (Olajide et al. 1992), calcitonin (Kumar et al. 1998), OPN (Apparao et al. 2001, Aplin 2006), homeobox A-10 transcription factor (HOXA-10) (Godbole et al. 2007), and MMP-3 (Fazleabas et al. 2004) are progesterone mediated and important in appropriate decidualisation.

Knock-out mouse model studies (Lydon et al. 1995, Conneely et al. 2000) have demonstrated that lack of PRA and PRB causes an influx of leukocytes into the endometrium, thus highlighting an anti-inflammatory role of progesterone (Lydon et al. 1995, Conneely et al. 2000, Jabbour et al. 2006). Progesterone is also a potent inducer of IL-4, IL-5, LIF, and macrophage- colony stimulating factor (M-CSF), as well as aids in differentiation of T cells into Th2 phenotype, which has been associated with better pregnancy outcomes in mouse models as well as in human studies (Hill et al. 1992, Wegmann et al. 1993, Piccinni et al. 1995, Piccinni et al. 2001, Miyaura et al. 2002).

Hormone levels and controlled ovarian stimulation

Natural cycle serum concentration of progesterone in the follicular phase approximates 500 pg/mL whereas the oestradiol concentration ranges from 20 to 300 pg/mL (Mishell et al. 1971, Paulson 2011).

Controlled ovarian hyperstimulation (COH) leads to non-physiologically high levels of oestrogen and progesterone, affecting the endometrial morphology and receptivity (Thomas et al. 2002b).

High oestrogen levels can lead to increased sensitivity to progesterone and, as such, may cause secretory advancement and dis-synchrony between the embryo and the endometrium (Simon et al. 1995). Data on pregnancy rates from oocyte recipients in non-stimulated cycles seems to support this hypothesis (Soares et al. 2005). In some patients with high oocyte yield related to multi-follicular development, excess growth of granulosa cells can cause elevated progesterone levels in COH cycles (Kyrou et al. 2009). This premature progesterone rise, can be associated with failed treatment outcome (Bosch et al. 2010, Kolibianakis et al. 2012), however the exact mechanisms of this are yet to be determined.

Other effects of oestrogen and progesterone

Additional down-stream mediators of oestrogen and progesterone action on the endometrium are CCAAT/Enhancer Binding Protein- β (C/EBPβ), Homeobox A10 (HOXA-10), Bone Morphogenetic Protein-2 (BMP2), Wingless 4 (Wnt4), Indian Hedghog (Ihh), and others (Ramathal et al. 2010).

CCAAT/Enhancer Binding Protein- β (C/EBPβ) is a transcription factor responsible for cell proliferation, differentiation, metabolic homeostasis, acute phase inflammation, and apoptosis (Ramji et al. 2002). *In vitro* studies have indicated that this factor is induced early in the decidualisation phase, implicating its important role in this cellular transformation (Christian et al. 2002, Ramathal et al. 2010). HOXA-10 transcription factor expression is also confined to the window of implantation (Ramathal et al. 2010). In HOXA-10 knock-out mice, a significant decrease in prostaglandin synthesis by COX-2 is noted (Lim et al. 1999).

BMP2 belongs to the TGF-β superfamily (Ramathal et al. 2010) and is responsible for multitude of cellular functions including proliferation, differentiation and apoptosis (Hogan 1996a, Hogan 1996b). Its expression in the stromal cells surrounding the embryo (Lee et al. 2007) is regulated by progesterone (Li et al. 2007). In the murine model (Lee et al. 2007), and similarly in the human

stromal cell cultures (Li et al. 2007), absence of BMP2 leads to complete failure of decidualisation.

Wnt signalling proteins also promote cellular division, differentiation and cell fate (Logan et al. 2004). Wnt4 seems to be under the control of BMP2 expression during decidualisation (Li et al. 2007). The Indian Hedgehog (Ihh) morphogen is a protein that seems to be responsible for epithelial and stromal cross-communication triggering decidualisation (Matsumoto et al. 2002b, Takamoto et al. 2002, Ramathal et al. 2010). It has been demonstrated that Ihh expression in the epithelium is under the control of stromal PR (Simon et al. 2009). Ihh converges on BMP2 expression to promote decidualisation (Kurihara et al. 2007, Ramathal et al. 2010).

Insulin, insulin-like growth factor 1 (IGF-1), IGFBP-1 and their respective receptors modulate the growth of endometrium and their actions seem to be dependent on the oestrogen priming (Strowitzki et al. 1993, Corleta et al. 2000, Klotz et al. 2002, Hewitt et al. 2005). Insulin receptors are found in greatest abundance in the secretory phase, other receptors are expressed continuously throughout the menstrual cycle (Strowitzki et al. 1993). Increasing expression of IGFBP-1 by decidualising stromal cells, might lead to a decreased local levels of bio-available IGF, which in turn restricts cellular proliferation in the luteal phase (Giudice et al. 1993, Jabbour et al. 2006). IGFBP-1 has been shown to stimulate the migration of the first trimester trophoblast, however high levels of this factor seem to inhibit trophoblast invasion into decidualising stromal cells giving IGFBP-1 an ambiguous function (Irving et al. 1995, Irwin et al. 1998, Gleeson et al. 2001, Fluhr et al. 2010).

Production of oxytocin seems to be effected by oestrogen and progesterone levels, with maximal local expression around the time of ovulation (Steinwall et al. 2004, Clancy 2009). Prokinetic action of oxytocin could stimulate myometrial contractions favouring transport of gametes and embryos to the site of fertilization and implantation, respectively (Steinwall et al. 2004).

1.3.4. **Human chorionic gonadotrophin (hCG)**

hCG and LH act on the same receptors due to their structural similarities (Licht et al. 1998, Licht et al. 2003, Cole 2010). When pregnancy occurs, hCG sustains the steroid production by the *CL* and this is the case for approximately 4 weeks following implantation (Cole 2010). hCG also promotes angiogenesis within the endometrial bed favouring appropriate placentation (Toth et al. 1994, Zygmunt et al. 2002, Berndt et al. 2009, Cole 2010), as well as appropriate syncytiotrophoblast differentiation (Shi et al. 1993, Cronier et al. 1994). Some observations also support the immunomodulatory action of hCG favouring implantation (Cole 2010).

Uterine myometrium also expresses the LH/hCG receptors and through the action of these, myometrial growth and relaxation throughout pregnancy can be achieved (Reshef et al. 1990, Eta et al. 1994, Zuo et al. 1994, Doheny et al. 2003, Edelstam et al. 2007). Activation of the human hCG/LH receptor induces the G-protein signalling pathway increasing intracellular levels of cyclic adenosine monophosphate (cAMP) (Segaloff et al. 1993). hCG also mediates the expression of prokineticin 1 (PROK1) and LIF. Following hCG exposure, PROK1 peaks first, followed by an increased expression of LIF (Evans et al. 2009).

1.3.5. **Menstruation**

If no conception occurs, endometrial changes leading to menstruation are commenced. Decrease of circulating progesterone levels affects PR-positive cells, and leads to increasing levels of immuno-active factors such as IL-8 (neutrophil chemotactic factor), β-chemokine CCL-2 (monocyte chemotactic peptide-1) and induction of COX-2, thus increasing prostaglandin synthesis (Critchley et al. 1996, Critchley et al. 1999, Jabbour et al. 2006).

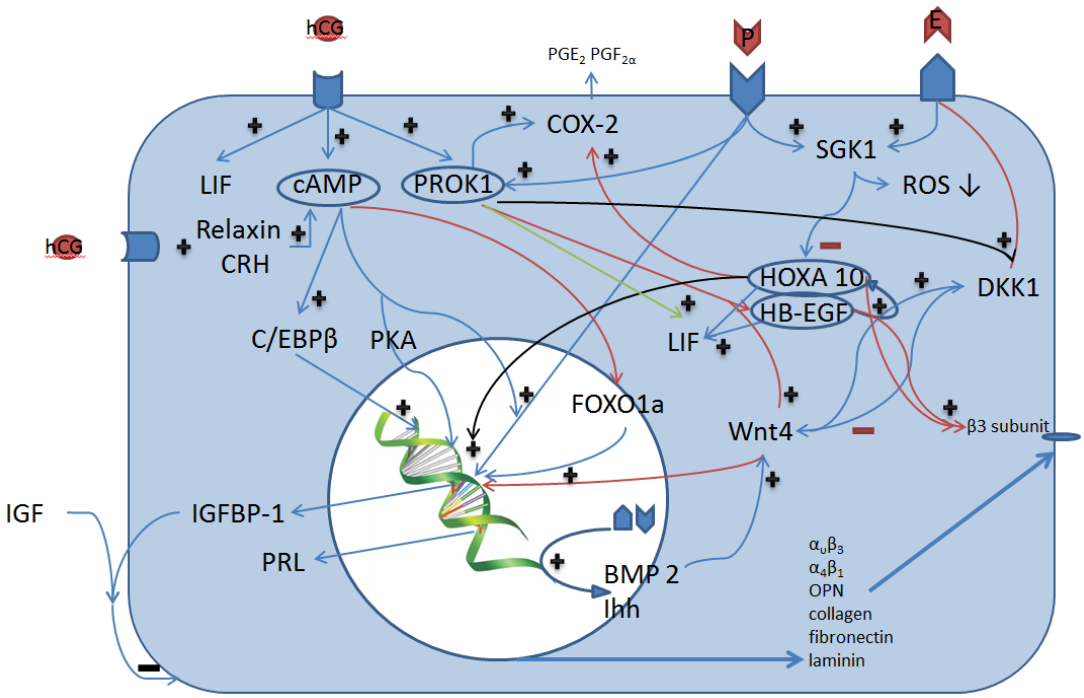


Figure 6: Simplified interactions of factors involved in endometrial stromal cell decidualisation.

Activation of hormone receptors regulates a multitude of factors associated with decidualization of endometrial stromal cells, both on a gene transcription level via induction of transcription factors, as well as increases synthesis of surface adhesion molecules.

$\alpha_u\beta_3$- integrin $\alpha_u\beta_3$; $\alpha_4\beta_1$- integrin $\alpha_4\beta_1$; E- oestrogen; P- progesterone; hCG- human Chorionic Gonadotropin; BMP2- Bone Morphogenetic Protein-2; cAMP- cyclic AMP; COX2- Cyclooxygenase 2; CRH- Corticotrophin Releasing Hormone; C/EBPβ- CCAAT/Enhancer Binding Protein- β; DKK1- Dickkopf related protein 1; IGF-1- Insulin-like Growth Factor 1; IGFBP-1- Insulin-Like Growth Factor Binding Protein 1; Ihh- Indian hedgehog; LIF- Leukaemia Inhibitory Factor; OPN- Osteopontin; PGE- prostaglandin E2; $PGF_{2\alpha}$- prostaglandin F2α; PKA- Protein Kinase A; PRL- Prolactin; PROK1- Prokineticin 1; ROS- Reactive Oxygen Species; SGK1- Serum- and Glucocorticoid- Inducible Kinase; **-** *inhibition of cell division;* **-** *inhibition of protein synthesis;* **+** *induction of protein synthesis;*  *- hCG receptor;*  *-progesterone receptor A and B;*  *- oestrogen receptor α and β.*

Production of prostaglandin PGE2, PGF2α and the expression of chemotactic factors, lead to perimenstrual influx of neutrophils, macrophages and other immuno-competent cells (Rampart et al. 1989, Colditz 1990, Salamonsen et al. 1999). PGF2α induces myometrial contractions and vasoconstriction of the endometrial spiral arteries leading to ischemia of the apical portion of the endometrium (Markee 1940, Ylikorkala et al. 1985). This in turn induces the expression of VEGF (vascular growth factor also affecting vascular permeability) (Popovici et al. 1999). These initial changes to the endometrium following withdrawal of progesterone can be reversed with add-back progesterone supplementation if initiated within 24-36 hours (Kelly et al. 2001, Brenner et al. 2002). The next phase of menstrual changes is caused by activation of lytic mechanisms and is irreversible (Jabbour et al. 2006).

Matrix metalloproteinase 1 (MMP-1) is thought to be the key enzyme in the degradation of the extracellular matrix leading to menstruation (Jabbour et al. 2006). Shedding of the upper two thirds of the endometrium heralds a new cycle of regeneration under the control of ovarian steroid hormones. Complete re-epithelialization of the endometrium following menstruation is normally complete by day 6 of the cycle (Nogales-Ortiz et al. 1978) and is under the control of such factors as epidermal growth factor (EGF), transforming-growth factor α (TGFα) and platelet-derived growth factor (PDGF) (Chan et al. 2004). Re-vascularization of the endometrium is in synchrony with the growing endometrium and ends with formation of spiral arterioles and sub-epithelial capillary plexus in the luteal (secretory) phase (Rogers et al. 1998, Gambino et al. 2002) allowing for appropriate tissue perfusion around the time of expected implantation.

1.3.6. **Uterine Natural Killer (NK) cells, IL-11 and IL-15**

Apart from the described histological and functional changes within the endometrium, the resident leukocyte populations also change

throughout the menstrual cycle. Up until the time of presumed WOI, a significant increase in the numbers of endometrial NK cells occurs. During WOI, NK cells- a component of the innate immune system, constitute approximately 70% of all endometrial leukocyte populations (Bulmer et al. 1991). Uterine NK (uNK) cells are phenotypically and functionally distinct from the peripheral NK (pNK) cells. Both of them express a common surface antigen CD56 (Moffett-King 2002). 80% of uNK cells are $CD56^{bright}$ and $CD16^{-}$ whereas 90% of pNK cells are $CD56^{dim}$ and $CD16^{+}$ (Nagler et al. 1989, King et al. 1991). CD16, or FcγRIII, expression is linked to the intrinsic ability of $CD56^{dim}$ NK cells to perform antibody-dependent cellular cytotoxicity (ADCC) (Manaster et al. 2010). Studies have shown that the $CD56^{dim}$ pNK cells have a significant cytotoxic activity against virus-infected and neoplastic cells (Bulmer et al. 2005, Dosiou et al. 2005).

$CD56^{bright}$ $CD16^{-}$ NK cells are the main source of immunoregulatory cytokines such as IL-10, IL-13, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-γ (IFN-γ), and tumour necrosis factor β (TNF-β) (Cooper et al. 2001). The main function of uNK cells seems to be the production of cytokines involved in trophoblast invasion and angiogenesis (Bulmer et al. 2005, Dosiou et al. 2005). pNK and uNK cells also differ in the expression of surface chemokine receptors and adhesion molecules (Manaster et al. 2010). $CD56^{dim}$ NK cells express high levels of killer cell immunoglobulin-like receptors (KIRs), CD57 (Eriksson et al. 2004), chemokine receptors CXCR1 and CX3CR1 (Manaster et al. 2010). Most of the $CD56^{bright}$ NK cells express KIRs preferentially binging to HLA –C (Chazara et al. 2011), and CD57, but also express high levels of CD94 receptors (Voss et al. 1998), CCR7, CXCR3, CXCR4 (Cooper et al. 2001, Hanna et al. 2003), and L-selectin (Cooper et al. 2001).

Within the endometrial stroma around the time of implantation, uNK cells are found as single cells or aggregates around spiral arterioles, near endometrial glands and in the extravillous trophoblast once

pregnancy has been established (Song et al. 1995, Salamonsen et al. 2000). The proximity and direct interaction of NK cells and developing trophoblast implies a significant role of these cells in pregnancy development (Moffett-King 2002). Though uNK cells have a cytotoxic potential (El Costa et al. 2008), they do not exhibit that effect towards cytotrophoblast. The possible explanations for this phenomenon might include the inhibitory effect of non-classical MHC class-I molecules (HLA- G and E) on NK cell receptors (i.e. KIR2DL4, CD94) (King et al. 2000a, El Costa et al. 2008), inability to form mature synapses allowing for perforin polarization (Kopcow et al. 2005), or interaction with local immune and non-immune cells which exert and inhibitory effect on the NK cell cytotoxic potential (Manaster et al. 2010).

Recent work attempting to describe the exact role of uNK cells has led to identification of two distinct subsets of NK cells. These are endometrial NK (eNK) cells and decidual NK (dNK) cells (Manaster et al. 2008). eNK cells seem to be inert NK cells without the ability to kill target cells or secrete NK-cell derived cytokines and growth factors, until the exposure to IL-15 induces that ability. dNK cells seem to resemble uNK cells but are found in the decidua (Manaster et al. 2008). The detailed role of this NK cell subset is still not fully described (Ramos-Medina et al. 2013), and in this thesis, we will not deal with these cells further.

Animal model work supports the important role of uNK cells at the feto-maternal interface. Abnormal implantation sites and inadequate remodelling of spiral arteries has been demonstrated in mice lacking uNK cells (Croy et al. 2003). Human uNK cells produce GM-CSF, CSF-1, TNF-α, LIF, IFN-γ (Jokhi et al. 1994), VEGF, placental growth factor (PLGF), angiopoetin-2, IL-10, and IP-10 (Hanna et al. 2006). The last two factors have been shown to bind to specific receptors on trophoblast causing its migration (Hanna et al. 2006). Overall, based on the secretory profile of NK cells, they seem to play an important role in endometrial spiral artery remodelling and trophoblast differentiation/ invasion.

The origin of uterine CD56^{bright} CD16^{-} NK cells is still a matter of debate. Two main possibilities are being suggested: self- renewal from local progenitor cells, or influx of peripheral NK cells and tissue-specific phenotypic differentiation. It is also possible, that resident eNK cells undergo maturation into the dNK cell phenotype upon exposure to decidualising stimuli (Manaster et al. 2010). Expression of L-selectin on peripheral CD56^{bright} CD16^{-} NK cells and high secretory phase endometrial tissue concentration of CSPG-2, the L-selectin ligand, might substantiate the blood-born NK cell theory (Frey et al. 1998, Yamaguchi et al. 2006). Presence of endometrial hematopoietic progenitor cells might indicate that blood-borne progenitor cells are a possible source of uNK cells which are trafficked into the endometrium by locally expressed chemokine (CXC) motif ligand 9, CXCL10, CXCL14 and trophoblast derived CXCL12 (van den Heuvel et al. 2005, Mokhtar et al. 2010). IL-11, IL-15 and IL-33 influence the proliferation and differentiation of the uNK cell subset (Ashkar et al. 2003, Godbole et al. 2010, Salker et al. 2012). Spatio-temporal expression of 11βHSD1 and associated steroid gradient may be responsible for uNK cell distribution within the endometrium irrespective of their origin (Kuroda et al. 2013a). Murine studies have demonstrated the potential of human endometrium to increase CD56 NK cells levels in NOD/SCID/γc^{null} mice (mice devoid of T and B lymphocytes and demonstrating extremely low NK cell levels) (Matsuura-Sawada et al. 2005).

Numerous studies have analysed the association of reproductive failure with peripheral (Aoki et al. 1995, Kwak et al. 1995, Beer et al. 1996, Matsubayashi et al. 2001b, Ntrivalas et al. 2001, Shakhar et al. 2003, Yamada et al. 2003) and uNK cell levels (Clifford et al. 1999, Quenby et al. 1999, Ledee-Bataille et al. 2005, Quenby et al. 2005, Matteo et al. 2007, Tuckerman et al. 2007). The results of these studies have yielded different conclusions in terms of association with pregnancy outcome. These controversies are possibly due to the different analysis methods used (immunohistochemistry versus flow-cytometry) (Lachapelle et al.

1996, Quenby et al. 1999), selection of different study populations and controls (Tang et al. 2011). Lack of uniformly accepted reference ranges for NK cell numbers and activity is another research goal that is yet to be achieved (Tang et al. 2011, Kuroda et al. 2013a). uNK cell normal level that has the widest acceptance is <5% of endometrial stromal cells (Quenby et al. 2005). pNK cell normal levels vary from <12% of peripheral lymphocytes (Coulam et al. 1995), to 16.4% (Yamada et al. 2003). A more recent and larger cohort study (n=318) has used the cut-off value of <18% as a marker defining the risk of reproductive failure with a sensitivity and specificity of 19% and 95.7% respectively (Ramos-Medina et al. 2013). It is certain, that we have not yet discovered all the fine interactions between the decidual, immuno-competent and throphoblastic cells responsible for pregnancy development.

Cytokines and interleukins

A multitude of cytokines are responsible for the paracrine and juxtacrine actions leading to decidualisation of the endometrial stroma (Evans et al. 2009). Among some of the better investigated are IL-15 and IL -11. IL-15 in *in vitro* and murine studies has been found to be an important inducer of endometrial stromal decidualisation and NK cell differentiation and proliferation (Carson et al. 1994, Croy et al. 2003, Manaster et al. 2008). Mice lacking IL-15 have a reduced number of uNK cells, have no spiral artery modification, and have an aberrant appearance of the decidua compared to normal mice (Ashkar et al. 2003).

IL- 11, which has been shown to promote progesterone- induced decidualisation (Dimitriadis et al. 2002, Fluhr et al. 2010) is expressed on all major cells types of the human endometrium (Singh et al. 2011a). Highest expression of this interleukin has been noted in the second half of the menstrual cycle in decidualised HESCs; however the maximal peak time has not been established (Dimitriadis et al. 2000, Cork et al. 2001, von Rango et al. 2004, Singh et al. 2011a). In ESC cultures, oestrogen and progesterone modulate the expression of IL-11 and IL-15 possibly via direct

action on the HOXA-10 transcription factor, which in turn negatively modulates the expression of these cytokines (von Rango et al. 2004, Godbole et al. 2010). Murine studies, have demonstrated the importance of IL-11 on maturation of decidual NK cells (Singh et al. 2011a). Biopsies of endometrium from women with recurrent miscarriage have demonstrated a decreased expression of this protein on the epithelial surface compared to normal, fertile controls (Linjawi et al. 2004).

The abundance of different factors and subtle interactions between these, make it very difficult to indicate the factor solely responsible for appropriate decidualisation. It is certain, that several factors simultaneously induce secretory changes within endometrial stromal cells, giving possible entry points for therapeutic modulation of this process.

1.4. Window of implantation (WOI) and embryo selection

Initiation and maintenance of pregnancy is a highly complex biological process depending on the interaction between the embryo and the uterine endometrium (Dey et al. 2004). Though oocyte and subsequent embryo quality is important, the contribution of the endometrium to successful pregnancy is estimated to equate to 31% to 64% (Walters et al. 1985, Rogers et al. 1986). The process of human implantation is thought to be highly conservative in evolutionary terms and is just starting to be unravelled (Hohn et al. 2003, Teklenburg et al. 2010a).

Based on studies by Wilcox et al. (Wilcox et al. 1995) it has been estimated that majority of pregnancies in humans result from coitus during a 6 day period preceding ovulation. This time frame however can vary between cycles and between couples and is termed the 'fertile window'. It is limited by the length of time the sperm and ovum remain viable within the female reproductive tract (Teklenburg et al. 2010a). The functional changes in the female

reproductive tract leading to fertilization are thought to be related to increasing oestradiol and progesterone levels influencing the cervical mucus and subendometrial contractions favouring sperm transport (Brosens et al. 1995, Brosens et al. 2009a).

Following fertilization, the developing embryo requires a primed environment to implant. In humans, the endometrium is decidualised around the time the blastocyst enters the uterine cavity and this process is independent of embryo presence (Kajihara et al. 2006, Gellersen et al. 2007, Leitao et al. 2010). Based on human studies, the WOI starts approximately 6 days following ovulation and lasts for a maximum of 5 days (Macklon et al. 2002, Horcajadas et al. 2007). Once the ESCs undergo decidualisation, they are fully dependant on progesterone support (Brosens et al. 1999, Gellersen et al. 2003, Gellersen et al. 2007). A prolonged WOI allows more embryos to implant, irrespective of their quality (Teklenburg et al. 2010a). This lack of selectivity might be responsible for subsequent biochemical or clinical pregnancy loss of developmentally incompetent embryos that would normally not be permitted to implant (Teklenburg et al. 2010a). Withdrawal of progesterone stimulation allows menstruation to occur due to breakdown of the *zona functionalis*, focal bleeding and cellular death, as described before (Salamonsen et al. 1999, Jabbour et al. 2006, Catalano et al. 2007).

Recent studies indicate the presence of an active cross talk between the embryo and decidualised cells (Teklenburg et al. 2010a). In their experiment, Teklenburg and colleagues (Teklenburg et al. 2010a, Teklenburg et al. 2010b) used hatched blastocysts that were cultured on decidualising ESCs in order to identify the possible factors involved in pregnancy establishment. No significant change in any factors, apart from a slight decrease in IL-5 expression, was noted when good quality embryos were used. When compromised embryos were introduced into the culture, the effect was considerably different- with decidual cells having stopped the production of major implantation markers and modulators: IL-1 β,

IL- 6, IL-10, IL-17, IL-18, eotaxin, and HB-EGF. The production and levels of IL-12, IL-15, TNF-α, monocyte chemotactic protein-I, and chemokine ligand 10 were not affected (Teklenburg et al. 2010a). When non-decidualised endometrial cells were used, embryos- irrespective of their developmental potential, did not elicit any noticeable maternal response. Brosens et at. (2010) has indicated that HESCs actively encapsulate the competent embryo contributing to implantation. These findings seems to indicate, that the developed decidual cells are sensors of appropriate embryo development (Teklenburg et al. 2010a). Inappropriate decidualisation can lead to early pregnancy loss or obstetric complications related to inappropriate placentation, depending on the severity of dysfunction (Brosens et al. 2002, Norwitz 2006, Brosens et al. 2009a).

1.5. Disorders of human reproduction

Human reproduction is a relatively ineffective process with only 20-25% of apparently fertile couples becoming pregnant during a single menstrual cycle assuming tubal patency, ovulation and viable sperm (Short 1979, Stevens 1997). Based on this generally accepted level of fecundity, the likelihood of achieving pregnancy has been estimated at 60%, 84% and 92% after 6, 12 and 24 months of unprotected, regular intercourse, respectively (Evers 2002a, Kamel 2010, Teklenburg et al. 2010a). The endometrial component is thought to account for up to 64% of the probability of successful pregnancy, whereas the remaining fraction can be attributed to oocyte development, embryo quality and other maternal factors, mainly age (Rogers et al. 1986, Bourgain et al. 2003).

1.5.1. **Infertility**

One of the major behavioural trends in western societies over the last few decades is the postponement of childbearing and an increase in the prevalence of subfertility and infertility (Broekmans et al. 2009, Mascarenhas et al. 2012) (see Figure 7). For example, in the UK, there has been a rise in the average age of women giving

birth from 26 and a half in 1971, to 29 and a half in 2009. 79% of births in 1971 were to women under 30, whereas in 2009 the same age group was responsible for only 53% of all births (Beaumont 2011). The current prevalence of subfertility is estimated at approximately 10-15% of couples trying to conceive (Evers 2002b, Evers 2002a).

The widely accepted definition of infertility is 'inability to conceive after 2 years of regular, unprotected intercourse' (Harper et al. 2008). The most common causes of infertility are male factors (sperm abnormalities), female factors, combined aetiology, and unexplained infertility (Kamel 2010). The basic investigations carried out on infertile couples should include a semen analysis, assessment of tubal patency and day 21 progesterone levels (Baird et al. 2000). Tubal factor is estimated to be responsible for approximately 14% of infertility cases; however this figure can vary and be significantly higher in places with prevalent sexually transmitted diseases such as *Chlamydia trachomatis* and *Neisseria gonorrhoeae* infections (Hull et al. 1985, Devroey et al. 2009, Kamel 2010). Uterine anomalies, both congenital and acquired, are present in approximately 40% of infertile women during pre-treatment investigations, however their actual effect on fertility remains debatable (Shamma et al. 1992, Doldi et al. 2005, Devroey et al. 2009).

Various treatment options exist and are based on the individual couple's needs and wishes. Depending on the cause of infertility, a conservative approach can be taken, followed by stimulated intrauterine insemination (IUI), through to IVF or ICSI treatments. If all these treatments fail, adoption remains as the final step.

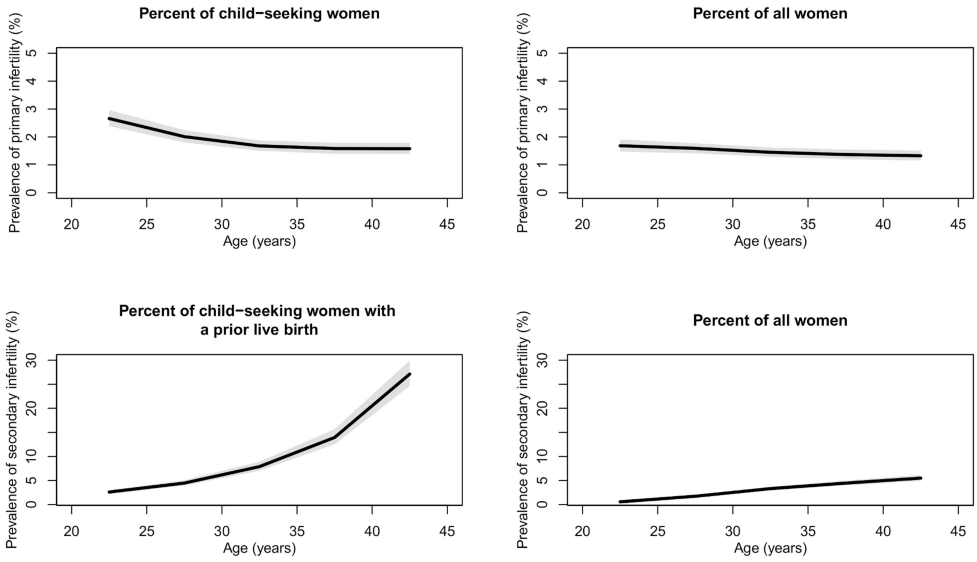


Figure 7. Global prevalence of primary and secondary infertility. From Mascarenhas et al. (2012).

1.5.2. **Miscarriage and recurrent miscarriage**

Miscarriage (spontaneous abortion) is the loss of pregnancy before it reaches a viable gestation, which has been arbitrarily set at 24 completed weeks of gestation (Regan et al. 2000). Depending on the advancement of pregnancy, it can be divided into biochemical pregnancy loss, where the only evidence of pregnancy is a positive pregnancy test, and clinical pregnancy loss, where sonographic evidence of intrauterine pregnancy has been demonstrated. The UK accepted definition of recurrent miscarriage (RM) is three or more consecutive spontaneous miscarriages (Jauniaux et al. 2006a, Rai et al. 2006). It is estimated, that approximately 60% of conceptions are early pregnancy losses- that is before the 6th week of gestation, with half of those being biochemical pregnancy losses (Chard 1991, Macklon et al. 2002). Following sonographic demonstration of pregnancy, only 10% of conceptions subsequently miscarry (Chard 1991, Macklon et al. 2002). The prevalence of RM is approximately 1- 3% of the general population (Quenby et al. 2002, Carrington et al. 2005, Jauniaux et al. 2006a, Rai et al. 2006).

The majority of spontaneous miscarriages are due to random chromosomal abnormalities of the foetus, which are mainly associated with maternal age- the main predictive factor for a successful, healthy pregnancy (Regan et al. 2000, Stephenson et al. 2002). Another factor predictive of pregnancy outcome is past obstetric history. Young women with no previous miscarriages or with a successful pregnancy have a 5% chance of miscarriage in the next pregnancy, whereas a woman over 35 with history of previous 4 unsuccessful pregnancies has a 54% risk of miscarriage (Regan et al. 1989, Knudsen et al. 1991, Regan et al. 2000). Other associated factors include smoking (Shiverick et al. 1999), cocaine use (Ness et al. 1999), alcohol consumption (Kesmodel et al. 2002), and exposure to teratogenic substances and/or drugs (Khattak et al. 1999). It is possible, and likely, that factors responsible for spontaneous miscarriage, can at least partially be responsible for aetiology of RM.

In cases of spontaneous RM, the histopathologic appearance of the products of conception and implantation site, suggest inflammatory and thrombotic changes (Kwak et al. 1999). This however, is likely to represent the effect, rather than the cause of miscarriage. After extensive diagnostic tests, approximately 50% of patients with RM will remain without a causative diagnosis (Kwak-Kim et al. 2009). According to current knowledge, possible causes of RM could be grouped into endocrinological, haematological, genetic, structural and immunological categories (Regan et al. 2000).

Endocrinological disorders

Dysfunctions of the thyroid gland and thyroid-directed autoimmunity (Abalovich et al. 2002, van den Boogaard et al. 2011), hyperprolactinaemia (Hirahara et al. 1998) and diabetes mellitus (DM) (Christiansen et al. 2008) are classical endocrine disorders linked with RM for which treatment options exist. Combination of PCOS and DM has been linked with RM (Rai et al. 2006). These conditions are thought to impair the fibrinolytic response in early pregnancy, thus resulting in miscarriage (Dale et al. 1998). In

PCOS, a syndrome characterised by LH hypersecretion, hyperandrogenism, polycystic ovarian morphology, insulin resistance and often infertility, abnormal oocyte gene expression and cytoplasm development is the main pathophysiological finding (Tesarik et al. 1995). Due to insulin resistance, premature follicle luteinisation occurs (Franks et al. 1999). These changes lead to abnormal oocyte development and subsequent lack of fertilization or pregnancy loss if ovulation occurs (Dumesic et al. 2008).

Haematological disorders

Anticardiolipin antibodies, *lupus* anticoagulant and anti-β2-glycoprotein I are antiphospholipid antibodies (APA), a group of over 20 antibodies directed against plasma proteins (Rai et al. 2006). Association of antiphospholipid syndrome (APS) defined as presence of antiphospholipid antibodies and three or more consecutive, unexplained miscarriages before the 10^{th} week of gestation; one or more unexplained deaths of morphologically normal foetuses at gestation over 10 weeks, and one or more premature births of morphologically normal foetuses at 34 weeks gestation or younger associated with severe pre-eclampsia or placental insufficiency (Wilson et al. 2001), with RM is quite convincing, with a 90% miscarriage rate if left untreated (Rai et al. 1995). *In vitro* studies have at least partially explained the pathology of APA action, which in essence interfere with endometrial decidualisation (Mak et al. 2002), decrease trophoblast fusion and invasion (Disimone et al. 1995, Sebire et al. 2003, Bose et al. 2004, Bose et al. 2005).

Factor V (Leiden) G1691A, factor II G20210A and methylene tetrahydrofolate reductase C677T are the most commonly encountered congenital thrombophilias causing a hypercoaguable state (Rai et al. 2006). Physiological hypercoagulation in pregnancy combined with the effect of congenital thrombophilia, leads to excess generation and deposition of fibrin and fibrinogen-split products (Isermann et al. 2003), in turn leading to thrombosis of the utero-placental bed, throphoblast apoptosis and subsequent miscarriage (Dizon-Townson et al. 1997, Isermann et al. 2003).

Genetic disorders

Trisomy, polyploidy and monosomy X are the most common genetic abnormalities noted in patients with RM (Stirrat 1990, Kalousek et al. 1993, Fritz et al. 2001, Stephenson et al. 2002). The majority of these abnormalities are thought to be the result of faulty meiotic divisions of the oocyte (Rai et al. 2006), however genetic sperm disorders have also been implicated (Giorlandino et al. 1998, Robinson et al. 1999). Approximately 4% of couples with RM have been found to be carriers of balanced reciprocal translocation or Robertsonian translocations (Clifford et al. 1994), which gives them a 50-65% chance of a healthy live-birth (Carp et al. 2004).

Structural anomalies of the reproductive tract

Congenital uterine abnormalities are noted in RM patients with a frequency of 1.8% to 37.6% (Grimbizis et al. 2001, Woelfer et al. 2001, Salim et al. 2003). Overall, the prevalence of congenital uterine anomalies in the RM population is approximately 3-fold higher compared to low-risk population, with the sub-septate uterus being the most common finding (77% of cases) (Salim et al. 2003). Uterine fibroids have been found in approximately 30% of women with RM (Hart et al. 2001). The increased prevalence of uterine abnormalities (both congenital and acquired) in the RM population, compared to normal women, might indicate a causative relationship, which at the moment remains unexplained.

Immunological factors

The *conceptus*, being a semi-allograft in immunological categories, must elicit a state of immunological tolerance in order to evade the maternal immune system (Rai et al. 2006). uNK cells are one of the main immune cells implied in maintenance of pregnancy as well as its failure. High levels of uNK cells have been associated with subsequent pregnancy loss, however a yet-to-be determined level of these cells is necessary to maintain an intact feto-maternal interface throughout pregnancy (Lachapelle et al. 1996, Clifford et al. 1999, Quenby et al. 1999). In women with RM, the state of immunologic balance has been reported to be shifted towards the Th-1 type

response (TNF-α mediated), in contrast to women with successful pregnancy, where Th-2 type response dominates (mediated by IL-4, 6 and 10) (Reinhard et al. 1998, Lim et al. 2000, Raghupathy et al. 2000, von Wolff et al. 2000, Rai et al. 2006).

Interventions

Based on the aetiology of RM, various treatments have been explored. Low molecular weight heparin, with or without aspirin, has been reported to decrease the miscarriage rate in women with APS (Hoppe et al. 2011). Use of these drugs in women without APS, did not improve the pregnancy outcome as judged by studies and a Cochrane review (Kaandorp et al. 2009, Akhtar et al. 2013a). A more recent review of three RCTs encompassing 386 women undergoing ART did however show a beneficial effect of perimplantation administration of LMWH. The authors did however advise caution when interpreting the results as the quality of included studies was poor and the result depended on the statistical analysis used (Akhtar et al. 2013b). Use of intravenous immunoglobulins to treat unexplained or immunologic RM, has not proven to be beneficial (Ata et al. 2011), and the potential side-effects of this drug, make this treatment not recommended for use outside of a research setting (Sherer et al. 2001, 2004b, Polanski et al. 2013). Long-term use of immunomodulatory steroids has been associated with increased risk of preterm delivery due to premature rupture of membranes, development of pre-eclampsia and gestational diabetes (Laskin et al. 1997). Pre-implantation genetic screening (PGS) has been suggested when genetic abnormalities are suspected, however its use has not conferred any benefit in terms of increasing live birth and decreasing the miscarriage rate (Musters et al. 2011). Progesterone supplementation in early pregnancy might benefit patients with RM, however until the results of a well-designed RCT are available, no firm conclusions can be drawn (Coomarasamy et al. 2011).

1.5.3. **Recurrent implantation failure**

Introduction of ART into clinical practice has created an iatrogenic phenomenon - implantation failure. Subfertility and infertility is on the increase and is estimated to affect 10-15% of couples trying to conceive (Evers 2002a, Shreeve et al. 2012). Current ART success rates in Europe, reported as clinical pregnancy rates are 28.9% and 32.9% per aspiration and embryo transfer for IVF respectively, and 28.7% and 32.0% per aspiration and embryo transfer for ICSI, respectively (de Mouzon et al. 2012, Ferraretti et al. 2013). Causative factors common to RIF could be divided into embryo related, embryo transfer related, and maternal factors. Recent developments in genetic testing (complete genomic hybridization-CGH and pre-implantation genetic screening and diagnosis-PGS/PGD) allow for selection of chromosomally normal embryos and their replacement. This approach should, at least in theory, lead to improved chances of pregnancy, purely due to exclusion of chromosomal abnormality as cause of implantation failure or miscarriage, however embryo mosaicism might provide falsely reassuring results. Despite repetitive replacements of genetically normal embryos, appropriate transfer techniques, and normal maternal investigations, a proportion of infertile couples remain childless. This situation created by ARTs, has been termed recurrent implantation failure (RIF).

The most common definition of RIF in the current literature and in infertility treatment centres is three failed IVF cycles without achieving pregnancy (Margalioth et al. 2006, Shufaro et al. 2011). A systematic review of literature performed as part of this thesis aimed to identify the current definitions of this condition and formulate a single, widely accepted and evidence based one. The conclusion of the review is that no clear definition of RIF exists, which causes diagnostic bias and confounds the results of studies on factors related to repeated failure of ART (Polanski et al. 2014).

The cumulative pregnancy rates after consecutive cycles of IVF or ICSI have never exceeded 90%, even when patients were followed up for twelve cycles (De Vries et al. 1999) (Guzick et al. 1986). Some authors demonstrate a decrease in the per cycle pregnancy rate after the second failed attempt, with clinical pregnancy rates of 19%- 26.8% for first, 17.4%- 24.2% for second and 11.8%-18.9% for third attempt, respectively (Roest et al. 1998, Schroder et al. 2004), whereas other studies have found no such decline (Guzick et al. 1986, Sharma et al. 1988, Padilla et al. 1989, Alsalili et al. 1995a, Dor et al. 1996). Where blastocysts were transferred, one study demonstrated a significant decrease in pregnancy rates following the first unsuccessful cycle, from 36% in the first attempt, to 19% in the second (Shapiro et al. 2001). A more recent study of 6164 patients where women were followed up for 6 cycles, showed cumulative pregnancy rates between 72% (95% CI 70-74) or 51% (95% CI 49-52) for an optimistic analysis or conservative analysis, respectively. The reported per cycle live birth rates decreased from 24.5% in the first cycle, to 13% in the sixth cycle. The authors concluded that age and associated ovarian reserve can not be overcome by ART (Malizia et al. 2009).

The actual prevalence of RIF is very difficult to estimate, as it is asymptomatic and represents treatment failure. Patients might give up further IVF attempts due to financial, health or relationship reasons, and not just due to RIF (Tan et al. 1992, Dor et al. 1996, de Mouzon et al. 2012). The estimated dropout rates for subsequent cycles of IVF are 15% to 36% of patients attempting an initial treatment cycle (Roest et al. 1998, De Vries et al. 1999). According to available literature the proportion of patients undergoing the second, third and fourth cycle can range from 28% to 59%, 7.5% to 34% and 15% to 20%, respectively (Alsalili et al. 1995a, Alsalili et al. 1995b, Dor et al. 1996, Roest et al. 1998, De Vries et al. 1999, Shapiro et al. 2001).

Numerous factors have been associated to a greater or lesser extent with repetitive failure of IVF or ICSI treatment. In approximately

50% of cases despite extensive investigations, no identifiable cause can be demonstrated (Shufaro et al. 2011). Each of the suspected causative factors will be briefly addressed in turn.

Gamete and embryonic factors

Studies on oocytes and embryos derived from couples suffering with RIF have demonstrated that genetic abnormalities are the leading cause of RIF. Autosomal translocations and sex chromosome abnormalities were the leading chromosomal alteration found in 2.5% of parental genes compared to 1.3% in a historic infertile population and 0.3% in a neonatal screening programme (Stern et al. 1999). Current review articles and recommendations, suggest that genetic evaluation of the couple with RIF should be considered in all cases (Sharif et al. 2010, Shufaro et al. 2011, Das et al. 2012).

Using fluorescence in-situ hybridization (FISH) for chromosomes 13, 18, 21, X and Y, poor sperm parameters in couples with RIF have been correlated with increased rate of sperm diploidy and disomy for sex chromosomes and chromosomes 18 and 21 (Rubio et al. 2001). An aneuploidy rate of 44.0% was present during the first and second PGS cycle when assessing polar bodies in RIF women (Vialard et al. 2008). Other authors have demonstrated an equally significant oocyte genetic abnormality rate ranging from 48.3% to 69% (Platteau et al. 2006, Vialard et al. 2007). Approximately 2/3 of all cleavage stage and day 3 embryos from RIF couples have been found to be chromosomally abnormal when FISH for chromosomes 13, 16, 18, 21, 22, X, and Y was used (Pehlivan et al. 2003, Wilton et al. 2003), with a control subjects abnormality rate of 36% (Pehlivan et al. 2003). CGH revealed an abnormality rate of 65% for oocytes and 45% for blastocyst in a study by Fragouli et al. (2010) suggesting a natural selection of developmentally competent embryos (Fragouli et al. 2010). Northrop et al. (2010) found that 58% of aneuploid embryos on day 2 can become euploid at the blastocyst stage confirming the possibility of compartmentalization or cellular selection within an embryo (Northrop et al. 2010).

Younger women are more likely to have a genetically normal embryo compared to older women (40 years old or less; average of 3.8 normal embryos in the older age group versus 4.9 in the younger) (Taranissi et al. 2005). Described earlier changes in the follicular environment linked to PCOS, can play a role in RIF and systemic conditions should be investigated and treated is possible.

The unnatural environment of IVF culture, has been shown to negatively affect the *zona pellucida* potentially contributing to the occurrence of RIF (De Vos et al. 2000). Suboptimal culture conditions have the potential to compromise embryonic development, hence strict monitoring is enforced in all IVF clinics (Gardner et al. 2005, Das et al. 2012). Combination of poor laboratory conditions and poor or difficult embryo transfers can negatively influence the IVF or ICSI treatment outcome (Schoolcraft 2001, Schoolcraft et al. 2001), and if repetitive errors occur, these can be responsible for RIF.

Maternal uterine factors

Approximately 40% (20-60%) of implantation failures are thought to be caused by the endometrial component in the form of structural uterine anomalies, endometrial development, and immune factors resident within the endometrium (Rogers et al. 1986, Bourgain et al. 2003, Dalal et al. 2011).

The exact mechanisms of why congenital and acquired uterine anomalies are associated with RIF are not known, however speculations suggest impaired endometrial vascularity, aberrant expression of cytokines and mechanical obstruction (Pabuccu et al. 2004, Aletebi 2010, Dalal et al. 2011). In women with at least 2 failed IVF cycles, some form of intrauterine pathology was found in 36.6% to 59.5% of patients using hysteroscopy as a diagnostic tool (Arefi et al. 2008, Makrakis et al. 2009, Aletebi 2010). Makrakis et al. (2009) reported presence of endometrial polyps in 16.7% of cases, endometrial adhesions in 12.5%, uterine septa in 0.9%, and submucous myomas in 0.8% (Makrakis et al. 2009). Uterine

leyomyomata can cause distortion of the endometrial cavity and affect the mechanical abilities of the uterine myometrium, which by some authors has been shown to negatively affect the IVF treatment outcome and live birth rates (Farhi et al. 1995), with other studies showing no such association (Eldar-Geva et al. 1998, Sunkara et al. 2010). Some authors recommend evaluation of the uterine cavity in all patients with failed attempts of IVF/ICSI or RM using either three-dimensional sonography or hysteroscopy, as correction of abnormalities has been shown to have a beneficial effect on subsequent pregnancy outcome (Shufaro et al. 2011, Simon et al. 2012). A hydrosalpinx has been shown to interfere with implantation either by direct toxic effects on the embryo (communicating hydrosalpinx) (Katz et al. 1996, Strandell 2000), or by affecting the vascularity of the endometrium, thus interfering with normal endometrial development (Ng et al. 2006b). Surgical removal of diseased oviducts has been shown to improve the reproductive outcome of patients undergoing ART (Johnson et al. 2010).

The minimal endometrial thickness below which implantation is unlikely to occur is between 6-8 mm (Maugey-Laulom et al. 2002, Amir et al. 2007, Singh et al. 2011b). Endometrial volume of 2 cm^3 as assessed by 3D ultrasound, seems to be a generally accepted lower cut off volume for successful implantation (Ng et al. 2006d). Thin, unresponsive endometrium is a recognised cause of RIF (Simon et al. 2012). The exact aetiology of this condition remains unknown. Incidence of thin endometrium in natural cycles is reported to be <5% in women under 40 and >25% in the older age group, which could be related to hormonal imbalance and/or relative tissue resistance to growth factors (Sher et al. 1991).

Maternal haematological disorders

Possibly the most controversial factors causing RIF are congenital or acquired thrombophilias. The action of antiphopsholipid antibodies on the developing trophoblast is through interference with fusion, invasion and increased induction of trophoblast apoptosis (Lyden et al. 1992, Disimone et al. 1995, Sebire et al. 2003, Bose et al. 2004,

Bose et al. 2005), as well as a direct embryo-toxic effects (Kaider et al. 1999). Congenital thrombophilias and associated hypercoaguable state can cause thrombosis of the utero-placental unit, leading to acceleration of trophoblast apoptosis through fibrinogen split products (Isermann et al. 2003, Rai et al. 2006). Most studies on association of thrombophilias and RIF show an increased frequency of these factors in the RIF population (Stern et al. 1998, Grandone et al. 2001, Coulam et al. 2002, Azem et al. 2004, Martinuzzo et al. 2005, Qublan et al. 2006, Bellver et al. 2008, Simur et al. 2009), with an accepted incidence of 20% to 30% (Quenby et al. 1993, Roussev et al. 1996). Small sample numbers, different definitions of RIF (two or more, three or more failed cycles), different assays used, and lack of normal control populations create a poor evidence base to unequivocally link thrombopilias with RIF (Bohlmann 2011). Meta-analysis on association of ART failure with thrombophilias, suggests a 3-fold increase in risk of treatment failure, when one or more antiphospholipid antibody is present. Significant heterogeneity of data was however noted (Di Nisio et al. 2011). Review of data by ASRM has come to a similar conclusion, stating that "*Antiphopsholipid antibodies do not affect IVF success*" (2004a) and routine use of heparins to improve treatment outcomes is not supported (Bohlmann 2011).

Other maternal factors associated with RIF

Presence of anti-thyroid auto-antibodies: anti-thyroid peroxidise (anti-TPO) and anti-thyroglobulin (anti-TG) in euthyroid women, is associated with an increased risk of implantation failure and miscarriage following IVF treatment (Revelli et al. 2009, Toulis et al. 2010). Where treatment was instigated with levothyroxin, aspirin and prednisolone, clinical pregnancy rates of 25.6% were noted, compared to 7.5% in the untreated controls (Revelli et al. 2009).

Interactions between the embryo and the mother are mediated through HLA system and multitude of cytokines. A well-recognized paradigm exists of a generalized shift in the maternal immune system during pregnancy towards T helper type 2 reactions (Th2),

with increased expression of IL-4 and IL-10. Miscarriage and implantation failures tend to be associated with a Th1 predominance marked by pronounced expression of IFN-γ and IL-2 (Wegmann et al. 1993, Kwak-Kim et al. 2005). Th1 type response stimulates cellular immunity, whereas Th2 response shifts the immune system towards humoral immunity.

A marked decrease in the expression of LIF in the glandular epithelium of women with RIF compared to control subjects was noted when samples were obtained in the mid-luteal phase (Laird et al. 1997, Hambartsoumian 1998, Mariee et al. 2012a). A decrease in LIF levels has been associated with an increased chance of pregnancy in one study where samples were obtained on day 26 of menstrual cycle (Ledee-Bataille et al. 2002a), which is the time of this cytokines' normal decrease (Mariee et al. 2012a). Therefore, its persistence during the WOI, might contribute to implantation failure (Mariee et al. 2012a).

Expression of IL-15 has been demonstrated to be maximal in the glandular epithelium in the mid luteal phase in normal women (Chegini et al. 2003, Mariee et al. 2012a). Some data suggests that IL-15 mRNA is lower in women with RIF compared to fertile controls (using PCR) (Ledee-Bataille et al. 2005), whereas another study using immunohistochemistry and semi-quantitative analysis suggests an increased expression of IL-15 in the endometrial stroma of RIF women compared with controls (Mariee et al. 2012a). Discrepancies could be due to analytical differences as biopsies in both studies were obtained during the luteal phase. Endometrial stromal levels of IL-15 have been found to be positively correlated with uNK cell levels (Ledee-Bataille et al. 2005, Mariee et al. 2012a), suggesting a link between these two components of the immune system.

NK cells constitute an important part of the innate immune system and can broadly be divided to pNK cells and uNK cells. Due to different reference ranges used and different analysis methods, no

clear correlation between the pNK cells an RIF can be drawn at present (Tang et al. 2011). Assessment of uNK cells in women with implantation failure has also not revealed any significant correlations between their levels and treatment outcome (Fukui et al. 1999, Ledee-Bataille et al. 2004a). A systematic review of literature has concluded that the prognostic value of testing both peripheral and uterine NK cells in women with RIF remains uncertain (Tang et al. 2011).

Environmental factors

Obesity is an increasing epidemic affecting around 1.4 billion adults worldwide (WHO 2012). USA and Germany based population studies suggest a significant negative impact of female obesity on IVF treatment outcomes, especially in women under the age of 35 (Luke et al. 2011). Where male factor obesity was present, the clinical pregnancy rate was noted to be 30.38% compared to 28.15% in less obese controls (Kupka et al. 2011). Authors conclude, that increase in pregnancy rates where male partner was obese, was related to life style factors associated with higher social status of these couples (2008a, Kupka et al. 2011). Active and passive cigarette smoking is an important modifiable factor causing failure of ART. Meta-analysis of studies shows a lower odds of live birth per cycle in smokers (OR 0.54, 95% CI 0.30-0.99) and higher likelihood of miscarriage (OR 2.65, 95% CI 1.33- 5.30) compared to non-smoking women (Waylen et al. 2009). Passive smoking is also associated with poor IVF treatment outcome, as nicotine metabolites can be demonstrated in the follicular fluid. This was associated with a 52% increase in the risk of implantation failure (Benedict et al. 2011). All women experiencing RIF should be advised to modify their lifestyles, meaning weight loss to the normal range (18.5-24.9 kg/m2), and smoking cessation.

1.5.4. **Recurrent reproductive failure**

Mentioned conditions- RM and RIF have recently been defined collectively as recurrent reproductive failure (Quenby et al. 2009).

In light of new evidence suggesting the endometrium as the gatekeeper of implantation, it is possible that severe deregulation of decidualization could lead to implantation failure. A milder form of decidualization defects could initially permit implantation, but further impaired development of the feto-maternal interface could lead to clinical pregnancy loss (Quenby et al. 2009, Teklenburg et al. 2010a, Teklenburg et al. 2010b).

1.6. Animal models of reproduction

Animals have been extensively studies in the context of reproduction. This approach allows for relatively easy testing of hypotheses and pathways that are otherwise not accessible in the human subjects. Primate models are in limited quality, are expensive and confined only to units with available expertise. Mouse models, though on average more affordable and accessible, have the limitation related to differences in placentation when compared to humans (Bonney 2013).

The baboon, chimpanzee and human placentas are hemochorial, indicating direct contact of maternal blood with fetal trophoblast (Enders et al. 1999). Rodent placentas are referred to as hemotrichorial, due to the presence of a giant cell layer in addition to the cytotrophoblast and syncytial trophoblast. Guinea pig placentas consist of only one layer of trophoblast, hence this type of pacentation is referred to as hemomonochorial. Anatomical differences between rodent and human uteri are another major dissimilarity, with two-horned uteri in the former group (Bonney 2013). The length of pregnancy in humans allows for low number of well-developed fetuses, whereas rodent pregnancies result in numerous and less developed off-spring (Clancy et al. 2007). The short pregnancy duration allows for multiple interventions to be carried out within a reasonable period of time- an option not possible in humans.

Though differences in the diversity of the major histocompatibility (MHC) molecules (Madeja et al. 2011), as well as phenotypic

differences between human and mouse uNK cells exist (Croy 1994), both models have been successfully used to investigate the role of this innate component of the immune system on reproductive outcomes (Murphy et al. 2009, Hiby et al. 2010). The ability to vary genetic differences between the mother and fetus is a great advantage of the mouse model, and as such, has been utilized as a model for examination of T cell regulation during pregnancy (Bonney 2013).

When considering recurrent pregnancy loss, the mouse has been investigated the most. The commonly utilized model of recurrent miscarriage involves breeding the female mouse of a CBA strain with a male mouse of a DBA strain (Bonney 2013). Pregnancies obtained during this process can be affected by high levels of resorption (Ho et al. 1994) and infiltration by immune cells, such as NK cells (Haddad et al. 1995) related to inflammation (Toder et al. 1991), deranged cytokine levels (Chaouat et al. 1995) and abnormal vascular development within the placental bed (Dixon et al. 2006). Loss of pregnancy related to antiphospholipid syndrome has been investigated in rabbit models (Pierangeli et al. 1993).

Direct assessment of clinical observations in humans may not always be possible. Animal models should therefore be utilized as a compliment and not a substitute, which allows us to better understand the physiology and pathology of reproduction.

1.7. Assessment of endometrial receptivity

Assessment of the endometrial receptiveness can be conducted by directly sampling the tissue, indirectly by aspiration of fluid from the endometrial cavity or with ultrasound. Detailed molecular analysis of the sample would allow for unequivocal categorisation of the sample; however this carries ethical dilemmas and is contraindicated in women undergoing ART just prior to embryo replacement (Karimzade et al. 2010). Uterine flushing and analysis of the aspirate (cells and fluid) has been shown in a research setting to categorise the endometrium into a receptive or non-receptive

state (Chan et al. 2013), with minimal impact on the subsequent ART outcome, even if the procedure is carried out in the embryo replacement cycle (van der Gaast et al. 2003, Boomsma et al. 2009).

Technologies used in the abovementioned case, are beyond everyday use and will take years to perfect. In this situation, clinicians are left with indirect, surrogate markers of endometrial receptivity. These are easily provided by minimally invasive approach using transvaginal ultrasound (TVUS).

1.7.1. **Ultrasonography**

Ultrasound (US) is one of the most commonly used medical investigations worldwide. The widespread availability of US and its minimally invasive nature has led to its use as an endometrial receptivity assessment tool. Assessment of the endometrial pattern (Fanchin et al. 2000, Singh et al. 2011b), endometrial thickness or volume (Dietterich et al. 2002, Amir et al. 2007, McWilliams et al. 2007, Martins et al. 2008), uterine artery pulse-wave Doppler (UA PWPD) patterns (Jinno et al. 2001, Chien et al. 2002, Adakan et al. 2005, Singh et al. 2011b), and 3DPD imaging (Schild et al. 2000, Maugey-Laulom et al. 2002, Wu et al. 2003, Ng et al. 2006b, Ng et al. 2006c, Ledee et al. 2008, Merce et al. 2008, Zackova et al. 2009) have been carried out to describe markers indicative of endometrial receptivity. Different study designs, populations and days of assessment have confounded the overall picture, rather than clarified it. In view of this, in the current literature there seems to be no single sonographic feature unequivocally predictive of pregnancy in an IVF setting.

The endometrium undergoes cyclical changes that can be reflected in its sonographic appearance. In the menstrual phase of the cycle, a thin (<5mm), uniform, hyperechoic line represents the *zona basalis*. Occasionally, mixed echogenic fluid in the endometrial cavity can be observed at this stage, representing menstrual blood and sloughed cells. Proliferation of the endometrium during the

follicular phase leads to its thickening. The echogenicity also changes to resemble that of the myometrium (isoechogenic) (Figure 8).

In the peri-ovulatory phase of the menstrual cycle, the endometrium has a characteristic triple-layer appearance with a hyperechoic outer band encircling a hypoechoic inner *zona functionalis* and a hyperechoic inner line representing the apposition of two endometrial surfaces (Figure 9 A) (Sher et al. 1991). Progesterone driven changes in the endometrium cause it to become hyperechoic (bright) in the luteal phase of the cycle starting from the periphery (Fanchin et al. 1999), likely due to the increasing glycogen stores and stromal oedema (Grunfeld et al. 1991, Fleischer 2011). The thickness of the endometrium can measure in excess of 14 mm (Fleischer 2011).

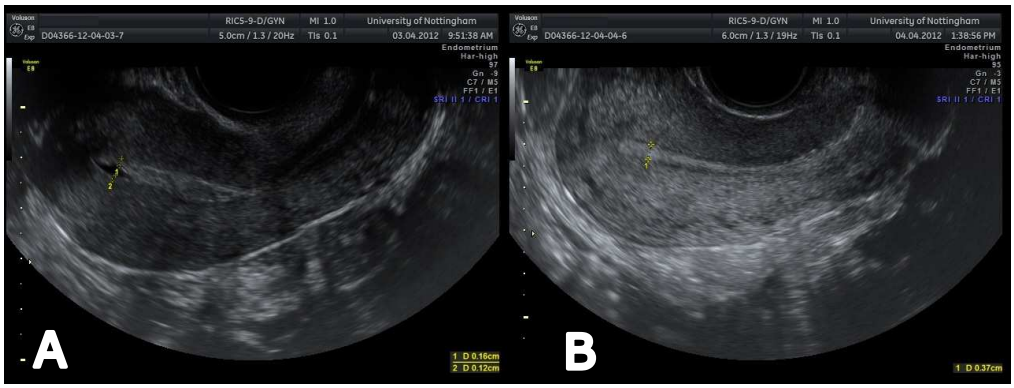


Figure 8: Menstrual and early follicular phase endometrium.

Menstrual endometrium (A): hyperechoic endometrial strip encompasses the mixed echogenicity cellular debris and anechoic (black) fluid most likely representing menstrual blood. Early follicular phase endometrium (B): an isoechoic stripe of endometrium is visible.

Endometrial pattern

In a study assessing frozen embryo replacement cycles by Zackova et al. (Zackova et al. 2009), the endometrial appearance on day of embryo transfer in an unstimulated cycle has been analysed using two distinct methods- subjectively by the observer, and objectively using VOCAL software and mean grey scale (MGS) values. Triple line pattern was significantly more prevalent in the pregnant group. MGS values of the endometrium and subendometrium were not different in pregnant and non-pregnant patients (Zackova et al. 2009). The small group of patients in the mentioned study (30) limits the generalizability of the results. A larger study by Fanchin et al. (Fanchin et al. 2000) also using computer software to assess endometrial echogenicity, indicated a poorer prognosis in patients with hyperechogenic endometrium on the day of hCG injection in a stimulated IVF cycle.

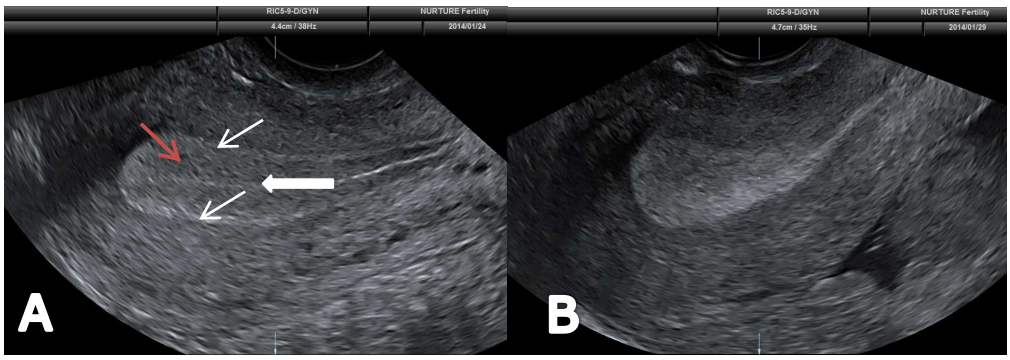


Figure 9: Late follicular and luteal phase endometrium.

Triple layer appearance of peri-ovulatory endometrium (A). Small arrow- hyperechoic zona basalis; red arrow- zona functionalis; large arrow- interface between the two endometrial surfaces. Hyperechoic appearance of luteal phase endometrium (B).

In patients with triple layer endometrium (hyperechogenic transformation <30%), the clinical pregnancy rates were 59% compared to hyperechogenic endometrium, where they were 23% (Fanchin et al. 2000). In patients undergoing IVF, a triple-layer appearance on day of hCG, can be an important positive prognostic factor for pregnancy (Coulam et al. 1994, Serafini et al. 1994,

Kupesic et al. 2001, Jarvela et al. 2002, Ng et al. 2006b), however it needs to be assessed alongside embryo quality and endometrial thickness (Singh et al. 2011b). Hyperechoic pattern found on the day of hCG injection can be associated with premature luteinisation, leading to an out-of-phase endometrium and subsequent lower conception rates, both in ART and natural cycles (Cohen et al. 1992, Fanchin et al. 2000). Due to population selection differences (i.e. oocyte recipients) and study design methodology (retrospective studies versus prospective ones) such association has not been demonstrated in other publications (Noyes et al. 2001, Rashidi et al. 2005, Ng et al. 2006c, Merce et al. 2008).

Endometrial thickness

Endometrial thickness (ET) is the next sonographic feature that has been extensively investigated as a predictor of pregnancy. Two approaches to the ET measurement exist with one assessing the thickest visible part (Sit et al. 2004), and the other measuring the ET at the junction of the upper third with the lower two thirds of the endometrial cavity (Raine-Fenning et al. 2004a). There is no evidence which method is better and depends on the practitioner's preference and experience. The reproducibility of ET measurement has been reported as exceeding 95% for one and more observers (Delisle et al. 1998). Woman's age and cause of infertility have been found to correlate with endometrial thickness, with thickest endometria found in younger women, women with good ovarian reserve and women with male factor infertility (Amir et al. 2007, McWilliams et al. 2007). In a study by Singh et al. (2011b), ET of less than 6 mm on day of hCG injection resulted in one pregnancy, with a mean endometrial thickness of 8.1mm in the studied population (Singh et al. 2011b). Maugley-Laulom et al. (2002) estimated a 92.3% risk of IVF treatment failure with endometrial thickness of <8 mm (Maugey-Laulom et al. 2002). Another study has used cut-off values of 7 mm and 14 mm as the 5^{th} and 95^{th} percentile of ET. Lowest pregnancy rates were observed with ET <7mm and highest with ET >14 mm (Amir et al. 2007). Some

authors have found a detrimental upper value of endometrial thickness above which pregnancy was less likely to occur, and if such event did take place, an increased risk of subsequent miscarriage was noted (Weissman et al. 1999). One of the largest observational studies so far (1294 women), reports a clinical pregnancy rate of 53% in women with ET of <9 mm and a 77% clinical PR in women found to have an ET of ≥16 mm on day of embryo transfer. This finding was independent of women's age and embryo quality (Richter et al. 2007). Significantly larger incremental changes in endometrial thickness at defined time points (at down-regulation, on day 6 of gonadotropins and on day of HCG) during IVF treatment have been found to be predictive of pregnancy (McWilliams et al. 2007). Other authors have failed to identify any similar cut-off values (Friedler et al. 1996, Schild et al. 2000, Chien et al. 2002, Dietterich et al. 2002, Wu et al. 2003, Ng et al. 2006b, Ng et al. 2006d, Martins et al. 2008, Merce et al. 2008, Zackova et al. 2009). A recent systematic review of 22 observational studies concluded that endometrial thickness of less than 7 mm as a decision tool has limited capacity to identify women with a low chance of conception (Kasius et al. 2014). Inclusion of retrospective studies, different study populations and methodological differences were the rationale behind this conclusion.

Endometrial volume

The endometrial and subendometrial volume as a marker of endometrial receptivity assessed using 3D US has been found to be reproducible with higher ICCs then endometrial thickness (Martins et al. 2008). Numerous studies have found a positive correlation of endometrial volume (EV) with IVF outcome (Raga et al. 1999, Martins et al. 2008, Merce et al. 2008) with a cut-off volume of 2 cm^3 below which pregnancy was least likely to occur (Raga et al. 1999). Some other authors indicated a lower still volume sustaining implantation (1.59 cm^3) (Schild et al. 2001). Other studies however, did not identify statistical significance between EV and IVF treatment outcome (Schild et al. 2000, Wu et al. 2003, Ng et al. 2006b, Ng et

al. 2006d, Zackova et al. 2009). Reasons for this discrepancy should once again be sought in different population selection and methodology (Heger et al. 2012). EV changes between the day before embryo transfer and 7 days post embryo transfer have been positively correlated with a successful pregnancy in a study by Martins et al. (Martins et al. 2008), with an average increase in EV of 0.7 cm^3 in pregnant women.

Endometrial and subendometrial blood supply

It is thought that, appropriate endometrial blood supply during the WOI is essential for implantation, hence it is logical to assess uterine and endometrial vascularity as a marker of endometrial receptivity (Ng et al. 2006d). Development of Doppler sonography allowed for subjective, visual description of blood flow, advent of pulsed- wave Doppler allowed for quantitative, objective description of vascular blood flow characteristics. 3DPD sonography allows for quantification of blood flow within a region of interest (ROI), thus depicting global vascularity in organs such as the endometrium and subendometrium. Newest addition to the Doppler sonography family is Spatio-Temporal Image Correlation (STIC) modality that combines the advantages of 3DPD with real time sonography, allowing for assessment of blood flow during a complete cardiac cycle. Up to date, no studies correlating ART outcome with STIC vascularity indices have been performed.

The pulsatility index (PI) and resistance index (RI), both representing impedance of blood flow, are the two most often used PWPD derived indices describing vascular blood flow (Ng et al. 2007). PI equates to the peak systolic velocity (PSV) minus end-diastolic velocity (EDV) and divided by the mean velocity. The RI is a ratio of PSV minus EDV divided by PSV (Ng et al. 2007).

$$PI = \frac{PSV - EDV}{(PSV + EDV)/2}$$

$$RI = \frac{PSV - EDV}{PSV}$$

High PI on the day of embryo transfer defined as >3.0, predicted a 35% chance of implantation failure (Steer et al. 1992). In another study, PI >3.3 was reported to have a high negative predictive value and sensitivity (88-100% and 96-100%, respectively) (Coulam et al. 1994). In a study by Maugley-Laulom et al. (2002), no pregnancies were achieved when the UA PI>3.4 was observed on the day of embryo transfer (Maugey-Laulom et al. 2002). The difference in study populations and timing of assessment has possibly lead other authors to find no correlation (Schild et al. 2000, Jinno et al. 2001, Dietterich et al. 2002, Ng et al. 2006a, Ng et al. 2006b, Ng et al. 2006d, Ledee et al. 2008). Waveform appearance of the UA Doppler signal has also been analysed in terms of predictive value for pregnancy. Absence of protodiastolic notch corresponds to the arterial elasticity and good uterine perfusion (Fleischer et al. 1992, Maugey-Laulom et al. 2002) and has been associated with better chances of pregnancy compared to patients with a notch present (Maugey-Laulom et al. 2002) (see Figure 10). Spiral arteries PWPD features have also been assessed to predict pregnancy, but this can be a difficult and time consuming procedure with no or limited predictive value (Zaidi et al. 1995, Yuval et al. 1999, Schild et al. 2001).

Absent or reduced endometrial blood flow as demonstrated by application of colour Doppler has been associated with reduced pregnancy rates (Chien et al. 2002, Maugey-Laulom et al. 2002) or no pregnancy (Zaidi et al. 1995, Battaglia et al. 1997, Yuval et al. 1999), likely due to inadequate nutrient and oxygen supply.

3DPD sonography has the advantage of increased sensitivity to low-velocity blood flow and is almost angle independent compared to PWPD imaging. 3DPD also enables assessment of the vascularity of the entire endometrium and subendometrium and its quantification.

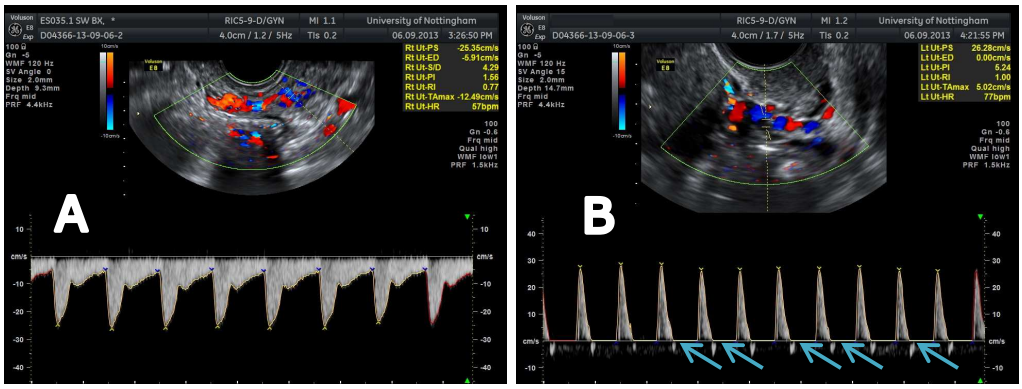


Figure 10: Examples of normal and abnormal pulse wave Doppler of uterine arteries.

Image depicting a normal, low resistance UA blood flow pattern (A). Image B demonstrates a high resistance UA with absent and reversed diastolic blood flow (arrows).

In vitro and *in vivo* studies (Graham et al. 2000, Raine-Fenning et al. 2004b, Raine-Fenning et al. 2008a, Raine-Fenning et al. 2008b, Martins 2010) have found this modality to be reproducible and reliable. Acquisition of 3DPD image is relatively easy, however factors related to the uterine version, presence of leyomyomas, bowel or movement artefacts, and patients' body habitus can make the examination difficult, and sometimes impossible. Blood flow characteristics when using 3DPD sonography are represented as vascularization index (VI), flow index (FI) and vascularization flow index (VFI), with the VI representing the number of Doppler voxels compared to all voxels, FI representing the Doppler signal intensity, and the VFI being a combination of both former indices (Pairleitner et al. 1999).

No single value of VI, FI or VFI has been identified as a 'normal' cut-off. This is the result of different equipment used, different populations studied and different timing of examination. Schild et al. (2000) has examined women on the first day of COH and demonstrated a lower subendometrial VI, FI, and VFI in patients with successful outcome, with FI being the strongest predictor

(Schild et al. 2000). Kupesic et al. (2001) performed the scan on day of embryo transfer and found higher subendometrial FI in pregnant women (Kupesic et al. 2001). Studies assessing the endometrial vascularity on the day of oocyte retrieval have found no difference in vascularity indices between pregnant and non-pregnant women (Dorn et al. 2004, Jarvela et al. 2005), and lower endometrial VI and VFI in pregnant subjects (Ng et al. 2006c). Assessment of subendometrium on day of hCG injection has revealed higher VFI (Wu et al. 2003), and higher VI, FI and VFI in pregnant women with an area under the curve of 0.813, 0.862 and 0.750, for each of the indices respectively (Merce et al. 2008). The VFI of the subendometrium above 0.24 on day of hCG administration, gave a positive predictive value for pregnancy of 93.8% (Wu et al. 2003). In a study evaluating natural cycle frozen embryo replacements and endometrial and subendometrial vascularity indices, no correlation with successful treatment has been demonstrated (Ng et al. 2006d, Zackova et al. 2009). Similarly, natural cycle analysis of subendometrial blood flow indices by Ledee et al. (Ledee et al. 2008) did not reveal a correlation with pregnancy outcome in patients with RIF when assessed on day 21-23 of cycle (see Table 1).

In a subpopulation of patients with hydrosalpinges, when assessing the endometrial vascularity on the day of oocyte retrieval, a significantly lower endometrial and subendometrial VI and VFI has been demonstrated, suggesting impaired endometrial blood supply, and this also correlated with decreased pregnancy rates in the hydrosalpinx group (Ng et al. 2006b).

Study	Timing of assessment	Assessed index	Association with pregnancy outcome
Schild et al. (2000)	Day 1 of ovarian stimulation N=75 cycles	Subendometrial FI 10.8±3.3	Lower in pregnant population
Kupesic et al. (2001)	Day of embryo transfer N=89 women	Subendometrial FI 13.2±2.2	Higher in pregnant population
Dorn et al. (2004)	Day of oocyte retrieval N=42 women	Subendometrial VI, FI and VFI	No difference between pregnant and non-pregnant women
Ng et al. (2006c)	Day pf oocyte retrieval N=451 women	Endometrial VI 0.707 (0.3-1.78) Endometrial VFI 0.161 (0.063-0.427)	Lower in pregnant population
Wu et al. (2003)	Day of hCG administration N=54 women	Subendometrial VFI >0.24	83.3% sensitivity and 88.9% specificity form predicting receptive endometrium
Merce et al. (2006)	Day of hCG administration N=80 women	Endometrial: VI 21.19±8.91 FI 28.12±3.90 VFI 6.30±4.46	All indices higher in the pregnant population
Ng et al. (2006d)	1 day after LH surge in frozen embryo cycles N=193 women	Endometrial and subendometrial VI, FI and VFI	No difference between pregnant and non-pregnant women
Zackova et al. (2009)	Day of embryo transfer in frozen embryo cycles N=30	Endometrial and subendometrial VI	No difference between pregnant and non-pregnant women
Ledee et al. (2008)	Women with RIF, 7-9 days after LH surge in a natural cycle N=40 women	Subendometrial VFI	No difference between RIF and control populations

Table 1. Study characteristics assessing endometrial and subendometrial vascularity using three-dimensional ultrasound in correlation with pregnancy outcomes.

FI- flow index; VI- vascularization index; VFI- vascularization flow index; LH- luteinizing hormone, hCG- human chorionic gonadotrophin

Only a few groups have investigated correlation between the sonographic appearance of the endometrium and expression of endometrial cell surface implantation markers. Studies by Ledee et al. (Ledee-Bataille et al. 2004a, Ledee et al. 2008) assessed the expression of IL-18, IL-15 and IL-12 in correlation with Doppler imaging in patients with RIF. Synthesis of IL- 15 mRNA has been found to be correlated with sub-endometrial blood flow and NK cell numbers. IL-18 levels were negatively correlated with the vascularity of the endometrium as measured by Doppler sonography. These relations were not observed in the control groups of fertile women (Ledee-Bataille et al. 2005, Ledee et al. 2008). In a study by Quenby et al. (Quenby et al. 2009), the authors have elegantly demonstrated a correlation between uterine NK cells and PWPD characteristics. Higher uNK cell levels, lower UA resistance indices and better blood flow features were present in women with RM and RIF. This can possibly explain the pathology behind some reproductive failures, as increased endometrial blood flow causes early oxidative stress to the embryo and its subsequent demise (Quenby et al. 2009).

Paucity of data exists assessing the 3DPD indices in correlation with 'new' markers of implantation, such as earlier mentioned PROKI. No data exists which correlates STIC Doppler indices with markers of endometrial receptivity.

1.7.2. **Endometrial biopsy**

The direct method of assessment of the endometrium is performed by means of obtaining a tissue fragment. This can be performed by Pipelle de Cornier endometrial sampler, targeted hysteroscopic biopsy, or more traditionally, by curettage. When endometrial malignancy is suspected, endometrial biopsy is the universal screening tool allowing for early diagnosis. In current IVF practice, endometrial biopsy is not routinely performed, even though a multitude of information can be obtained. As described earlier, markers of decidualization (PRL, IG-FBP1), NK cell levels,

histological stages of endometrial development, and endometritis can be diagnosed using this technique. In the past, endometrial biopsy has been used as the standard tool for LPD diagnosis (Lessey et al. 1995) and endometrial dating in keeping with endometrial morphology as described by Noyes et al. (Noyes et al. 1950). Recent well-designed studies have proven that assessment of endometrial morphology is an imprecise method, which cannot distinguish fertile and infertile women and cannot be used to diagnose LPD (Murray et al. 2004, Myers et al. 2004).

Recent transcriptomic work carried out on endometria of women from natural and stimulated cycles, seems to be a promising tool to accurately diagnose the endometrial stages irrespective of the morphological appearance of the specimen (Horcajadas et al. 2007). Though some differences remain due to study design and analysis method, a general consensus exists that transcriptomic profiles are able to accurately allocate the stage of endometrial development (Ruiz-Alonso et al. 2012) allowing for personalized timing of embryo transfer (Ruiz-Alonso et al. 2013). Possibly one of the most promising works incorporating personalized medicine is the endometrial receptivity assay (ERA), where serial endometrial biopsies obtained on days LH+1, LH+3, LH+5 and LH+7 have led to description of genes associated with appropriate endometrial receptivity and successful ART outcome. The authors have identified 238 genes differentially expressed in the prereceptive and receptive samples. Differentially expressed gene groups included genes involved in immune response, response to stress and injury, cell cycle regulators, regulators of cellular adhesion, and cell-to-cell signalling. Based on the gene expression, the receptivity assay is able to predict a receptive endometrium with a specificity and sensitivity of 0.8857 and 0.99758, respectively (Diaz-Gimeno et al. 2011). Application of the endometrial receptivity assay in women with RIF, has led to a very high clinical pregnancy rate of 51.7% in this poor prognosis population (Ruiz-Alonso et al. 2013). The presented results still require further validation, but hint at the

possible future of reproductive medicine with genomics and proteomics at the forefront.

1.7.3. **Serum markers**

Progesterone, being the one of the most important markers driving secretory endometrial changes, can be used as a screening marker of decidualisation. Prior to pregnancy, progesterone is secreted in a pulsatile manner reflecting LH pulses (Filicori et al. 1984). Maximal serum level of progesterone is noted 6 to 8 days after ovulation, if no conception occurred (Speroff L. 2005). However, to assess the day of maximal progesterone levels, the exact timing of ovulation has to be known. This can be achieved by urinary LH kits, but these can produce a false positive result in 7% of normally cycling women. Lack of normograms correlating progesterone levels with fertility and appropriate decidualisation, make progesterone a poor marker of decidualisation and limit its use in this context (McGovern et al. 2004). Day 21 progesterone above 20nmol/L is suggestive of ovulation and, as such, is performed especially when anovulation is suspected as the cause of infertility. At present, no serum markers of decidualisation have been clinically validated.

1.7.4. **Magnetic Resonance Imaging**

Magnetic resonance imaging (MRI) has been employed as an imaging modality to assess uterine pathologies as well as endometrial receptivity. No differences in MRI markers (appearance, thickness) were identified between women who conceived and the ones that did not (Turnbull et al. 1994). The limited availability of high resolution MRI scanners, time consuming process of image acquisition, and more importantly, high cost of this investigation, makes MRI a modality currently not suited for routine assessment of endometrial receptivity (Pierson 2003).

1.8. Modification of the endometrial environment

Modification of endometrial receptivity is an important step towards improving the pregnancy outcome in the infertile population. Multiple approaches to improve the endometrial milieu have been proposed.

1.8.1. **Oral medication**

The ease of administration of oral medications makes them an appealing modality aiming to improve endometrial receptivity. Aspirin- a non-steroidal anti-inflammatory drug, has been used with varying success as means to improve the endometrial receptivity, especially in women with thin endometrium (<8 mm) (Weckstein et al. 1997, Rubinstein et al. 1999, Lok et al. 2004, Waldenstrom et al. 2004, Hurst et al. 2005, Pakkila et al. 2005, Gelbaya et al. 2007, Ruopp et al. 2008). At present, the use of this medication remains at the discretion of the treating physician.

Pentoxifylline and tocopherol have also been used in a group of oocyte recipients with thin, unresponsive endometrium with limited success (Ledee-Bataille et al. 2002b). Vaginal use of sildenafil has been shown to increase endometrial blood flow and thickness, (Sher et al. 2000, Sher et al. 2002), however this did not convert to an increased pregnancy rate in oocyte recipients (Frattarelli et al. 2006). Modification of stimulation protocol (El-Toukhy et al. 2004), increased dose or alternate route of oestrogen supplementation (Fanchin et al. 2001, Tourgeman et al. 2001), and hCG administration (Ben-Meir et al. 2010) have also been assessed with varying success as therapies to improve endometrial receptivity.

1.8.2. **Endometrial biopsy**

Apart from its diagnostic use, it is thought that endometrial biopsy and elicited endometrial injury can induce a localized inflammatory reaction altering the endometrial milieu to one favouring

implantation. Studies assessing the effect of endometrial biopsy on the local microenvironment, have used different inclusion criteria, different timing of the biopsy and different biopsy methods ranging from Pipelle biopsy, to directed, hysteroscopic endometrial sampling (Huang et al. 2011, Nastri et al. 2012). The possible mechanisms responsible for the beneficial modification include: mechanical distension of the uterine cavity during hysteroscopy, induction of an aseptic inflammatory process within the endometrium, associated hyperaemia, neo-angiogenesis, changes in the endometrial immunecompetent cell populations, and delayed endometrial maturation (decidualisation) of the injured tissue allowing synchrony with the embryo (Zhou et al. 2008, Quenby et al. 2009, Almog et al. 2010, Gnainsky et al. 2010, Huang et al. 2011).

In 1906, a beneficial effect on decidualization following endometrial injury (scratching) was observed in guinea pigs (Loeb 1906, Loeb 1908). Subsequent rodent studies have shown that other forms of injury, such as suturing the uterine horns (Wood et al. 1970) and intrauterine infusion of oil (Finn et al. 1972) induce decidual transformation. Human studies also indicate that endometrial injury may improve chances of live birth following IVF treatment in the RIF population (Nastri et al. 2012), as well as in an unselected group of women undergoing ART (Nastri et al. 2013). In a paper by Gnainsky et al. (2010), the authors suggest that mechanical injury caused by biopsy initiated a localized inflammatory reaction. Marked influx of macrophages and dendritic cells was noted. TNF-α, growth-regulated oncogene-α (GRO-α), macrophage inflammatory protein 1B (MIP-1B), IL-15, and OPN were significantly increased in biopsy specimens from women that underwent an endometrial sampling procedure earlier in the cycle (Gnainsky et al. 2010). The authors postulate that dendritic cells (DCs) and their persistence within the endometrium following the biopsy create an endometrial milieu favouring implantation in the subsequent cycle (Gnainsky et al. 2010). TNF-α, as a pro-inflammatory factor, by itself is responsible for initiation of inflammatory response and increased secretion of

MIP-1B, GRO-α and IL-15 by endometrial stromal and epithelial cells *in vitro* (Haider et al. 2009, Gnainsky et al. 2010). MIP-1B has been shown to positively affect the trophoblast migration, potentially improving implantation (Hannan et al. 2006). MIP-1B and GRO-α attract macrophages and DCs (Menten et al. 2002) to the site of injury facilitating wound healing (Luster 1998).

Animal studies have shown that endometria deprived of active DCs exhibit impaired decidualisation caused by reduced vessel formation, proliferation and differentiation of ESCs leading to lack of embryo attachment and invasion (Plaks et al. 2008, Granot et al. 2012). DCs secrete IL-15 and IL-12 (Blois et al. 2011), interleukins that are crucial for uNK cell differentiation, thus creating a functional link between these two immune cell populations. OPN also seems to be involved in recruitment of macrophages and DCs (Giachelli et al. 1998, Renkl et al. 2005) with its effect mediated by MIP-1B (Zheng et al. 2009). This cytokine also serves as an adhesion molecule mediating interactions between the endometrial epithelium and trophoblast allowing for attachment of the blastocyst to the endometrium (Apparao et al. 2001, Johnson et al. 2003). An increase in the abovementioned factors following endometrial injury, might lead to improved implantation rates in the subsequent cycle.

Endometrial injury inevitably leads to the development of an inflammatory process acting as an inducer of epigenetic changes (Backdahl et al. 2009, Munro et al. 2010). Epigenetic changes, such as methylation of the proximal promoter of the HOXA10 gene have been demonstrated upon induction of endometriosis and inflammation in a baboon model (Kim et al. 2007). Similar epigenetic changes secondary to injury can also control endometrial stem cell proliferation, migration and differentiation leading to formation of a more receptive endometrium (Granot et al. 2012, Tollervey et al. 2012).

Based on the available literature, the optimal timing for the endometrial biopsy is the mid to late-luteal (LH +7 to LH+9) phase

of the cycle preceding IVF stimulation. This timing allows ample time for the injury to heal and has not resulted in decreased pregnancy rates in subsequent IVF treatment (Spandorfer et al. 2002b, Tuckerman et al. 2004). A more detailed review of literature and studies related to the effect of endometrial injury on IVF treatment outcome is presented in the appropriate chapter.

1.8.3. **Endometrial flushing**

Recent studies have focused on direct administration of medications into the uterine cavity with the hope to improve the endometrial environment in women with unresponsive, thin endometrium. One such drug is granulocyte colony-stimulating factor (G-CSF). Intrauterine administration of this drug in the embryo transfer cycle has resulted in an increased endometrial thickness and pregnancy rates (Gleicher et al. 2011). Randomized controlled trials of this intervention are underway. Until then, intrauterine G-CSF infusion remains experimental; however it represents a promising way to alter the endometrial environment.

Endometrial flushing with 2ml of follicular fluid at the time of oocyte collection has also been assessed as means of improving the endometrial receptivity due to the high content of various growth factors within the aspirate. One study reported a non-significant increase in clinical pregnancy rate compared to controls when endometrial flushing with follicular fluid was carried out (34% vs. 31%, P=0.718) (Hashish et al. 2014). Introduction of hCG into the endometrial cavity prior to embryo transfer (500 IU) has improved the clinical pregnancy rates in women undergoing first IVF or ICSI cycle from 60% in the control populations to 75% ($P<0.05$), according to a prospective randomized study. Smaller doses of hCG did not produce a significant increase in pregnancy rates (Mansour et al. 2011).

Addition of compounds increasing viscosity of the embryo culture medium (Bontekoe et al. 2014), flushing the endometrial cavity with culture medium prior to embryo transfer (Berkkanoglu et al. 2006)

or low molecular weight heparin, as well as co-culture protocols (Parikh et al. 2006) are being explored as possible modalities aiming to improve the endometrial micro-environment prior to embryo replacement and produce higher pregnancy rates.

1.9. Rationale for the study and Hypothesis

The evidence suggests that alteration of the endometrial environment in women with previous failed IVF or ICSI by inflicting endometrial injury improves the outcome of the subsequent treatment cycle. The selected population assessed in the studies supporting this statement prevents from generalizing this to all women undergoing IVF or ICSI treatment. The mechanisms responsible for alteration of the endometrial environment following biopsy remain still unclear. If proven effective, endometrial injury could serve as a beneficial adjunct for all couples undergoing ART.

The hypothesis forming the basis of the work reported in this thesis was that endometrial injury in the cycle directly preceding an embryo transfer cycle, be it fresh or frozen, improves the outcome of that treatment irrespective of previous reproductive history. In order to support or refute this hypothesis, a clinical trial of endometrial biopsy prior to IVF or ICSI treatment has been designed. Additional objectives that allow examining the mechanisms responsible for the beneficial effects of the biopsy on the endometrium are:

1. Determine the inter and intra observer reliability of application of a novel sonographic technique- STIC, in the context of the effect of endometrial injury on endometrial and subendometrial blood flow as a predictor of treatment outcome.
2. Using all available sonographic modalities, examine the predictive value of any indices on ART outcome. Same sonographic modalities will be used to determine the effect of endometrial injury on endometrial and subendometrial blood flow.
3. Examine the value of uterine natural killer (uNK) cell numbers as a predictor of ART outcome.
4. Examine the relationship between ART outcome and expression of molecular markers of endometrial function.

CHAPTER 2. Materials and Methods

This chapter describes the materials and methods used to evaluate the reliability of methods used, followed by methods used to perform the clinical and laboratory components of the study, including the endometrial biopsy, ultrasound image acquisition and analysis, cell culture, and molecular analyses used to determine factors responsible for success or failure of IVF/ICSI treatment.

2.1. Research Ethics Committee application

The research studies were conducted in accordance with the ethical principles originating from the Declaration of Helsinki on Ethical principles for Medical Research Involving Human Subjects, adopted by the General Assembly of the World Medical Association (1996), and the principles of the International Conference on Harmonization guideline on Good Clinical Practice.

The ethical application process was carried out through the Integrated Research Application System (IRAS) website (https://www.myresearchproject.org.uk/). All study templates for protocols, patients information leaflets, consent forms and posters were obtained from Nottingham University Hospitals Research and Development website (http://nuhrise.org/) or University of Nottingham Clinical Trials Unit website (http://ctu.nottingham.ac.uk/ctu/default.asp).

Nottingham Research Ethics Committee has granted ethical approval for the studies carried out within the remits of this thesis (Reference numbers: 12/EM/0345; 13/EM/0277). Nottingham University Hospitals NHS Trust and The University of Nottingham, as study sponsors have granted approval for the studies conducted (Ref: 12GY005; 13GY009 and 13069 respectively). The randomised controlled trial of endometrial sampling prior to ART (Endoscratch study) was registered on www.clinicaltrials.gov website (Ref: NCT01882842).

Informed and written consent was obtained prior to enrolment of the study participants.

2.2. Ultrasound Technique

2.2.1. **Image acquisition**

All ultrasound investigations were carried out via the trans-vaginal route using Voluson E8 Expert BT12 (GE Healthcare, Zipf, Austria) equipped with trans-vaginal transducer (5-9MHz). Data identifiers were entered according to the study protocol. Patients were asked to empty their bladder before the procedure was carried out for comfort and best visualization of the pelvic organs. Before each scan, the ultrasound transducer was cleaned with ultrasound equipment sanitizer wipes, following which sonographic gel was applied to the transducer. Latex or non-latex probe covers were then used depending on the patients' sensitivity. All scans were conducted in the supine position, with hips abducted and knees flexed. The assessment consisted of conventional 2D ultrasound of the pelvis to identify the pelvic organs and exclude any pathology. The uterus was identified and measurement of the endometrial thickness in the sagittal (longitudinal) section was obtained. Acquisition of a static 3D uterine volume was subsequently conducted. The multiplanar display of the uterus was examined to confirm the inclusion of the endometrium in its entirety. 3D power Doppler volumes of the uterine and endometrial blood flow were carried out with the settings described below. The uterus was again identified in the sagittal section.

The 4D STIC datasets of endometrial blood flow were acquired using the settings described below. The uterus was visualized in the sagittal section. Prior to the ultrasound scan, radial pulse was measured over 30 seconds in order to compare the STIC heart rate value with the woman's actual pulse. Patients were asked to lie as still as possible during the scan and then the automatic sweep was activated. If the estimated heart rate as determined by the STIC modality was within +/-10% of the radial pulse value, the dataset was stored. All images prior to storage on the machines internal hard disk drive (HDD) were analysed for motion Doppler artefacts, if these were present, the image acquisition was

repeated. On more than one occasion, multiple acquisitions were made, and the best ones were selected for analysis.

PWPD was used to measure the blood flow to the uterus through the ascending branch of the uterine artery (right and left). Datasets were transferred from the machines' HDD to a portable HDD device.

Ultrasound settings were selected for their best depiction of two-dimensional, three-dimensional and STIC details allowing for accurate delineation of planes. These were as follows:

Two-dimensional ultrasound settings:

- Gain: adjusted to obtain the best image possible
- Tissue harmonics: On
- Power: 100%
- Focus: 5 cm deep (dependant on patient)

Static three-dimensional ultrasound settings:

- Quality: high
- Angle of acquisition: 120 degrees

3D Power Doppler settings:

- Smooth rise/fall: 2/2
- PRF: 0.6 kHz
- Angle of acquisition for 3D PD: 120 degrees
- Gain: -2

STIC settings (determined as described in Chapter 7):

- Sweep time: 15 sec (max)
- Angle of acquisition: 90 degrees (max)
- Smooth rise/fall: 2/2

- PRF: 0.6
- WMF: low 1
- Balance: >225
- Frequency: med.

2.2.2. **Data display and analysis**

All ultrasound images were analysed offline on a personal computer (PC) using 4D View™ software (version 10.5 BT12, GE Medical Systems). The 3D volumes were displayed using the multiplanar view which demonstrates three sectional planes (the A, B and C planes). All these planes are mutually related, so that manipulation of one plane produces equivalent movement of the other planes. This allows for standardisation of the analysed region of interest, in the context of this thesis- the endometrium. Plane A displays the sagittal section of the endometrial cavity, plane B- the transverse section and plane C- the coronal reconstruction of the endometrial cavity. The magnification of the image was adjusted to allow for accurate delineation of the endometrium. Volume of the endometrium was measured using virtual organ computer aided analysis (VOCAL) on 4D View software. This was done by delineating the endo-myometrial junction on the A plane as the volume was rotated through 180^{o} using varying rotational steps (Figure 11). Only images where the ROI was no deeper than 5 cm from the transducer and delineation of the endometrium was possible without doubt were included. Presence of fluid within the endometrial cavity excluded the image from further analysis. PWPD indices were recorded from the stored datasets for each uterine artery.

PD indices (vascularisation index-VI, flow index- FI and vascularisation flow index- VFI) were obtained using the histogram facility applied to the rendered image obtained using the VOCAL software (Figure 12).

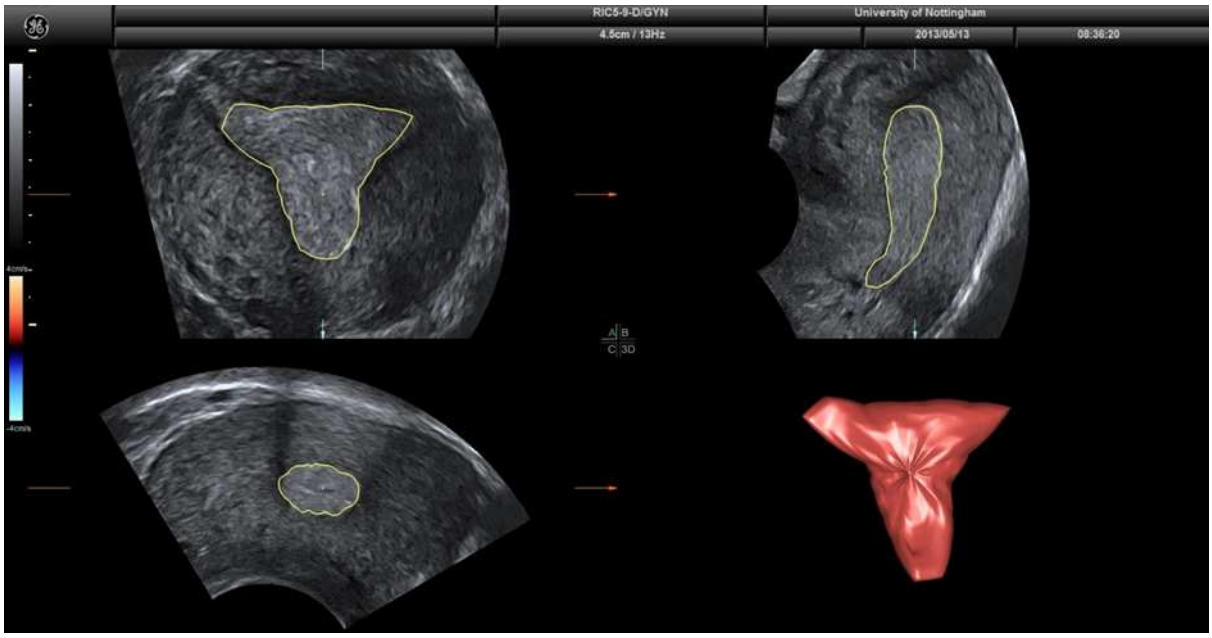


Figure 11: Multiplanar view.

The image demonstrates the multiplanar view of a 3D volume of the endometrium with rendering of the endometrium presented in the bottom right corner of the image.

STIC datasets are composed of a series of static 3D acquisitions. Each image of the series was analysed in a manner described above using 15^{o} rotational steps when analysing the volume of the region of interest. STIC vascularity indices were recorded in an Microsoft Excel spreadsheet.

Spherical sampling of the endometrium using STIC modality

Using the multiplanar view, the endometrium was aligned in the sagittal, transverse and coronal planes respectively. Using VOCAL software, a spherical contour was applied so that pole 1 was touching the top of the endometrial cavity on reference images A and C (see Figure 13).

To allow reproducibility, 1 cm^3 sphere was drawn (see Figure 14). The histogram modality was applied to produce the FI, VI and VFI results of the ROI included in the sphere.

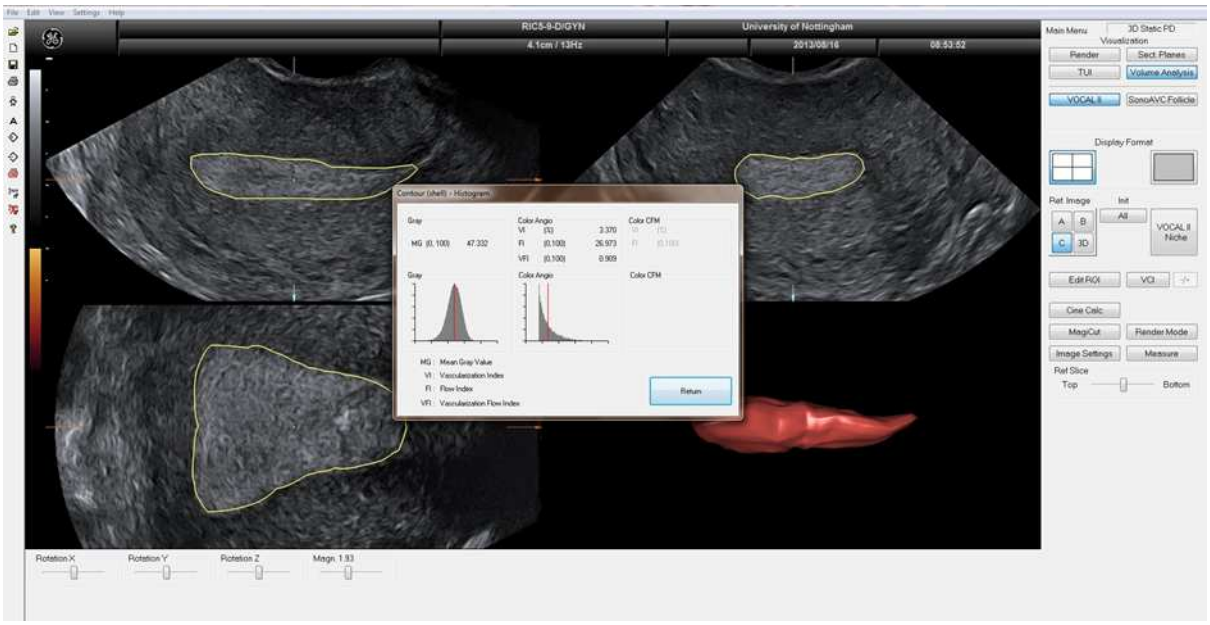


Figure 12: Histogram facility.

Once the region of interest has been determined using VOCAL software, the histogram facility displays the mean grey score (MGS), vascularisation index (VI), flow index (FI), and vascularisation flow index (VFI).

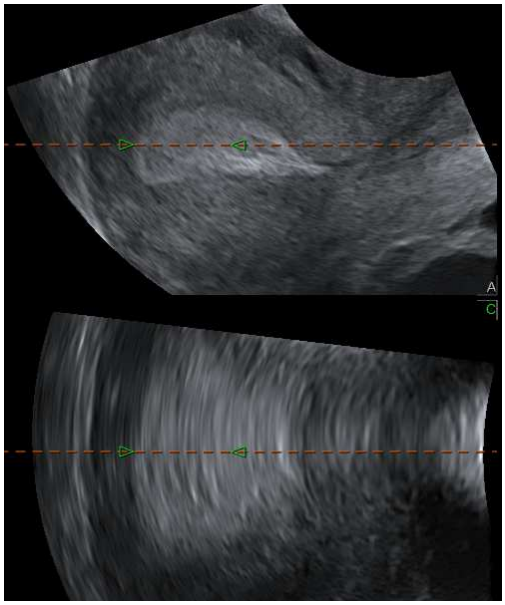


Figure 13. Multiplanar view of the endometrial cavity (plane A and C) demonstrating placement of the spherical ROI at the fundus (green arrows).

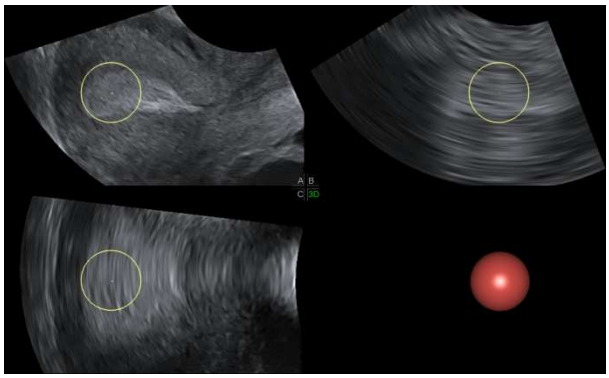


Figure 14. Sphere ROI demonstrating the placement in the top portion of the cavity.

2.2.3. **Reproducibility of ultrasound image measurements**

3D ultrasound measurements have been found to be reproducible by many authors (Raine-Fenning et al. 2002, Raine-Fenning et al. 2004b). To assure accurate and reproducible results of analyses carried out within this thesis a single 3D endometrial dataset has been analysed 10 times by a single observer using 30^{o}, 15^{o}, 9^{o}, and 6^{o} rotational steps. For every dataset, the 6^{o} volumes were used as the 'true' endometrial volume and served as control. The corresponding limits of agreement are presented in graphs in Figure 15.

To compromise between the timing required to analyse one dataset, and the accuracy of the endometrial volume measurement as compared to the control volume, 9^{o} rotational steps were chosen for analysis of 3DPD endometrial ultrasound images (Figure 16). Endometrial volume measurements did not differ when measured using 6^{o}, 9^{o} and 15^{o} rotational steps ($p>0.05$); results obtained using the 30^{o} rotational steps were significantly higher compared to other methods ($p<0.05$). For this reason, endometrial analysis using 30^{o} rotational steps was not used in this thesis.

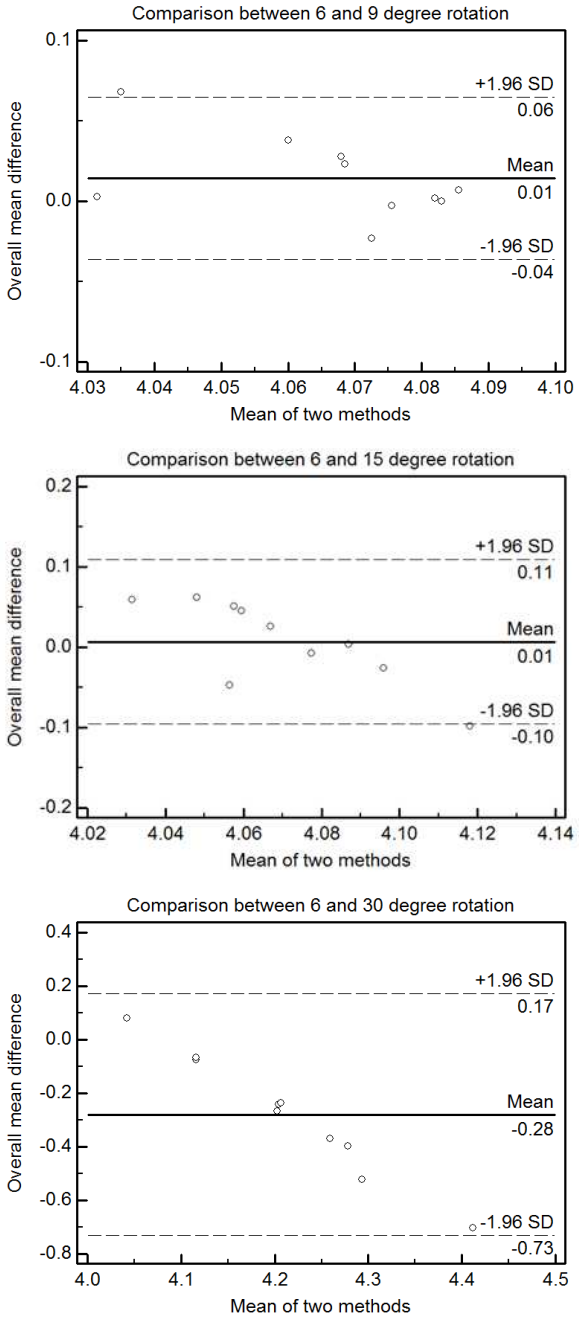


Figure 15: Bland-Altman plots of endometrial volume measurements.

Bland-Altman plots showing performance of different methods of measurement of the endometrial volume when compared to the control volume as assessed by 6^o rotational volume measurements. The widest LOA are demonstrated for 30^o rotational steps.

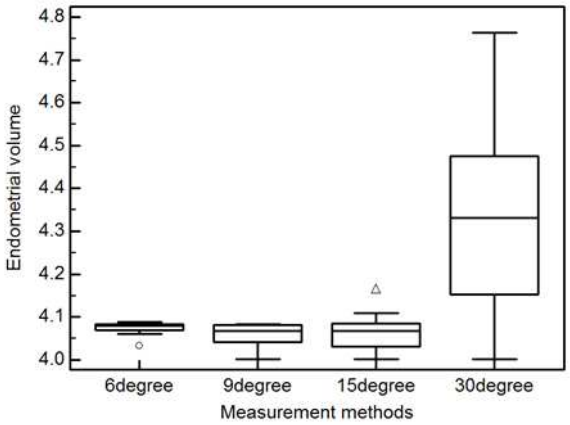


Figure 16: Box and whisker plot demonstrating the similarities of endometrial volumes as obtained using different methods of measurement.

STIC datasets have been analysed using the 15° rotational steps. In order not to compromise the results of the analysis, the obtained volume was compared to the reference volume obtained using 6° rotational steps and was only included if it was within ±10% of the reference volume. If this criterion was not met, the image was reanalysed. As each STIC dataset contains a minimum of 10 static 3DPD volumes, the 15° rotational analysis was carried out to allow timely image analysis with minimal or no compromise to the results.

2.3. Timed endometrial sampling

Timed endometrial samples were collected from participants enrolled into the studies performed within the remits of this thesis. All participants monitored daily urinary luteinising hormone (LH) levels using ovulation kits (Clearblue Digital Ovulation Test, SPD, Switzerland). Timed endometrial biopsies were obtained 7 to 9 days after the pre-ovulatory LH surge (LH+7 to LH+9) or on day 18 to 23 of a non-ovulatory cycle following a negative urinary pregnancy test.

All biopsies were performed using Pipelle endometrial sampler (CCD, Paris). The cervix was visualized using a Cusco's speculum and any mucous was cleaned away using cotton swabs. The Pipelle was then

introduced into the uterus until resistance from the fundus was felt. The piston was withdrawn to generate negative pressure and the device was rotated 360° to cover all the walls of the endometrial cavity. Subsequently, the device was moved in and out by 2 cm while rotating simultaneously. This was done for a maximum of 30 seconds or until the Pipelle was filled with endometrial tissue. On occasions, in order to gain entry into the endometrial cavity, a stabilising device (Vulsellum) was used. A malleable embryo transfer catheter (Cook Medical, UK) was used as the second choice device to perform the endometrial injury if this was not possible using the Pipelle (i.e. due to acute cervico-uterine angle, pin-point cervical os). In this case, the catheter was introduced to the fundus under US guidance, the malleable stylet was withdrawn and a 10 ml syringe was attached to the catheter to generate the negative pressure. Subsequently, the catheter was moved in and out by 2 cm for 30 seconds. Any obtained sample was divided into two parts if sufficient material was obtained. For establishment of primary stromal cell culture, the tissue was collected into 10% DCC/FCS DMEM/F12 culture medium supplemented with antibiotic/ antimycotic, insulin and L-Glutamine. Samples used for immunohistochemistry were collected into 4% paraformaldehyde (PFA) and fixed for 24 hours.

2.4. Laboratory analysis

All human endometrial stromal cells were cultured in standard conditions (humid atmosphere with 5% v/v CO2 maintained at 37° C) using Incu Safe Incubator (Sanyo). Handling of endometrial sample and HESCs was carried out in a Class II Microbiological Safety Cabinet (NuAire, MN, USA). 70% ethanol was used to clean the working surface of the cabinet, following which it was left running for 5 minutes to allow the airflow to stabilise. Sterile plastic cell culture flasks (Corning), plugged disposable sterile pipettes (Corning) and plastic universal tubes (Sterilin) were used in the culturing process.

2.4.1. **Preparation of culture media**

As fetal bovine serum (FBS) contains endogenous steroid hormones with the potential to mask the effect of added reagents, removal of these was

necessary prior to culture media preparation. Dextran coated charcoal (DCC) treatment was used to strip the serum of small molecules. 1.25 g of charcoal and 125 mg of dextran were added to a 500 ml bottle of FBS, which was then incubated in a water bath at 56^{o} C for 2 hrs and inverted every half hour. The media was then transferred into 50 ml Flacon tubes and centrifuged at 4000 x g for 30 minutes. Following centrifugation, the supernatant was pre-filtered using a 45μm sterile syringe filter (Sartorius, UK) into a sterile bottle. Final filtration was performed using a Nalgene filter unit (Nalge Nunc, NY, USA). The filtered media was aliquoted into sterile 50 ml Falcon tubes and stored at -20° C. DMEM/F12 culture medium (500 ml) supplemented with 10% or 2% v/v of DCC/FCS, 5 ml antibiotic/ antimycotic, 5 ml 200mM L-Glutamine, 100μl (10mg/ml) Insulin, and 5μl of 10^{-4}M solution of β-Estradiol was used for cell cultures.

2.4.2. **Establishment, maintenance and storage of human endometrial stromal cell cultures**

The fresh biopsy was transferred onto a Petri dish with the excess media being discarded. Using sterile scalpels, the sample was finely minced. Digestion media consisting of additive free DMEM containing 0.5 mg/ml collagenase type IA (Sigma) and 0.1 mg/ml DNAse I (Roche) was syringe filtered onto the minced sample. This was then aspirated and transferred into a sterile T-25 cm^2 flask. The sample was incubated for 1 hr at 37° C with vigorous shaking every 20 minutes. The collagenase IA disintegrates the extracellular matrix and the DNAse I eliminates the viscous DNA released from dead cells. Following incubation, 10 ml of 10% DCC/DMEM was added to stop the collagenase activity, the sample was transferred into 20 ml Falcon tubes and centrifuged at 215 x g for 6 minutes. The supernatant was discarded and the pellet containing epithelial, stromal, red blood cells and glands was re-suspended in 6 ml or 12 ml of 10%DCC/ DMEM depending on the size of the flask (T-25 cm^2 or T-75 cm^2, respectively). The sample was incubated for 1hr. After that time, the media with blood cells and epithelial cells was removed; cells were washed with PBS and replenished with fresh 10%DCC/ DMEM. This method has been validated and produces very pure HESCs (Brosens et al.

1999). The cells were maintained in 10% DCC/DMEM culture media until 90% confluence was achieved (Figure 17). This took 2 to 14 days. At this point, the cells were harvested and frozen for use in further experiments.

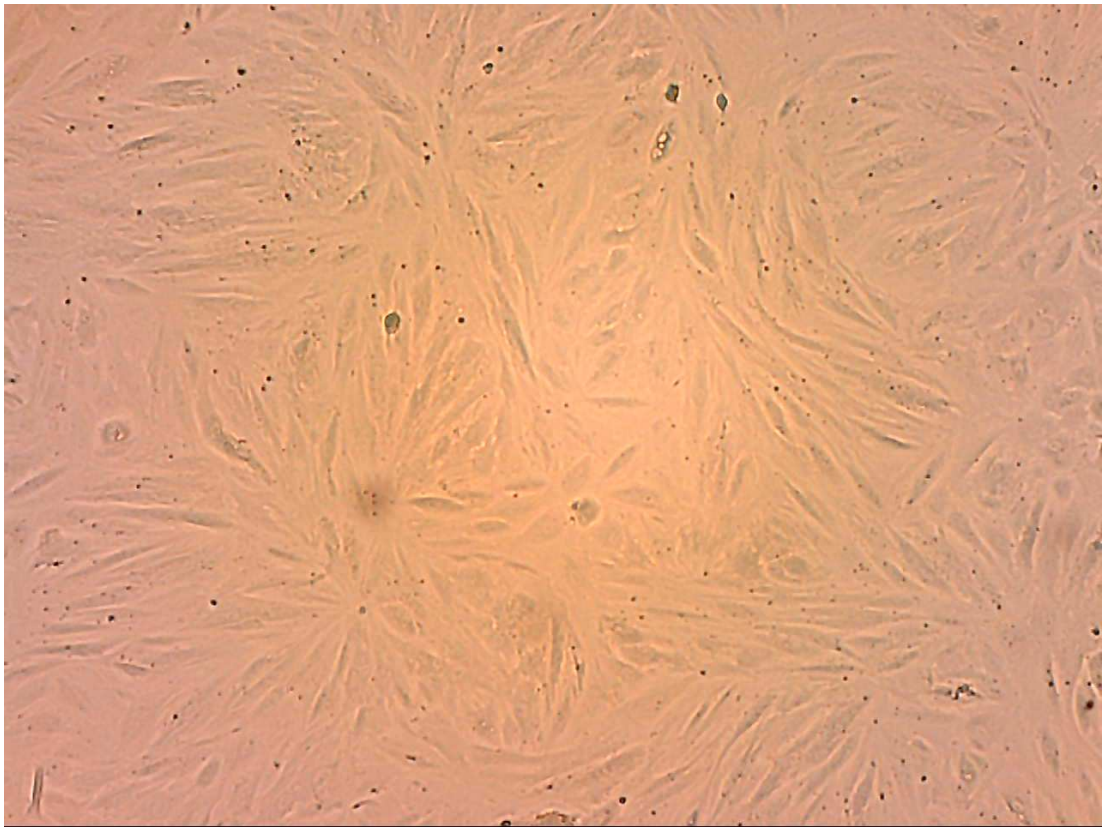


Figure 17: Confluent human endometrial stromal cells.

Briefly, the media was removed and the cells washed in pre-warmed PBS. Trypsin-EDTA was added to the flask and incubated for 5 minutes in standard conditions. The flask was then tapped to dislodge any attached cells (Figure 18). 10 ml of 10% DCC/ DMEM was added to stop the trypsin activity, and the sample was transferred to a 50 ml Falcon tube for centrifugation at 215xg for 6 minutes. The obtained cell pellet was re-suspended in 90% FBS and 10% dimethyl sulfoxide (DMSO). Pre-labelled cryogenic vials were filled with 1.8 ml of cell suspension and frozen using CoolCell® (BioCision) alcohol free cell freezing container by placing it in a freezer at -80° C for at least 4 hours. The cryogenic vials were subsequently transferred into liquid nitrogen for long-term storage. Frozen samples were transported on dry ice from the University of

Nottingham, Nottingham, to University of Warwick, Coventry, where subsequent sample analysis took place.

Cells recovered from liquid nitrogen were thawed rapidly at 37° C and transferred to 10 ml of 10%DCC/DMEM media for centrifugation at 215xg for 6 minutes in a 20 ml Falcon tube. The cell pellet was re-suspended in 10% FBS/DMEM media and transferred to a T-25 cm^2 culture flask. 4-6 hours later the media was refreshed. These cells were left to reach confluence (2-14 days) with media change every other day. Upon confluence, cells were trypsinized and pelleted as above. The re-suspended cells were counted using a haemocytometer and transferred to a 6-well plate for decidualisation.

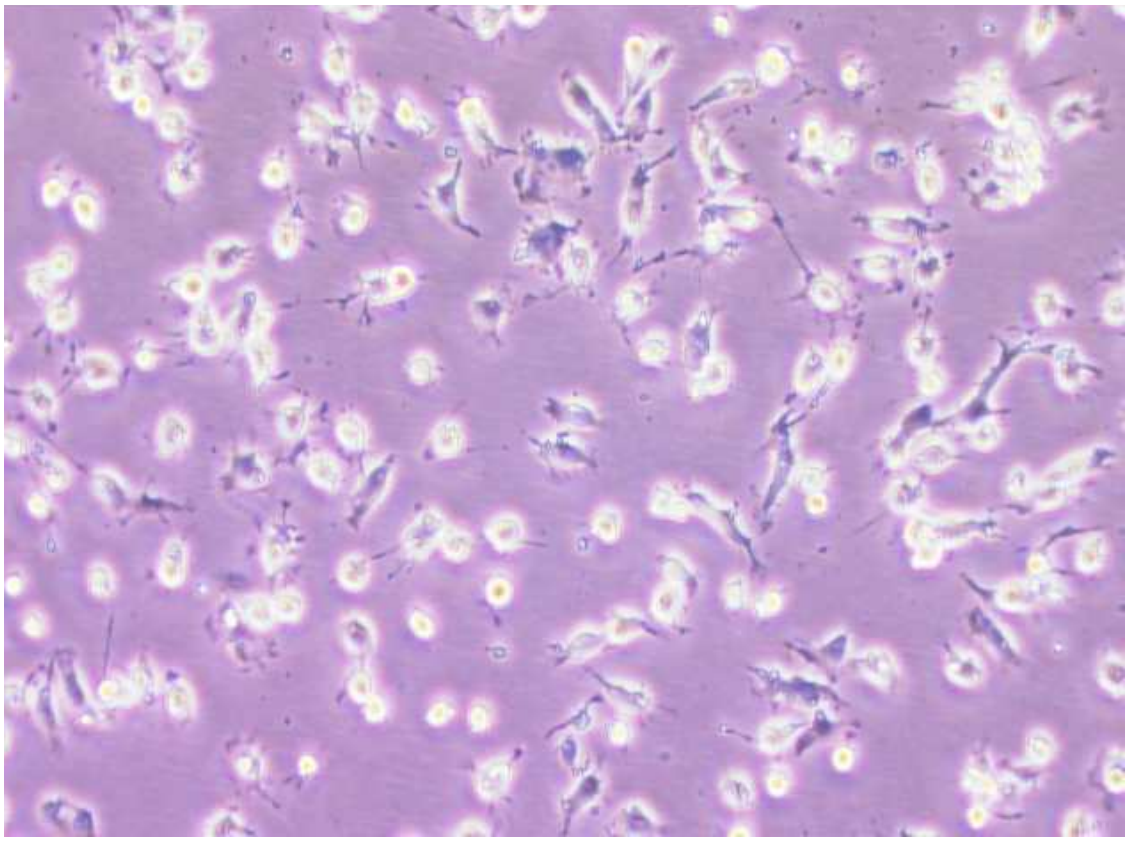


Figure 18: EDTA-Trypsin exposed human endometrial stromal cells.

2.4.3. **Hormone treatment of cells**

Upon reaching 90% confluence, the growth media was replaced with 2% DCC/DMEM media for 24 hrs. Control cells were left untreated in the presence of 1μM ethanol (vehicle). Treated cells were maintained in 2%

DCC/DMEM media containing a combination of 0.5 mM 8-Br-cAMP and 1μM medroxyprogesterone acetate (MPA). Treated and control cells were harvested 2 and 8 days after initiation of treatment (see Figure 19).

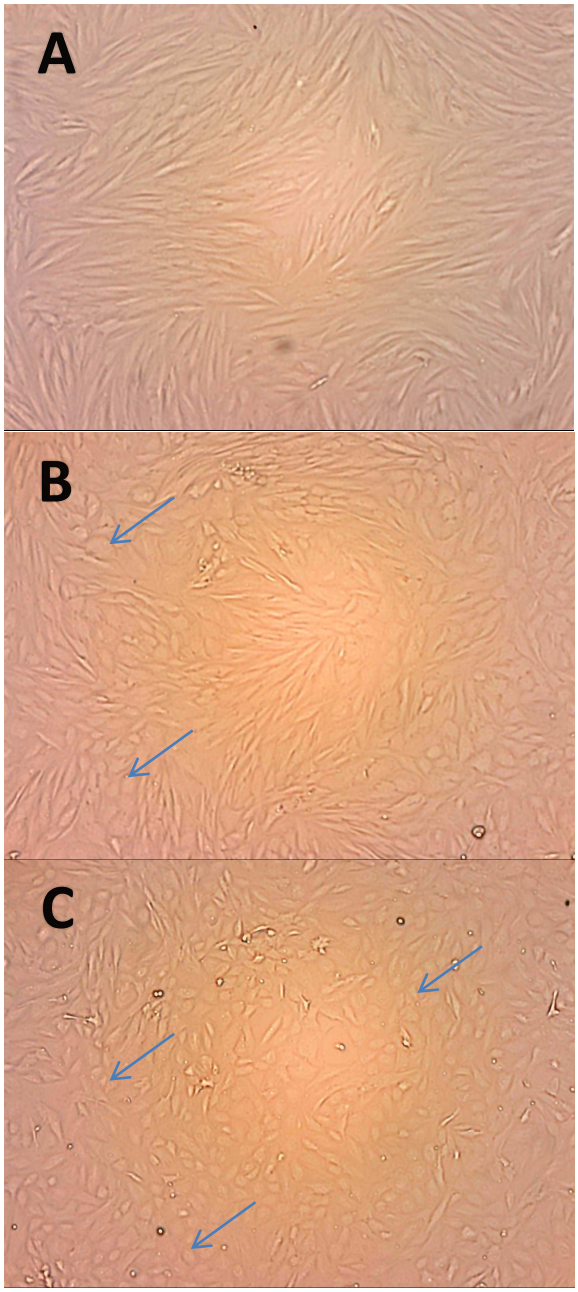


Figure 19. Human endometrial stromal cells.

Day 0 of decidualisation (control sample; image A), day 2 (image B) and day 8 (image C). Arrows indicate rounded secretory phenotype of ESCs. 10 x magnification.

2.4.4. **Gene Expression Analysis using Real Time Quantitative PCR (RTQ-PCR)**

Principle

RTQ-PCR is based on detection of fluorescence produced proportionally to the PCR product in real time. This method allows for a wide dynamic range of quantification, high sensitivity and precision (Klein 2002). The simplest and most economically sound approach uses SYBR green which is a dye binding all double stranded DNA (dsDNA) molecules. In its free, unbound form, it has an undetectable fluorescence. Laser light used in the detection equipment leads to excitation of fluorophores in the sample, and the resulting fluorescence signal is directed to a spectrograph. Due to its non-specific dsDNA binding characteristics, SYBR green can also detect non-specific PCR products such as primer-dimers generated during the PCR process or genomic DNA contamination from RNA purification, hence careful optimization of the assay is required.

In the 7500 Fast Real-Time PCR System (Software v.2.0.6; Applied Biosystems) used, the change in fluorescence intensity (ΔRn) related to amplification is calculated by the software. ΔRn is calculated using the following equation ΔRn=Rn+ - Rn-, where Rn+ is the product fluorescence at any given time, and Rn- is the baseline background fluorescence estimated during the first 3-10 PCR cycles. During the early cycles of PCR, ΔRn does not exceed the baseline values. As the amplification proceeds, the fluorescence intensity reaches a threshold, which is defined as a statistically significant point above the baseline, usually determined as 10 times the standard deviation of the baseline fluorescence. The threshold cycle (Ct) is calculated by determining the point at which the fluorescence crosses the threshold limit correlating to the initial amount of target gene quantity. Thus, Ct values decrease linearly with increasing initial target gene quantity (Heid et al. 1996).

Primer design and optimization

Primers were designed against reference sequences (NCBI; http://www.ncbi.nlm.nih.gov/genome/guide/human/) using the Roche Universal Probe Library assay design centre

(http://lifescience.roche.com/shop/CategoryDisplay?catalogId=10001&tab=Assay+Design+Center&identifier=Universal+Probe+Library&langId=-1), unless otherwise stated. Intron-spanning primers were used wherever possible in order to allow discrimination of RNA- and gDNA-derived amplicons. Primer sequences are given in Table 24.

Primers were validated using melting curve analysis and agarose gel confirmation of correct product size. Products were purified using the QIAquick Gel Extraction Kit (QIAGEN) and 8-log standard curves were prepared in order to determine primer efficiencies.

RNA extraction

In order to minimize the risk of RNA degradation, only RNase-free plastic ware was used. The bench used for RNA work was decontaminated using RNaseZAP (Ambion).

ESC cultures in 6-well plates were directly lysed using 200 µl of STAT-60 reagent per well according to the manufacturer's protocol, followed by the use of a cell scraper to ensure all cells were adequately exposed to the lysis solution. STAT-60 is a monophasic solution of phenol and guanidine isothiocynanate that maintains RNA integrity whilst simultaneously disrupting cells and cellular components. After 5-minute incubation at room temperature, samples were transferred to 1.5 ml RNase free tubes and 0.2 volumes of chloroform were added. The tube was shaken vigorously for 15 sec and stored at -80 °C. Tubes were centrifuged at 13,000 x g for 30 minutes at 4 °C. This separated the solution into a superficial aqueous phase containing RNA, DNA containing interface and lower red, phenol-chloroform protein containing compartment. The aqueous top layer was transferred carefully into new tubes and one volume of ice-cold isopropanol was added in order to precipitate the RNA. The sample was vortexed and stored at -80 °C for 30 minutes. Subsequently the samples were centrifuged at 13,000 x g for 30 min at 4 °C. The supernatant was discarded and the RNA pellet was washed with 500 µl of 70% ethanol and centrifuged at 13,000 x g for 15 min. This was repeated once more. Following the second spin, the supernatant was discarded, the purified RNA pellet was air dried and

dissolved in 20 µl of Tris-EDTA buffer pH 8.0. RNA concentration was determined using spectrophotometer at 260Å wavelength (ND-1000, NanoDrop). Pure RNA has an absorbance 260Å/280Å ratio of 1.8-2.1, with lower values suggesting protein and phenol contamination.

cDNA Synthesis

Reverse transcription was performed using the Quantitect Reverse Transcription Kit (QIAGEN) according to the manufacturer's instructions. A total of 500 ng RNA from each sample was used for reverse transcription, diluted in RNAse free water to give a total volume of 12µl RNA solution. In order to remove contaminating genomic DNA, which would be amplified during the PCR and would lead to false results, each sample was treated with 2µl of gDNA Wipeout Buffer for 2 min at 42°C. The reaction was stopped by placing the sample on ice.

A mastermix was prepared for reverse transcription, containing reverse transcriptase (RT), RT buffer and primer mix. A mastermix volume of 6 µl was added to each sample, mixed and incubated at 42°C for 30 min. RT activity was terminated by heating the samples at 95°C for 3 min. RNAse free water was added to give a final volume of 50 µl of cDNA solution. Samples were stored at -20°C.

Amplification of target cDNA

Power SYBR green Master Mix (Applied Biosystems) was used to prepare PCR reactions. All reactions were carried out in 96 well plates each containing 1µl of cDNA solution, 7.8µl of RNAse free water, 10 µl of Master Mix, and 1.2µl of primers. Negative controls without cDNA were included. All measurements were performed in triplicate using a 7500 Fast Real-Time PCR System (Applied Biosystems). The plate was heated to 95°C for 10 min in order to activate the DNA polymerase. Subsequently, 40 cycles of 95°C for 15 sec (denaturation), 60°C for 1 min (annealing and extension) were performed. The expression levels were calculated using the ΔCt method with efficiency correction, and expressed in arbitrary units. Expression of L19 - a non-regulated human ribosomal house-keeping gene was used to normalize for minor variances in input RNA amount or RT efficiencies. This approach is adequate for

most purposes, where changes in gene expression levels are investigated. The units are irrelevant in this case, and the relative quantities can be compared across multiple RT-PCR experiments (Orlando et al. 1998). Though multiple housekeeping genes are routinely required to normalize RNA input variances, only L-19 gene was analysed due to prior experience in the hosting laboratory and thorough validations of the genes' performance as a reference gene (Kuroda et al. 2013b).

2.4.5. **Paraffin block preparation and staining**

The fresh endometrial sample was transferred to 4% PFA and fixed for 24 hours, routinely processed and embedded in a paraffin block. The research team in the histopathology department in Queen's Medical Centre, Nottingham, performed this step. Subsequent processing of samples took place at the University of Warwick, Coventry. For NK cell analysis, three-micrometre thick sections were mounted on slides and stained with anti-CD56 monoclonal antibody (Novocastra, Leica Biosystems, Newcastle Upon Tyne, UK) using the Avidin-Biotin Complex (ABC) staining method according to the specifications provided by the antibody manufacturer. The sample was incubated for 60 minutes, developed with 3,3'-Diaminobenzidine (DAB) containing 0.01% H_2O_2 to produce brown reaction product. Mayer's haematoxylin was used to counterstain the sample. Following dehydration and cleaning, the sample was mounted in DPX synthetic resin (Raymond A. Lamb Ltd, London, UK).

2.4.6. **Natural killer cell analysis**

There is no universally agreed method of counting uNK cell levels. Historically, these levels have been expressed as mean numbers of uNK cells (Clifford et al. 1999), percentage of total stromal cells (Quenby et al. 1999, Tuckerman et al. 2004) and as percentage of CD45+ cells (Michimata et al. 2002). A close correlation between the total NK cell numbers and percentage of total cells has been confirmed by Tuckerman et al. (Tuckerman et al. 2007) hence these methods can be used interchangeably.

When analysing the uNK cells, manual cell counting using computer software has been described as the gold standard (Drury et al. 2011), against which other counting methods have been assessed. Computer aided analysis methods have been shown to be more reproducible and observer independent and are the preferred method of analysis in the hosting unit (Coventry).

In this thesis, the method of choice for NK cell counting was the computer aided method using 20x magnification fields and reported as percentage (%) of area stained. To assess the reproducibility and reliability of the method, ten sequential samples were anonymised and analysed twice in forward and reverse order by one observer (LTP) and once by two observers (LTP and AN).

Image acquisition and analysis

Images were acquired using Pannoramic SCAN (3DHISTECH Ltd, Hungary) with a 20x objective. Obtained images can be manipulated and magnified in the software up to x 200. Pannoramic Viewer™ software (v. 1.15.2, 3DHISTECH Ltd, Hungary) was used to randomly select a minimum of three, maximum of five images from each sample using 20x magnification, depending on the sample size. The following criteria were followed when selecting the region for analysis:

- Epithelial edge had to be included in the image.
- Care was taken to avoid blood vessel inclusion (if this was unavoidable, then these regions were subsequently excluded from analysis (see below).
- The least possible number of glands were included.

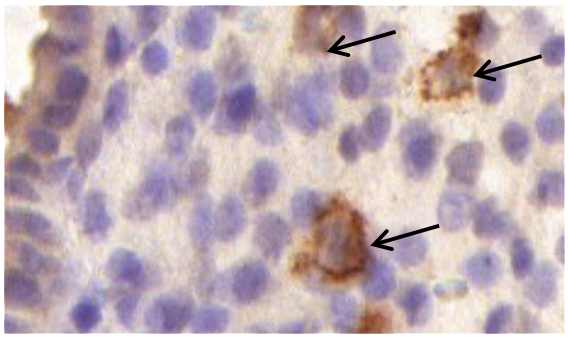


Figure 20: Subepithelial NK cells. H-DAB staining: 100x magnification. Arrows indicate NK cells.

Once selected, the screenshot was saved as .tiff format labelled with the study number and consecutive image numbers. Subsequently, each image was analysed using ImageJ software (v. 1.47, Wayne Rasband, NIH, USA). Using the freehand selection tool, the epithelium, glands and blood vessels were outlined along the basal membrane and subsequently cut out from the image (Figure 21).

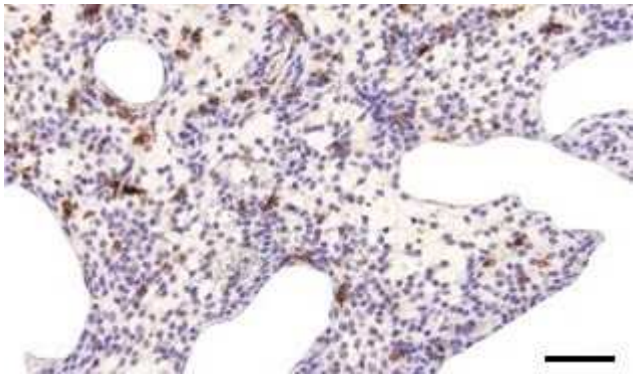


Figure 21: Endometrium with exclusion of glands and epithelial edge. 20 x magnification.

Scale 100 μm.

The Colour Deconvolution plugin was then applied to the image. This plugin produces images generated by colour subtraction implementing a method described by Ruifrok in 2001 (Ruifrok et al. 2001). This produces three images: Haematoxylin, DAB and a third complementary component indicating match between the two former vectors (Figure 22). The deconvolution tool allows measuring the area taken up by each of the separate dyes producing a percentage value. First, the histogram threshold for the DAB image was adjusted to minimize background noise.

With the threshold set arbitrarily at 100, the image was a good representation of the areas suggestive of NK cell presence and allows for standardisation of measurement. For the haematoxylin image, the threshold value was left as automatically determined by the software as this appropriately delineated the nuclei. Secondly, the automatic measurement of the surface area covered by dye was obtained from both images. These values taken together produced a percentage value of the area occupied by DAB stained cells (NK cells).

The results obtained from each image were averaged over the entire sample to produce the NK cell count expressed as percentage value (%) of area stained.

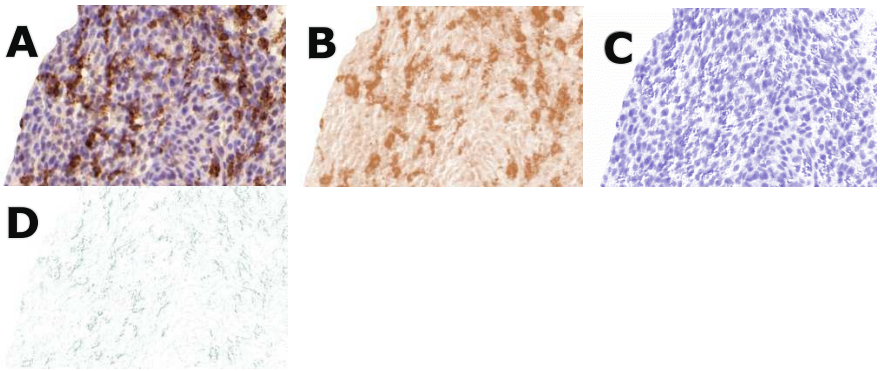


Figure 22: Haematoxylin and DAB separation. Original image (40 x magnification) with epithelial border removed (A), DAB (B), Haematoxylin (C), third component (D).

Duration of analysis

The mean time taken for analysis was 116±53 seconds.

Intraobserver analysis

The intraobserver reliability was high with an ICC >0.93 (95% CI 0.771-1.0; p<0.001) for all samples.

Interobserver analysis of images acquired by a single observer

The interobserver reliability was high for all samples analysed, with an ICC of 0.99 (95% CI 0.96- 1.0; p<0.001).

2.4.7. **Blood sample analysis**

Blood collection followed a standard phlebotomy protocol. The blood tube was centrifuged (20 minutes, 4000rpm) and the supernatant serum was used for analysis.

Oestradiol and progesterone levels were measured using the Architect System from Abbott Diagnostics®. The assay used a delayed one step immunoassay to determine the presence of oestradiol and progesterone in serum by Chemiluminescan Microparticle Immunoassay technology. In the first step, the sample is combined with specimen diluents and assay diluents, and coated paramagnetic microparticles (rabbit, monoclonal for oestradiol and mouse, monoclonal for progesterone). Oestradiol and progesterone present in the sample will bind to the anti-oestradiol and anti-progesterone coated microparticles. After a period of incubation oestradiol and progesterone acridinium labelled conjugate is added. After the second incubation a Pre-trigger and Trigger solution are added. The resulting chemiluminescent reaction is measured as relative light units (RLUs). An inverse relationship exists between the amount of oestradiol and progesterone present in the sample and the RLUs detected by the optical system.

For oestradiol, the functional sensitivity in the product information of the assay (REF7K72) was reported as =<25pg/mL, this is the lowest concentration to be measured with a coefficient of variation of less than or equal to 20%. In our individual laboratory this was found to be <37pg/mL. The progesterone assay sensitivity is reported to be ≤ 0.1ng/mL and a coefficient of variation of ≤10%. Quality control runs were done on a daily basis for all assays and comparative testing was performed for the machine on a quarterly basis against another lab.

2.5. Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (version 21; SPSS, Chicago, IL, USA). Distribution of data was assessed using Kolmogorov-Smirnov test. For parametric un-paired data student's t test was used and for non-parametric data, Mann-

Whitney U test was carried out. For paired samples, student's t test was used for parametric data and Wilcox signed rank test was used for non-parametric data. A P value of <0.05 was considered statistically significant.

Changes over time were analysed using repeated-measures ANOVA, reported as *F*-ratio with corrected degrees of freedom if data sphericity was violated. Data sphericity was assessed using Mauchly's test, with $P<0.05$ signifying lack of sphericity. Greenhouse-Geisser correction (ε) was automatically applied for data with minimal violation of sphericity (ε approaching 1). When significant violation of sphericity was confirmed, MANOVA was performed and reported as *F*-ratio with degrees of freedom.

Linear regression analysis was used to assess the effect of different variables for prediction of the outcome of assisted reproduction treatment. The effect of the same variable on treatment outcome was assessed using binary logistic regression analysis. Receiver operating characteristic (ROC) curve analysis was performed to quantify the ability of any significant factors to discriminate between pregnant and non-pregnant study participants.

Inter and intra- observer reliability was assessed by two-way mixed Intraclass Correlation Coefficients (ICCs) with absolute agreement and their 95% confidence intervals (95%CI). Coefficient of determination (R^2) was used to assess the performance of two analysis methods. Limits of agreement were used to demonstrate intra and inter observer reliability and compare the different methods (Bland et al. 1995). ICC is a measure of reliability which evaluates the proportion of variance between observations (McGraw et al. 1996). ICC values under 0.20 were classified as poor, 0.21- 0.40 fair, 0.41-0.60 moderate, 0.61-0.80 good, and 0.81-1.00 very good according to classification of the reliability values as described by Altman et al. (Altman et al. 1983, Bland et al. 1999). Limits of agreement are estimated from the mean and standard deviation of the differences with 95% of the differences laying within two standard deviations either side of mean. These limits, like confidence intervals, give an idea of the spread of variance between the methods.

CHAPTER 3. The Definition of Recurrent Implantation Failure

3.1. Introduction

Human reproduction is a relatively ineffective process with only 20-25% of apparently fertile couples becoming pregnant during a single menstrual cycle assuming tubal patency, ovulation and viable sperm (Short 1979, Stevens 1997). Based on this generally accepted level of fecundity, the likelihood of achieving pregnancy has been estimated at 74%, 93% and 100% after 6, 12 and 24 months of unprotected, regular intercourse respectively (Evers 2002a, Teklenburg et al. 2010a).

Events leading to a successful pregnancy commence with fertilization of the oocyte. Research suggests that the early embryo undergoes the same stages of implantation as an activated, migrating leukocyte when transgressing vascular endothelium namely rolling, apposition, adhesion, and invasion (Genbacev et al. 2003). Taking this model into consideration, implantation should be considered only until the invasion of the embryo is complete and the latter is formally embedded within the endometrium. This phase will be heralded by an increase in serum hCG levels. *In vitro* studies confirm secretion of the beta subunit of hCG by the developing embryo seven days after fertilization (Marshall JR 1968, Dokras et al. 1991, Woodward 1993). It is unclear, however, at what time point implantation can be formally confirmed and, by default therefore, when implantation failure can be diagnosed. This is obviously a contentious issue but an important one at least as far as semantics are concerned. The ability to unequivocally state when implantation occurred, would allow differentiating between the failure of implantation and miscarriage. These clinically distinct entities are often confused and in combination referred to as a collective term 'recurrent reproductive failure' suggesting they are different manifestations of the same underlying problem (Farquharson et al. 2005). This is not a universal view, however, and yet many couples with recurrent implantation failure are investigated and managed in a similar, if not identical way to those with recurrent miscarriage (Christiansen et al. 2006).

The pressures of society, modern lifestyles and changes within our environment all combine to make it more difficult for couples to start a

family and subfertility appears to becoming more common with a current, estimated prevalence of 10-15% (Evers 2002a, Shreeve et al. 2012). Since the introduction of *in vitro* fertilization in the 1980's, some couples, that may have otherwise remained childless, have had an opportunity to become parents. ARTs are, however, no more efficient, and possibly less effective, than normal human reproduction with implantation rates consistently in the region of 20-30% (Voullaire et al. 2002b). Life table analyses of cumulative pregnancy rates following multiple cycles of IVF have shown that ART is not the panacea it was once proposed to be and that a proportion of couples remain childless despite multiple cycles of treatment. Cumulative pregnancy rates after ART have been estimated to range from 40% to 55% after 3 IVF cycles (Guzick et al. 1986, Simon et al. 1993, Roest et al. 1998) to 51% to 75% after six consecutive cycles (Guzick et al. 1986, Simon et al. 1993, Alsalili et al. 1995a, Dor et al. 1996). Cumulative pregnancy rates do not significantly increase thereafter and pregnancy rates per cycle tend to fall after the third unsuccessful treatment (Croucher et al. 1998, Osmanagaoglu et al. 1999, Sharma et al. 2002). Mathematical modelling has also demonstrated a steady decrease in live birth following a failed IVF cycle. A 50% reduction in live birth was noted after the 10th failed cycle for double embryo transfers and lower rates were associated with single embryo transfer (Roberts et al. 2010, Roberts et al. 2011, Roberts et al. 2012). Failure to conceive after repeated attempts of ART is often referred to as RIF. The exact definition of this entity remains unclear however and current descriptions incorporate the number of previously failed cycles and whether these were fresh or frozen, the number of embryos transferred and / or their respective quality, or a combination of these factors.

When devising this review, the aim was to present an up to date overview of the definitions of recurrent implantation failure currently being used in the scientific literature. Based on the evidence gathered and expert opinion, we have attempted to clarify the terminology used and standardize the inclusion criteria in future studies of RIF to allow a more appropriate comparison between patient populations.

3.2. Materials and Methods

3.2.1. **Eligibility criteria**

Inclusion criteria were any type of study where the definition of recurrent implantation failure in human subjects was used. Case reports and conference abstracts, if deemed appropriate, were included. Review articles and letters to the editor were excluded. There was no limitation on language, publication date, or publication status. In cases where suitability for inclusion could not be ascertained after analysis of the abstract, the complete article was obtained. Reference lists from included articles were manually screened for articles that that could have been missed during the initial search.

3.2.2. **Information sources and search**

The following databases were searched electronically by in a systematic manner by two researchres (Lukasz Polanski- LP and Miriam Baumgarten- MB): Cochrane Central Register of Controlled Trials (CENTRAL), Medical Literature Analysis and Retrieval System Online (MEDLINE) and Embase, spanning years from 1946 to June 2013. In addition, a hand search of the reference list of the included articles and similar reviews in order to find additional data of interest for the review was conducted.

Titles and abstracts were reviewed checking for duplicates and using the *a priori* criteria for inclusion. Full-text manuscripts of studies considered potentially eligible for inclusion were obtained from on-line sources, from the library or via interlibrary loans. There were no limitations on language, publication date or publication status.

3.2.3. **Search terms**

The following search terms were used, adjusting for each database as necessary:

Recurrent implantation failure*, recurrent failure to implant, repeat failure to implant, implantation failure*, repeat* implantation failure, recurrent failed implantation, and repeat failed implantation*, RIF,

recurrent reproductive failure*, repeat reproductive failure, poor implantation, Artificial Reproductive Treatment*, ART, in vitro fertilization, IVF, intra-cytoplasmic sperm injection, ICSI. MESH terms were expanded appropriately to gain the maximum number of options.

3.2.4. **Study selection**

Two independent reviewers (LP and MB) screened the retrieved titles and abstracts selecting and excluding those that clearly did not meet the eligibility criteria; disagreements between reviewers were resolved by consensus or a third party (Nick Raine-Fenning- NRF). One author (LP) obtained full articles of all potentially relevant studies, which were examined for eligibility independently by two reviewers (LP and MB). Disagreements between the reviewers were again resolved by consensus, or when not possible, by consulting a third author (NRF).

Duplicates were removed and only studies with human subjects were included to undergo further analysis.

3.2.5. **Data collection process**

One review author (LP) extracted the data from included studies using a data extraction form designed and pilot-tested by the authors. One author (MB) independently checked the extracted data. If there were data queries the corresponding author of the study was contacted. Disagreements were resolved by consensus. The names of article authors and titles of the included studies were juxtaposed to identify duplicate publication; in case of duplicates both articles were considered as a unique study. Analysis of the abstracts and full text articles was subject to the use of the term 'Recurrent Implantation Failure' or 'RIF' within the article. Full text articles were analyzed in detail by two assessors (MB and LP). Definition of recurrent implantation failure along with supporting information was extracted from the article and recorded for further analysis. No meta-analysis was performed and only descriptive statistics were applied.

3.2.6. **Risk of bias in individual studies**

The studies were not assessed for bias and heterogeneity as meta-analysis was not performed due to the nature of this review.

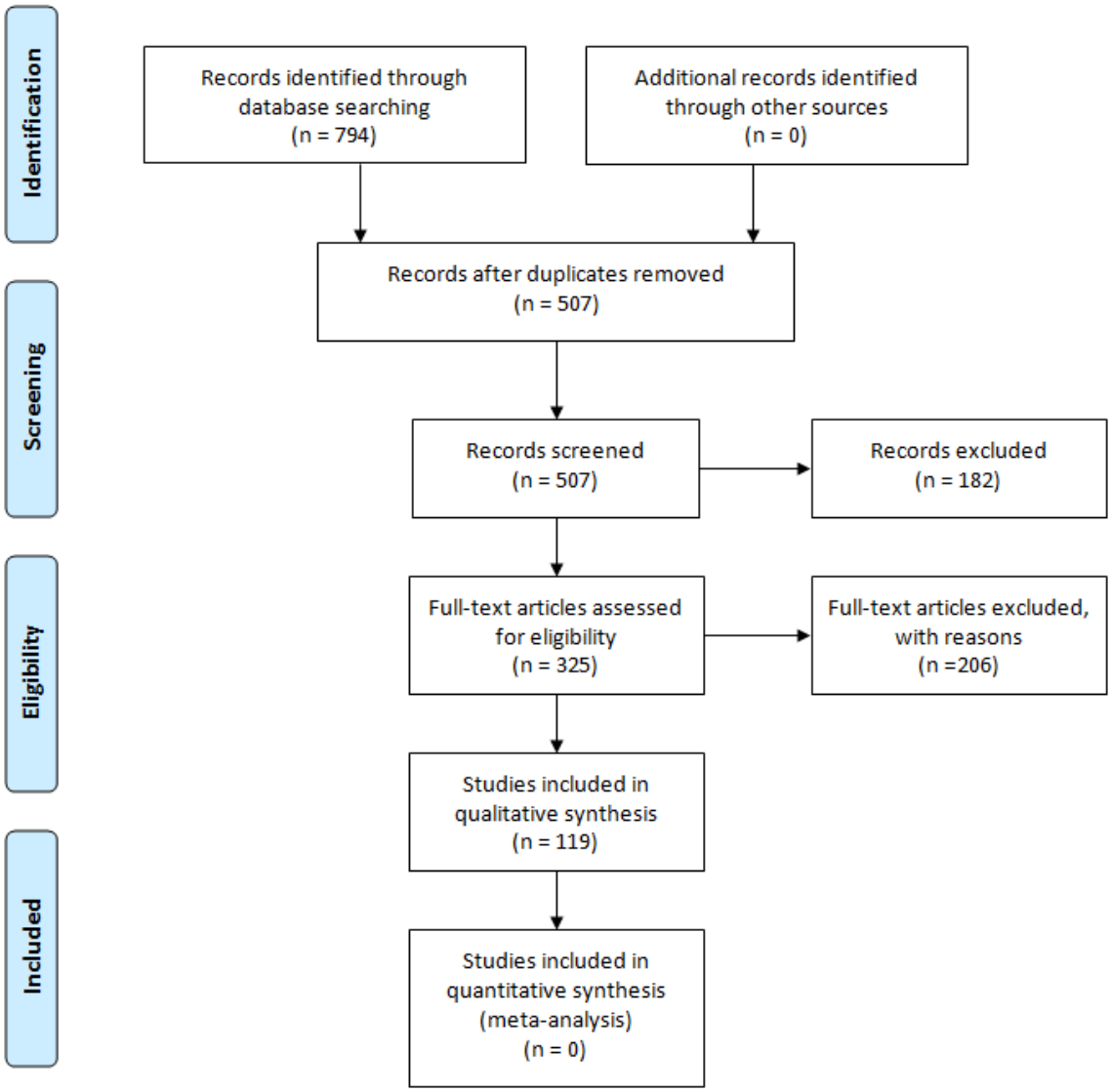


Diagram adapted from: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(6): e1000097. doi:10.1371/journal.pmed1000097

Figure 23. The selection process flow-chart for selection of suitable studies.

3.3. Results

The electronic database search identified 794 potential papers. No further papers were identified by manual searches of reference lists. Removal of duplicates left 507 articles. Following review of titles and abstracts, 325 papers were selected for review of the full manuscript. 206 of these studies were subsequently excluded (Figure 23). 119 studies met the inclusion criteria and were included in the systematic review. In three articles, neither 'RIF' nor 'recurrent implantation failure' were used by the authors to define the populations studied but these included patients with more than three failed ART cycles (Raziel et al. 2002, Pehlivan et al. 2003, Raziel et al. 2007). None of the studies provided a sound rationale for the number of cycles, the number of transferred embryos, the use of fresh or frozen embryos or embryo quality, or a combination of these factors as the basis for defining RIF (see Table 2).

Definition of recurrent implantation failure		**Number of studies**
Three or more unsuccessful or failed cycles		37/119
Two or more unsuccessful or failed cycles		25/119
Number of embryos transferred	Cumulative transfer of 8 or more cleavage stage embryos or four blastocysts over several cycles of ART	7/119
	Ten or more embryos	5/119
	Five or more good quality embryos	2/119
	Other numbers of embryos replaced	3/119
Number of failed treatment cycles in combination with number of embryos transferred		39/119

Table 2. Number of studies using various definitions of recurrent implantation failure.

ART- assisted reproductive treatment

3.3.1. **Number of unsuccessful cycles alone**

Three different definitions based on the number of unsuccessful cycles were used. Three authors used consecutive treatment cycles in the definition (Rufas-Sapir et al. 2004, van den Heuvel et al. 2007, Kling et al. 2008a). The most commonly used definition of RIF was 'three or more unsuccessful or failed cycles', which was stated in 37 of the 119 (31.6%) articles. The type of assisted reproductive treatment used was described in 31 of these 37 studies. In 22 articles the definition only included unsuccessful IVF cycles (Levran et al. 2002, Ledee-Bataille et al. 2004a, Ledee-Bataille et al. 2004b, Pantos et al. 2004, Rufas-Sapir et al. 2004, Ledee-Bataille et al. 2005, Qublan et al. 2006, Mantzouratou et al. 2007, Matsubayashi et al. 2007, van den Heuvel et al. 2007, Weissman et al. 2007, Kling et al. 2008b, Kling et al. 2008a, Pagidas et al. 2008, Prakash et al. 2008, Brosens et al. 2009b, Debrock et al. 2009, Jee et al. 2009, Achache et al. 2010b, Achache et al. 2010a, Choi et al. 2011, Guan-Gui et al. 2011), IVF or ICSI in five (Loutradis et al. 2004, Ghobara et al. 2006, Platteau et al. 2006, Kim et al. 2011, Sacks et al. 2012), embryo transfer cycles in three (De Placido et al. 1991, Germeyer et al. 2010, Tiboni et al. 2011), one of which included gamete intra-fallopian transfer (GIFT) and/ or trans-cervical embryo replacements (De Placido et al. 1991) and ICSI cycles only in one case (Rubio et al. 2001). Eight studies commented on the embryos transferred as being 'fresh and/or frozen' (Levran et al. 2002, Weissman et al. 2007, Kling et al. 2008b, Prakash et al. 2008, Yakin et al. 2008, Debrock et al. 2009, Achache et al. 2010b, Sacks et al. 2012) and the embryos were described as being of good quality in eight studies (Ledee-Bataille et al. 2004a, Loutradis et al. 2004, Ledee-Bataille et al. 2005, Prakash et al. 2008, Brosens et al. 2009b, Achache et al. 2010a, Germeyer et al. 2010, Kim et al. 2011). The day of embryo transfer was not provided in any of the studies included.

The second most common definition used was 'two or more failed treatment cycles' which was used in 25 studies. In this group, the treatment cycle involved the use of IVF in eleven cases (Gianaroli et al. 1997, Matsubayashi et al. 2001a, Spandorfer et al. 2002b, Martinuzzo et al. 2005, Fukui et al. 2006, Vaquero et al. 2006, Kalu et al. 2008, Chou

et al. 2011, Huang et al. 2011, Ivanov 2012, Sermondade et al. 2012), ICSI in two cases (Berker et al. 2011, Oliveira et al. 2011), IVF or ICSI in two cases (Eyheremendy et al. 2010, Lodigiani et al. 2011) and was not clarified in 10 studies (Kahraman et al. 2000, Stephenson et al. 2000, Petersen et al. 2005, Foulk et al. 2007, Hiraoka et al. 2008, Valojerdi et al. 2008, Urman et al. 2009, Schoolcraft et al. 2010, Tsoumpou et al. 2010, Takahashi et al. 2011). Six of the authors identified the transferred embryos as being 'fresh and or frozen' (Stephenson et al. 2000, Petersen et al. 2005, Hiraoka et al. 2008, Urman et al. 2009, Eyheremendy et al. 2010, Takahashi et al. 2011). Stage of embryos transferred was mentioned in one study as 'cleavage stage' but precise data were not given (Hiraoka et al. 2008).

One study defined recurrent implantation failure as unsuccessful treatment after 'three or more fresh cycles' or 'two or more fresh cycles and two or more frozen embryo replacement cycles' (Tuckerman et al. 2010).

No other definitions were given.

3.3.2. **Number of embryos transferred**

The number of embryos transferred was used to define recurrent implantation failure in 16 studies. All of these studies considered the cumulative number of embryos transferred. The quality of the embryos replaced was also only used in three studies (Hapangama et al. 2008, Quenby et al. 2009, Tremellen et al. 2011).

The most common definition included the 'cumulative transfer of eight cleavage stage embryos or four blastocysts over several cycles of ART' (two to six) (Karimzadeh et al. 2009), which was used in seven studies (Coulam et al. 2002, Coulam et al. 2006, Coulam et al. 2008, Goodman et al. 2008, Firouzabadi et al. 2009, Goodman et al. 2009, Sauer et al. 2010). The next most popular definitions were the cumulative transfer of 'ten or more embryos', which was used by five authors (Inagaki et al. 2003, Wilton et al. 2003, Vialard et al. 2007, Voullaire et al. 2007, Vialard et al. 2008), and 'five or more good quality embryos' which was

given as the definition in two studies (Hapangama et al. 2008, Quenby et al. 2009).

Three other definitions were used including the cumulative transfer of 'three or more good quality embryos' (Tremellen et al. 2011), 'four to six cleavage stage embryos' (Firouzabadi et al. 2009), and 'ten or more cleavage stage embryos or five blastocysts' (Russell et al. 2011).

3.3.3. **Number of unsuccessful cycles in combination with number of transferred embryos**

The combination of the number of failed treatment cycles and the number of embryos transferred without achieving pregnancy was used to define recurrent implantation failure in 40 articles. The most common number of unsuccessful cycles considered was three or more and then two or more. Several other definitions were also given. The characteristics of these studies are contained in the Appendix in Table 1, Table 2 and Table 3.

Unsuccessful treatment after 'three or more treatment cycles' in combination with the number of embryos transferred was used by 18 authors (Carp et al. 1994, Stein et al. 1995, Creus et al. 1998, Pehlivan et al. 2003, Primi et al. 2004, Taranissi et al. 2005, Matteo et al. 2007, Quenby et al. 2007, Blockeel et al. 2008, Thum et al. 2008, Koler et al. 2009, Simur et al. 2009, Chernyshov et al. 2010, Fragouli et al. 2010, Maritnez-Zamora et al. 2011, Rajaei et al. 2011, Sudoma et al. 2011, Scarpellini et al. 2012). IVF was the treatment modality in 13 of these studies (Carp et al. 1994, Stein et al. 1995, Creus et al. 1998, Pehlivan et al. 2003, Taranissi et al. 2005, Matteo et al. 2007, Blockeel et al. 2008, Thum et al. 2008, Koler et al. 2009, Simur et al. 2009, Chernyshov et al. 2010, Fragouli et al. 2010, Scarpellini et al. 2012), IVF or ICSI in two cases (Quenby et al. 2007, Rajaei et al. 2011) or not stated in three cases (Primi et al. 2004, Maritnez-Zamora et al. 2011, Sudoma et al. 2011). The term 'fresh embryos' in the definition was used once (Matteo et al. 2007). The most commonly stated numbers of embryos transferred during three or more failed cycles were 'two or more embryos' (Creus et al. 1998, Primi et al. 2004, Matteo et al. 2007, Thum et al. 2008,

Chernyshov et al. 2010, Sudoma et al. 2011) and the 'cumulative transfer of ten or more embryos' (Pehlivan et al. 2003, Taranissi et al. 2005, Blockeel et al. 2008, Koler et al. 2009, Fragouli et al. 2010, Rajaei et al. 2011) both of which were used in six studies. The next most common definition in this category included the transfer of 'three or more embryos', which was used in three studies (Carp et al. 1994, Stein et al. 1995, Simur et al. 2009). Other definitions included the transfer of 'one or more embryos' during three cycles (Maritnez-Zamora et al. 2011), transfer of 'seven or more embryos' (Scarpellini et al. 2012) and the 'cumulative transfer of nine embryos' (Quenby et al. 2007) which were used once each. The quality of the embryos used was not stated in four articles (Carp et al. 1994, Creus et al. 1998, Taranissi et al. 2005, Rajaei et al. 2011), whereas the remaining authors described the embryos as being of 'good quality' but exact grades were not stated.

Unsuccessful treatment after 'two or more treatment cycles' in combination with the number of embryos transferred was used by 9 authors (Kwak-Kim et al. 2003, Kahraman et al. 2004, Koscinski et al. 2006, Arefi et al. 2008, Fukui et al. 2008, Aletebi 2010, Johnston-MacAnanny et al. 2010, Sharif et al. 2010, Yang et al. 2010). These studies included only IVF cycles in five cases (Kwak-Kim et al. 2003, Koscinski et al. 2006, Fukui et al. 2008, Johnston-MacAnanny et al. 2010, Yang et al. 2010), IVF or ICSI in two studies (Arefi et al. 2008, Sharif et al. 2010) and was not stated in the other two studies (Kahraman et al. 2004, Aletebi 2010). The number of embryos transferred included 'one or more' (Johnston-MacAnanny et al. 2010), 'two or more' (Kwak-Kim et al. 2003, Koscinski et al. 2006, Yang et al. 2010), 'or three or more' (Kahraman et al. 2004, Arefi et al. 2008, Aletebi 2010). The cumulative transfer of 'four or more' (Fukui et al. 2008) and 'six or more' were also used (Sharif et al. 2010). The quality of embryos was not stated in three studies (Kwak-Kim et al. 2003, Fukui et al. 2008, Yang et al. 2010).

Unsuccessful treatment after the transfer of 'at least two embryos' in 'four or more treatment cycles' (Farhi et al. 2000, Castelo-Branco et al. 2004, Friedler et al. 2007, Raziel et al. 2007, Kremenska et al. 2010) or 'five or more IVF or ICSI cycles' were also used to define recurrent

implantation failure (Huang et al. 1999, Varla-Leftherioti et al. 2007). Other definitions included the cumulative transfer of 10 or more embryos in 'six or more', 'seven or more', 'several', and 'multiple' ART cycles (Raziel et al. 2002, Voullaire et al. 2002b, Elram et al. 2005, Ledee et al. 2008, Dos Santos et al. 2012). 'Two to six' failed IVF cycles without clinical pregnancy during which 'ten or more' embryos were transferred was used by one group (Karimzadeh et al. 2009) whilst another defined RIF as unsuccessful treatment after 'two or more or three or more IVF or ICSI cycles where two or more or one or more embryos were transferred each time, respectively' (Brinsden et al. 2009).

3.3.4. **Additional factors included in the definition**

Several other factors were mentioned as inclusion or exclusion criteria for defining recurrent implantation failure. Good ovarian reserve, defined as an 'FSH less than 8 IU/L' was used as an inclusion criterion, whereas poor response to previous treatment, defined as less than four follicles on day of hCG or less than four oocytes retrieved in previous cycles, were used by eight authors as exclusion criteria (Ledee-Bataille et al. 2004a, Ledee-Bataille et al. 2004b, Matteo et al. 2007, Jee et al. 2009, Karimzadeh et al. 2009, Achache et al. 2010a, Oliveira et al. 2011, Dos Santos et al. 2012). The consecutivity of previous failed treatment cycles was only mentioned in three studies (Rufas-Sapir et al. 2004, van den Heuvel et al. 2007, Kling et al. 2008a). The day of embryo transfer and, by default the stage of embryo development, were stated by 8 authors in their inclusion criteria (Coulam et al. 2002, Coulam et al. 2006, Coulam et al. 2008, Goodman et al. 2008, Hiraoka et al. 2008, Firouzabadi et al. 2009, Goodman et al. 2009, Sauer et al. 2010).

The outcomes of treatment, which defined each unsuccessful cycle, were stated in 39 of the all reviewed studies. The most frequently stated outcome was 'failure to achieve a pregnancy' which was used in twenty one studies (Carp et al. 1994, Stein et al. 1995, Creus et al. 1998, Raziel et al. 2002, Voullaire et al. 2002a, Wilton et al. 2003, Taranissi et al. 2005, Friedler et al. 2007, Quenby et al. 2007, Raziel et al. 2007, van den Heuvel et al. 2007, Kalu et al. 2008, Ledee et al. 2008, Vialard et al.

2008, Yakin et al. 2008, Quenby et al. 2009, Achache et al. 2010b, Sharif et al. 2010, Tsoumpou et al. 2010, Tremellen et al. 2011, Dos Santos et al. 2012). A negative serum beta-hCG two weeks following embryo transfer was used to define treatment outcome by eight authors (Coulam et al. 2006, Goodman et al. 2008, Debrock et al. 2009, Firouzabadi et al. 2009, Goodman et al. 2009, Urman et al. 2009, Sauer et al. 2010, Sacks et al. 2012). Other treatment outcomes included 'conception' (Fragouli et al. 2010, Chou et al. 2011, Huang et al. 2011, Takahashi et al. 2011), 'clinical pregnancy' (Huang et al. 1999, Johnston-MacAnanny et al. 2010), 'implantation' (Vialard et al. 2007, Lodigiani et al. 2011, Sermondade et al. 2012), 'positive serum hCG and subsequent miscarriage' (Germeyer et al. 2010), 'negative urinary pregnancy test or chemical pregnancy loss' (Coulam et al. 2008), 'failure to maintain a pregnancy' (Varla-Leftherioti et al. 2007) and 'failure to implant, biochemical pregnancy failure or spontaneous abortion of less than 8 weeks gestation' (Stephenson et al. 2000).

3.4. Discussion

This systematic review has highlighted the enormous variability in the definition of recurrent implantation failure. Three generic definitions appear to be in current use and these are based on the number of unsuccessful treatment cycles, the number of embryos transferred, or a combination of both factors. Very few definitions consider all the aspects of the IVF treatment process and even the most comprehensive definitions lack pertinent embryological data or meaningful outcome definitions (Matteo et al. 2007, Sharif et al. 2010, Maritnez-Zamora et al. 2011). All definitions used remain hypothetical and no justification was given for the use of any selected definition of recurrent implantation failure.

As a descriptive systematic review, the results of this study are solely for the purpose of discussion. As with every definition, it needs to evolve with time and should do so in the light of new developments. A significant limitation of this review is the time period over which the articles have been sourced which spans more than two decades. Within that time frame, we have observed a shift from cleavage stage embryo to blastocyst stage embryo replacements, double to single embryo transfers- all factors contributing to an overall increase in ART success rates. This is reflected, at least partially, in the evolution of the definitions of RIF, which is primarily evident in the decreasing number of embryos transferred, which fell from three or more in the early 1990s (Carp et al. 1994, Stein et al. 1995) to the elective transfer of one embryo in 2011 (Maritnez-Zamora et al. 2011). Different modalities of ART- IVF and ICSI result in comparable delivery rates ranging from 21.9% in Europe to 33.3% in U.S.A for IVF and 19.9% in Europe and 32.5% in U.S.A. for ICSI (de Mouzon et al. 2012, CDC November 2009). Hence differentiation between these two treatment modalities when defining RIF is not required.

A single embryo transfer (SET) policy has been introduced in many countries to decrease multiple pregnancy rates and their associated health risks. In 2007, 2.5% of embryo transfers in Europe included four

or more embryos whilst 21.4% were single embryo transfers (SET), with Sweden, Finland and Belgium having the highest proportion of SETs (de Mouzon et al. 2012). The trend towards single embryo replacement, suggests that the number of embryos transferred without achieving pregnancy should be closer to a cumulative of two or three, after which RIF should be diagnosed. Single blastocyst transfer has been associated with similar live-birth rates as the transfer of two cleavage stage embryos (Gardner et al. 1998, Gardner et al. 2004, Blake et al. 2007, Csokmay et al. 2011, Zander-Fox et al. 2011), thus equating the developmental potential of a single blastocyst to that of two cleavage stage embryos- a factor which also should be considered when defining RIF.

Irrespective of the stage of embryo development and numbers of embryos transferred, their quality is another relevant predictor of outcome. The rate of cleavage, as well as the degree of fragmentation, have also been associated with the chance of pregnancy in the reference ART cycle. Day two embryos at the 4-cell stage with less than 21% fragmentation have been shown to exhibit a higher developmental potential than chronologically comparable embryos demonstrating alternate growth patterns and characteristics (Fauque et al. 2007, Pelinck et al. 2010). This was highlighted in two of the studies when patients were considered for RIF investigations following a transfer of 10 or more fresh or frozen embryos, each with fragmentation rate below 20% and at the 4-cell stage on day two (Ledee et al. 2008, Dos Santos et al. 2012). The same relationship between morphology and success of treatment exists when considering blastocyst quality (Balaban et al. 2000).

Several other potentially contributory factors have been omitted from the definitions of RIF in the literature analysed, most notably, culture conditions and the embryo transfer itself (Das et al. 2012). The former could be rectified by rigorous monitoring of the embryo culture conditions (Gardner et al. 2005, Das et al. 2012) but these are still likely to vary from unit to unit which itself is associated with different success rates. The same is true for embryo transfer which is operator dependent and influenced by certain patient characteristics. Difficult embryo transfer most often encompasses at least one of the following steps: resistance of

advancement of embryo transfer catheter, necessity for cervical dilatation, cervical traction and blood on the outer sheath of the transfer catheter (Tur-Kaspa et al. 1998, Thomas et al. 2002a). Any of these steps have been associated with a 30% relative decrease in clinical pregnancy rates (Phillips et al. 2013). Ultrasound-guided embryo transfer and appropriate unit experience are undoubtedly important (Brown et al. 2010, Das et al. 2012, Penzias 2012) but hard if not impossible to control for.

As frozen embryos are the product of stimulated oocyte retrieval cycle, their replacement does contribute to the cumulative pregnancy rate (de Mouzon et al. 2012) and should be pooled together when defining RIF. Cumulative pregnancy rates remain relatively stable until the third ART cycle with the highest pregnancy rates being achieved in the first two cycles (Shapiro et al. 2001, Schroder et al. 2004, Silberstein et al. 2005, Rinehart 2007).

Limited and often contradictory recommendations exist to guide the necessary and appropriate investigations of RIF. Considering suspected etiological factors of RIF, various therapies to improve endometrial receptivity, embryo developmental potential and complex maternal conditions have been suggested (Margalioth et al. 2006). Hysteroscopic investigations of the endometrial cavity (Demirol et al. 2004), endometrial biopsy (Nastri et al. 2012), and low molecular weight heparins (Fiedler et al. 2004) have shown benefit in the form of increased pregnancy rates in couples with failed ART cycles. Preimplantation genetic diagnosis, embryo co-culture and preferential blastocyst transfers can be implemented to minimize the embryonic component in RIF causality (Margalioth et al. 2006). Medical treatment of endometriosis-related RIF has been shown to benefit affected couples (Sallam et al. 2006) however surgical treatment of the disease still remains controversial (Adamson 2005). Where hydrosalpinx is suspected as the cause of treatment failure, it should be removed prior to embarking on further treatment (Johnson et al. 2004). Due to the lack of sound scientific evidence, often an empirical approach to investigations and treatment of RIF is taken, resulting in varying success rates.

The findings reported in this article are to be expected, and suggest that definition of recurrent implantation failure should be based on consensus and expert opinion.

3.4.1. **Suggestions for definition**

In a widely accepted and implemented international definition of RIF each step of the treatment process, from fertilization through to an unambiguous definition of the treatment end-point, should be considered. For clarification, when defining 'implantation' failure, we refer to the lack of formation of a physical attachment between the embryo and the decidualising endometrium resulting in a negative serum beta hCG.

The definition can be based on the number and quality of embryos used for treatment. As there is a significant trend towards single embryo replacement and preferential use of blastocyst stage embryos, these should be used as 'the gold standard' in the proposed definition of RIF.

One foreseeable benefit of standardizing the definition would be the uniformity of collected research data. As the overall numbers of patients with RIF per clinic are small, in order to perform good quality research into uncertainties surrounding this phenomenon, multicentre and international studies need to be put in place. Data heterogeneity will be minimized when same study populations are considered, making meta-analyses more reliable and accurate. Expert opinion needs also to be pursued and pilot studies using this definition need to be launched in order to test its feasibility in practice. This systematic review is the first step in improving the quality of research into recurrent implantation failure.

Combining all the aspects of the definition, the authors of this review suggest that recurrent implantation failure should be defined as the absence of implantation, itself defined by a negative serum hCG 14 days after oocyte collection, after two consecutive cycles of IVF, ICSI or frozen embryo replacement where the cumulative number of transferred embryos was no less than 4 for cleavage stage embryos and no less than 2 for blastocysts, with all embryos being of 'good quality' and of

appropriate developmental stage. Patients in the oocyte donation scheme failing to achieve pregnancy and meeting the above criteria, should also be treated as being affected by recurrent implantation failure. This definition incorporates all of the relevant information necessary to completely define recurrent implantation failure, including the outcome. Whether the scientific community will acknowledge the suggested definition, depends on results of pilot studies and ease of inclusion of patients according to these new, stricter yet more inclusive and comprehensive criteria.

The proposed definition unfortunately may still be biased and open to criticism, as it is based on expert opinion. It would be important to mention, that a possible adjustment for age could be considered when labelling a patient with RIF, so that younger women would require more failed cycles to be considered for investigations and possible treatment compared to older counterparts. This approach could be supported by the higher monthly fecundity rate of younger women in natural conceptions as well as during ART (Wang et al. 2008b). This approach however, breeches the attempt to standardise the definition of RIF and would still prevent the combination of data from different studies in order to formulate meta-analysis conclusions.

3.4.2. **Potential biases in the review process**

The significant difference in publication dates of analysed studies spanning two decades has been considered as the main confounding factor.

3.4.3. **Agreements and disagreement with other studies or reviews**

No previous systematic reviews were performed assessing the definition of recurrent implantation failure or any studies attempting to provide basis for the definition exist. Rinehart and El-Toukhy et al. in two separate articles attempted to standardize the definition of recurrent implantation failure in their respective publications (El-Toukhy et al. 2006, Rinehart 2007). These articles were not, however, systematic

reviews and as such only represent the authors' opinion on the subject. No definition was suggested in either article. Tan et al., aimed to determine the definition of recurrent implantation failure through a questionnaire survey (Tan et al. 2005). 79 centers form the HFEA database were asked to participate with an 82% reply rate. The definitions of RIF ranged from two to six failed previous treatment cycles but the majority of centers (63.1%) used three fresh IVF cycles in their definition. Only 18% of centers included frozen embryo transfers in their definition. This survey highlights the discrepancies in defining recurrent implantation failure even within a small geographic area and, by extrapolation, suggests that worldwide differences in defining this iatrogenic condition would be even greater. A more recent opinion suggests RIF should be defined as at least three fresh or frozen unsuccessful cycles with a cumulative transfer of at least four good quality embryos in a woman under 40 years of age (Li 2012), but no rationale for this definition is given.

3.4.4. **Conclusions**

This review of literature has revealed a significant heterogeneity when defining recurrent implantation failure. Various factors are responsible for this iatrogenic condition, however no definition was identified that fully describes these. A uniform definition of RIF will improve the quality of the information gathered and help couples undergoing repeated cycles of ART to achieve a successful, healthy pregnancy. The most likely way to lead to a successful definition of RIF would however, be through specialist consensus. We propose a new definition, which is still arbitrary as it is based mainly on opinion, which does however consider the key determinants of implantation and the IVF treatment process.

An earlier revision of this chapter has been published in Reproductive Biomedicine Online (2014).

CHAPTER 4. Review of literature on the effect of endometrial biopsy on ART outcome.

4.1. Introduction

Infertility is an increasing problem among the population, affecting 10-15% of couples of reproductive age (Thoma et al. 2013). Current options for these couple include various modalities of ART, where the success, defined as a live birth, can be achieved in up to 30% of patients (de Mouzon et al. 2012). Any intervention aiming to improve this rate will be welcome, as failure of treatment is the main source of psychological stress and a cause for drop out form subsequent treatment (Verberg et al. 2008, Pasch et al. 2012).

A relatively simple procedure- endometrial injury- has been shown to be a promising intervention allowing for improvement of the live birth rate in a population of women with failed IVF or ICSI cycles. This beneficial association in the ART setting has been described for the first time in 2003 by Barash et al. (2003). Previous studies on animal models have shown a beneficial effect of endometrial scratching on endometrial stromal cell decidualisation in guinea pigs (Loeb 1906, Loeb 1908) and rats (Lejeune et al. 1981). The exact effect the injury has on the endometrium is not fully understood. Most authors seem to think that an inflammatory response caused by the insult leads to an influx of immune cells and production of various cytokines and growth factors, which in turn alter the endometrial milieu to that favouring implantation (Gnainsky et al. 2010). Another theory suggest that the injury leads to a delay in closing the WOI and allowing for synchronisation of the endometrium and embryo in ART cycle (Li et al. 2009).

Endometrial biopsy has been performed in a few studies in the ART setting, however the inclusion criteria, timing of the procedure and study design differed. Up to date, only six studies reported on the live birth rate in an IVF or ICSI cycle following endometrial scratching (Barash et al. 2003, Zhou et al. 2008, Narvekar et al. 2010, Baum et al. 2012, Inal et al. 2012, Nastri et al. 2013). All studies have focused on couples with failed previous attempts at ART, with the exception of the study by Nastri et al. (2013) where a population of women undergoing their first IVF cycle was included. Frozen embryo replacement cycles have not been

assessed in any of the studies, and yet these form a significant part of work load and contribute to the cumulative pregnancy rate.

The timing of the procedure is another uncertain issue. In the majority of cases, the endometrial injury was performed in the cycle preceding the embryo replacement cycle. When a biopsy is performed in the embryo replacement cycle (during COH) or at oocyte recovery, the pregnancy rates achieved seem to be lower compared to the control groups (Karimzade et al. 2010, Motana et al. 2013). This observation is likely related to the lack of sufficient amount of time for the injury to heal, or the aforementioned immune effects to take place.

The objective of this chapter was to collate the available evidence supporting or refuting the use of endometrial scratching in the IVF or ICSI setting as means to improve the outcome of treatment. Description of complications has also been sought. The data obtained has served as theoretical foundation for the subsequent endometrial biopsy trial design (see Chapter 5).

4.2. Materials and Methods

The literature search was conducted using Ovid Gateway (www.ovidsp.ovid.com) on the 16.11.2013. The searched databases included Medline, Embase, PsycINFO, and Cochrane Library from the database inception to present. Search terms used included endometri*, biops*, injur*, *in vitro* fertili*ation, IVF, intracytoplasmic sperm injection, ICSI. Where necessary, MESH terms were expanded and included in the search. The initial search was limited to human subjects and English language only. Selection of only human studies allowed for extrapolation of data onto human subjects and helped with subsequent study design methodology. Only articles looking at endometrial injury in relation to an IVF or ICSI cycle where an outcome of treatment was stated were sought. Preference was given to RCTs; however matched control, prospective and retrospective studies were also included. Included articles were obtained in full and used in further analysis. The remaining articles were not considered, as they did not meet the inclusion criteria.

Outcome data retrieved included live birth rates (primary outcome), clinical pregnancy rates, miscarriage rates, timing of biopsy, number of biopsies performed, and any procedure related complications (secondary outcomes). A live birth is defined by consensus as any delivery of a live infant after 24th completed week of gestation (Births and Deaths Registration Act 1953, paragraph 41, modified by Still Birth Definition Act 1992). Implantation rate is defined as the number of gestation sacs visible on an early ultrasound scan divided by the number of embryos transferred. Clinical pregnancy is defined as the number of gestation sacs containing a fetus with visible cardiac activity on a first trimester scan. Miscarriage is a loss of pregnancy before 24 completed weeks of gestation.

Retrieved data was analysed using RevMan 5.2 (Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2012). For every considered outcome Mantel-Haenszel risk ratios (RR) were used with corresponding 95% confidence intervals (95% CI). If a zero cell count was observed or prevalence < 1% was noted, Peto fixed-effect odds ratio

(OR) with corresponding 95% CI was used. Forrest plot graphs were used to graphically represent the relationship. Heterogeneity of data was presented in the form of I^2 statistics.

4.3. Results

4.3.1. **Study selection**

As a result of the literature search, 163 articles were obtained. Duplicates were removed and the resulting citations were screened for eligibility. This resulted in 31 articles being retrieved in full and scrutinized. Fifteen studies were excluded and the respective reasons for exclusion are presented in Table 3.

Study	***Reason for exclusion***
Francisco et al. 2011 ***Huang et al. 2011*** ***Kumbak et al. 2012*** ***Shohayab et al. 2012*** ***Kovacs et al. 2013*** ***Nayar et al. 2013***	Studies assessed the effect of endometrial sampling during a diagnostic hysteroscopy.
Kalma et al. 2009 ***Gnainsky et al. 2010***	No outcome data was presented.
Tiboni et al. 2011	No control population was described.
Peavey et al. 2011	The study assessed the effect of saline infusion sonography on IVF treatment outcome without endometrial sampling.
Szlit et al. 2011	The study assessed the effect of endometrial stromal cell co-culture on IVF outcome where both examined groups of women had endometrial sampling.
Hayashi et al. 2013	A 3 mm curette was used to perform the endometrial scratching.
Chang et al. 2013	Mild and complete endometrial injury was performed in study participants.
Faghih et al. 2013	No clear timing of the procedure was stated.
Dadras et al. 2012	The biopsy was performed in a timed intercourse cycle.

Table 3. Characteristics of excluded studies.

Of the included studies, five were randomised controlled studies (Karimzadeh et al. 2009, Narvekar et al. 2010, Baum et al. 2012, Inal et al. 2012, Nastri et al. 2013). Remaining articles included prospective studies (Barash et al. 2003, Zhou et al. 2008, Karimzade et al. 2010, Bonavita et al. 2011, Jung et al. 2013), retrospective studies (Spandorfer et al. 2002b, Kara et al. 2012), matched case-control studies (Ubaldi et al. 1997, Motana et al. 2013), or had no study description (Raziel et al. 2007, Safdarian et al. 2011).

Included populations differed between studies and included patients with recurrent failed treatment cycles (two or more) (Spandorfer et al. 2002a, Raziel et al. 2007, Karimzadeh et al. 2009, Huang et al. 2011), patients with at least one failed cycle (Barash et al. 2003, Karimzade et al. 2010, Narvekar et al. 2010), good responders to previous treatment (Ubaldi et al. 1997, Zhou et al. 2008, Bonavita et al. 2011, Safdarian et al. 2011) or not commented upon (Bonavita et al. 2011).

Timing of the procedure also differed between studies and single or multiple biopsies were performed. Barash et al. (Barash et al. 2003) and Jung et al. (Jung et al. 2013) performed four biopsies on days 8, 12, 21 and 26 of a natural cycle preceding IVF treatment. Narvekar et al. (Narvekar et al. 2010) have performed two biopsies- on days 7-10 directly following a hysteroscopy, and on day 24-25 of the cycle preceding IVF treatment. Baum et al. (Baum et al. 2012) have also performed the biopsy twice- on days 9-12 and 21-24, with a cervical pipelle in the control population. Raziel et al. (Raziel et al. 2007) have performed the biopsy on days 21 and 26 of the spontaneous cycle. Two samples one week apart in the luteal phase of the non-transfer cycle were performed in a study by Inal et al. (2012). Remaining authors have performed single biopsies (Spandorfer et al. 2002a, Zhou et al. 2008, Karimzadeh et al. 2009, Karimzade et al. 2010, Bonavita et al. 2011, Huang et al. 2011, Safdarian et al. 2011, Kara et al. 2012, Motana et al. 2013, Nastri et al. 2013). Endometrial biopsy in the cycle preceding the ovarian stimulation was performed in eleven studies (Spandorfer et al. 2002a, Barash et al. 2003, Raziel et al. 2007, Karimzadeh et al. 2009, Bonavita et al. 2011, Safdarian et al. 2011, Baum et al. 2012, Inal et al.

2012, Kara et al. 2012, Jung et al. 2013, Nastri et al. 2013) and was performed on days 21-26 (Raziel et al. 2007, Karimzadeh et al. 2009, Bonavita et al. 2011, Safdarian et al. 2011) or day 1 to 12 post LH surge (Spandorfer et al. 2002a). Zhou et al. performed the biopsies during the ovarian stimulation cycle- on day 5 to 22 (Zhou et al. 2008). Karimzade et al. (Karimzade et al. 2010), Ubaldi et al. (Ubaldi et al. 1997) and Motana et al. (Motana et al. 2013) have performed the endometrial biopsy on the day of oocyte retrieval.

In all analysed studies, Pipelle de Cornier (CCD, Paris), or a similar device, was used to perform the injury to the endometrium.

Antibiotics post procedure were administered in four studies (Zhou et al. 2008, Narvekar et al. 2010, Inal et al. 2012, Kara et al. 2012). The use of non- hormonal contraception in the biopsy cycle was mentioned in three studies (Barash et al. 2003, Narvekar et al. 2010, Kara et al. 2012), and oral contraceptive pill was prescribed in two studies (Safdarian et al. 2011, Nastri et al. 2013).

4.3.2. **Effect of endometrial biopsy on live birth rate**

Of the analysed studies, only six authors have presented the data on this outcome (Barash et al. 2003, Zhou et al. 2008, Narvekar et al. 2010, Baum et al. 2012, Inal et al. 2012, Nastri et al. 2013). Majority of studies have shown a significant benefit of the endometrial biopsy on live birth in the study populations. Live birth rates ranged from 22.4% (Narvekar et al. 2010) to 48.9% in the biopsy groups (Barash et al. 2003) versus 9.8% (Narvekar et al. 2010) to 23% (Barash et al. 2003, Zhou et al. 2008) in the control populations. The estimated average difference of live birth rates between the control and study populations was 17%.

The performed meta-analysis on the available data has shown a significant improvement in the live birth rate in patients undergoing IVF after an endometrial biopsy in the cycle directly preceding ovarian hyperstimulation. Odds ratio for a live birth was 2.20 (95%CI 1.55-3.12; P<0.0001) (see Figure 24).

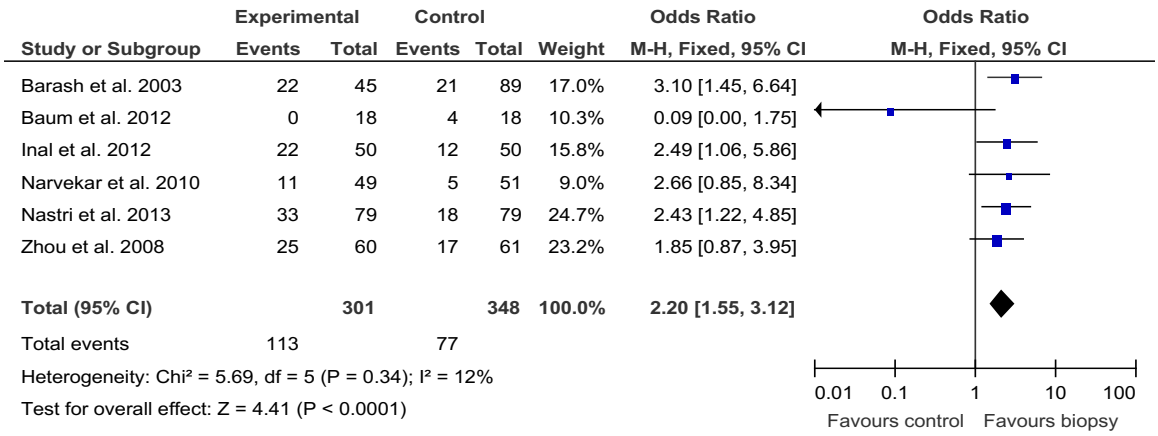


Figure 24. Effect of endometrial biopsy on live birth rates.

4.3.3. **Effect of endometrial injury on implantation rate**

Meta-analysis of this IVF treatment outcome was only possible in four studies (Ubaldi et al. 1997, Safdarian et al. 2011, Kara et al. 2012, Nastri et al. 2013). The OR for implantation based on the data from the mentioned studies was 1.52 (95% CI 1.04-2.21; P=0.03) (see Figure 25).

4.3.4. **Effect of endometrial injury on clinical pregnancy rate**

Of the assessed studies, fifteen provided enough information to perform a meta-analysis (Ubaldi et al. 1997, Barash et al. 2003, Raziel et al. 2007, Zhou et al. 2008, Karimzadeh et al. 2009, Karimzade et al. 2010, Narvekar et al. 2010, Bonavita et al. 2011, Huang et al. 2011, Safdarian et al. 2011, Baum et al. 2012, Inal et al. 2012, Kara et al. 2012, Jung et al. 2013, Motana et al. 2013, Nastri et al. 2013).

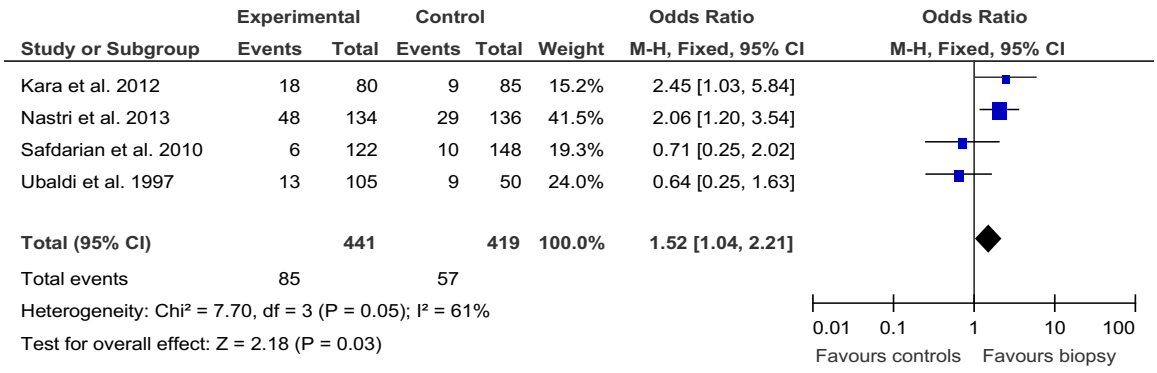


Figure 25. Effect of endometrial biopsy on implantation rates.

The results indicate a highly significant improvement (P<0.00001) in the clinical pregnancy rate in patients that have undergone endometrial biopsy with an OR of 1.64 (95%CI 1.33-2.03) (see Figure 26). Heterogeneity of the populations analysed is significant (I^2=78%). This is probably due to different study designs and study populations.

As the heterogeneity of data was high, a subgroup analysis of studies employing similar methodology has been performed (single biopsy in a cycle preceding the embryo transfer cycle) (Zhou et al. 2008, Karimzadeh et al. 2009, Bonavita et al. 2011, Safdarian et al. 2011, Kara et al. 2012, Nastri et al. 2013). This demonstrated a very highly significant (P<0.00001) positive correlation between endometrial biopsy and clinical pregnancy rates with an OR of 2.28 (96%CI 1.63- 3.21) and a minimal data heterogeneity (I^2=0%) (see Figure 27).

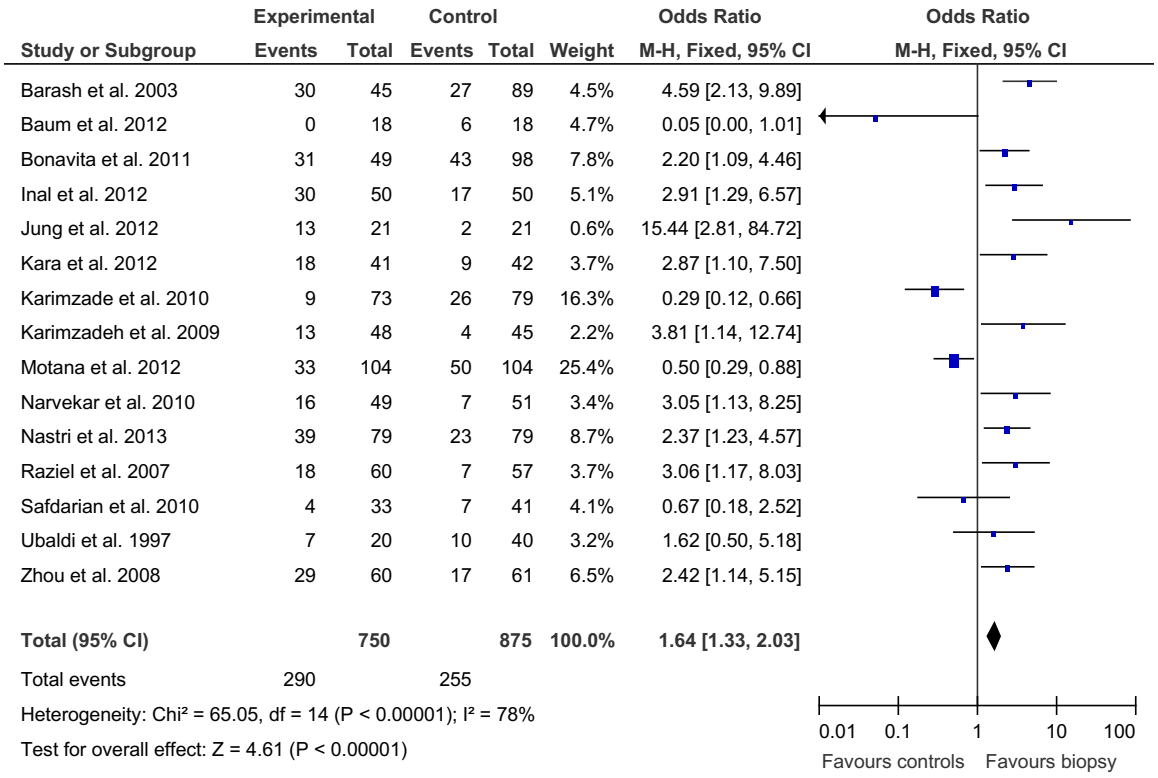


Figure 26. Effect of endometrial biopsy on clinical pregnancy rates.

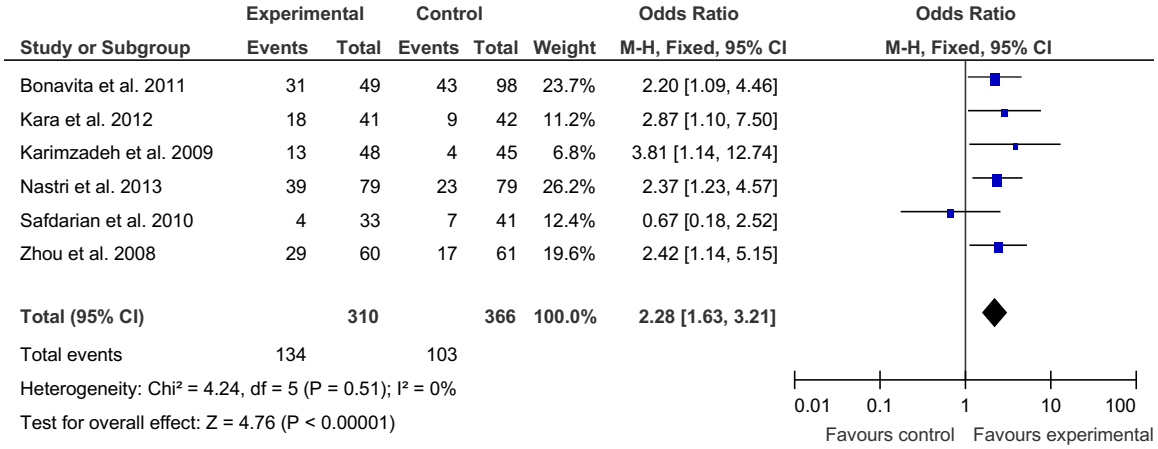


Figure 27. Effect of endometrial injury on clinical pregnancy rates in studies with same methodology.

4.3.5. **Effect of endometrial injury on miscarriage rate**

Of the assessed studies, only five provided enough information to assess the miscarriage rates following the biopsy (Ubaldi et al. 1997, Barash et al. 2003, Raziel et al. 2007, Narvekar et al. 2010, Nastri et al. 2013). The conducted meta-analysis has shown no statistically significant difference (P=0.85) in this outcome between the control and experimental groups (OR=0.93, 95%CI 0.45-1.95) (see Figure 28).

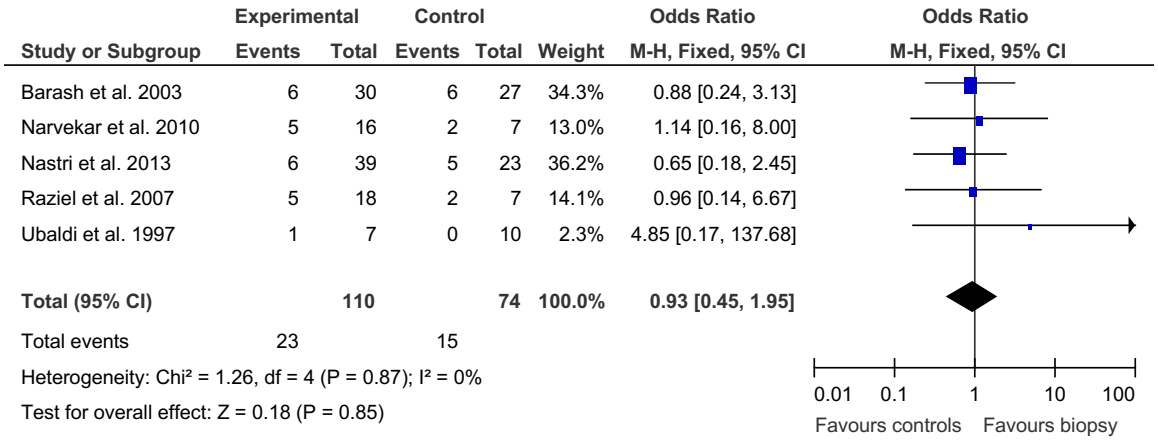


Figure 28. Effect of endometrial injury on miscarriage rates.

4.3.6. **Impact of single versus multiple biopsies on clinical pregnancy rates**

One of the main differences in the design of the studies was the number of biopsies performed in the cycle preceding the IVF stimulation cycle. This ranged from one (Raziel et al. 2007, Karimzadeh et al. 2009, Bonavita et al. 2011, Huang et al. 2011, Safdarian et al. 2011, Tiboni et al. 2011) to four (Barash et al. 2003). Data from these studies was combined in order to assess the impact of single versus multiple biopsies (two or more) on clinical pregnancy rates.

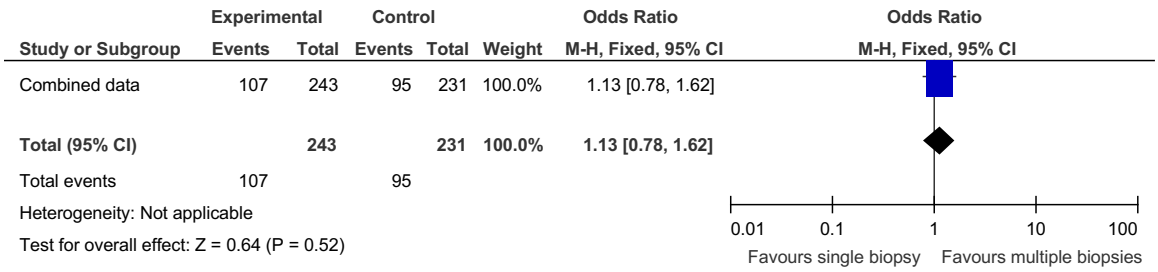


Figure 29. Effect of single versus multiple biopsies on clinical pregnancy rates.

Combined data analysis indicates no significant difference in clinical pregnancy rates and live birth rates between single or multiple biopsies when the procedure was performed in the menstrual cycle directly preceding embryo replacement, with an OR for clinical pregnancy of 1.13 (95% CI 0.78-1.62) (see Figure 29) and 0.72 (95% CI 0.39-1.32) for live birth (see Figure 30).

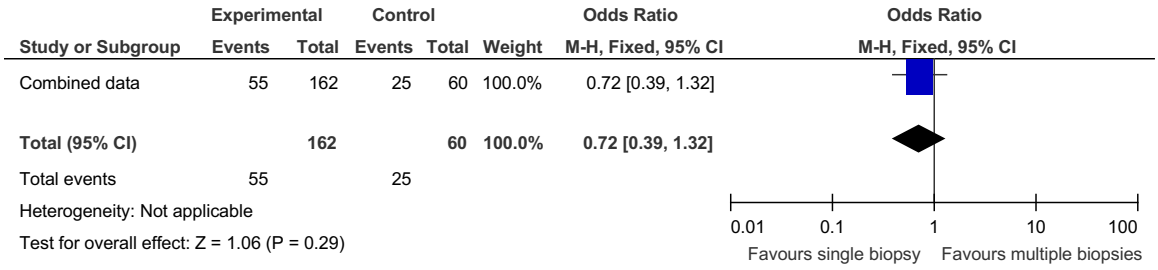


Figure 30. Effect of single versus multiple biopsies on live birth rates.

4.3.7. **Complications of endometrial biopsy**

In the reviewed articles, only six authors comment on the immediate complications of endometrial biopsy (Raziel et al. 2007, Karimzadeh et al. 2009, Karimzade et al. 2010, Narvekar et al. 2010, Safdarian et al. 2011, Nastri et al. 2013). Vaginal spotting for two days after the procedure has been reported by Narvekar et al. (2010). Pain and a small amount of bleeding during the procedure were reported by Nastri et al. (2013). Infectious complications were feared, however no such complications have occurred in any of the six studies.

No major fetal anomalies were observed in the study population following the endometrial injury. This has, however, been reported only by one author where the intervention group consisted of 79 women (Nastri et al. 2013).

4.4. Discussion

As demonstrated in this chapter, endometrial scratching performed in the cycle directly preceding an embryo replacement cycle, seems to have a beneficial effect on the implantation rates, clinical pregnancy rates and live birth rates with and OR of 1.52 (95% CI 1.04-2.21), 2.28 (96%CI 1.63- 3.21), and 2.43 (95%CI 1.63-3.62), respectively. Apart from pain and minimal vaginal bleeding, the procedure was well tolerated and has an acceptable side effect profile.

As long as the scratch is performed in the non-transfer cycle, the benefits are significant. Endometrial injury during oocyte collection has a negative impact on pregnancy rates (Karimzade et al. 2010). This has been also assessed in a recent systematic review of the subject (Nastri et al. 2012), which recommends that the scratch not be performed during the COH cycle. A more recent update of the abovementioned systematic review, indicates that an improvement in clinical pregnancy rates and ongoing/ live birth rates can be observed in women with at least two failed ART cycles, provided the endometrial biopsy is performed between day 7 of the previous cycle and day 7 of the embryo replacement cycle. The quality of the evidence was however moderate only (Nastri et al. 2015). Thus, women with previous failed IVF or ICSI cycles seem to benefit the most from this intervention, however a study by Nastri et al. (2013), indicated that an unselected population of women undergoing IVF can also increase their chances of success from 22.8% in the control population, to 41.8% in the scratch group. Where the study populations consisted of RIF patients (two or more failed cycles), the clinical pregnancy rates were 8.9% to 12% in the control populations, and 27.1% to 30% in the biopsy arms (Raziel et al. 2007, Karimzadeh et al. 2009). The number of biopsies performed (one or more) does not seem to have any measureable additive effect on pregnancy rates according to the results presented in this chapter. Though not reported, the increasing number of biopsies might lead to an increased number of potential complications, and unnecessarily and repeatedly expose the woman to a painful procedure.

Thought the effect of the biopsy seems favourable, the issue of generalizability remains. This is due to varying COH treatment regimens used by different clinics, the use of additional medications (i.e. oral contraceptive pill- OCP), and differing demographic details of participant enrolled in the studies up to date. Currently used gonadotropins seem to exhibit a similar effect on the endometrial proliferation and subsequent treatment outcomes (Ku et al. 2002, Chang et al. 2011). Use of OCP in the context of IVF is related to convenience of cycle scheduling, with no detrimental effect on outcomes. The only observed drawbacks of OCP use were related to longer duration of stimulation and need for a higher total dose of gonadotropin (Pinkas et al. 2008). The general consensus is that OCP pre-treatment is favourable for women with failed multiple attempts at IVF (Nastri et al. 2012, Nastri et al. 2013). As OCP pre-treatment is routine practice in some centres, data from these studies was included in the analyses presented in this chapter.

More work is necessary to elucidate the effect of endometrial biopsy on women undergoing their first treatment cycle and couples undergoing frozen embryo replacement. An approach looking at a general infertile population can also be taken, and this should include women undergoing clomiphene citrate ovulation induction and intrauterine insemination. Further basic science work should be carried out contemporaneously aiming to improve our understanding of the mechanisms responsible for normal and abnormal pregnancy development.

CHAPTER 5. Effect of endometrial biopsy on outcome of ART in an unselected population of women- a pilot randomised control study

5.1. Introduction

The demand for assisted reproductive treatment (ART) is increasing, however the live birth rates achieved using these techniques are consistently within the region of 30% (de Mouzon et al. 2012, Ferraretti et al. 2013). Procedures aiming to improve the success rates of ART are necessary to limit the significant emotional stress on the couple and the financial burden to the healthcare systems and couples, where the state does not reimburse these treatments. Higher doses of gonadotropins and use of modified treatment protocols aim to maximize the oocyte yield, risking the development of ovarian hyperstimulation syndrome (OHSS) and associated complications. Double embryo transfer, blastocyst transfer and embryo cryopreservation are other methods used to increase ART efficacy.

A simple procedure- an endometrial scratch (or biopsy) has been associated with an improvement in the ART outcome in a population of infertile women with previous failed treatments (see chapter 4 and references therein). The exact mechanism of this effect remains uncertain, with the possible mechanisms being induction of an aseptic inflammatory process (Gnainsky et al. 2010, Granot et al. 2012), and a delay in endometrial decidualization allowing for better synchronicity between the endometrium and the transferred embryo (Li et al. 2009).

Initial human studies have focused on women with recurrent implantation failure (RIF) or previous failed cycles (Barash et al. 2003, Raziel et al. 2007, Karimzadeh et al. 2009, Karimzade et al. 2010, Narvekar et al. 2010, Huang et al. 2011) and, in all cases but one (Karimzade et al. 2010), significant improvements in ART outcomes were observed. More recent studies have expanded the recruitment scope to include an unselected population of infertile women (Nastri et al. 2013, Yeung et al. 2014). These two works provide however contradictory results. A recent Cochrane systematic review update has concluded that endometrial biopsy can be beneficial for women with at least two failed cycles provided the procedure is performed up to day 7 of the embryo transfer cycle (Nastri et al. 2015). Studies where women undergoing frozen

embryo replacement (FER) cycles were never considered and, currently, no evidence exists regarding the effect the endometrial biopsy has on frozen embryo replacement cycles.

In view of the available evidence base, the objective of this study was to assess the effect of endometrial injury on the *in vitro* fertilization (IVF) and/or intra-cytoplasmic sperm injection (ICSI) outcome in an unselected population of infertile women, including women undergoing their first treatment cycle and women undergoing FERs. The assessment of procedure related complications was also carried out.

5.2. Materials and Methods

5.2.1. **Study design and participants**

The study was designed as a pilot randomized controlled trial (RCT) with a 1:1 recruitment ratio. All participating women were screened and recruited from a university affiliated infertility clinic- Nurture Fertility, University of Nottingham, Nottingham, UK, from January 2012 to July 2014. Women <49 years of age with no major uterine anomalies and sufficient ovarian reserve (antral follicle count- AFC≥6) undergoing treatment using own oocytes in fresh or cryopreserved cycles were eligible. Women willing to take part in the study have given written consent prior to any procedure. Research ethics committee has approved the study protocol (12/EM/0345) and the study was registered on ClinicalTrials.gov website (NCT01882842).

5.2.2. **Randomisation and blinding**

A computer generated pseudo-random code using random permuted blocks of randomly varying size provided by the Clinical Trials Unit, University of Nottingham, Nottingham, UK was used to allocate participants to either group A (intervention) or B (control). The participants were informed of the randomization outcome on the day of the procedure. The clinician and the participants were not blinded to the allocation due to the nature of the study. In accordance with good clinical practice, the participant was allowed to drop out from the study at any stage.

5.2.3. **Screening and endometrial biopsy**

Screening for eligibility was performed prior to ART at the initial ultrasound scan on day 1 to 5 of a natural menstrual cycle. The study information leaflet was given to women meeting inclusion criteria.

A home urinary ovulation (luteinizing hormone- LH) kit was given to each participant with instructions to test morning urine from day 8 of the cycle directly before the embryo replacement cycle. Endometrial biopsy was performed 7 to 9 days after a positive LH surge, or as close to day 21 of

the menstrual cycle (day 18 to 23) if no LH surge was detected. All women were advised to use barrier methods of contraception or abstain from sexual intercourse in the biopsy cycle.

Prior to the study procedures, a urinary pregnancy test was carried out. If this was positive, the patient was withdrawn from the study. A blood sample for oestrogen and progesterone levels was obtained and an endovaginal ultrasound scan was carried out in every participant as described on page 79.

Following the scan, an endometrial biopsy was carried out using Pipelle de Cornier (CCD Paris, France) in group A as described on page 86. Group B had no procedure performed apart from a blood test and ultrasound (US) scan. In my absence, endometrial biopsies and scans were performed by another experienced physician (MB).

5.2.4. **Ovarian stimulation protocols**

Following the mid-luteal appointment, all recruited participants have undergone IVF treatment using standard protocols. In brief, during a long protocol, pituitary down-regulation with GnRH agonist, buserelin (Aventis Pharma, Kent, UK) or nafarelin (Pfeizer, Belgium) was started seven days prior to the expected date of next menstrual bleed (e.g. day 21 of 28 day cycle). Following a withdrawal bleed, a transvaginal ultrasound scan was performed to confirm down regulation (quiescent ovaries with follicles <10 mm diameter, endometrium ≤5mm in thickness and serum oestradiol level <200 pmol/l). Ovarian stimulation was commenced subsequently with Human Menopausal Gonadotrophin (HMG, Menopur, Ferring, UK) or recombinant FSH- follitropin α (Gonal-F, Merck Serono, UK) at a daily dose (adjusted to the individual patient) administered by subcutaneous injection. From day 6 of stimulation, subjects were monitored for their ovarian response using ultrasound and serum oestradiol levels. When a short antagonist protocol was used, stimulating medications (Menopur or Gonal-F) were commenced on day 2 of period. GnRH antagonist, cetrorelix (Cetrotide, Merck Serono, UK) was commenced when a lead follicle began to develop. Once women met the criteria for oocyte retrieval (≥3 leading follicles ≥17 mm), 250 μg of

choriogonadotrophin α, (Ovitrelle, Merck Serono, UK), was administered subcutaneously to trigger oocyte maturation. Transvaginal ultrasound guided oocyte retrieval (TVOR) was performed 36 hours later directly preceded by ultrasound assessment of the endometrium.

In natural FER cycles, the participant was assessed on day 6 of the cycle. Following this, ultrasound examinations and/or serum LH testing were carried out every 2 to 3 days. When a follicle ≥ 18 mm was identified, LH surge was simulated by administering 5,000 IU of hCG, or the cycle was monitored for LH surge using serum LH monitoring or home urinary LH detection kits. No luteal support was administered. If artificial endometrial preparation in FERs was required, oestradiol valerate 6 mg/day was administered. When ET was ≥ 7.0 mm, micronized progesterone (Cyclogest, 400mcg twice daily, Actiavis, UK) was administered vaginally.

Depending on the number of oocytes, the fertilization rate and number of day 2 embryos, one or two embryos were transferred on day 2, 3 or 5. Prior to embryo transfer, another transvaginal ultrasound scan of the endometrium was carried out. In all stimulated cycles, luteal support was necessary and continued until the 9th week of gestation, if pregnancy was achieved. A urinary pregnancy test was carried out 2 weeks following the embryo transfer to confirm implantation.

5.2.5. **Outcome measures**

The main outcome of the study was a clinical pregnancy per allocated woman, defined as evidence of a viable fetus on a transvaginal scan performed at 6-8 weeks gestation.

Secondary outcomes included multiple pregnancy rates (presence of more than one fetus on a transvaginal ultrasound scan at 6-8 weeks gestation), biochemical pregnancy rate, first trimester miscarriage rate, implantation rate, and procedure related complications. Live birth rate will also be sought.

5.2.6. **Sample size**

Assuming a 10% difference in the clinical pregnancy rates between the placebo and intervention groups (a clinically significant difference), 80% power and α=0.05, the required total sample size would be 766 women. Taking into consideration the available timeframe (18 months) and a recruitment rate of 50%, a pilot study aiming to recruit a population of 160 women (80 in each group) was designed. An interim analysis was carried out after recruitment of 80 women to assess that no negative effect of the procedure on the outcomes was observed.

5.2.7. **Statistical analysis**

Statistical analysis was carried out using SPSS (version 21) on an intention-to-treat (ITT) and per protocol basis. Distribution of data was assessed using Kolmogorov-Smirnov test. For parametric unpaired data Student's t test was used and for non-parametric data, Mann-Whitney U test was carried out. For paired samples, student's t test was used for parametric data and Wilcox signed rank test was used for non-parametric data.

Logistic regression analysis was used to assess the effect of different variables for prediction of the outcome of ART. The effect of the same variable on treatment outcome was assessed using binary logistic regression analysis. A P value of <0.05 was considered as statistically significant.

5.3. Results

The target population of 160 women was not achieved in the allocated timeframe. The recruitment to the study was on target, until staff changes and a commercial decision to offer the endoscratch by the clinic lead to a drop in recruitment rates.

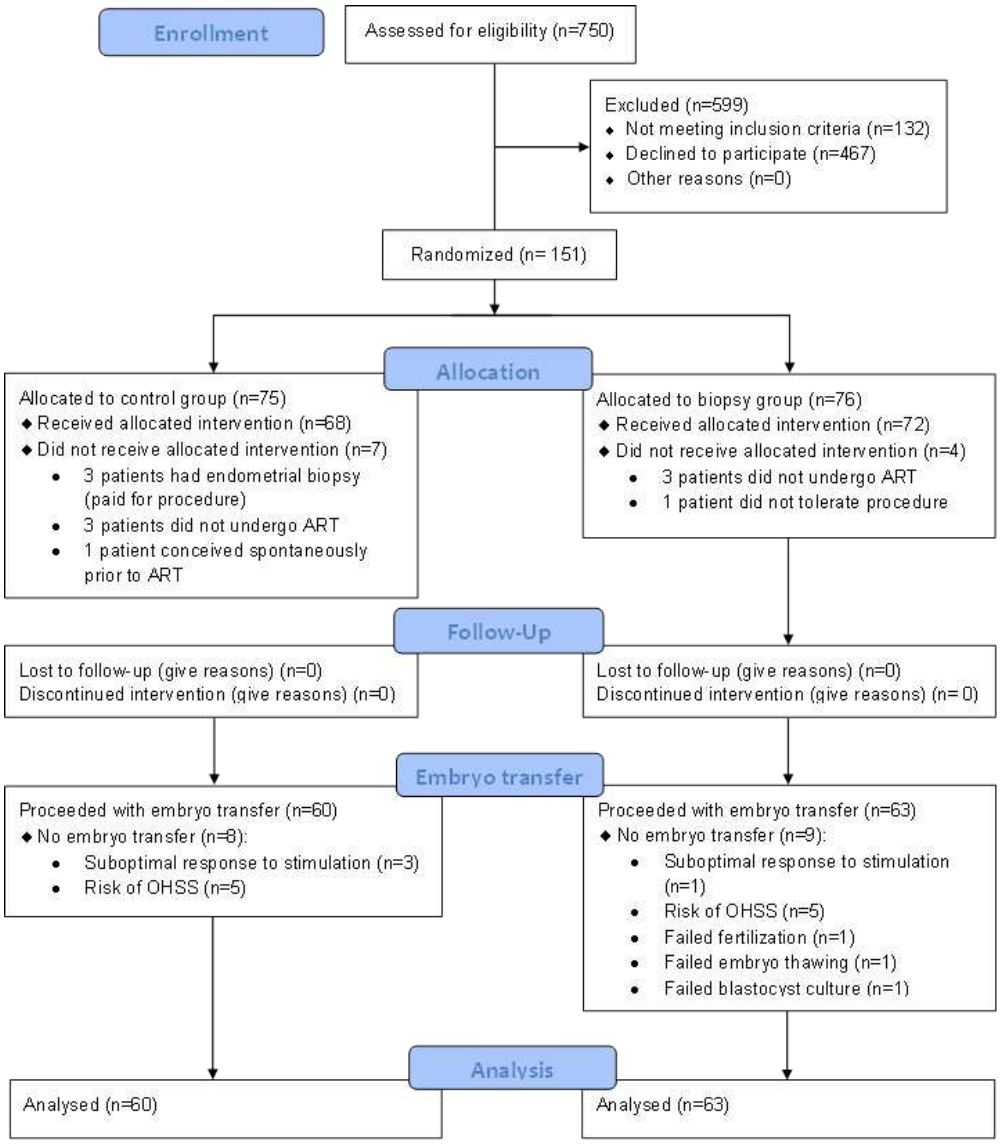


Figure 31. Endoscratch study participant flow diagram. Diagram adapted from CONSORT.

5.3.1. **Population demographics**

Between January 2013 and July 2014, 151 women were recruited: 76 in the study group and 75 in the control group. The study flow diagram is

presented in Figure 31. Baseline demographics of study participants are presented in Table 4.

Variable	***Control group***	***Biopsy group***
Age (years)	32.6(±4.2)	33.3(±4.0)
BMI (kg/m^2)	24.9(±4.0)	24.7(±3.3)
Smoking		
Yes	0	1(1.3%)
No	70(93.3%)	67(88.2%)
Ex-smoker	5(6.7%)	8(10.5%)
Menstrual regularity		
Regular	59(78.7%)	65(85.5%)
Irregular	16(21.3%)	11(14.5%)
Duration of infertility (months)	35(24-48)	36(28.5-48)
Causes of infertility		
Male	25(33.3%)	27(35.5%)
Anovulation	9(12%)	5(6.6%)
Tubal	3(4%)	4(5.3%)
Endometriosis	6(8%)	1(1.3%)
Unexplained	12(16%)	23(30.3%)
Mixed causality	20(26.7%)	16(21.1%)
Number of previous live-births		
0	69(92%)	64(84.2%)
1-2	6(8%)	12(15.8%)
≥3	0	0
Previous embryo transfers		
0	57(76%)	50(65.8%)
1-2	15(20%)	21(27.6%)
≥3	3(4%)	5(6.6%)
AMH (pmol/L)	16.9(10.5-29.5)	18.8(10.3-32.1)
AFC	20(12-32)	23(14-32)
Timing of procedure		
Positive LH surge	7.1(±0.9)	7.4(±0.8)
No LH surge	21.2(±1.1)	21.4(±1.2)

Table 4. Baseline demographics of study participants.

*Data reported as mean (±SD), median (25th- 75th centile) or number (percentage) as appropriate. *P<0.05*

		Control group (N=71); N(%)	***Biopsy group (N=73); N(%)***
Type of protocol	*Long agonist*	60(84.5)	60(82.2)
	Short antagonist	8(11.3)	6(8.2)
	FER	3(4.2)	7(9.6)
Suboptimal response n(%)		3(4)	1(1.3)
Days of stimulation		12(10-12.75)	12(11-13)
Gonadotrophin dose (units)		2526(±1065)	2586(±910)
Number of oocytes retrieved		13(8-18)	13(9-20.5)
Type of	*IVF*	26(38.2)	31(43.1)
treatment n(%)	*ICSI*	38(55.9)	34(47.2)
Day of ET n(%)	*Day 2*	3(4.8)	5(7.8)
	Day 3	3(4.8)	1(1.6)
	Day 5	57(90.5)	58(90.6)
Freeze all due to OHSS risk n(%)		5(7.0)	5(6.8)
SET n(%)		48(76.2)	41(64.1)
DET n(%)		15(23.8)	23(35.9)
Good quality embryos n(%)		54(69.2)	62(71.3)
Poor quality embryos n(%)		24(30.8)	25(28.7)
Easy transfer n(%)		50(79.4)	50(78.1)
Number of embryos frozen		0(0-3)	0(0-2.5)
E2 levels at ET (ng/mL)		4989(±3300)	4720(±3428)
Progesterone levels at ET (pmol/L)		121(±72)	117(±63)

Table 5. Treatment characteristics.

*Data presented as number (%), mean(±SD) or median(25^{th}- 75^{th} centile) as appropriate. SET- single ET; DET- double ET; *P<0.05;*

There was no difference between the control and biopsy populations in age, BMI, smoking status, menstrual regularity, duration and cause of infertility, ovarian reserve markers and past reproductive history (P>0.05). No differences in the treatment parameters (days of stimulation, number of oocytes retrieved, day of ET, single or double embryo transfer, embryo quality, number of embryos frozen, hormone levels) were identified between the biopsy and control groups (P>0.05) (Table 5). A difficult embryo transfer as reported by the clinician was

more common in the negative outcome group, however statistical significance was not reached (P=0.065). Sonographic data including endometrial thickness, volume and pattern, MGS, and 3DPD vascularity indices was no different between groups at embryo transfer (P>0.05) (Table 6).

		Control group	***Biopsy group***
ET at embryo transfer (mm)		10.6(±3.4)	10.9(±2.9)
Endometrial pattern N(%)	*Triple layer*	1(1.9)	1(1.8)
	Intermediate	34(63)	32(58.2)
	Homogenous	19(35.1)	22(40)
Mean Grey Scale (MGS)		43.47(±8.46)	43.77(±8.41)
Endometrial volume (cm^3)		3.298(2.37-5.33)	3.558(2.48-5.08)
3DPD VI		0.96(0.37-3.56)	1.59(0.58-3.16)
3DPD FI		26.45(±6.33)	26.46(±5.64)
3DPD VFI		0.25(0.09-1.14)	0.41(0.15-0.91)

Table 6. Sonographic data at embryo transfer.

*Data presented as number (%), mean(±SD) or median(25^{th}- 75^{th} centile) as appropriate; *P<0.05.*

5.3.2. **Primary outcomes**

There was no significant difference in the clinical pregnancy rates between the biopsy and control groups both on an ITT basis [53.9%(41/76) versus 44.0%(33/75); RR 1.23(95%CI=0.88-1.70); P=0.256] and per embryo transfer [65.1%(41/63) versus 55%(33/60); RR 1.18 (95%CI=0.88-1.58); P=0.254].

5.3.3. **Secondary outcomes**

There was no significant difference in the biochemical pregnancy rates between the biopsy and control populations on an ITT basis [59.2% (45/76) versus 52.0% (39/75); RR 1.14 (95% CI=0.86-1.52), P=0.415] and per embryo transfer [71.4% (45/63) versus 65% (39/60); RR 1.1 (95% CI=0.86-1.4), P=0.587].

Biochemical pregnancy losses were 8.9% (4/41) and 15.4% (6/33) in the biopsy and control groups, respectively (P=0.359) with an RR 0.58 (95%CI=0.18-1.9). First trimester miscarriage rates were 7.3% (3/41) and 15.2% (5/33) in the biopsy and control groups, respectively (P=0.281) with an RR 0.41 (95%CI=0.11-1.58). Multiple pregnancy rate in the biopsy group was 19.5% (8/41) and 6.1% (2/33) in the control group (RR 3.22; 95%CI=0.73-14.15; P=0.092). Implantation rates did not differ between groups [44/87(50.6%) in biopsy group vs. 37/78(47.4%); P=0.687].

Regression coefficient for clinical pregnancy adjusted for age, number of previous ETs, BMI, AMH, and AFC was 1.536 (95%CI=0.695-3.39; P=0.289). Further control for cause of infertility, presence of ovulation, treatment characteristics, sonographic endometrial characteristics at oocyte collection and ET, and E2 and P levels made little difference to the effect estimate.

5.3.4. **Outcomes per cycle number**

Due to the limited numbers of cycles meeting RIF criteria (as defined in Chapter 3) in the control group (n=3), a comparison between first cycle and RIF patients was not possible. Therefore a comparison between women undergoing first and second or subsequent cycles was performed. In the biopsy population, 49 women underwent first ET cycle and 23 second or subsequent. Respective numbers for the control population were 55 and 13 women. The pregnancy outcomes are presented in Table 7. No statistically significant differences were noted in any of the analysed outcomes (P>0.05).

5.3.5. **Outcomes per type of treatment**

Biochemical and clinical pregnancy rates stratified per type of treatment received (IVF, ICSI or FER) did not differ between groups (Table 8; P>0.05).

	First cycle		Second or subsequent cycle	
	Biopsy group (n=49)	*Control group (n=55)*	*Biopsy group (n=23)*	*Control group (n=13)*
Biochemical pregnancy rate	61.2(30)	58.2(32)	65.2(15)	53.8(7)
Clinical Pregnancy rate	55.1(27)	50.9(28)	60.9(14)	38.5(5)
Biochemical pregnancy loss	10(3)	12.5(4)	6.67(1)	28.6(2)
Clinical pregnancy loss	7.4(2)	14.3(4)	7.1(1)	20(1)

Table 7. Pregnancy outcomes per ET cycle.

*Data reported as N(%); * P<0.05.*

	Pregnancy rates in biopsy group N(%)		***Pregnancy rates is control group N(%)***	
Type of treatment	Biochemical	Clinical	Biochemical	Clinical
IVF	16(59.3)	14(52.9)	15(60)	14(56)
ICSI	24(77.4)	23(74.2)	25(71.4)	21(60)
FER	6(85.7)	5(71.4)	2(66.7)	1(33.3)

Table 8. Pregnancy rates depending on treatment type.

P<0.05.

5.3.6. **Procedure related complications**

One woman (1.4%) did not tolerate the procedure due to pain. In one case (1.4%), no visible tissue was obtained despite appropriate uterine instrumentation confirmed by ultrasound. No significant bleeding or infections were reported following the endometrial biopsy. Vasovagal episodes requiring brief hospital observation were experienced by four (5.6%) women.

5.4. Discussion

This study demonstrates that endometrial biopsy performed in a menstrual cycle preceding ART does not improve biochemical or clinical pregnancy rates in an unselected population of infertile women. Clinical pregnancy rates in women with two or more failed ART cycles were higher in the biopsy group compared to the control population (60.9% versus 38.5%), however due to low numbers statistical significance has not been achieved. No major immediate complications related to the procedure were observed. The procedure was well tolerated by majority of participants, with an overall complication rate of 8.3%.

Initial animal studies performed on guinea pigs have demonstrated improved decidualization of the endometrium (Loeb 1906). Almost a century has elapsed between this initial research and first human study (2003), which has shown improved live birth rates following endometrial biopsy in the non transfer cycle. The included population consisted of good responders with at least one previous failed ART cycle (Barash et al. 2003). Subsequent randomised controlled studies on women with failed ART have shown a similar improvement in outcomes, provided the biopsy was performed in the non-transfer cycle (Karimzadeh et al. 2009, Narvekar et al. 2010). Intentional endometrial injury seems to have a detrimental effect on pregnancy rates, when it is performed on day of oocyte retrieval (Karimzade et al. 2010). The results of these studies have indicated that endometrial biopsy can improve pregnancy rates following ART in a selected population of women. The hypothesis of improvement of endometrial receptivity following endometrial biopsy has been subsequently explored in more recent studies (including the one reported in this chapter), where unselected populations of infertile women were included.

The first randomized controlled study reporting on the effects of biopsy on ART outcome in an unselected population was reported in 2013 by Nastri et al. The authors of that trial have recruited 158 women, of which only 18 (11.4%) did not have a previous embryo transfer. Oral contraceptive pre-treatment (to synchronise the cycles) was also used in

this study. The reported live birth rates were in favour of the procedure (41.8% versus 22.8%; P=0.01) (Nastri et al. 2013). A more recent study included a larger proportion of first cycle participants (69.7%; 209 women), demonstrated a clinical pregnancy rate of 34% versus 38% in the biopsy and control groups, respectively (RR 0.895; 95% CI=0.661-1.211; P=0.548) (Yeung et al. 2014). The results of the study reported in this chapter are therefore in agreement with the findings of Yeung et al. (2014). Subgroup analysis correlating cycle rank with ART outcomes demonstrated that following an endometrial biopsy, women undergoing repeated cycles had a lower chance of conception compared to women in the control population (OR 0.886; 95%CI=0.799-0.983; P=0.022) (Yeung et al. 2014). This finding does not correlate with the findings of the current study, where pregnancy rates in women undergoing second or subsequent cycles were higher in the biopsy population. Another potential difference between the study by Yeung et al. (2014) and the current work is related to the fact that our participants did not undergo any uterine instrumentation within 3 months prior to ART. This could form a potential source of bias, as hysteroscopy (El-Toukhy et al. 2008) or even saline sonography could alter the endometrial milieu to a one favouring implantation.

In the current study, we have chosen to perform a single biopsy in the control population in the mid luteal phase of the cycle preceding ART. This approach has been taken by numerous previous studies with proven benefit (Barash et al. 2003, Zhou et al. 2008, Karimzadeh et al. 2009, Nastri et al. 2013) as timing closer to embryo transfer could be detrimental to the outcome of treatment. As demonstrated in the meta-analysis reported on page 131, single or multiple biopsies do not significantly impact the treatment outcome, hence single biopsy was performed to minimise the potential discomfort and necessity for additional visits.

The current study is the first one where frozen embryo replacement cycles have been included in the analysis. Previous works have only included fresh IVF or ICSI treatments. Low numbers of such participants (11 in the biopsy group and 3 in the control population) precludes

drawing conclusion on the effect of endometrial biopsy on FER cycle outcomes. Provided the timing of the procedure is temporally separate from ET, endometrial biopsy might have a beneficial effect on this type of treatment, potentially improving the cumulative pregnancy rates of couples with stored frozen embryos. Further work is required to support this statement. In the same study, an answer should be sought as to the necessity to perform a single biopsy prior to the first FER, or prior to every FER in order to maximally improve the chances of conception.

The limitations of this pilot study are related to missing the recruitment target for the reasons detailed in the results section. Prominent medial coverage of the endometrial biopsy as means of improving the chances of pregnancy has made recruitment to the current trial difficult and longer than expected. Lack of blinding can be perceived as a potential source of bias, however due to the nature of the procedure under investigation, this step was not possible. The use of different stimulation regimes (long versus short, fresh versus frozen) in the current study, could have contributed to the observed lack of benefit on pregnancy outcomes, however it does support the 'unselected' population nature of the current study. A recognised suppressor effect of hMG or FSH used in the stimulation regimes on the endometrium has been previously reported, which might have had a negative effect on endometrial development and receptivity (Ku et al. 2002, Chang et al. 2011), contributing to the observed results. This limitation however can only be bypassed in natural cycle FER treatments. Single centre setting of the current work, minimizes the confounding factors related to laboratory handling of gametes and clinicians experience. In future works, these factors, including the difficulty of embryo transfer, should also be covered.

Despite the mounting evidence related to endometrial injury and subsequent pregnancy outcomes, there is still insufficient data to support the routine use of this procedure in an unselected population of women. Our data, though small in numbers, contributes to the overall evidence. A recent Cochrane systematic review of evidence concluded that endometrial biopsy, if performed within one month of embryo transfer and up to day 7 of embryo transfer cycle, is associated with improved live

birth and clinical pregnancy rates in a population of women with failed two or more embryo transfers (OR 2.46; 95%CI 1.28-4.72 and OR 2.61; 95%CI 1.71-3.97, respectively) (Nastri et al. 2015). As the quality of evidence is moderate, further well designed studies are still necessary.

Guided by the Hippocratic '*primum no nocere'* motto, as clinicians we need to assure that any new procedures are safe and effective, and not just driven by semi-evidence or financial gains. Limited data presented in this chapter, indicates that endometrial biopsy does no harm. The beneficial effect though is still debatable. Further research is required to ascertain if the cause of infertility, previous obstetric history and type of treatment (IVF, ICSI, FER, or intrauterine insemination- IUI) can determine if the endometrial scratch will have a beneficial or detrimental effect on treatment outcome.

CHAPTER 6. Sonographic markers of endometrial receptivity

6.1. Introduction

Medical ultrasound is a safe and minimally invasive modality allowing for assessment of the structure and function of various organs. Since the introduction of US in gynaecology and infertility, various sonographic markers have been sought which would be useful as predictive for successful pregnancy.

The endometrium remains receptive to the embryo only in a narrow timeframe termed the window of implantation (WOI) (see page 39). Oestrogen and progesterone drive the cyclical changes in *zona functionalis* of the endometrium allowing for normal embryo-maternal communication. Proliferation of the endometrium and decidualization can be observed macroscopically using US. Normal progression of sonographic endometrial appearance follows a pattern described on page 58. In current ART practice, endometrial thickness, endometrial pattern, endometrial volume (EV), and vascular indices are used to differentiate a receptive from non-receptive endometrium. Both two-dimensional (2D) and three-dimensional (3D) ultrasound (US) has been shown to be a reliable and reproducible tool to describe the abovementioned parameters in the context of assisted reproductive treatment (ART) (Delisle et al. 1998, Raine-Fenning et al. 2002, Raine-Fenning 2004, Makker et al. 2006). Multiple studies assessing these markers in various infertile populations have provided often-contradictory evidence in terms of their respective predictive values. To date, there is no single sonographic factor that is unequivocally predictive of successful *in vitro* fertilization (IVF) treatment outcome (Heger et al. 2012, Kasius et al. 2014). This is related to different timing of the examination, use of varying US equipment, study populations and ART regimes.

In this chapter, the analysis of 2D and 3DUS markers has been performed in a population of infertile women undergoing IVF or intra-cytoplasmic sperm injection (ICSI) treatment at three set time points. This study design was used to prospectively assess sonographic changes in the endometrium during ART and correlate these with treatment outcome in hope of identifying patterns predictive of clinical pregnancy.

Assessment of the endometrial sonographic markers following endometrial biopsy was a secondary objective.

6.2. Materials and Methods

6.2.1. **Study design and participants**

The study was designed as a prospective observational study and was carried out as part of the Endoscratch study protocol. All participating women were screened and recruited from Nurture Fertility, Nottingham, from January 2012 to July 2014. Women <49 years of age with no major uterine anomalies and sufficient ovarian reserve (antral follicle count- AFC≥6) undergoing treatment using own oocytes in fresh or cryopreserved cycles were eligible. Women willing to take part in the study had given written consent prior to any procedure. The research ethics committee had approved the study protocol (12/EM/0345).

6.2.2. **Variables and ultrasound measurements**

Demographic data including age, duration of infertility, cause of infertility, past reproductive history, number of previous failed ART cycles, ovarian reserve markers (anti-Mullerian hormone- AMH and AFC), presence of uterine pathology, were recorded at recruitment. ART cycle details were recorded including, the type of treatment, dose of gonadotropins, duration of stimulation, number of oocytes collected, day and number of embryos transferred, grade of embryos transferred. ART outcomes included biochemical and clinical pregnancy rates, and first trimester miscarriage rates. Sonographic data was collected at three time points including the mid-luteal phase of the menstrual cycle directly preceding the ART cycle, directly before TVOR and directly before embryo transfer. The collected measurements included endometrial thickness, EV, endometrial pattern, MGS, 3DPD values, 3DHD values, and UA PWPD indices.

Ultrasound investigations and analyses of images were carried out as described in the Materials and Methods chapter (see page 79). The main outcome for this study was a clinical pregnancy, defined as presence of a fetal heart beat at the 6-week transvaginal ultrasound scan. First trimester miscarriage was a secondary outcome defined as absence of fetal cardiac activity following its prior visualization.

6.2.3. **Bias**

Inability to obtain or measure the sonographic data due to anatomical differences forms a potential source of bias.

6.2.4. **Study size**

Study size estimation is described on page 142, however no formal power calculations were conducted when designing the work reported in this chapter.

6.2.5. **Statistical analysis**

SPSS version 21 was used for statistical analyses. Normality of data was tested using Kolmogorov-Smirnov test. Data normally distributed was analysed using independent student's t test. Mann-Whitney U test was used for not normally distributed data. When paired samples were considered, student's t test was used for parametric data and Wilcox signed rank tests was used for non-parametric data. Changes across time were analysed using repeated measures ANOVA. Greenhouse-Geisser correction (ε) was used for data with minimal violation of sphericity and MANOVA was used for data with significant violation of sphericity. For comparing more than three groups, the data were analysed using ANOVA test, followed by the *t*-test with Bonferroni adjustment. Corrected binary logistic regression was used to assess the effect of known variables on the outcome of ART. Pearson's r was used to correlate vascularity indices obtained using two different methods. Statistical significance for all tests was assumed when $P<0.05$.

ROC curve analysis with AUC and appropriate sensitivity and specificity values were used to determine the predictive value of each vascularity index for outcomes specified.

6.3. Results

6.3.1. **Participants**

Between January 2012 and July 2014, 151 women were recruited: 76 in the biopsy group and 75 in the control group of the Endoscratch study. Baseline demographics of study participants are presented in Table 4 on page 144. Twenty-one women did not attend for the initial mid-luteal scan appointment prior to ART cycle and were excluded from analysis.

A positive LH surge was detected by 66 women. In these cases, the ultrasound scan was carried out at LH+7 (7-8) days. If LH surge was not detected, the scan was carried out on day 21 (21-22) of the menstrual cycle directly preceding ART.

6.3.2. **Mid-luteal sonographic appearance of the endometrium**

Presence of fluid in the endometrial cavity in one case and an axial uterus in six women precluded 3D endometrial analysis in these participants.

The endometrial cavity shape based on 3D reconstruction was defined as normal in 114 women (75.5%), arcuate in 36 (23.8%) and T-shaped in one woman (0.7%). Adenomyosis defined as disruption of EMJ, discrepancy in thickness of anterior and posterior uterine wall, presence of subendometrial inclusion cysts or parallel shadowing was present in 34 women (22.5%). Unequivocal diagnosis of adenomyosis was not possible in 3 cases (2.0%).

Sonographic details are presented in Table 9.

6.3.3. **Mid-luteal endometrium in pregnant and non-pregnant women.**

All assessed mid-luteal endometrial markers of receptivity did not differ between women that achieved and did not achieve a clinical pregnancy, and between the ones that experienced a first trimester miscarriage and had an on-going pregnancy (P>0.05) (see Table 10).

Variable		***Value (N=145)***
Endometrial thickness (mm)		8.4(7.25-10.1)
Endometrial pattern	*Triple layer*	15(11.3%)
	Intermediate	82(61.7%)
	Homogenous	36(27.1%)
Endometrial volume (cm^3)		2.36(1.69-3.41)
MGS		44.3(±7.44)
3DVI		1.61(0.71-3.24)
3DFI		27.0(±3.85)
3DVFI		0.43(0.19-0.92)
3DHDVI		2.29(1.0-4.55)
3DHDFI		52.58(±4.76)
Average UA Vmax (cm/s)		32.25(±9.34)
Average UA Vmin (cm/s)		4.16(1.96-5.78)
Average UA RI		0.87(0.83-0.94)
Average UA PI		2.53(2.16-3.28)
Average UA S/D		7.0(5.41-13.7)

Table 9. Sonographic data in the mid luteal phase of the cycle in the biopsy and control populations, combined.

*Data presented as mean(±SD) or median(interquartile range) as appropriate; *P<0.05. MGS- mean grey scale; VI- vascularisation index; FI- flow index; VFI- vascularisation flow index; UA- uterine artery; Vmax- maximal systolic velocity; Vmin- minimal diastolic velocity; RI- resistance index; PI- pulsatility index; S/D- systole to diastole ratio.*

6.3.4. **Endometrium at TVOR in biopsy and control groups**

In total, 99 datasets were analysed. Fluid in cavity precluded analysis in two women; images were not acquired in 16 women due to patients being late for procedure or technical issues; 16 images were not analysed due to suboptimal response to stimulation or risk of OHSS and subsequent cycle cancellation (no outcome).

Triple layer appearance of the endometrium was present in 29 women (29.3%), intermediate in 53 (53.5%) and hyperechogenic in 17 (17.2%). The endometrial pattern in the biopsy group, compared to the control group, more often had a triple layer appearance (38.8% versus 20%, respectively; P=0.03). Other sonographic variables did not differ significantly between groups (see Table 11).

Variable	***Positive group (N=66)***	***Negative group (N=47)***
Endometrial thickness (mm)	8.52(±2.72)	8.76(±2.53)
Endometrial volume (cm3)	2.24(1.58-3.29)	2.42(1.7-3.54)
MGS	44.42(±7.88)	44.03(±7.39)
3DVI	1.87(0.75-3.1)	1.36(0.57-3.35)
3DFI	26.92(±3.69)	26.79(±4.14)
3DVFI	0.47(0.19-0.89)	0.32(0.15-0.96)
3DHDVI	2.76(1.44-4.38)	2.08(0.77-4.7)
3DHDFI	52.15(49.46-54.53)	52.98(49.43-56.89)
Average UA Vmax (cm/s)	32.03(±9.17)	31.9(±9.19)
Average UA Vmin (cm/s)	4.12(±2.53)	4.27(±3.45)
Average UA RI	0.88(±0.07)	0.88(±0.07)
Average UA PI	2.66(±0.81)	2.81(±0.85)
Average UA S/D	6.74(5.46-14.65)	7.05(5.68-12.74)
Progesterone (ng/mL)	13.17(±11.75)	14.41(±12.34)
Oestradiol (pmol/L)	424.0(333.0-598.0)	552.0(344.5-670.3)

Table 10. Sonographic data in the mid-luteal phase of the cycle in the populations with and without clinical pregnancy.

*Data presented as mean(±SD) or median(interquartile range) as appropriate; *P<0.05.*

6.3.5. **Endometrium at TVOR in pregnant and non-pregnant women**

Women with a clinical pregnancy were more likely to have a triple pattern endometrium compared to women that did not have a viable intrauterine pregnancy (P=0.034). All other assessed endometrial markers at TVOR

did not differ between women that achieved and did not achieve a clinical pregnancy, or between the populations with or without a first trimester miscarriage (P>0.05) (see Table 12).

Variable	***Biopsy group (N=49)***	***Control group (N=50)***
Endometrial thickness (mm)	9.73(±3.21)	10.03(±2.64)
Endometrial volume (cm3)	3.64(2.27-4.89)	2.97(1.88-4.59)
MGS	42.74(±7.3)	44.88(±6.71)
3DVI	1.27(0.58-3.37)	1.8(0.86-4.18)
3DFI	26.88(±4.17)	27.98(±3.66)
3DVFI	0.37(0.14-1.01)	0.47(0.23-1.24)
3DHDVI	2.19(1.15-3.81)	2.73(1.16-5.49)
3DHDFI	52.16(48.84-54.81)	52.24(49.83-55.64)
Average UA Vmax (cm/s)	34.3(±10.07)	36.6(±9.68)
Average UA Vmin (cm/s)	4.91(±2.85)	5.23(±2.39)
Average UA RI	0.87(±0.09)	0.86(±0.06)
Average UA PI	2.32(±0.5)	2.32(±0.42)
Average UA S/D	6.96(5.18-9.27)	7.16(5.8-8.27)

Table 11. Sonographic data at TVOR in the biopsy and control populations.

*Data presented as mean(±SD) or median(interquartile range) as appropriate; *P<0.05.*

6.3.6. **Endometrium at embryo transfer in control and biopsy groups**

In total, 95 datasets were analysed. 22 uteri were not analysable due to axial position or significant displacement of the uterus by enlarged ovaries. Images were not acquired in 27 women due to patients being late for procedure or technical problems; 16 images were not analysed due to suboptimal response to stimulation or risk of OHSS and no clinical outcome.

All analysed sonographic parameters did not differ between biopsy and control groups (P>0.05) (see Table 13).

Variable	***Positive group (N=53)***	***Negative group (N=39)***
Endometrial thickness (mm)	9.7(±2.97)	10.15(±2.87)
Endometrial volume (cm3)	3.52(2.01-5.03)	3.26(2.16-4.2)
MGS	43.96(±7.14)	43.64(±7.02)
3DVI	1.57(0.82-3.07)	1.92(0.78-4.61)
3DFI	27.25(±3.	27.69(±4.63)
3DVFI	0.42(0.2-0.88)	0.53(0.2-1.41)
3DHDVI	1.98(1.04-3.37)	3.11(1.29-5.78)
3DHDFI	52.52(48.53-54.83)	52.13(49.55-55.22)
Average UA Vmax (cm/s)	35.5(±9.84)	35.36(±10.1)
Average UA Vmin (cm/s)	4.81(±2.74)	5.43(±2.45)
Average UA RI	0.88(±0.09)	0.85(±0.06)
Average UA PI	2.41(±0.52)	2.19(±0.35)
Average UA S/D	7.17(5.24-10.65)	6.29(5.13-8.02)
Progesterone (ng/mL)	8.76(±4.85)	12.69(±14.75)
Oestradiol (pmol/L)	3288.0(2228.0-5204.3)	3548.0(2499.0-4933.0)

Table 12. Sonographic data at TVOR in the populations with and without a clinical pregnancy.

*Data presented as mean(±SD) or median(interquartile range) as appropriate; *P<0.05.*

6.3.7. **Endometrium at embryo transfer in pregnant and non-pregnant women**

All assessed endometrial markers prior to embryo replacement did not differ between women that achieved and did not achieve a clinical pregnancy, and the populations that had and did not have a first trimester miscarriage (P>0.05). The oestradiol and progesterone levels did not differ between the populations (P>0.05) (see Table 14).

Variable		***Biopsy group (N=49)***	***Control group (N=37)***
Endometrial thickness (mm)		10.9(±2.92)	10.6(±3.42)
Endometrial pattern	*Triple layer*	1(1.8%)	1(1.9%)
	Intermediate	32(58.2%)	34(63%)
	Homogenous	22(40%)	19(35.1%)
Endometrial volume (cm3)		3.56(2.48-5.08)	3.3(2.37-5.33)
MGS		43.77(±8.41)	43.47(±8.46)
3DVI		1.59(0.58-3.16)	0.96(0.37-3.56)
3DFI		26.46(±5.64)	26.45(±6.34)
3DVFI		0.41(0.15-0.91)	0.25(0.09-1.14)
3DHDVI		3.05(0.75-5.44)	1.85(0.85-3.79)
3DHDFI		53.78(±5.15)	51.9(±6.1)
Average UA Vmax (cm/s)		39.66(±10.55)	37.3(±11.81)
Average UA Vmin (cm/s)		8.29(±3.65)	7.19(±3.57)
Average UA RI		0.79(±0.09)	0.81(±0.07)
Average UA PI		1.83(1.6-2.21)	2.0(1.6-2.27)
Average UA S/D		4.67(3.84-5.62)	4.86(4.09-6.42)

Table 13. Sonographic data at embryo transfer in the biopsy and control populations.

*Data presented as mean(±SD) or median(interquartile range) as appropriate; *P<0.05.*

Variable	*Positive group (N=54)*	*Negative group (N=32)*
Endometrial thickness (mm)	10.82(±3.17)	10.64(±3.2)
Endometrial volume (cm3)	3.08(2.27-5.33)	4.03(2.56-5.09)
MGS	42.96(±8.92)	44.7(±7.48)
3DVI	1.3(0.34-3.43)	1.13(0.5-3.0)
3DFI	26.54(±5.95)	26.32(±5.94)
3DVFI	0.41(0.08-1.05)	0.3(0.1-0.87)
3DHDVI	*2.71(0.71-4.91)*	1.95(0.85-4.13)
3DHDFI	54.58(50.62-56.99)	52.12(47.53-55.39)
Average UA Vmax (cm/s)	39.02(±10.92)	37.73(±11.67)
Average UA Vmin (cm/s)	8.07(±3.41)	7.28(±3.98)
Average UA RI	0.79(±0.08)	0.81(±0.08)
Average UA PI	1.93(±0.48)	2.18(±0.87)
Average UA S/D	4.71(4.07-6.23)	4.86(3.82-6.38)
Progesterone (ng/mL)	119.65(±59.39)	117.69(±77.91)
Oestradiol (pmol/L)	4412(2217-6333)	3913.5(2213.75-7409)

Table 14. Sonographic data at embryo transfer in the populations with and without a clinical pregnancy.

*Data presented as mean(±SD) or median(interquartile range) as appropriate; *P<0.05.*

6.3.8. **Effect of endometrial biopsy on endometrial appearance over time**

Overall, endometrial thickness increases significantly between the mid-luteal phase and TVOR (P<0.001), and TVOR and embryo transfer (P=0.019). EV follows a similar pattern with a significant increase between the mid-luteal phase and TVOR (P<0.001). Subsequently, the increase in EV slows down towards embryo transfer (P>0.05) (see Figure 32). No significant differences in ET and EV value progression between the biopsy and the control groups were observed (P>0.05) (see Figure 33).

Endometrial pattern differs between the three analysed time points with the endometrium being more frequently triple layer at TVOR compared to the mid-luteal phase and embryo transfer (P<0.001). Overall, there was no difference in the endometrial pattern progression between the biopsy and control groups at the assessed time points (P>0.05). MGS did not differ overall or per group at all the assessed time points (P>0.05).

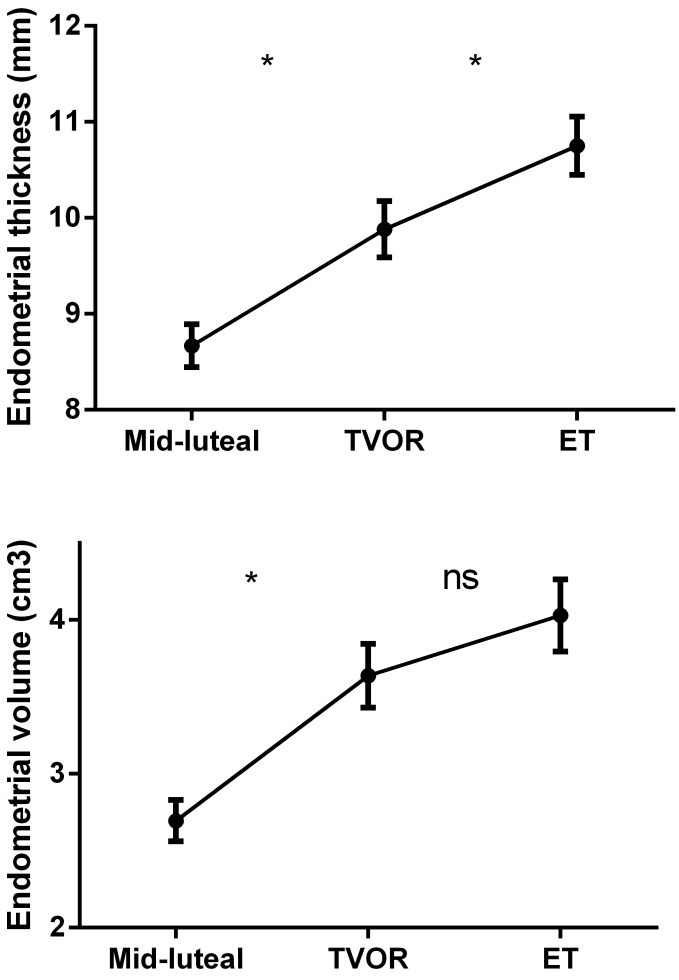


Figure 32. Endometrial thickness and volume at assessed time points.

*Data presented as mean ±SEM. TVOR- transvaginal oocyte retrieval; ET- embryo transfer; ns- non significant for overall effect; * P<0.05.*

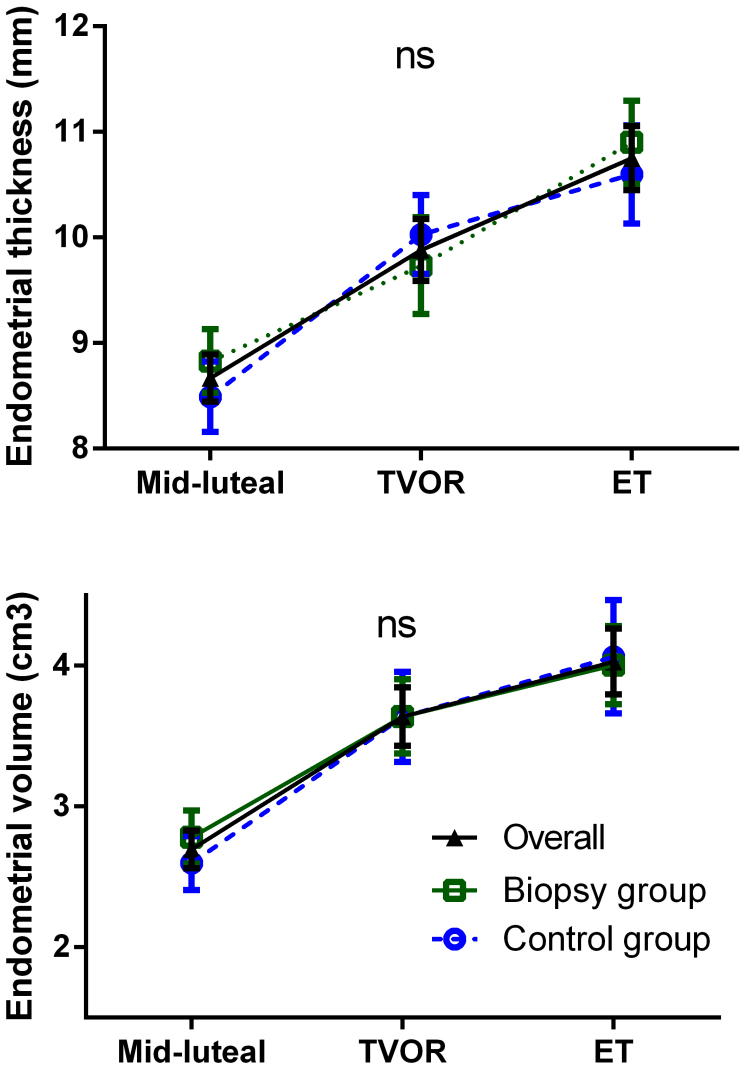


Figure 33. Endometrial thickness and volume at assessed time points in the biopsy and control groups.

Data presented as mean ±SEM. TVOR- transvaginal oocyte retrieval; ET- embryo transfer; ns- non significant for overall effect.

The overall endometrial vascularity, as examined by 3DPD, varied between the three time points with only FI being significantly less between TVOR and embryo transfer (P<0.001). There were no significant differences between the biopsy and control groups at the time points assessed, though the VI and VFI appeared to be lower in the biopsy group (P>0.05) (see Figure 34).

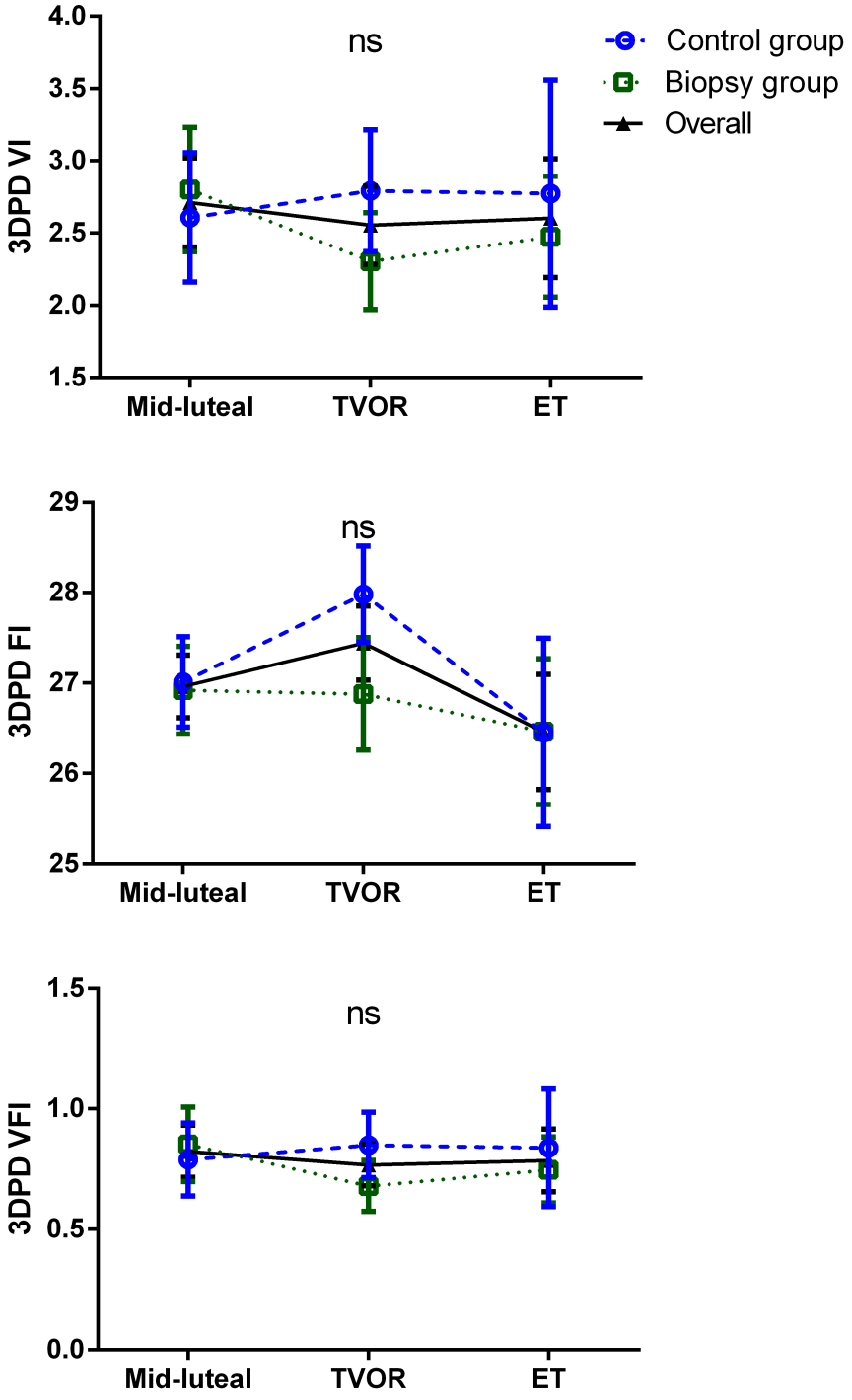


Figure 34. 3DPD endometrial vascularity at assessed time points.

Data presented as mean ±SEM. TVOR- transvaginal oocyte retrieval; ET- embryo transfer; ns- non significant.

Exploration of endometrial vascularity using high definition PD imaging, has demonstrated a relative decrease in Doppler signal intensity at TVOR and a subsequent rise in the VI and the FI indices (P>0.05). At embryo

transfer, the 3DHD FI was found to be significantly lower in the control arm of the study (P=0.027), with no such difference in the 3DHD VI (P>0.05). ANOVA analysis of the changes of vascularity indices between the control and biopsy groups has failed to demonstrate statistical significance (P>0.05; see Figure 35). This pattern of vascular change progression appears to differ from the one observed when using 3DPD indices.

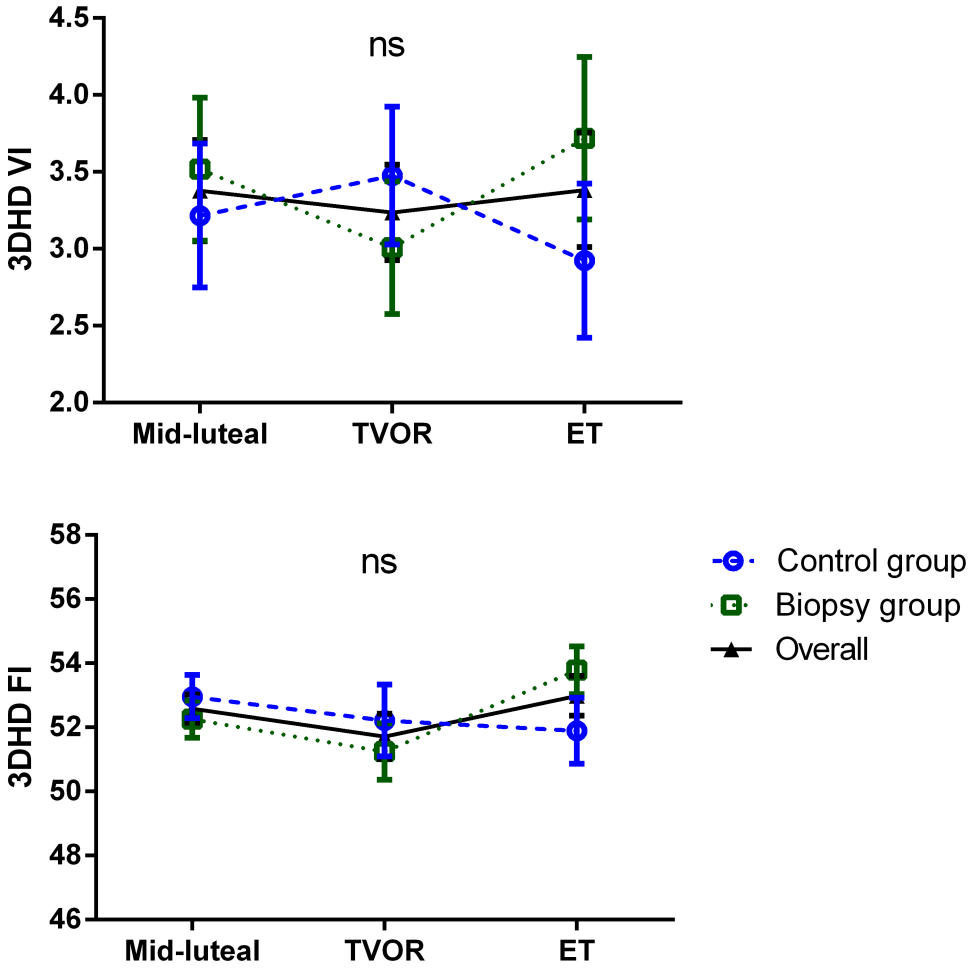


Figure 35. 3D high definition endometrial vascularity at assessed time points.

Data presented as mean ±SEM. TVOR- transvaginal oocyte retrieval; ET- embryo transfer; ns- non significant.

Comparison of VI values obtained using 3DPD and 3DHD has demonstrated significant correlation in the mid-luteal phase, at TVOR and at embryo transfer (P<0.01). FI values have been only correlated at

embryo transfer (P<0.01) (see Figure 36). Overall, the vascularity indices obtained using 3DHD modality, were significantly higher compared to same indices obtained using 3DPD modality (P<0.001), which indicates that the modality is more sensitive to low velocity, low intensity blood flow.

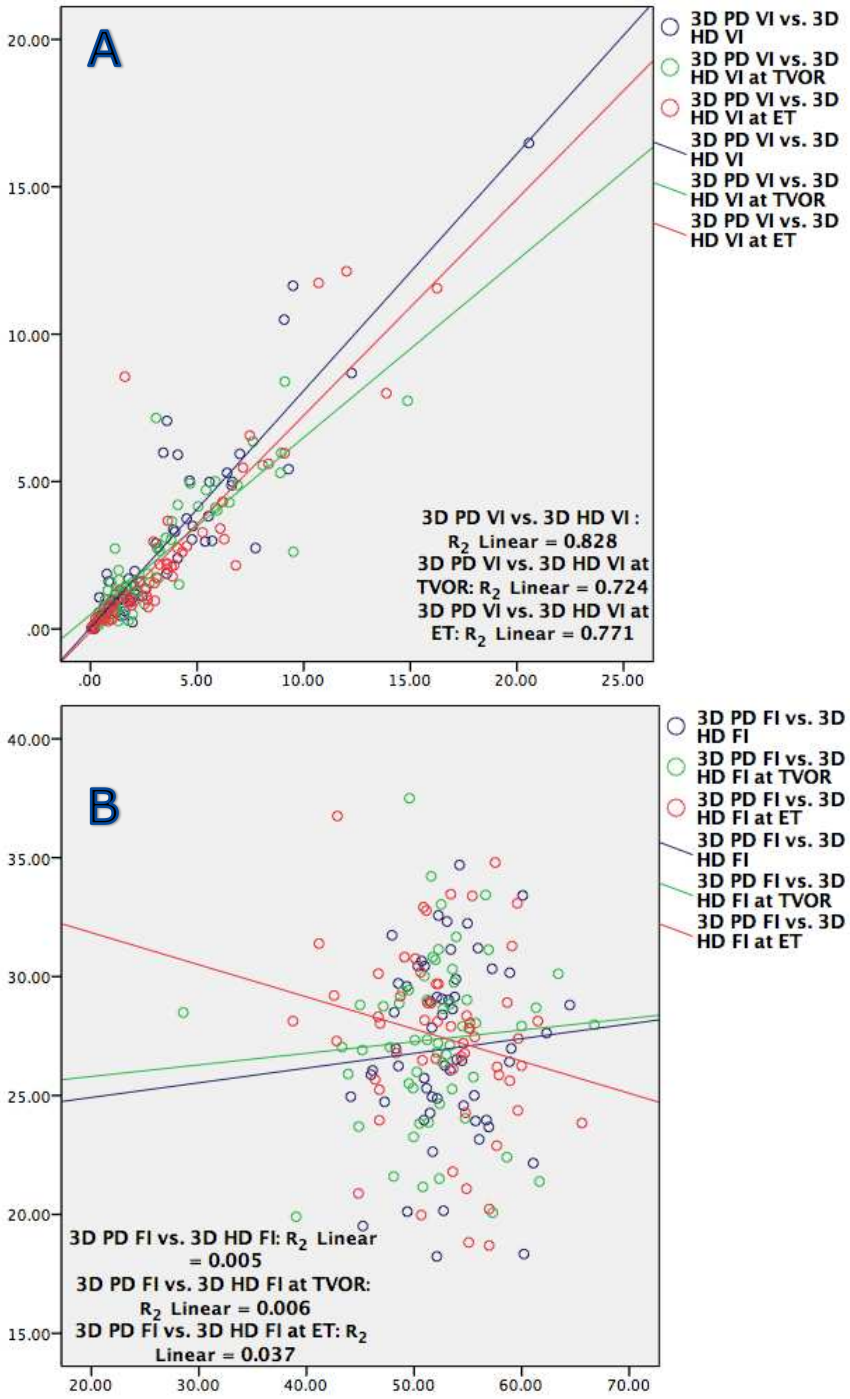


Figure 36. Correlation between 3DPD and 3DHD VI (A) and FI (B) in the mid-luteal phase, at TVOR and at embryo transfer (ET).

Average UA minimal and maximal blood flow velocities increased towards embryo transfer (P=0.031 and P<0.001, respectively), however the S/D ratio did not differ (P>0.05). The PI was significantly different between all time points and was decreasing towards embryo transfer (P=0.032). The RI was only significantly different between TVOR and embryo transfer, with a significant drop at embryo transfer (P<0.001).

Between the control and biopsy groups, the only significantly different index was a UA Vmax between TVOR and embryo transfer, with Vmax being significantly higher in the biopsy group (P<0.05) (see Figure 37).

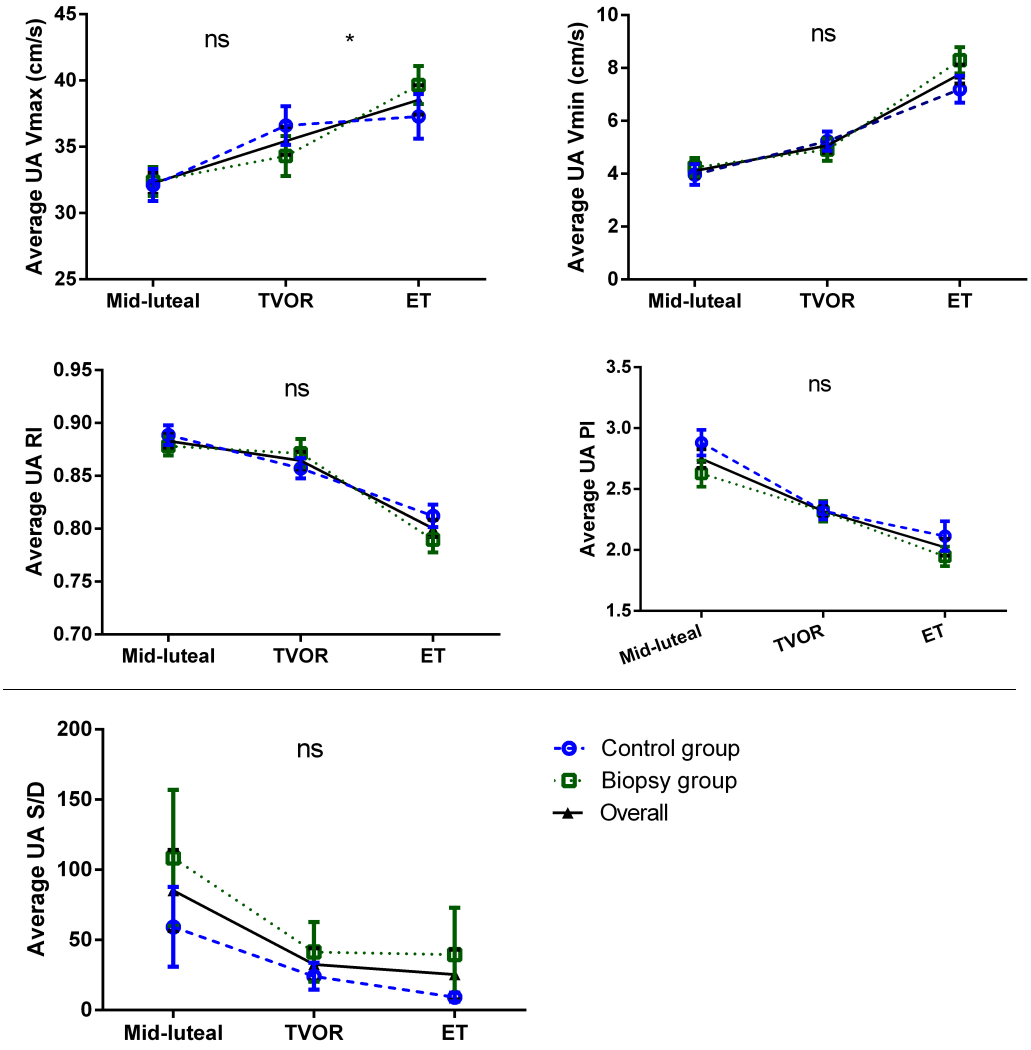


Figure 37. UA PWPD vascularity indices at assessed time points.

*Data presented as mean ±SEM. TVOR- transvaginal oocyte retrieval; ET- embryo transfer; ns- non significant; * P<0.05.*

6.3.9. **Sonographic changes of the endometrium in cycles with a clinical pregnancy over time**

Endometrial thickness progression did not different between the groups that achieved and did not achieve a clinical pregnancy (P>0.05). The endometrial volume did not differ in the mid-luteal phase and at TVOR in the successful and unsuccessful cycles (P>0.05), however at embryo transfer the group that achieved a clinical pregnancy had a significantly lower EV compared to the control (3.87cm^3 vs. 4.28cm^3;P=0.044) (see Figure 38).

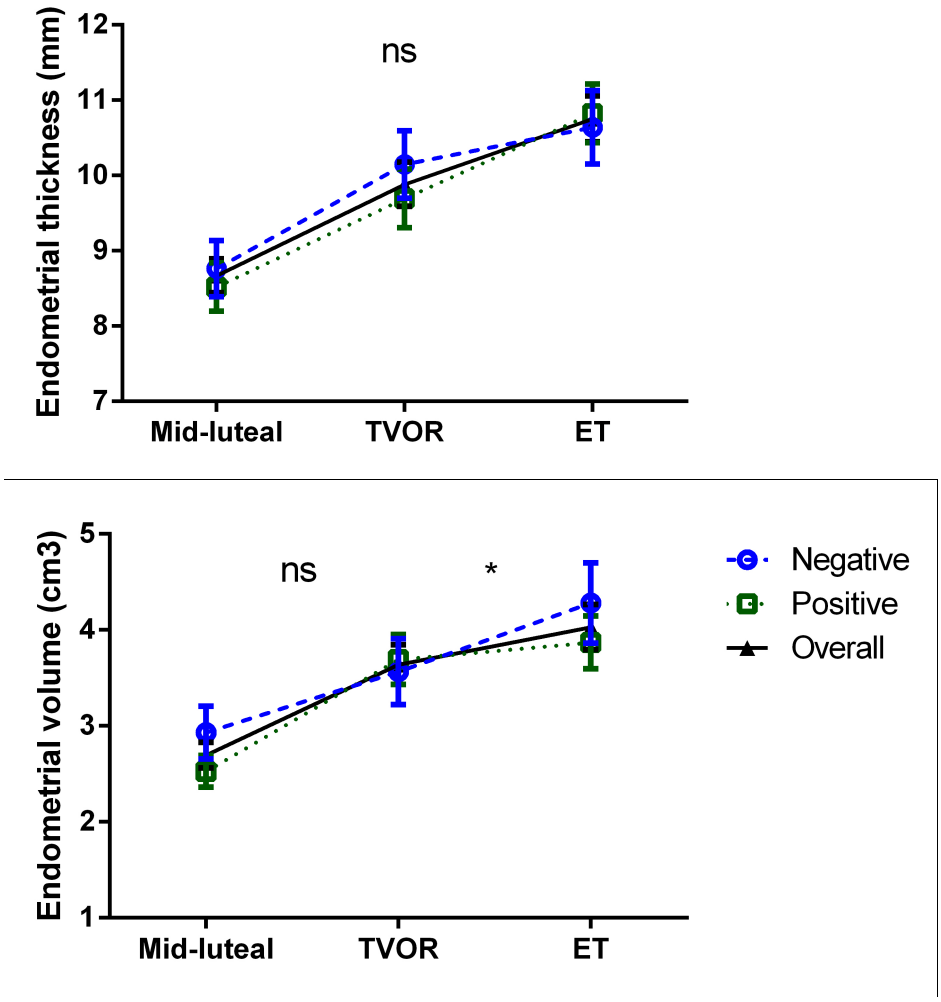


Figure 38. Endometrial thickness and volume at assessed time points in cycles with and without a clinical pregnancy.

*Data presented as mean ±SEM. TVOR- transvaginal oocyte retrieval; ET- embryo transfer; ns- non significant; * P<0.05.*

Triple layer and intermediate endometrium was more prevalent at TVOR in the group that achieved a clinical pregnancy compared to the negative group (P=0.043). There were no significant differences in the pattern in the mid luteal phase and at embryo transfer (P>0.05) (see Figure 39).

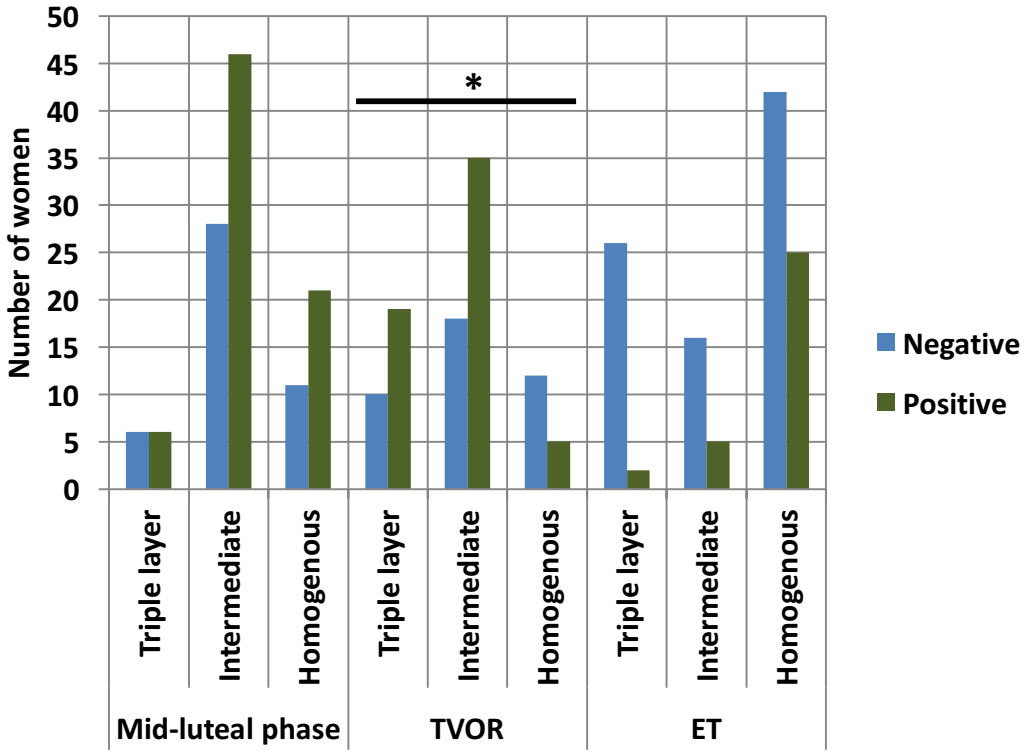


Figure 39. Endometrial patterns in cycles with and without a clinical pregnancy.

*Data presented as absolute values. TVOR- transvaginal oocyte retrieval; ET- embryo transfer; * P<0.05.*

MGS, 3DPD and 3DHD vascularity indices did not differ at any of the time points between the successful and negative groups (P>0.05). Average UA Vmax, Vmin, PI, and S/D indices did not differ significantly between the two populations. The average UA RI was lower in the successful population at embryo transfer compared to the negative group, however statistical significance was not achieved (P=0.082) (see Table 10, Table 12, Table 14 and Figure 40). Contemporaneous hormonal changes between the pregnant and non-pregnant population have shown no difference in levels of oestradiol and progesterone at any of the given

time points (P>0.05) (see Figure 41). 3DPD and HDPD changes over time did not differ between the pregnant and non-pregnant populations (P>0.05) (see Figure 42).

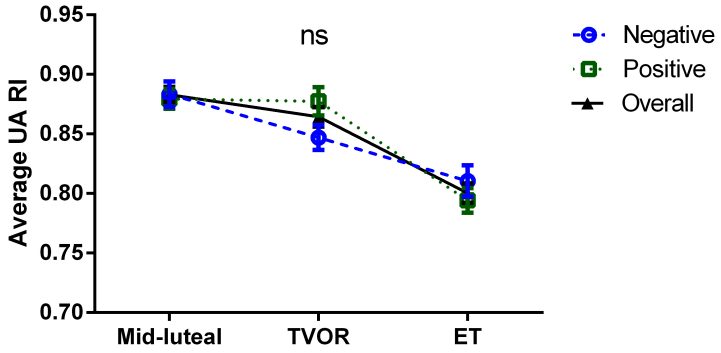


Figure 40. Average UA RI at assessed time points in cycles with and without a clinical pregnancy.

Data presented as absolute values. TVOR- transvaginal oocyte retrieval; ET- embryo transfer; ns- non significant.

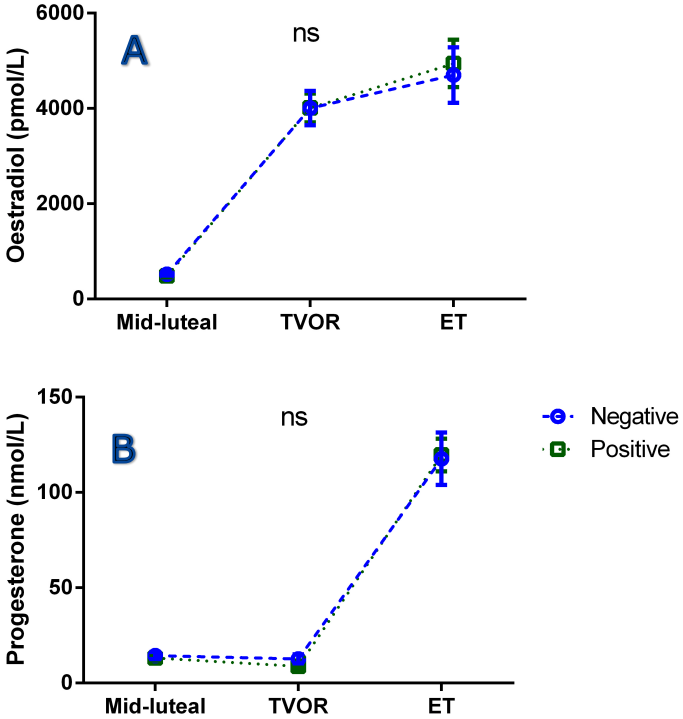


Figure 41. Oestradiol (A) and progesterone (B) level changes over time in the pregnant and non-pregnant populations.

Data presented as absolute values. TVOR- transvaginal oocyte retrieval; ET- embryo transfer, ns- non significant.

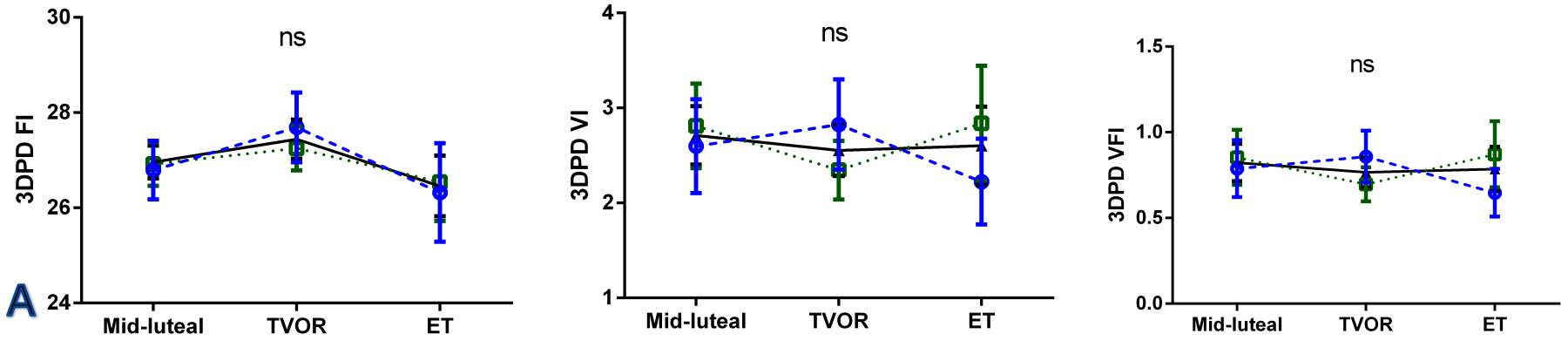
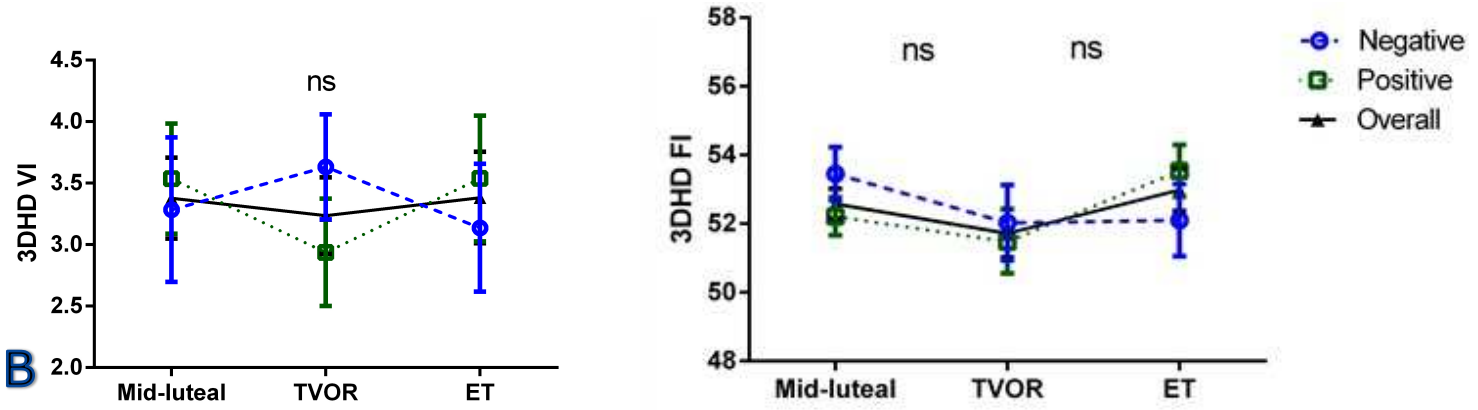


Figure 42. Endometrial vascularity using 3DPD (A) and HDPD (B) at assessed time points.

*Data presented as mean ±SEM. TVOR- transvaginal oocyte retrieval; ET- embryo transfer; ns- non significant; * P<0.05.*

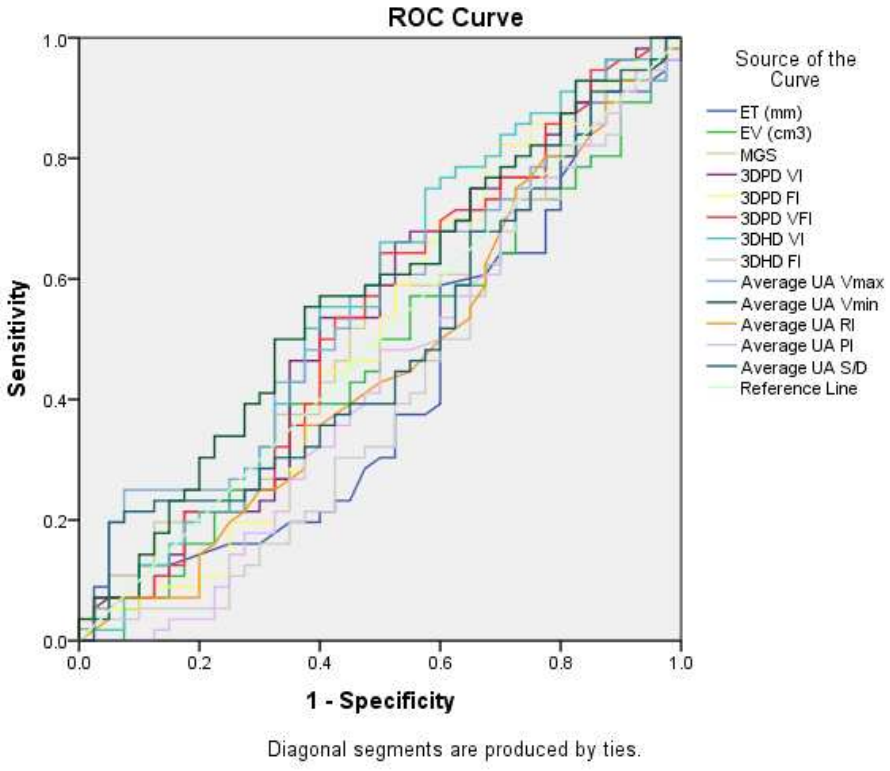


6.3.10. **Sonographic markers and clinical pregnancy**

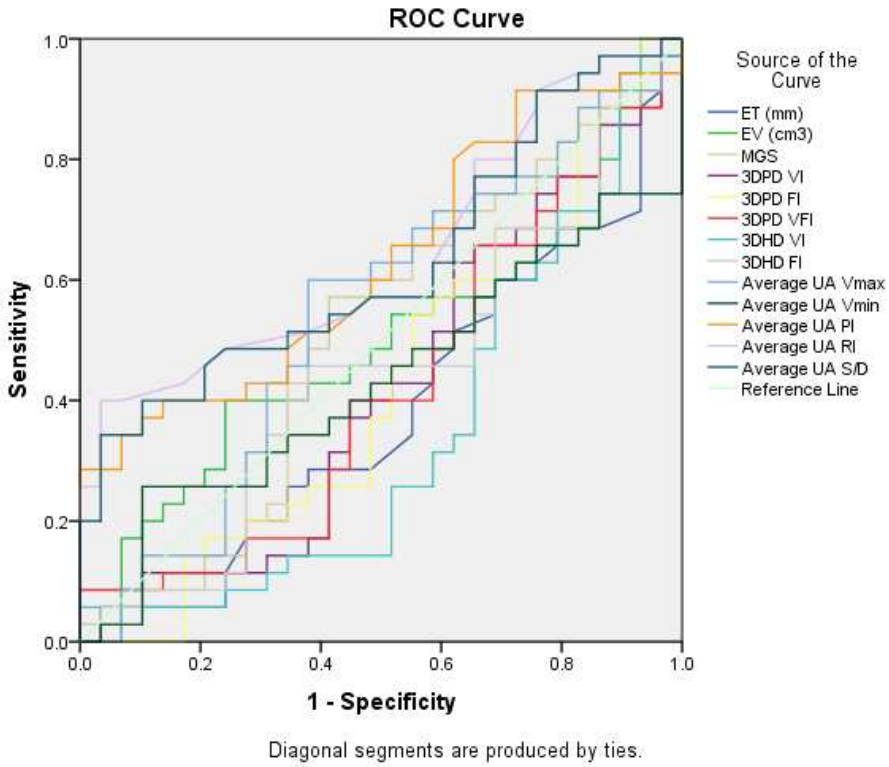
Of all the analysed sonographic markers, the best predictors for a clinical pregnancy following ART, were UA blood flow indices at TVOR, with the RI having the highest AUC of 0.645, followed by PI (AUC 0.632) and S/D (AUC 0.629) (see Table 15). All other indices at any given time point have proven worse predictors of clinical pregnancy (see Figure 43).

Marker	***AUC***	***Value***	***Sensitivity (%)***	***Specificity (%)***
Average UA PI at TVOR	0.632	2.16	87.5	41.1
Average UA RI at TVOR	0.645	0.83	87.5	32.1
Average UA S/D at TVOR	0.629	6.22	87.5	41.1

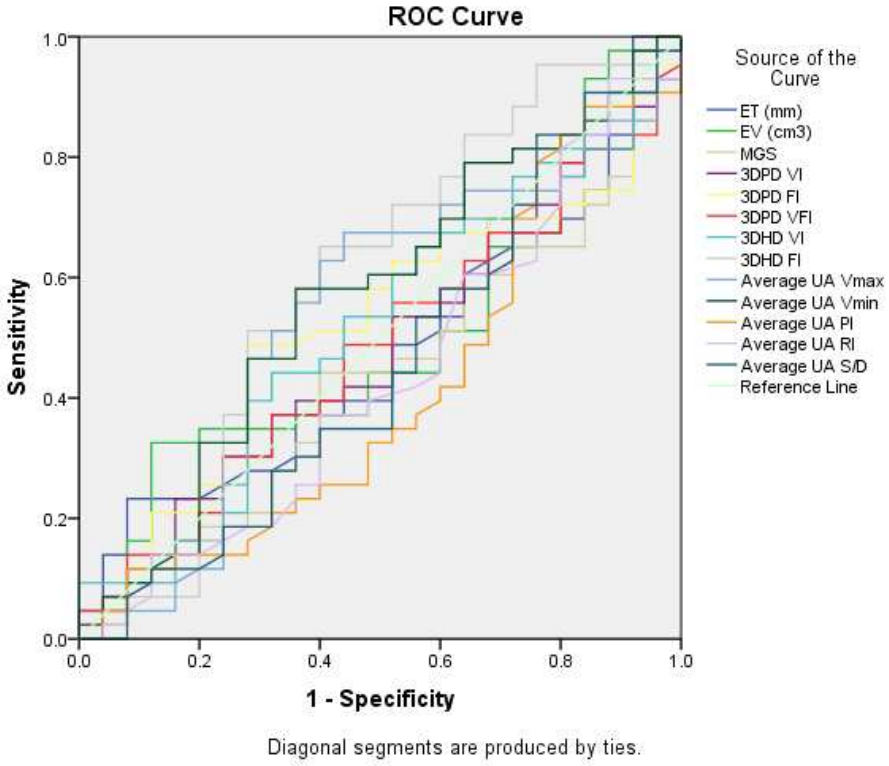
Table 15. Sensitivities and specificities for prediction of clinical pregnancy.



A



B



C

Figure 43. ROC curves for sonographic markers and clinical pregnancy (A- mid-luteal phase; B- TVOR; C- embryo transfer).

The best predictor of a first trimester miscarriage was MGS at TVOR with an AUC of 0.810. Average UA S/D in the mid-luteal phase with an AUC of 0.621, average UA PI at TVOR (AUC 0.673), average UA RI at TVOR (AUC 0.653), average UA S/D at TVOR (AUC 0.680), endometrial thickness at embryo transfer (AUC 0.613), and average UA RI at embryo transfer (AUC 0.601) were the other markers with some predictive value for first trimester miscarriage (see Table 16).

Marker	***AUC***	***Value***	***Sensitivity (%)***	***Specificity (%)***
Average UA S/D (mid-luteal phase)	0.621	5.69	84.6	26.5
MGS at TVOR	0.810	43.73	87.5	58.9
Average UA PI at TVOR	0.673	2.16	87.5	41.1
Average UA RI at TVOR	0.653	0.83	87.5	32.1
Average UA S/D at TVOR	0.680	6.2225	87.5	41.1
Endometrial thickness at embryo transfer (mm)	0.613	10.55	83.3	53.2
Average UA RI at embryo transfer	0.601	0.78	83.3	40.3

Table 16. Sensitivities and specificities for prediction of first trimester miscarriage.

6.4. Discussion

The overall findings of this study suggest a temporal increase of endometrial thickness and volume from the mid-luteal phase of a non-transfer cycle, through TVOR to embryo transfer. Triple layer endometrial pattern at TVOR was found to be associated with endometrial biopsy and subsequent successful ART outcome ($P<0.05$). UA RI at TVOR of 0.83 was the strongest predictor of clinical pregnancy with an AUC of 0.645 (sensitivity 87.5%, specificity 32.1%), with other 2D and 3D markers demonstrating worse performance. Apart from higher prevalence of a triple layer endometrium at TVOR, endometrial biopsy had no effect on any other sonographic markers of endometrial function and vascularity.

The results of our study, do not clarify the already complicated area of ultrasonography as a predictive tool for ART outcome. In agreement with other studies, UA blood flow parameters did not differ between the pregnant and non-pregnant populations (Dickey 1997, Schild et al. 2001, Kim et al. 2014). Endometrial thickness and volume were not significantly different between the populations in agreement with other authors (Kim et al. 2014). Unlike other reports where the threshold for successful pregnancy was estimated to be at 2-3.3 cm^3 (Raga et al. 1999), we were unable to determine a cut-off value, which with a good sensitivity and specificity, would be predictive of successful outcome.

In this study, we have observed similarities in endometrial pattern between the biopsy group and the group that achieved a clinical pregnancy. This might be related to the postulated effect of a delay in endometrial development following an endometrial biopsy in the form of a more prevalent triple pattern at TVOR, which allows synchrony with the embryo (Bourgain et al. 2003). A similar finding of more prevalent triple pattern endometrium following endometrial biopsy was observed by Wang et al. (Wang et al. 2012). Presence of homogenous endometrium at TVOR may conversely be associated with premature luteinisation and as such, precludes embryo implantation (Detti et al. 2011).

Uterine blood flow in our participants did not differ between the pregnant and non-pregnant women. When considering absolute values, UA RI, PI

and S/D indices were marginally higher in the pregnant population in the mid luteal phase of the cycle (P>0.05). This relationship was reversed at the time of embryo transfer with no statistical significance. In our population, in both pregnant and non-pregnant women, the average UA PI was below the optimum value (≥3) indicating good prognosis (Haapsamo et al. 2009, Zollner et al. 2012), however the usefulness of this index as predictor of successful outcome was found to be at best moderate (AUC<0.6). Similarly, all other UA PWPD indices were inadequate as sole predictors of ART outcome.

Analysing the temporal progression of vascularity indices, we have demonstrated a drop in the VI and VFI values between the mid-luteal phase and TVOR, with a subsequent rise at embryo transfer. Taking into consideration the outcome (clinical pregnancy), the group achieving clinical pregnancy had a higher mid-luteal VI and VFI than the negative population, subsequently getting less at TVOR, with an increase at embryo transfer (P>0.05, see Figure 39). This is similar to a description of natural cycle vascularity progression, when endometrial blood flow reaches a peak approximately 3 days before ovulation and a nadir 5 days after ovulation (Raine-Fenning et al. 2004d). The observed results might indicate, that the mid-luteal phase of the cycle is a useful starting point to assess blood flow progression and determine if subsequent transfer of poor quality embryos was likely to lead to a clinical pregnancy. If a poor embryo had developed and suboptimal vascularity was detected, an option to freeze all of the embryos with a subsequent replacement in a natural cycle could be considered. However, one would have to bear in mind the poor survival rate of suboptimal quality embryos in these circumstances. Such an approach requires larger studies with determination of endometrial vascularity normograms.

In contrast to a recent study employing the same data collection and analysis methodology, we were unable to demonstrate significant differences when assessing the endometrial vascularity parameters at embryo transfer. Kim et al. (2014) found significantly lower VI, FI and VFI index values in the non-pregnant group. In the abovementioned study, the AUC and respective predictive value for clinical pregnancy

outperformed our results with a cut-off for VI of 0.95 (AUC 0.76, 71.7% sensitivity and 68.9% specificity, P<0.001), FI of 12.94 (AUC 0.727, sensitivity 72.5%, specificity 68.2%, P<0.001) and VFI of 0.15 (AUC 0.790, sensitivity 65.4%, specificity 72.8%, P<0.001). In our population the highest AUC was obtained for endometrial FI (AUC 0.537), with respective vascularity indices marginally less in the pregnant population (see Table 11). The fact that Kim et al. used only fresh cycle and long GnRH agonist protocols might be the obvious cause of the observed differences (Kim et al. 2014). Mercee et al. (2008) has found significantly higher VI, FI and VFI values at hCG administration in 80 infertile women undergoing IVF/ICSI, with a FI≥26.1 predictive of pregnancy with a sensitivity of 85.7% and a false-positive rate of 27.6% (Merce et al. 2008). As the timing of scanning was different compared to our study, this could significantly contribute to the observed discrepancies.

In this study, we have also employed the high definition (HD) flow imaging which has better axial resolution, improved sensitivity to small vessel blood flow and fewer blooming artefacts compared to standard PD (Kim et al. 2008). Physical differences between the methods include different Doppler pulses with HD modality using short broadband US pulses, and PD narrowband long pulses (Hata 2006). In agreement with reported data (Alcazar et al. 2010), our HD indices were higher compared to PD indices. Though more sensitive and theoretically able to detect finer vascularity details, HD modality did not outperform standard 3DPD modality in predicting clinical pregnancy. We did however observe that patients following an endometrial biopsy and with a clinical pregnancy had a similar HDVI and HDFI progression (see Figure 32 and Figure 39). One can speculate, that the endometrial biopsy influences the very small vascular beds within the endometrium causing detectable changes. These can only be detected however by the more sensitive to low blood flow 3DHD modality. No evidence up to date is able to substantiate this finding. It is debatable if the observed changes are related directly to the effect of the endometrial injury and subsequent molecular and cellular changes, or the endometrium determines the outcome of the pregnancy

at the onset of treatment. The latter would support the notion that the endometrial component acts as a gatekeeper of implantation.

The selection of the time points for investigation, though arbitrary, was chosen for two reasons. First- to minimize additional visits to the unit, and secondly- as TVOR and embryo transfer are the most crucial time points in the ART cycle, assessment of endometrial events during that period is justified. As some patients did not attend all appointments (frozen embryo cycles) or analysis of some datasets was not possible due to suboptimal image quality or uterine displacement, this data was not included, decreasing the overall count of analysed images and forming a potential source of selection bias. This can also be considered as a strong point of the study, as confounding factors such as poor image quality and subsequent forced analysis have been excluded. We have chosen not to perform US scans on the day of hCG injection, as this would require an additional visit to the unit, to which the patients did not consent.

In conclusion, when analysing the sonographic endometrial parameters in the unselected population of women undergoing ART (fresh and frozen cycles) at different time points, single or multiple sonographic markers of endometrial receptivity unequivocally predictive of successful pregnancy have not been identified. This is despite a great homogeneity of participants in the study populations and single observer analysis. Further research is however still required, as the sensitivity of the equipment continues to improve, and the identification of non-invasive markers predictive of successful pregnancy outcome following ART would be invaluable. In future studies in order to maintain homogeneity with available literature, US assessment should also be carried out on the day of hCG administration. Though troublesome for participants, this time point could provide additional early markers of endometrial receptivity and indicate the need for embryo cryopreservation in order to optimize the chances of conception.

CHAPTER 7. Spatio-Temporal Image Correlation (STIC) of the endometrium: methodology, feasibility, reproducibility and correlation with assisted reproductive treatment outcome.

7.1. Introduction

3DPD angiography allows for examination as well as quantification of organ and tissue blood flow (Pairleitner et al. 1999). *In vitro* and *ex vivo* phantom studies have shown correlation between indices obtained using this modality and actual flow/perfusion of the specimen (Jones et al. 2009, Morel et al. 2010). The vascularity index (VI), flow index (FI) and vascularisation flow index (VFI) however, lack standardisation and depend on the equipment setup (Raine-Fenning et al. 2008b, Martins et al. 2010), attenuation (Raine-Fenning et al. 2008a, Jones et al. 2009) and sampling volume (Kudla et al. 2010). This creates difficulty when comparing results obtained by different research groups, as the abovementioned factors may vary. When a three-dimensional power Doppler (3DPD) volume is acquired, the overall tissue perfusion is averaged over the duration of the acquisition, meaning that vascularity indices are averaged across a few cardiac cycles, or different phases of a cardiac cycle if the volume of interest (VOI) is very small and/or the acquisition time very short (Martins et al. 2011).

Spatio-temporal image correlation (STIC) is a new ultrasound modality allowing for assessment of the blood flow characteristics throughout the entire cardiac cycle within a given VOI. STIC acquisition is an automated process, where the transducer array records a volume consisting of numerous 2D frames. Due to this, the recorded B-mode frame rate is very high (up to 150 frames/second) (DeVore et al. 2003). Combination of this technology with PD imaging allows for acquisition of a series of 3DPD volumes, each representing a different phase of the cardiac cycle. This is displayed as a cine loop, which can be stopped at any time allowing for detailed analysis of the single volume. Each volume contained within a single STIC acquisition produces a VI, FI and VFI result representing amplitude of blood flow. As expected, the vascularity assessed using STIC is greater during cardiac systole than diastole, with significantly different vascular index values (Alcazar et al. 2010, Welsh et al. 2012). Due to the characteristics of the technology allowing for step-by-step depiction of blood flow during an entire cardiac cycle, STIC is

almost exclusively used to assess fetal cardiac anomalies *in utero* (DeVore et al. 2003).

When analysing spectral Doppler, introduction of PWPD indices has helped to overcome the technical inconsistencies, such as angle of insonation and vessel diameter. These indices are derived from ratios involving the peak systolic velocity (PSV), end-diastolic velocity (EDV) and the mean velocity. Most commonly used indices are the pulastility index (PI), resistance index (RI) and systole to diastole ratio (S/D). PI is reported to be the most sensitive of the three, as it takes into account the mean velocity (Nelson et al. 1988). Some authors hope, that a similar approach to STIC imaging might overcome limitations of this modality (Martins et al. 2011). Following the complete analysis of all the images from a STIC dataset, the following volumetric indices derived from 3D signal intensity changes have been suggested:

- *Volumetric pulsatility index (vPI)= ([maximum value]-[minimum value]/(mean value)*
- *Volumetric resistance index ($vRI_{max-min}$)= ([maximum value]-[minimum value])/(maximum value)*
- *Volumetric S/D ratio ($vS/D_{max-min}$)= (maximum value)/(minimum value)*

Due to technical limitations, up until recently it has only been possible to analyse a small fragment of the endometrium, placenta or ovarian stroma using spherical VOI sampling (Alcazar et al. 2010, Kudla et al. 2012, Welsh et al. 2012). Recent advancements allow for an angle of acquisition of 90 degrees, which is sufficient for the entire endometrium to be included in one STIC acquisition. This advancement has however some limitations which need to be taken into consideration. Due to the acquisition time, respiratory movements or transmitted pulsatility from the iliac vessels can induce motion artefacts distorting the obtained image. This can limit the number of good quality images appropriate for analysis, as has been demonstrated in a study assessing the fetoplacental blood flow using spherical sampling, where 75 of 90 volumes were analysable (Welsh et al. 2012). As some planes are

computer-generated reconstructions (C-plane), analysis of these can be troublesome or even impossible due to lack of clearly defined tissue planes.

In contrast to 3DPD sonography, there are no studies assessing the correlation of volumetric vascularity indices with ART outcome. Previous studies utilising STIC, have only focused on assessment of selected spherical VOI within the endometrium, placenta or ovarian stroma in order to validate the methodology and compare to static 3DPD modality (Alcazar et al. 2010, Kudla et al. 2012, Welsh et al. 2012). Recent advancements allow a 90-degree angle of acquisition, which enables assessment of a significantly larger volume of interest. As demonstrated in the current work, this angle of acquisition is sufficient for analysis of the entire endometrium using STIC acquisition in most cases.

As averaging the perfusion of the endometrial vasculature when applying 3DPD sonography has been found to be positively correlated with ART outcome by some authors (Ng et al. 2006d, Ng et al. 2007, Singh et al. 2011b), we have postulated that the ability to obtain a more accurate description of blood flow through the application of STIC, will allow more precise prediction of ART outcome.

The aim of the first part of this study was to identify optimum machine settings for acquisition of a whole endometrium STIC dataset; demonstrate the feasibility and inter- and intra-observer reproducibility of measurements of STIC of the entire endometrium and spherical samples; and assess the biological variation between different STIC acquisitions in the same patient. In the second part of the study, we have focused on the correlation of obtained STIC vascularity indices with ART outcome. To assess if such a correlation can be identified, we have employed whole endometrium and spherical STIC analysis of vascularity in the mid-luteal phase of the cycle preceding ART, on day of transvaginal oocyte retrieval (TVOR) and on day of embryo transfer. The vascularity indices obtained were correlated with presence or absence of clinical pregnancy and first trimester miscarriage.

7.2. Materials and Methods

7.2.1. **Study design**

The feasibility study was designed as a single centre, prospective observational cohort study. Local research ethics committee approval was not necessary, as Doppler assessment of the endometrium during scans prior to ART is routine practice in the hosting unit. The second part of the study with outcome correlation was designed as a single centre, prospective observational cohort study within the realms of the Endoscratch study protocol with local research ethics committee approval (12/EM/0345). Women prior to undergoing IVF or ICSI treatment in a tertiary university affiliated fertility treatment centre underwent an ultrasound assessment in the mid-luteal phase, on day of TVOR and day of ET. Recruitment to the study took place from February 2013 to July 2014.

7.2.2. **Participants and collected data**

All women under the age of 49 with no major congenital or acquired uterine anomalies precluding endometrial assessment were eligible. Written consent was obtained prior to any procedures being carried out. Following consent and the mid-luteal scan, all participants underwent IVF or ICSI treatment according to set protocols (see section 5.2.4).

Collected data included participant's age and past reproductive history. Sonographic data included endometrial pattern, thickness, volume, and STIC vascularity indices. Sonographic images were stored on the ultrasound machine and subsequently analysed off-line as described on page 81.

The main outcome of this study was clinical pregnancy in study subjects, defined as the presence of a fetal heartbeat at 6 weeks gestation on a transvaginal ultrasound scan. A biochemical pregnancy was defined as a positive urinary pregnancy test 14 days after ET. Biochemical pregnancy loss was defined as no evidence of pregnancy at the 6-week ultrasound scan following a positive pregnancy test. Miscarriage was defined as absence of fetal heartbeat on the scan following prior visualization of fetal

cardiac activity. On-going pregnancy was defined as a viable pregnancy past the 12th week of gestation.

7.2.3. **Pilot testing of machine settings**

STIC datasets from infertile women attending for a baseline ultrasound scan prior to undergoing IVF treatment at Nurture Fertility were obtained by one sonographer (LP) during days 1 to 5 of a menstrual cycle following verbal consent or during ART stimulation protocols. These datasets were used to optimise the STIC machine settings. All initial 2D and PD settings were selected based on optimal image quality and the available literature (Martins et al. 2011). Time of acquisition was 15 seconds (longest permitted by equipment). Within these acquisitions, one setting varied. The number of acquisitions varied depending on the amount of possible combinations. The determined optimal settings were used in subsequent image acquisitions.

7.2.4. **Image acquisition and analysis**

Ultrasound assessments were performed using a Voluson E8 Expert BT12 (GE Healthcare, Zipf, Austria) and 5-9MHz endovaginal transducer on day 1 to 5 of menstrual cycle to optimise machine settings, or on day LH+7 to LH+9, or day 21 ± 2 in case of absent LH surge in the cycle preceding COH; 36 hours after hCG injection (in fresh cycles); or prior to embryo transfer. Two experienced sonographers (LP, MB) conducted all ultrasound examinations. All ultrasound images were analysed offline using 4D ViewTM software (version 10.5 BT12, GE Medical Systems) as described on page 81, by means of manual analysis with 15º rotational steps and 1cm^3 spherical sampling. Only images where the ROI was no deeper than 5cm from the transducer and delineation of the endometrium was possible without doubt were included in order to minimise the effect of attenuation on Doppler signal and provide a reliable endometrial representation, respectively.

7.2.5. **Repeatability analysis**

Datasets from ten consecutive patients were independently analysed once by two observers (LP and MB). The demographic data was then

removed and images were analysed once more in forward order to assess reproducibility of measurements.

Eleven subsequent STIC acquisitions from three patients were acquired using 45-degree angle to assess repeatability of the assessed indices (variability across data acquisition, spherical analysis only). Smaller angle of acquisition and smaller VOI was chosen to allow for a more detailed depiction of the cardiac cycle (Welsh et al. 2012) allowing better cycle to cycle variability assessment. All obtained volumes were subsequently analysed by a single observer (LP).

7.2.6. **Statistical analysis**

Statistical analysis was performed using the Statistical Package for the Social Sciences (version 21; SPSS, Chicago, IL, USA). Distribution of data was assessed using Kolmogorov-Smirnov test. For parametric data, an unpaired data Student's t test was used and for non-parametric data, the Mann-Whitney U test was carried out. Where there were paired samples, a student's t test was used for parametric data and Wilcox signed rank test was used for non-parametric data. A P value of <0.05 was considered as statistically significant. For correlation with outcome assessment, a same approach has been used as described on page 157 in section 6.2.5.

Intra- observer reliability was assessed by two-way, mixed Intraclass Correlation Coefficients (ICCs) with absolute agreement and their 95% confidence intervals (95% CI); Bland-Altman plots were used to compare the differences between the two methods. Pearson's correlation coefficient was used to compare the results of the entire endometrial vascularity indices and spherical sampling vascularity indices. The repeatability of vascular indices between different acquisitions was assessed using the coefficient of variation (CV).

7.3. Results- Methodology, Feasibility and Reproducibility

7.3.1. Assessment of optimal ultrasound machine setting for STIC modality

Smooth rise and fall

This setting determines how long it takes for the Doppler signal to increase and persist on the screen following actual changes in the blood flow, respectively. The higher the setting, the longer it takes for the signal to increase and the longer it persists. The optimal image quality and accurate identification of cardiac cycle components was achieved with rise and fall of 2/2. In this setting, the Doppler signal takes a short time to increase and disappears quickly (Figure 44).

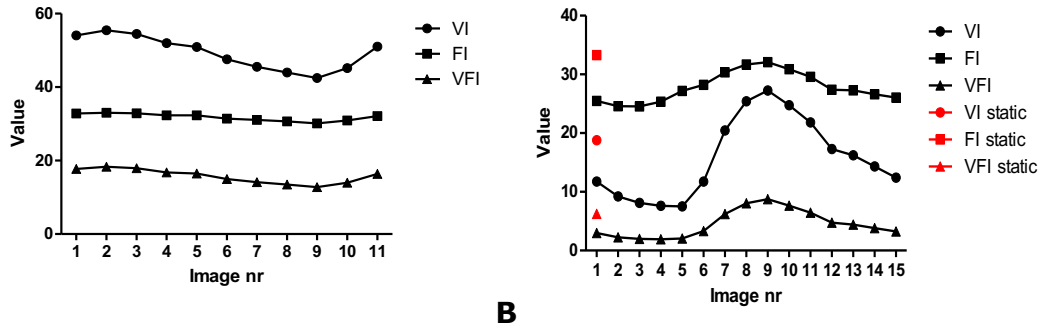


Figure 44: STIC acquisition of the subendometrium using 5 mm shell representing one dataset.

Rise and fall settings of 5/5 (A) and 2/2 (B). VI, FI and VFI values are depicted on the Y axis, image number of the analysed STIC dataset is on the X axis. Red markers represent static 3D power Doppler vascularity indices for comparison.

Pulse repetition frequency

Pulses of Doppler signal are emitted at a given sampling frequency, known as the pulse repetition frequency (PRF). This setting allows for assessment of vascular beds with different blood flow velocities. Low PRF

is more useful when assessing low velocity vascular beds (i.e. venous vessels), whereas higher PRF is used for assessment of vessels with higher blood flow velocities (i.e. uterine arteries) (Torp-Pedersen et al. 2008, Martins et al. 2011).

Analysis of this setting was performed on the basis of visual assessment of the acquired image. Low PRF of 0.1 did not allow for differentiation of any anatomical structures of the uterus due to the abundance of the Doppler signal (Figure 45). Highest assessed PRF (1.2) had almost no visible Doppler signal in the dataset (Figure 47). The most informative setting was PRF 0.6 as the background two-dimensional information was clearly defined and the differentiation between systole and diastole was easily identifiable (Figure 46 A).

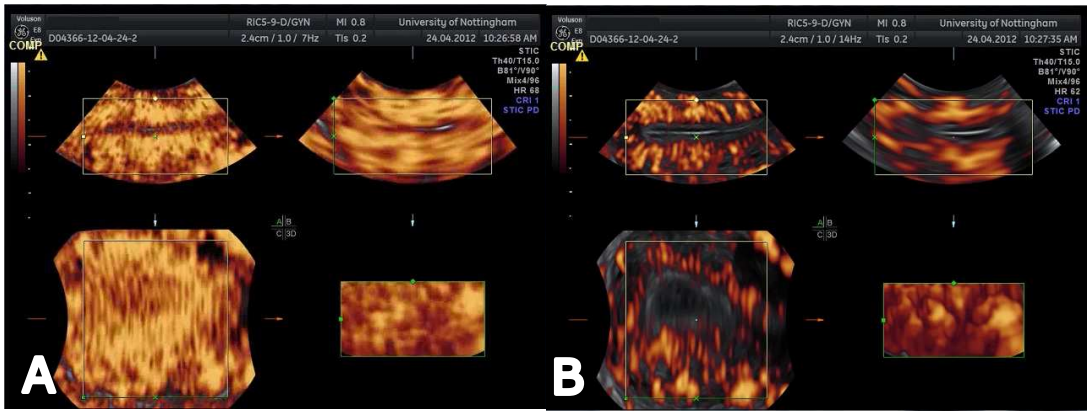


Figure 45: Images demonstrating a STIC dataset with PRF settings 0.1 (A) and 0.3 (B).

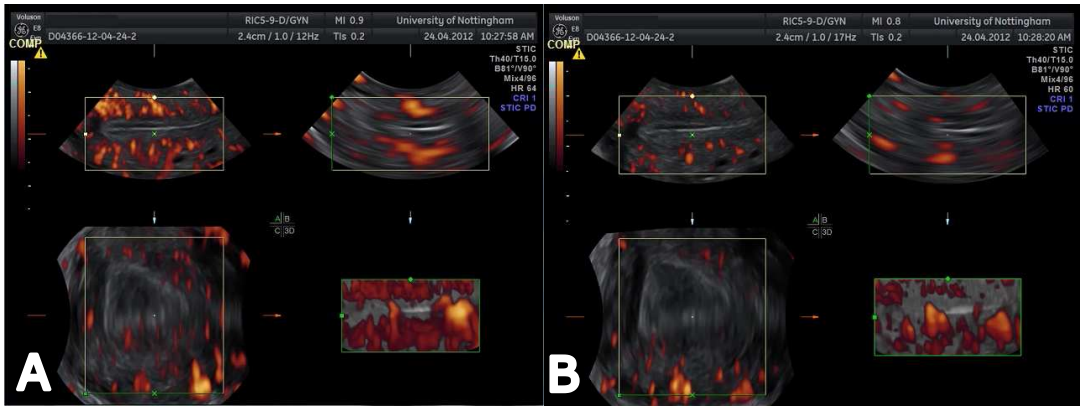


Figure 46: Images demonstrating a STIC dataset with PRF settings 0.6 (A) and 0.9 (B).

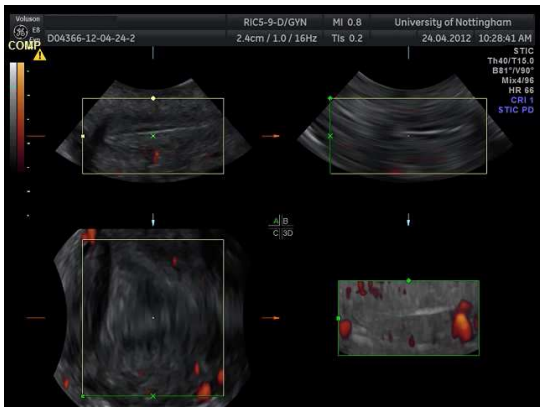


Figure 47: Image demonstrating a STIC dataset with PRF settings 1.2.

Ultrasound Signal Frequency

Lower frequency allows for deeper tissue penetration of ultrasound signal, compromising the resolution. Higher frequency allows assessment of tissue in more detail but this is achieved closer to the transducer. Higher frequency allows also for assessment of lower blood flow velocities. Subjective (visual) assessment was followed by objective assessment in the form of vascularity index calculation. This analysis using varying frequency settings, has demonstrated that the 'mid' setting produces most informative and distinguishable results (Figure 48). This setting is consistent with suggestions in the available literature (Martins et al. 2011).

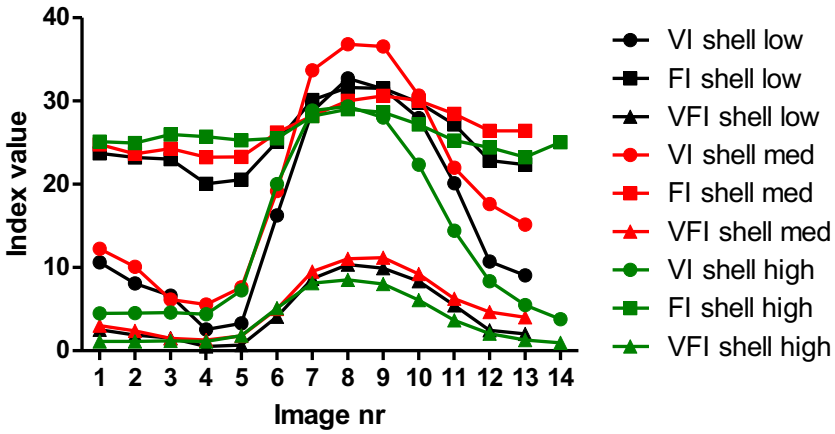


Figure 48: Vascularity index values in different frequency settings (one patient, three different acquisitions).

Grey/ colour balance

The recommended setting for Doppler grey/colour balance is >225 (Martins et al. 2011). The vascularity indices were objectively analysed using different balance settings of >225, 220, 210, 200, 190, 180 and 175 (data not shown). The value suggested in the literature allows for appropriate differentiation of systole and diastole, hence balance >225 will be used for study protocol (Martins et al. 2011).

Gain

The gain setting resembles brightness control. This function allows for signal amplification to be regulated. Studies assessing the effect of gain on 3D Doppler vascularity indices, suggests an increase in the values with increasing gain (Raine-Fenning et al. 2008b). Higher gain settings cause artefactual noise increase in the signal on the screen. Figure 49 demonstrates different vascularity indices depending on gain settings.

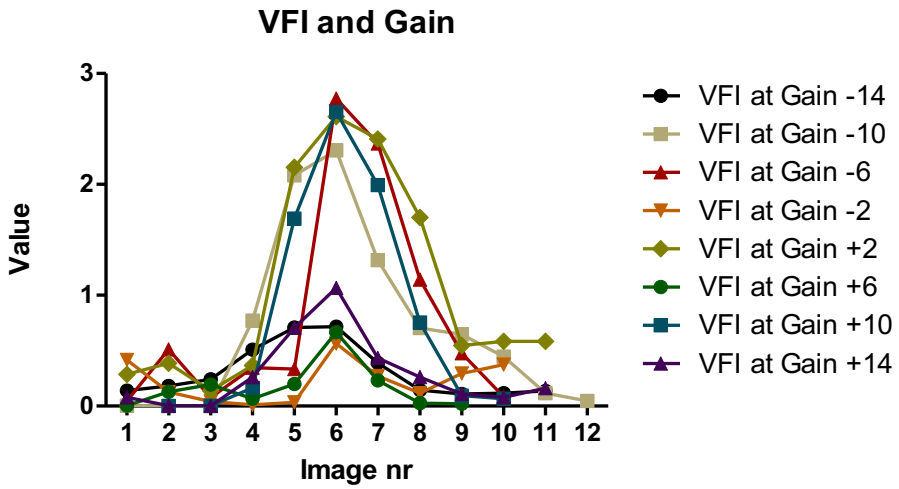


Figure 49: VFI values of the endometrium and the effect of different gain settings (one patient, eight different acquisitions).

To overcome the effect of gain on the Doppler indices, volumetric pulsatility index (vPI) was calculated from eight acquisitions obtained from the same patient using 2.0 gain steps, starting at value -14. vPI derived from FI is static throughout the different gain settings as well as during a cardiac cycle (Figure 50), and as such lacks reliability (Martins et al. 2011). Based on the increase of vascularity indices above gain value

of '2' and data available in the literature (Martins, Wesh et al. 2011), gain value of '-2' has been selected as the optimum setting.

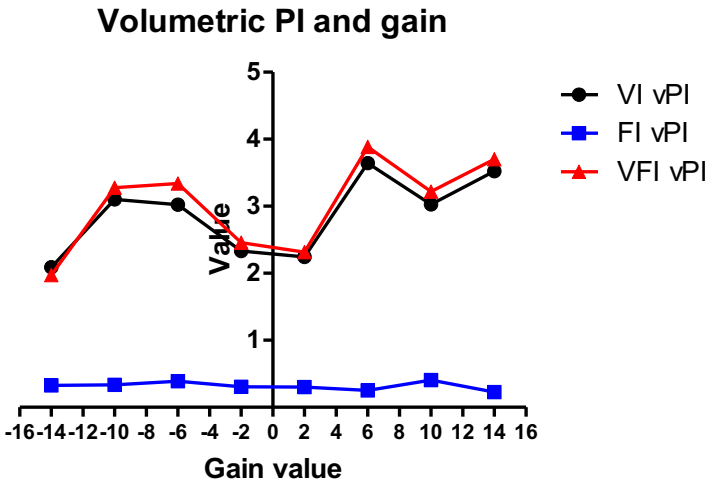


Figure 50: vPIs derived from VI, FI and VFI, and the effect of different gain settings (one patient, eight different acquisitions).

Overall optimal STIC US machine settings

Based on the performed analyses, settings for PDSTIC allowing for best differentiation of diastole and systole have been optimised, and were consistently set at:

- Smooth rise/fall: 2/2
- PRF: 0.6
- Frequency: mid
- Grey/colour balance: >225
- Gain: -2

7.3.2. **Duration of endometrial assessment using STIC**

The mean time taken for analysis of one STIC dataset was 1478.86 ± 290.99 s using the manual analysis method and 266.8 ± 39.31 s using spherical sampling ($P<0.05$). The mean number of 3DPD volumes in one STIC acquisition was 15.5 ± 2.5 (range 10-22).

7.3.3. **Intra and inter-observer reliability of measures**

In total 164 images from 10 participants were analysed twice by one (LP) and two observers (LP and MB). The endometrial vascularity indices were recorded and compared. The respective intra- and interobserver reliability measures are presented in Table 17.

Measurement method and vascularity index	***Intraclass correlation coefficient***	***95% Confidence intervals***	
		Lower bound	*Upper bound*
Single observer manual VI	0.963	0.929	0.981
Single observer manual FI	0.864	0.759	0.925
Single observer manual VFI	0.933	0.876	0.964
Single observer spherical VI	0.979	0.972	0.985
Single observer spherical FI	0.982	0.976	0.987
Single observer spherical VFI	0.969	0.956	0.978
Two observers manual VI	0.887†	0.826	0.932
Two observers manual FI	0.823†	0.735	0.892
Two observers manual VFI	0.923†	0.880	0.955
Two observers spherical VI	0.8†	0.737	0.85
Two observers spherical FI	0.507†	0.384	0.613
Two observers spherical VFI	0.94†	0.919	0.956

Table 17. Intra and inter-observer reliability analysis of manual versus spherical STIC of the endometrium.

† Lowest recorded value of the two for interobserver reliability analyses.

7.3.4. **Comparison of entire endometrial assessment and spherical sampling**

Vascularity indices (maximum, minimum and average VI, FI and VFI) and derived volumetric PI indices obtained using spherical sampling and whole endometrium delineation of 208 complete datasets were compared. Initial analysis revealed that minimum VI, maximum FI and minimum VFI values obtained using the two analysis methods were similar ($P>0.05$). Remaining indices were significantly different between

methods (P<0.05). Bland-Altman plots were constructed for the variables showing some degree of agreement (see Figure 51). Further exploration of the agreement using linear regression, has shown that both methods have no linear trend (there exists a significant variance of difference between methods) (P<0.008).

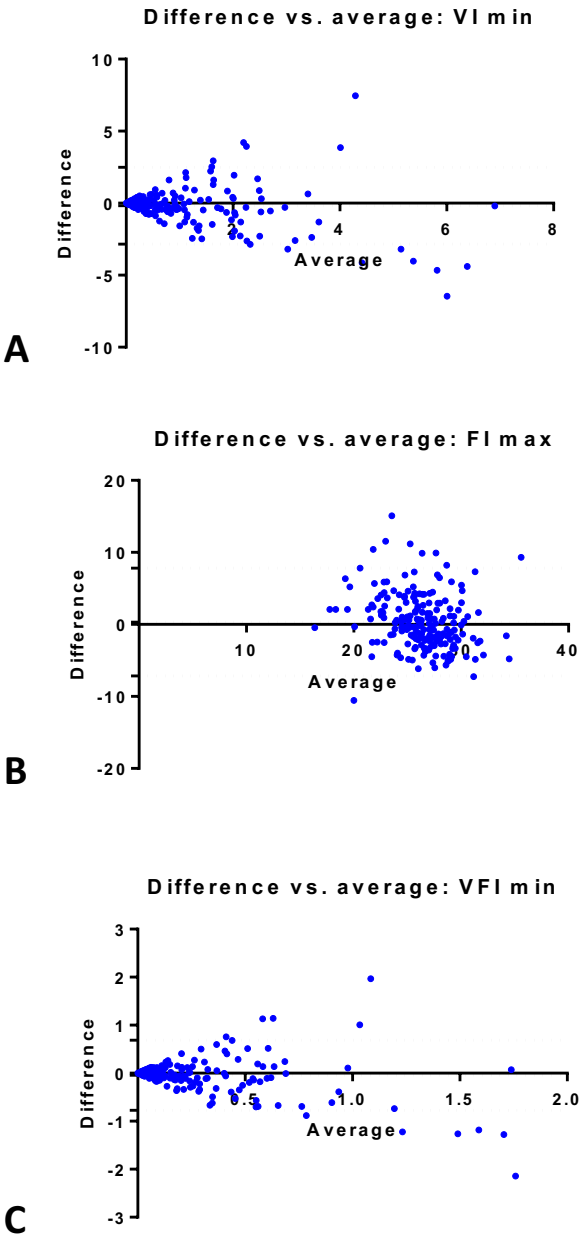


Figure 51. Bland Altman plots comparing performance of spherical sampling of the endometrium versus whole endometrial delineation for the maximal VI (A), maximal FI (B) and minimal VFI (C).

7.3.5. **Cyclical variations of vascularity indices**

Cyclical variations of VI, FI and VFI were present in all cases analysed, (Figure 52), and volumetric vascularity indices (vPI, VRI and vS/D) for all analysable volumes based on the equations given on page 183 were calculated. As some of the measured vascularity indices were equal to zero, it was not possible to derive the vRI and vS/D due to mathematical restrictions (division by zero). This restricted the number of available variables for analysis. Thus, the only volumetric vascularity index considered further will be the vPI.

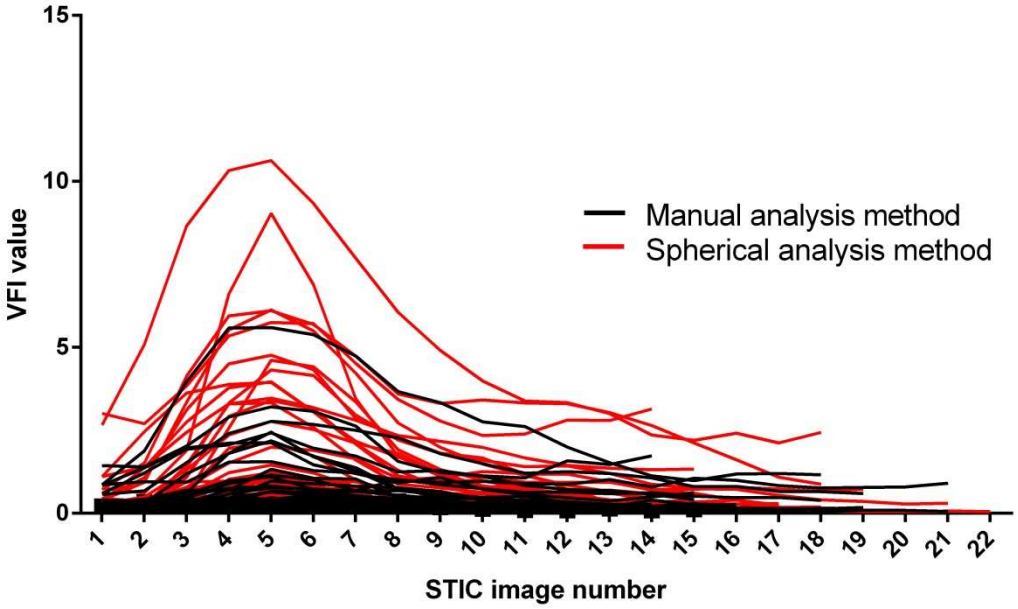


Figure 52. Values of volumetric vascular VFI STIC indices during a single cardiac cycle obtained using manual and spherical sampling (n=60).

7.3.6. **Cycle to cycle variability of STIC derived vascularity indices**

The acquisitions for this analysis consisted of a mean of 20.7 (range: 14-35) individual volumes. The CVs for the systolic, diastolic and average VI, FI and VFI using the spherical ROI ranged from 8.3% for diastolic FI to

76.3% for diastolic VFI. The CVs for vPI were 26.8%, 32.0% and 27.3% for VI, FI and VFI derived indices, respectively (see Figure 53). The heart rate variance was 4.7%.

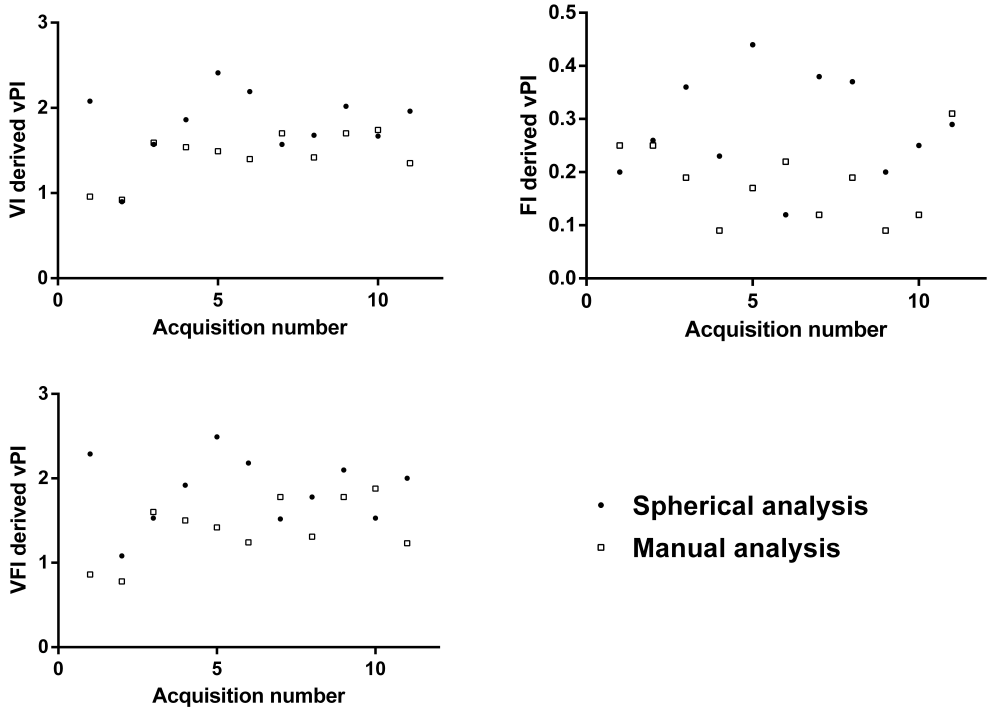


Figure 53. Scatter plots of VI, FI and VFI derived vPI obtained from one participant using spherical and manual analysis.

7.3.7. **Correlation of STIC vascularity indices with static PD vascularity indices**

The average VI, FI and VFI obtained using manual (207 pairs) and spherical (264 pairs) STIC analyses have been compared with VI, FI and VFI obtained using 3DPD analysis. 3DPD VI and FI were found to be significantly higher compared to the average values obtained from manual and spherical STIC ($P<0.05$). VFI values did not differ between methods (see Figure 54).

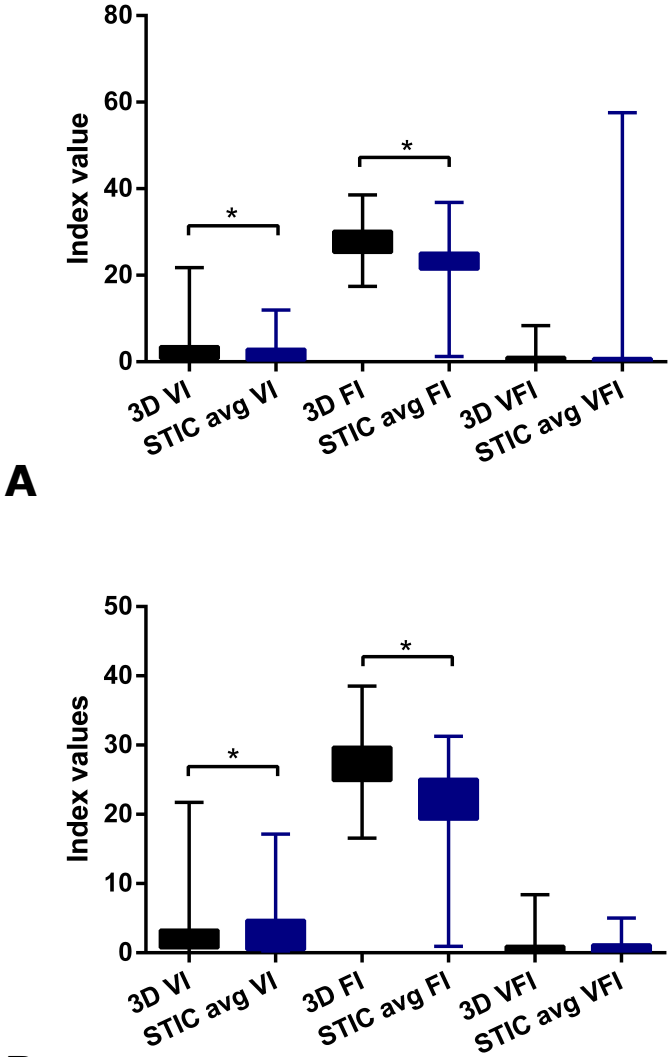


Figure 54. Relationship between endometrial VI, FI and VFI values obtained using static 3DPD and manual STIC (A; N=55) and spherical STIC (B; N=72).

*Data presented as mean (±SD); * P<0.05; P values assessed by unpaired t test.*

7.4. Results- correlation with assisted reproduction treatment outcome

7.4.1. **Population characteristics**

Between January 2012 and July 2014, 151 women were recruited into the Endoscratch study: 76 in the biopsy group and 75 in the control

group. Baseline demographics of study participants are presented in Table 4 on page 144. Twenty-one women did not attend for the initial mid-luteal scan appointment prior to ART cycle and were not included in analysis.

The preovulatory LH surge was recorded by 66 women, in which case the ultrasound scan was performed on day LH+7 (±1). If the LH surge was not detected, the scan was carried out on day 21 (±1) of the cycle. Biochemical pregnancy rate was 69.3% (88/127). Biochemical pregnancy loss affected 10 women (11.4%). Five of 78 (6.4%) women who achieved a clinical pregnancy subsequently miscarried before the 12th week of gestation. On-going pregnancy rate was 57.5% (73/127). Total pregnancy loss, including biochemical and clinical pregnancy loss, was 17%.

7.4.2. **Endometrial vascularity defined by STIC indices in the mid-luteal phase**

Manual analysis of the endometrium was possible in 102 cases (67.5%) and spherical sampling was possible in 119 (78.8%). Thin endometrium in 6 cases and inability to delineate the endometrium in 20 cases were the cause of analysis failure in the manual method. Thin endometrium in 2 women and inability to delineate the endometrium in 7 cases precluded spherical analysis of endometrial vascularity. Scan data was not available for 23 women.

Maximum FI and average FI values were similar between the manual and spherical sampling methods ($P>0.05$). Other vascularity indices and vPI values differed significantly between analysis methods ($P<0.05$) (see Table 18).

Vascularity index	*Manual analysis (N=102)*	*Spherical sampling (N=119)*
STIC VI max	2.71(1.1-5.56)**	7.4(2.54-15.08)**
STIC VI min	0.45(0.06-1.1)**	0.75(0.05-2.0)**
STIC VI average	1.39(0.37-2.7)**	2.88(1.05-6.91)**
STIC FI max	25.93(2.69)	26.39(3.48)
STIC FI min	20.58(18.16-22.66)*	20.4(17.48-22.18)*
STIC FI average	23.28(21.2-24.81)	23.47(20.96-25.76)
STIC VFI max	0.67(0.25-1.38)**	1.98(0.55-4.03)**
STIC VFI min	0.1(0.01-0.25)**	0.17(0.01-0.46)**
STIC VFI average	0.33(0.08-0.74)**	0.66(0.22-1.84)**
VI derived vPI	1.87(1.44-2.49)**	2.23(1.69-2.94)**
FI derived vPI	0.22(0.16-0.32)**	0.28(0.21-0.39)**
VFI derived vPI	1.98(1.49-2.57)**	2.34(1.87-3.1)**

Table 18. STIC vascularity indices in the mid luteal phase of the cycle.

*Data presented as mean(±SD) or median(interquartile range) as appropriate; VI- vascularisation index; FI- flow index; VFI- vascularisation flow index; vPI- volumetric pulsatility index;. *P<0.05; **P<0.001; P-values assessed by unpaired t test or Mann-Whitney test, as appropriate.*

7.4.3. **Endometrial vascularity in the mid-luteal phase and pregnancy outcome**

All STIC vascularity indices assessed were similar in women that achieved a clinical pregnancy and the women that had a negative outcome (P>0.05). There was no difference between STIC vascularity indices in the population with a miscarriage and on-going pregnancy (data not shown; P>0.05).

7.4.4. **STIC vascularity indices at TVOR**

Manual analysis was only possible in 52 cases. Thirty-nine images were not acquired due to technical issues or unavailability of staff to perform scan (21) and patients being late for TVOR (18). Eighteen datasets were

not analysable due to suboptimal response to stimulation (6), risk of OHSS and cycle cancellation (10), and FER (2).

Vascularity index	***Biopsy Group***		***Control group***	
	Manual method (n=26)	*Spherical Sampling (n=41)*	*Manual method (n=26)*	*Spherical sampling (n=36)*
Maximum VI	2.52(0.85-5.75)	4.14(1.54-10.22)	3.03(1.55-5.25)	5.71(1.37-11.5)
Minimum VI	0.53(0.16-1.53)	0.27(0.0-1.19)	0.36(0.09-0.81)	0.1(0.0-1.37)
Average VI	1.31(0.48-3.35)	1.65(0.61-3.56)	1.30(0.64-2.2)	1.84(0.3-5.06)
Maximum FI	26.72(2.98)	26.91(4.44)	26.97(2.26)	26.47(4.39)
Minimum FI	20.83(18.72-23.14)	19.56(16.33-22.22)	20.74(18.63-22.37)	18.11(0.0-21.34)
Average FI	24.23(21.31-25.51)	23.1(19.48-25.84)	23.7(22.66-24.99)	22.77(19.02-25.28)
Maximum VFI	0.63(0.22-1.54)	0.99(0.37-2.8)	0.74(0.39-1.29)	1.32(0.3-3.15)
Minimum VFI	0.13(0.03-0.4)	0.07(0.0-0.27)	0.08(0.02-0.19)	0.02(0.0-0.33)
Average VFI	0.33(0.1-0.83)	0.41(0.14-0.93)	0.31(0.16-0.55)	0.43(0.07-1.31)
VI derived vPI	1.45(1.12-2.26)	2.35(1.81-3.97)	1.76(1.51-2.61)	2.63(2.06-3.69)
FI derived vPI	0.25(0.19-0.33)	0.31(0.26-0.51)	0.22(0.18-0.34)	0.42(0.27-1.28)
VFI derived vPI	1.45(1.02-2.25)	2.42(1.94-4.13)	1.86(1.67-2.64)	2.80(2.32-3.8)

Table 19. STIC vascularity indices at TVOR in the biopsy and control groups.

Data presented as mean(±SD) or median(interquartile range) as appropriate.

Forty-two images were not analysed due to the endometrium being too broad to fit in 90-degree angle of acquisition (13), inability to trace the EMJ (12), fluid within the cavity (4), too thin endometrium (2), too axial uterus (3) and displacement of the uterus by enlarged ovaries (8). Spherical analysis was possible in 77 cases. No differences were observed between the biopsy and control populations irrespective of analysis method (see Table 19).

7.4.5. **Endometrial vascularity at TVOR and pregnancy outcome**

None of the analysed indices were different between the group, which achieved a clinical pregnancy, and the one that did not. Maximum FI in the manual method was approaching statistical significance (P=0.053), with lower values in the group with a successful outcome.

In a population of women with first trimester miscarriages, the spherical vascularity indices did not differ compared to on-going pregnancies (P>0.05). Manual minimal VI was lower in the on-going population compared to first trimester miscarriage [0.33 (0.07-1.04) versus 1.63 (0.62-3.0), respectively; P=0.036], as was average VI [1.08(0.31-3.03) versus 2.09 (1.67-6.0), respectively; P=0.048], and minimal FI [20.44 (17.96-22.89) versus 23.41 (20.66-25.11), respectively; P=0.041] (see Table 20).

7.4.6. **STIC vascularity indices at embryo transfer**

Manual analysis was possible in 55 cases. Thirteen images were not acquired due to technical issues or unavailability of staff to perform scan, seventeen due to patients being late for embryo transfer. Sixteen datasets were not analysable due to suboptimal response to stimulation (6), risk of OHSS and cycle cancellation (10). Fifty-one images were not analysed due to the endometrium being too broad to fit in 90-degree angle of acquisition (6), inability to trace the EMJ (16), too thin endometrium (1), and displacement of the uterus by enlarged ovaries (28). Spherical analysis was possible in 72 cases.

STIC vascularity indices did not differ between the biopsy and control populations irrespective of analysis method (P<0.05; see Table 21).

Vascularity index	***First trimester miscarriage (N=15)***		***On-going pregnancy (N=73)***	
	Manual method	*Spherical Sampling*	*Manual method*	*Spherical sampling*
Maximum VI	3.96(3.4-10.9)	5.49(2.3-11.73)	2.4(0.73-5.61)	3.73(1.4-9.02)
Minimum VI	1.63(0.62-2.99)*	0.59(0.15-1.62)	0.33(0.07-01.04)*	0.06(0.0-0.89)
Average VI	2.09(1.67-6.0)*	1.89(0.68-5.07)	1.08(0.31-3.03)*	1.33(0.36-3.41)
Maximum FI	27.56(±2.85)	28.48(±3.5)	26.89(±2.52)	26.14(±4.43)
Minimum FI	23.41(20.66-25.11)*	21.42(20.16-21.79)	20.45(17.96-22.89)*	17.29(0.0-21.4)
Average FI	25.89(22.53-27.06)	24.01(22.05-25.56)	23.38(20.72-24.75)	22.52(18.74-25.46)
Maximum VFI	1.00(0.97-1.83)	1.36(0.57-3.25)	0.58(0.17-1.38)	0.87(0.29-2.46)
Minimum VFI	0.4(0.13-0.79)	0.24(0.08-0.46)	0.07(0.01-0.25)	0.01(0.0-0.21)
Average VFI	0.56(0.39-1.65)	0.45(0.17-1.31)	0.26(0.07-0.74)	0.34(0.08-0.91)
VI derived vPI	1.5(0.95-1.83)	2.13(1.67-3.11)	2.0(1.47-2.67)	2.77(1.9-4.09)
FI derived vPI	0.19(0.14-0.22)	0.25(0.2-0.39)	0.25(0.19-0.33)	0.34(0.25-0.47)
VFI derived vPI	1.9(1.01-1.99)	2.44(1.8-3.08)	2.1(1.4-2.57)	2.9(2.1-4.02)

Table 20. Vascularity indices at TVOR in the group with first trimester miscarriage and an on-going pregnancy.

*Data presented as mean(±SD) or median(interquartile range) as appropriate; *P<0.05; P-values assessed by unpaired t test or Mann-Whitney test, as appropriate.*

Vascularity index	***Biopsy Group***		***Control group***	
	Manual method (n=31)	*Spherical Sampling (n=43)*	*Manual method (n=24)*	*Spherical sampling (n=29)*
Maximum VI	2.31(0.84-5.75)	3.96(0.92-6.16)	2.28(0.99-5.98)	1.96(0.91-5.48)
Minimum VI	0.45(0.05-2.01)	0.27(0.0-0.62)	0.22(0.01-1.59)	0.04(0.0-0.43)
Average VI	1.18(026-2.87)	1.8(0.23-2.85)	0.85(0.36-3.33)	0.6(0.23-2.65)
Maximum FI	33.78(±41.17)	24.86(±4.34)	27.76(±4.35)	25.0(±4.4)
Minimum FI	19.49(16.89-24.36)	18.66(0.0-20.75)	19.49(16.89-24.36)	17.39(0.0-19.56)
Average FI	23.53(21.75-27.5)	22.18(14.21-23.61)	23.1(21.68-26.33)	20.56(16.86-23.0)
Maximum VFI	0.55(0.21-1.15)	0.97(0.22-1.66)	0.54(0.27-1.52)	0.43(0.2-1.4)
Minimum VFI	0.12(0.01-0.27)	0.05(0.0-0.14)	0.04(0.0-0.45)	0.01(0.0-0.08)
Average VFI	0.31(0.06-0.75)	0.42(0.05-0.72)	0.19(0.09-0.85)	0.14(0.05-0.66)
VI derived vPI	1.5(1.03-2.45)	2.7(2.12-4.02)	1.92(1.17-2.73)	2.97(2.03-3.96)
FI derived vPI	0.24(0.2-0.32)	0.33(0.25-1.41)	0.32(0.22-0.46)	0.38(0.3-1.42)
VFI derived vPI	1.58(1.14-2.84)	2.83(2.15-4.47)	1.96(1.27-2.68)	3.12(2.05-4.15)

Table 21. STIC vascularity indices at ET in the biopsy and control groups.

Data presented as mean(±SD) or median(interquartile range) as appropriate.

7.4.7. **Endometrial vascularity at embryo transfer and pregnancy outcome**

There was no difference in vascularity indices when clinical pregnancy was considered as the primary outcome, irrespective of the analysis method (P>0.05). STIC vascularity indices, manual or spherical, did not differ between women with first trimester miscarriage and an on-going pregnancy (P>0.05).

7.4.8. **STIC vascularity changes over time**

Considering VFI as the most informative vascularity index, when spherical analysis was applied, its value had decreased from the mid-luteal phase to embryo transfer. Manual analysis of the overall trend has shown a reverse trend, however statistical significance was not achieved (P>0.05; see Figure 55). A trend was observed for VFI derived vPI increase following TVOR, (P=0.053). Analysis of the overall trend of volumetric vascularity indices has demonstrated significant differences between VFI derived vPI at all three assessed time points (P<0.05; see Figure 56).

7.4.9. **STIC vascularity index progression following endometrial biopsy**

STIC vascularity indices did not differ between the biopsy and control populations at any given time point (P>0.05). The obtained curves differed between the manual and spherical sampling, with spherical sampling showing a decreasing blood flow when approaching embryo transfer in both populations (P>0.05; see Figure 57). The manual analysis has clearly separated the two populations, with an increase in the average VFI at TVOR and embryo transfer in the biopsy group, however statistical significance was not achieved (P=0.08) (see Figure 58).

7.4.10. **Endometrial STIC vascularity index progression and pregnancy outcome**

Due to anatomical variations and uterine displacement by enlarged ovaries, only 11 STIC dataset series for the manual method and 12 datasets series for the spherical method including the mid-luteal, TVOR and embryo transfer scans were analysable in the miscarriage group and 49 and 60 datasets respectively, in the on-going pregnancy group.

When considering clinical pregnancy as the primary outcome, the mid-luteal phase maximal FI obtained using the manual analysis method was significantly higher in the pregnant group compared to the non-pregnant population (P=0.007). Average manual STIC FI at embryo transfer was higher in the non-regnant population compared to the pregnant women (P<0.05), with all other average vascularity indices not differing between the analysed populations (P>0.05; see Figure 59 and Figure 60). When considering first trimester pregnancy loss, the manual minimal VI, average VI and minimum VFI prior to TVOR were significantly higher compared to the on-going pregnancy group (P<0.05; see Table 20).

Binary logistic regression corrected for age, duration of infertility, ovarian reserve, biopsy or control group allocation, day of embryo transfer and embryo quality did not alter the significance of STIC vascularity indices as predictors of clinical pregnancy (P>0.05).

7.4.11. **Predictive value of STIC vascularity indices for clinical pregnancy**

Both analysis methods have proven to be poor predictive markers for clinical pregnancy with no index achieving an AUC of >0.65 at any of the assessed time points.

For first trimester miscarriage, the highest AUC of 0.8 was for the minimal manual VI at TVOR (cut-off value ≥0.7; sensitivity 80.0% and specificity 68.1%), followed by minimal manual VFI at TVOR (AUC 0.787, cut-off value ≥0.15; sensitivity and specificity of 80.0% and 66.0%, respectively) and average manual VI at TVOR (AUC 0.79, cut-off value

≥1.67; sensitivity and specificity of 80.0% and 63.8%, respectively) (see Figure 61). Vascularity in the population with first trimester miscarriages was higher compared to women with successful pregnancies (higher minimum VFI; P>0.05).

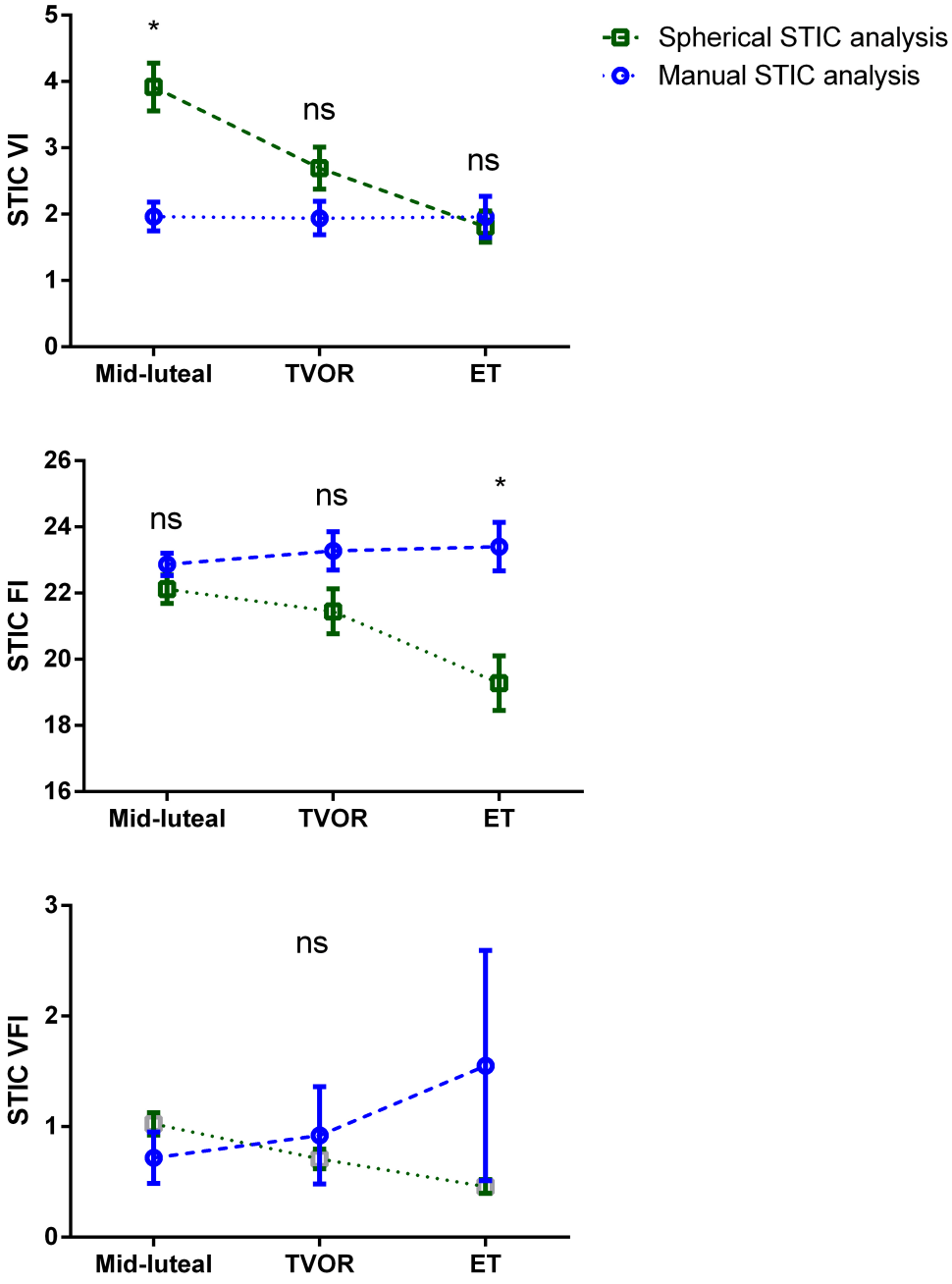


Figure 55. Average STIC vascularity indices using the manual and spherical sampling.

*Data presented as mean ±SEM. TVOR- transvaginal oocyte retrieval; ET- embryo transfer; ns- non significant; * P<0.05.*

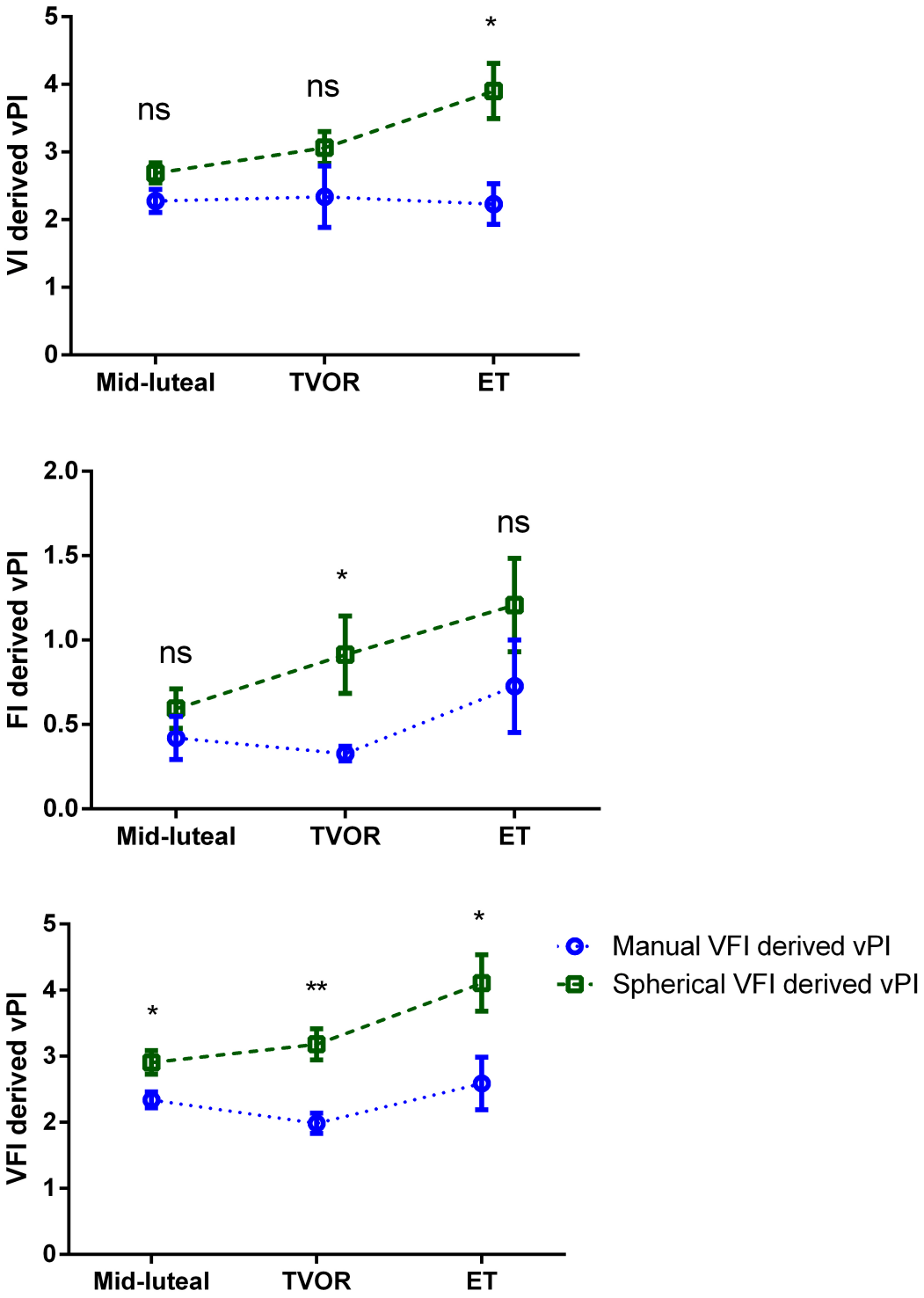


Figure 56. STIC vPI indices using the manual and spherical sampling methods at assessed time points.

*Data presented as mean ±SEM. TVOR- transvaginal oocyte retrieval; ET- embryo transfer; ns- non significant; * P<0.05; ** P<0.001.*

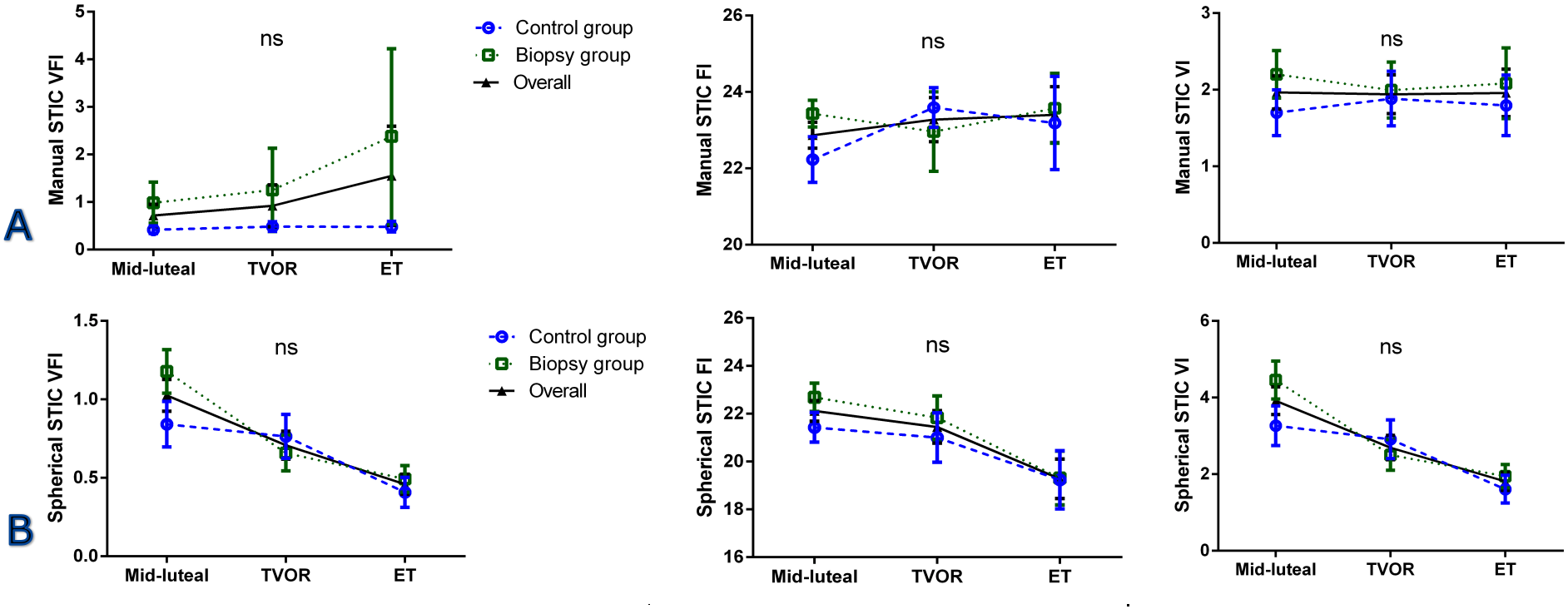


Figure 57. Average STIC vascularity indices obtained using manual (A) and spherical (B) analysis methods at the assessed time points in the biopsy and control populations.

Data presented as mean ±SEM. TVOR- transvaginal oocyte retrieval; ET- embryo transfer; ns- non significant.

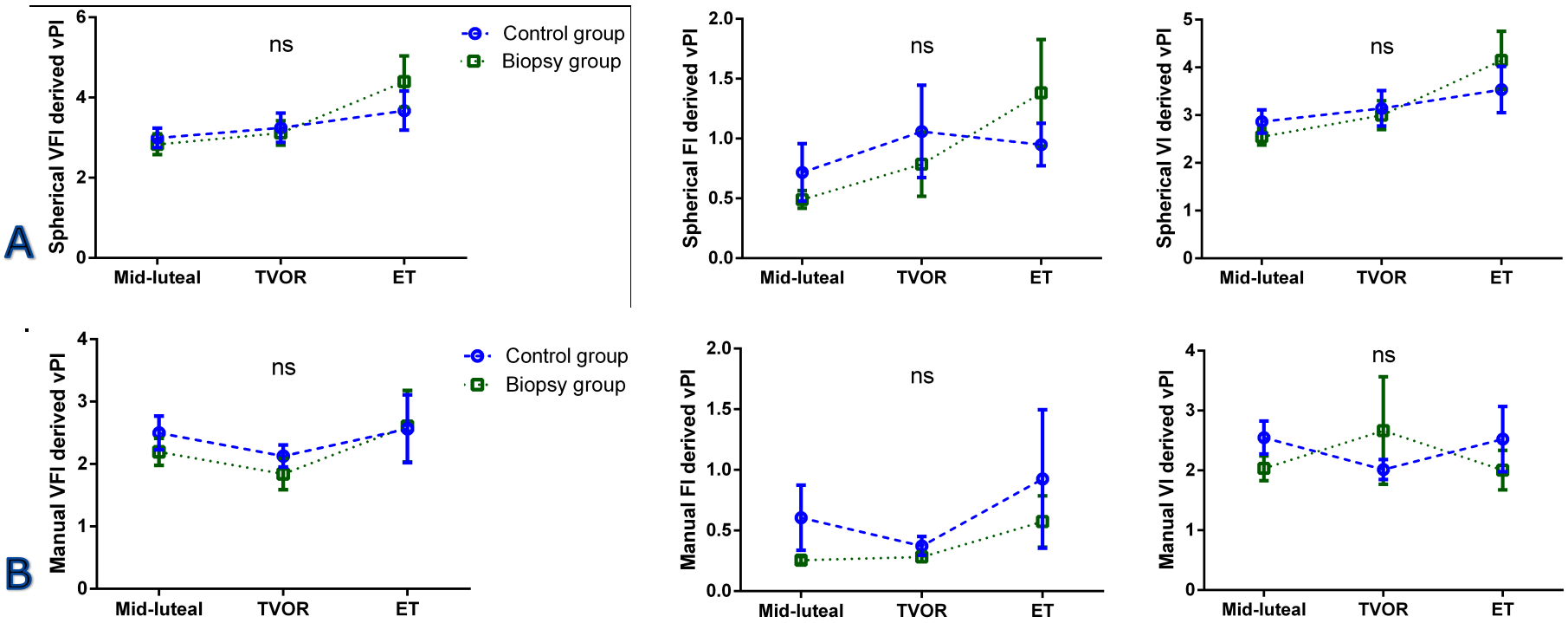


Figure 58. STIC vPI in the biopsy and control populations in the spherical (A) and manual (B) analysis methods at assessed time points.

Data presented as mean ±SEM. TVOR- transvaginal oocyte retrieval; ET- embryo transfer; ns- non significant.

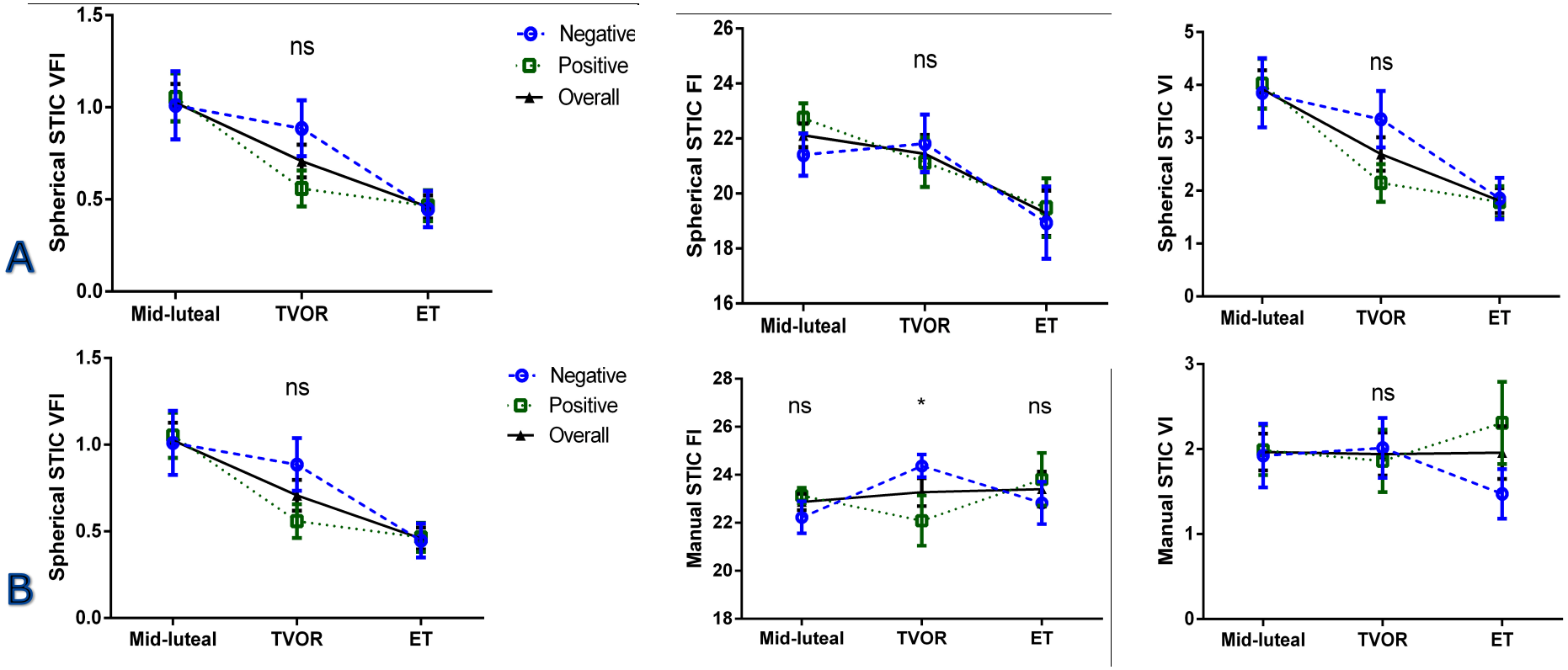


Figure 59. Average STIC vascularity indi es obtained using spherical (A) and manual (B) analysis methods at the assessed time points in the pregnan and non-pregnant populations.

*Data presented as mean ±SEM. TVOR- trans aginal oocyte retrieval; ET- embryo transfer; ns- non significant; * P<0.05.*

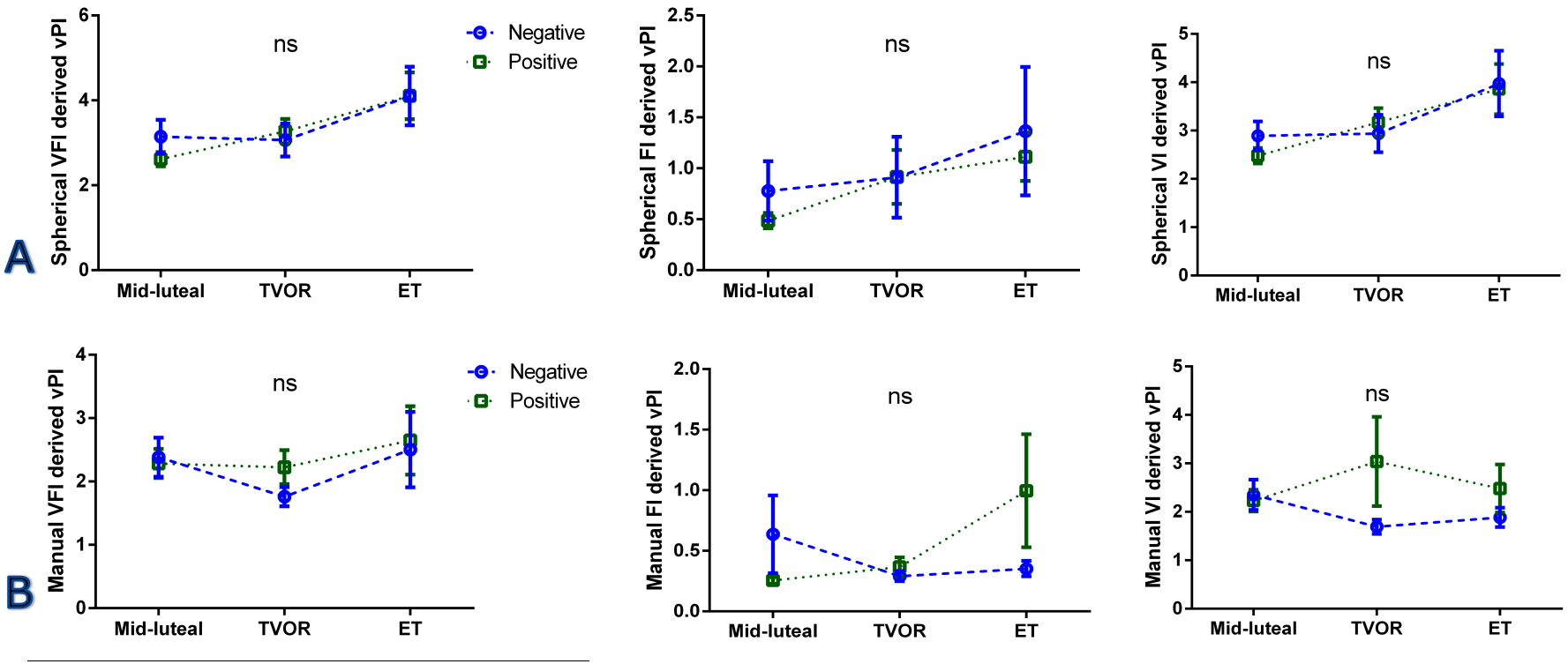


Figure 60. Average STIC volumetric PI vascularity indices derived from spherical (A) and manual (B) analysis methods at the assessed time points in the pregnant and non-pregnant populations.

Data presented as mean ±SEM. TVOR- transvaginal oocyte retrieval; ET- embryo transfer; ns- non significant.

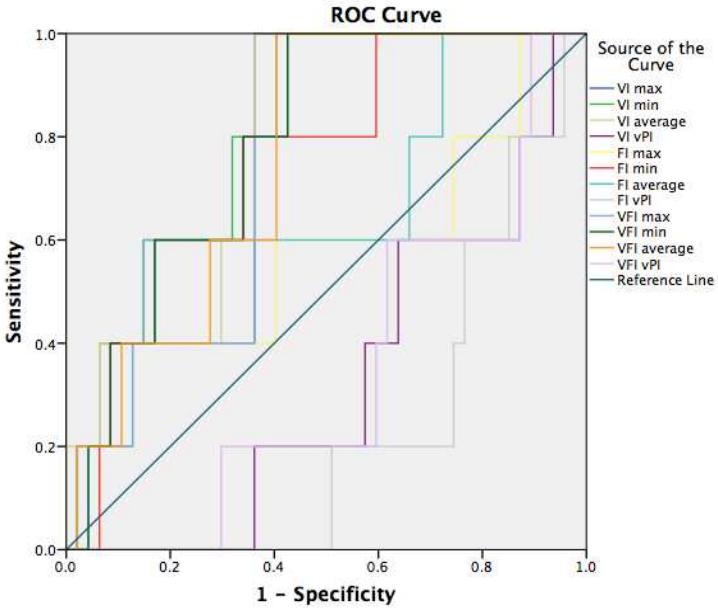


Figure 61. ROC curve analysis of manual STIC vascularity indices at TVOR when predicting first trimester miscarriage.

The greatest area under the curve is represented by minimal manual VI (VI min), followed by minimal manual VFI (VFI min) and average manual VI (VI average).

7.5. Discussion

In the first part of this study, the possibility to acquire a volume of the entire endometrium and measure it reliably by the same and different observers using STIC was demonstrated. We have described in detail, the most optimal settings for acquisition of endometrial STIC allowing for clear depiction of blood flow (=Doppler signal intensity) during cardiac systole and diastole. In all cases, the ICCs were above 0.8, suggesting a very good reliability of measures. When spherical samples of the endometrium were measured, inter and intra-observer reliability was acceptable, though lower (ICC >0.5 for FI). The difference in vascular indices obtained using spherical sampling is most likely due to the subjective selection of the 'true' uterine fundus by different observers. Vascularity indices obtained using whole endometrial and spherical analysis significantly differed and hence one method cannot be substituted for the other when analysing the endometrial STIC indices. We have also confirmed previous reports of the 3DPD index values differing from obtained average STIC values. In our study the VI and FI were higher when using 3DPD modality compared to STIC, which is in agreement with the study by Martins et al. (2011) where STIC VI and FI differed compared the static 3DPD counterpart, depending on phase of cardiac cycle.

To our knowledge, the second part of this work assessing the correlation of endometrial STIC vascularity indices with ART outcome is the first study reported to date. Endometrial vascularity was assessed using spherical and manual analysis, in an attempt to explore which method, if any, is superior as a minimally invasive predictor of implantation or early pregnancy failure. The results show that there were no differences in the individual STIC vascularity indices between the pregnant and non-pregnant groups, regardless of the day of data acquisition. Manual minimal VI, average VI and minimal FI were lower in the on-going population compared to first trimester miscarriage populations ($P<0.05$). Post- biopsy changes in the vascularity were no different when compared to the control population. In this regard, vascularity indices obtained

using STIC show little overall difference compared to conventional 3DPD analysis, thought absolute values do differ.

Studies assessing the reproducibility of 3DUS measurements have confirmed the modality to be reproducible, hence useful in a clinical and research setting (Raine-Fenning et al. 2004b). As STIC is a relatively new modality, no studies as yet have assessed the ability to acquire and measure the entire endometrium. As demonstrated in this chapter, it is possible to perform this with an ICC >0.8. When spherical sampling was performed, the ICC values were lower and in agreement with a previous paper on the subject (Martins et al. 2011). Other papers utilising spherical sampling for assessment of fetoplacental blood flow have also reported ICC values in excess of 0.8 (Welsh et al. 2012).

The time-consuming manual analysis of the entire dataset is however a significant limiting factor to the generalizability of the technology (Martins et al. 2006, Martins et al. 2011). Spherical sampling has an added benefit of shorter analysis time and assessment of the same spherical ROI, as the software keeps the ROI static throughout analysis of all static volumes included in one STIC dataset. Manual analysis has a potential to delineate a different ROI in each frame leading to introduction of measurement errors. Alterations of equipment settings and distance of the ROI from the transducer have an impact on the acquired vascularity indices; hence care needs to be given to standardisation of this technology, both in a research setting and in possible clinical applications.

The inability to analyse all images, also is a significant limiting factor of the technology. Lack of clear definition of the endo-myometrial border on numerous images does not allow delineation of the endometrium, and as such is prone to inclusion of a varying volume of the subendometrium with abundant spiral arteries. This is also the case when utilising spherical sampling, as in a proportion of the analysed images, the top part of the endometrium and a minimal volume of the adjoining subendometrium was analysed. This is related to arbitrarily setting the sphere volume to 1 cm^3 (to allow for reproducibility), which in cases of

small uteri led to inclusion of subendometrium. Potentially the most significant limiting factor of the technology is the significant cycle-to-cycle variability of obtained vPI in excess of 20% for each 3D vascularity index.

The only research application of STIC in the context of reproduction and female pelvic organ assessment was related to spherical sampling of the endometrium and ovarian stroma (Alcazar et al. 2010, Kudla et al. 2012). In the mentioned studies, no clinical outcome data was reported. Therefore, we have addressed this paucity of data related to the correlation of STIC indices with ART outcomes in the second part of this study.

As described, vascularity indices obtained using manual and spherical STIC analysis differed significantly, most likely due to the inclusion of a vascular subendometrium. The average vascularity indices decreased towards embryo transfer when spherical analysis was employed, suggesting that the vascularity within the subendometrium is depleted dramatically. The observed results may indicate that the subendometrial component within the spherical analysis constitutes a significant proportion of the Doppler signal within the VOI visibly influencing the results. This is supported by the increased vascularity indices obtained when only the endometrium was analysed using the manual method. A relative decrease of endometrial and subendometrial vascularity in known to take place both in natural (Raine-Fenning et al. 2004d) and ART cycles when 3DPD modality is used (Ng et al. 2009). Our data is in accordance with this observation, however only when spherical STIC vascularity indices are considered. Manual analysis shows an increasing vascularity overall from the mid-luteal phase of the cycle to the day of embryo transfer. Inclusion of the endometrium and subendometrium in the spherical analysis and averaging the vascularity within these two structures might explain the concordance with the 3DPD findings of Ng et al. (2009). Potential technical differences in STIC and static 3DPD modalities and related averaging of observed vascularity may be responsible for the discrepancies when the manual method results are considered, thus highlighting that STIC and static 3DPD cannot be used

interchangeably. The observed endometrial vascularity in on-going pregnancies has been lower, with higher vPI values, compared to endometrial vascularity when first trimester miscarriages occurred. This observation does concur with 3DPD observations by some authors (Ng et al. 2006d), but is in contradiction with other observations (Wu et al. 2003, Merce et al. 2008). The timing of the analysis seems to be important, as Ng et al. (2006) has performed the analysis at TVOR, but Wu et al. (2003) and Merce et al. (2008) have carried out their analyses on day of hCG injection.

The importance of endometrial and subendometrial vascularity in the process of embryo implantation has been well recognised (Jinno et al. 2001). Findings of lower vascularity and higher vPI prior to implantation seem to confirm the hypothesis of oxidative stress, which is related to an increased vascular perfusion to the conceptus overwhelming its defensive mechanisms, thus leading to a miscarriage (Jauniaux et al. 2000). When 3DPD vascularity indices were measured in the populations with and without a miscarriage, no such differences were observed (See Chapter 6). This discrepancy is difficult to explain, but might yet again be related to the sensitivity of the STIC modality compared to static 3DPD and averaging of the vascularity indices. The different vascularity index value progression between the manual and spherical analysis suggest that these two entities- the endometrium and the subendometrium- display different vascularity throughout the menstrual cycle. Higher predictive values for miscarriage when manual analysis was carried out, indicate that the endometrial vascularity is more important in preparation for embryo implantation.

Overall, predictive values for clinical pregnancy were suboptimal, with the highest AUC achieved for mid-luteal manual maximal FI=27.8 (AUC=0.643; sensitivity and specificity 76.0% and 44.4% respectively). STIC was much better at predicting first trimester miscarriage when manual analysis was carried out prior to TVOR. The recorded vascularity indices were significantly lower in the on-going pregnancies compared to the ones that terminated in the first trimester, with minimal VFI highlighting the difference best. The marker with best performance as a

predictor of miscarriage was the minimal VI with a cut-off at ≥0.7 with a sensitivity of 80% and specificity of 68.1%. Analysis at different time points did not achieve such good values.

From the observations contained within this Chapter, we can infer that endometrial biopsy does not affect the endometrial microvasculature to produce a phenotype resembling vascularity index progression observed in successful cycles. These patterns and conclusions however, are based on limited numbers of patients; hence validation is required on larger populations and in natural cycles.

In summary, the analysis reported in this chapter, indicates that STIC is a viable modality for analysis of the endometrium. It is however a time consuming and labour intensive modality with high biological variability between cardiac cycles posing a potential source of bias and indicating that the method will most likely remain an experimental novelty. The two available methods- the manual and spherical analysis, produce differing results the significance of which was assessed in the prospective study of correlation with ART outcome. It has been demonstrated that STIC modality does not improve prediction of ART outcome, and as such has no real advantage over static 3DPD. Manual STIC analysis seems more accurate however in predicting first trimester pregnancy loss, but only on the day of TVOR. As only a few cases were analysed, additional studies are needed to substantiate this assertion.

Future developments that overcome current drawbacks of the technology (i.e. time consuming analysis, limited availability, and lack of automated data analysis) and the increasing sensitivity of the US equipment especially when HDPD is used, might allow for a more accurate description of the blood flow pattern within even finer endometrial vessels and allow for better correlation of the vascular data with pregnancy outcome. If this proves to be useful, then STIC analysis of the endometrium will become a very valuable endometrial receptivity assessment tool.

CHAPTER 8. Uterine natural killer cells in a selected and unselected population of women and association with reproductive outcomes.

8.1. Introduction

In the human endometrium, $CD56^+$ uterine natural killer (uNK) cells are present from 3 to 8 days following the preovulatory LH surge (King et al. 2000b, Rieger et al. 2004). When no pregnancy occurs, these cells are the first to show signs of apoptosis (King et al. 2000b). When conception takes place, the uNK cells increase in numbers (King et al. 1996, Kammerer et al. 1999), peaking between week 6 and 12 of gestation and remaining elevated until at least week 20 (Bulmer et al. 2005). These endometrial immune cells are a rich source of cytokines and growth factors, which are thought to stimulate the appropriate development of the implantation site- both the maternal and fetal component. In contrast to their peripheral counterparts, uNK cells have almost no cytotoxic potential (Bulmer et al. 2005).

Inappropriately low or high levels of NK cells within the endometrium during the window of implantation (WOI) have been associated with reproductive problems. Murine studies with NK/uNK-cell deficient mice show that these animals have normal fertility with abnormal implantation sites (Croy et al. 2006). Spiral arteries maintain their pre-pregnant properties that prevent the physiological drop in blood pressure from occurring, allow for pulsatile blood flow to continue and respond to vasoconstrictive stimuli (Croy et al. 2006). Reversion of these changes was observed in NK cell deficient mice following marrow transplant, which allowed for reconstitution of the NK cell population (Guimond et al. 1998). The overall secretory profile of uNK cells, as described on page 34 of this thesis, supports the important role of these cells on spiral artery remodelling and trophoblast maturation.

Progesterone (Piccinni et al. 1996) and dendritic cells (DCs) (Borzychowski 2005) are thought to be involved in differentiation of the uNK cell population to a pregnancy promoting Th2 type. This has been confirmed by the expression of ST2L receptor (selective surface marker for type 2 immune response) on NK, NKT and Th cells in a healthy pregnancy (Chan et al. 2001, Borzychowski et al. 2005). Low cytotoxicity of uNK cells and production of cytokines, such as colony-stimulating

factor-1, granulocyte and macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-γ, tumour necrosis factor (TNF)-α, transforming growth factor (TGF)-β, leukaemia inhibitory factor (LIF), platelet-derived growth factor (PGF), and vascular endothelial growth factor (VEGF)-C (Saito et al. 1993, Li et al. 2001), are similar in human and murine uNK cells. Secretion of these factors promotes neo-angiogenesis within the endometrium and placental bed. For example, VEGF-C derived from uNK cells has been shown to be responsible for lymphatic and arterial vasculature development within the endometrium (Croy et al. 2006).

Data on the exact prevalence of elevated uNK cells in selected and unselected populations of women is scarce. The normal levels of these cells have been based on small numbers of healthy patients, and require further inquiry (Quenby et al. 2005). Though uncertainties exist, immunosuppressant therapy in women with elevated uNK cells has been reported to improve reproductive outcomes, suggesting a possible cause-effect relationship (Quenby et al. 2002, Quenby et al. 2005). Human studies on women with RM (Lachapelle et al. 1996, Clifford et al. 1999, Quenby et al. 1999) and implantation failure assessing the pNK (Aoki et al. 1995, Kwak et al. 1995, Beer et al. 1996, Matsubayashi et al. 2001b, Ntrivalas et al. 2001, Shakhar et al. 2003, Yamada et al. 2003) and uNK cell levels (Clifford et al. 1999, Quenby et al. 1999, Ledee-Bataille et al. 2005, Quenby et al. 2005, Matteo et al. 2007, Tuckerman et al. 2007) come to different conclusions in terms of association with subsequent pregnancy outcome. Sample analysis methods, different study populations, and lack of uniformly accepted reference ranges for NK cell numbers may all contribute to these uncertainties.

The aims of this study were to assess the prevalence of elevated uNK cells in a population of women with reproductive problems: infertile women undergoing first cycle of assisted reproductive treatment (ART) and women with reproductive failures, and correlate the levels with ART pregnancy outcomes, as well as assess the uNK cell levels in two consecutive menstrual cycles.

8.2. Materials and Methods

8.2.1. **Study design and participants**

To address the prevalence of elevated uNK cell levels in women with recurrent implantation failure (RIF) and recurrent miscarriage (RM), an observational comparative cohort study was conducted. Women under the age of 49 with RM or RIF attending Nurture Fertility, Nottingham for Comprehensive Implantation Screening Service from July 2014 to July 2014 served as population of women with reproductive failure; women recruited into the biopsy arm of the Endoscratch study with no history of RM or RIF undergoing first ART cycles served as the control population. RM was defined as two or more early trimester miscarriages and RIF was defined as previous two or more failed embryo transfer cycles. Uterine instrumentation in the previous three months constituted an exclusion factor for recruitment.

To address the uNK cell levels in subsequent cycles, a prospective observational study was carried out (13/EM/0277) in women with RM, RIF or infertile women recruited between December 2013 to July 2014.

8.2.2. **Interventions**

Verbal or written consent was obtained prior to any intervention, as appropriate. In all cases, a home urinary ovulation (luteinising hormone-LH) kit was given to each participant to use from day 8 of the biopsy cycle. Endometrial biopsies were carried out 7 to 9 days following a detected LH surge or on day 19 to 24 if no LH surge was apparent. All women were advised to use barrier methods of contraception or abstain from intercourse in the biopsy cycle. If a biopsy was performed in two successive cycles, the timing of the second biopsy was the same as in the first cycle. A positive pre-procedure urinary pregnancy test, excluded the participant from the study. A transvaginal ultrasound scan prior to the biopsy was performed using a GE Voluson E8 ultrasound machine equipped with a 5-9MHz endocavitary transducer (GE, Sipf, Austria) to exclude any gross pathology. The endometrial biopsy was carried out by two experienced physicians (LP and MB) using Pipelle de Cornier (CCD

Paris, France) in a manner described on page 86. The endometrial sample was then transferred into a container with 4% PFA.

8.2.3. **Sample preparation and analysis**

The PFA sample preparation and CD56 staining was carried out as described on page 95.

Slide analysis was carried out by one observer (LP) using the semi-automated 20x magnification method described on page 95. The uNK cell results were reported as percentage (%) of sample area occupied by diaminobenzidine (DAB) in relation to the sample area occupied by nuclei of endometrial stromal cells (ESCs).

8.2.4. **Outcome variables**

Collected demographic data included age, cause of infertility, body mass index (BMI), and past reproductive history. Sonographic data including endometrial thickness, volume, three-dimensional power Doppler (3DPD) vascularity, and uterine artery (UA) Doppler indices was collected for women in the repeat biopsy study and the Endoscratch study. Timing of the biopsy according to LH was recorded.

For assessing the correlation of uNK cell count with ART outcome, the clinical pregnancy rate, defined as presence of a fetal heart beat at a 6-week transvaginal ultrasound scan, and miscarriage rate were recorded.

8.2.5. **Statistical analysis**

Statistical analysis was performed using the Statistical Package for the Social Sciences (version 21; SPSS, Chicago, IL, USA). Distribution of data was assessed using Kolmogorov-Smirnov test. For parametric un-paired data student's t test was used and for non-parametric data, Mann-Whitney U test was carried out. For paired samples, student's t test was used for parametric data and Wilcox signed rank test was used for non-parametric data. Pearson's r statistics was used to assess correlation between samples. A P value of <0.05 was considered as statistically significant.

Receiver operator characteristics (ROC) curve analysis with area under the curve (AUC) and appropriate sensitivity and specificity values were used to determine the predictive value of uNK cell levels for outcomes specified.

8.3. Results

8.3.1. **Participants**

Seventy-six women were enrolled into the biopsy group of the Endoscratch study. Forty-five women were undergoing their first *in vitro* fertilization (IVF) treatment cycle, of which four had prior RM, leaving 41 women meeting inclusion criteria for the control population. The demographic details of this group are presented in Table 22.

Sixty-three women that attended the host unit for endometrial biopsy between February 2012 and July 2014 as part of implantation failure screening and endoscratch study, which met the inclusion criteria of having RIF or RM, were included. Endometrial sample in two cases was too small to be analysed. The demographic details of the participants are presented in Table 22.

Twenty-two women were recruited to have repeated biopsies performed between December 2013 and July 2014. One woman did not have a repeat biopsy due to severe vasovagal reaction following the first biopsy. One sample was too small to be analysed. Three women did not attend for the study appointments and were withdrawn from the study. One woman was found to have a hydrosalpinx at the initial appointment and was withdrawn from the study. Complete data was available for sixteen women.

8.3.2. **Prevalence of elevated uNK cell levels**

There was no statistical difference in uNK cell levels between women with RM or RIF and women undergoing their first IVF treatment cycle [4.25 (1.8-7.6) vs. 2.56 (1.4-7.4) respectively; Mann- Whitney test P=0.246]. Though the number of subjects in the repeat biopsy group was small (n=16), the uNK cell count tended to be lower when compared to the reproductive failure group (P>0.05, see Table 23).

Clinically significant result defined as >5% of ESC was greater in women with reproductive complications compared to controls undergoing first IVF cycle (42.2% vs. 28.3%, respectively; P>0.05).

Variable		*Control population (n=41)*	*Women with RIF and RM (n=63)*	*Women having repeat biopsies (n=18)*
Age (years)		32.5(±3.6)[a]	37.0(±4.6)[a]	35.6(±4.4)
BMI (kg/m2)		24.5(±3.3)	25.0(±3.8)	22.4(±4.6)
Previous biochemical pregnancies	*0*	33(71.7%)[a]	9(17%)[a]	5(27.8%)
	1-2	13(28.3%)	22(41.5%)	9(50%)
	≥3	0	20(41.5%)	4(22.2%)
Previous clinical pregnancies	*0*	34(73.9%)[a]	14(26.4%)[a]	7(38.9%)
	1-2	12(26.1%)	22(41.5%	8(44.5%)
	≥3	0	15(32.1%)	3(16.6%)
Previous miscarriages	*0*	38(80.9%)[a]	16(30.2%)[a]	11(61.1%)
	1-2	8(19.1%)	21(39.6%)	5(27.8%)
	≥3	0	14(30.2%)	2(11.1%)
Previous live births	*0*	42(91.5%)	41(77.4%)	15(83.3%)
	1-2	4(8.5%)	10(18.9%)	3(16.7%)
	≥3	0	0	0
Previous EPs or TOPs		2 EPs and 2 TOPs	4 EPs and 1 TOP	2 EPs and 4 TOPs
Previous IVF or ICSI treatments	*0*	46(100%)[a]	14(26.4%)[a]	9(50%)
	1-2	0	22(41.6%)	8(44.4%)
	≥3	0	15(32%)	1(5.6%)

Table 22. Demographic details of women with known NK cell count.

Data presented as mean (±SD), median (interquartile range) or number (%) as appropriate. BMI- body mass index; EP- ectopic pregnancy; TOP- termination of pregnancy; ICSI- intracytoplasmic sperm injection; †- levels reported in repeated samples for 16 women; [a] P<0.05 for comparison between control and RIF population; P-values assessed by unpaired t test or Mann-Whitney test, as appropriate.

Variable	Control population (n=46)	Women with RIF and RM (n=64)	Women having repeat biopsies (n=18)
NK cell count (%)	2.56(1.4-7.4)	4.25(1.8-7.6)	2.4(0.78-4.8)[a] 0.7(0.4-0.91)[a]
Percentage of clinically significant NK result (>5%)	13(28.3%)	27(42.2%)	4(22.2%) 2(12.5%)

Table 23. NK cell results in assessed populations.

Data presented as mean (±SD), median (interquartile range) or number (%) as appropriate; [a] P<0.05 assessed using Mann-Whitney test.

8.3.3. **Correlation of uNK cell levels with pregnancy outcomes**

Women in the biopsy arm of the Endoscratch study were included in this analysis. Sixty-eight women underwent the biopsy. uNK cell results were available for sixty-six women, as two samples were too small to be analysed. Embryo transfer was carried out in fifty-eight women. Stimulation protocol and embryo quality details are presented in Table 24. The median NK cell count in this population was 2.43% (1.05-7.65%). Clinically significant uNK cell levels were found in 19 (32.8%) of women.

In women with elevated uNK cell levels, ART outcomes were unrelated to the uNK cell count, with biochemical pregnancy rates of 68.4% (13/19) in women with uNK cell count >5% versus 74.4% (29/39) in the population with uNK cell levels of <5% (P>0.05). Clinical pregnancy rates were 63.2% (12/19) and 69.2% (27/39), respectively (P>0.05). Miscarriage rates were 5.3% (1/19) and 12.8% (5/39), respectively (P>0.05).

Variable		***Endoscratch biopsy population (N=76)***
Age (years)		33.3(±4.0)
Duration of infertility (months)		36(28.5-48)
BMI (kg/m2)		24.7(±3.3)
AMH (pmol/L)		18.75(10.3-32.1)
Cycle number	*First cycle*	50(65.8)
	Second or subsequent	26(34.2)
Duration of stimulation (days)		12(11-13)
Dose of gonadotropin (IU)		2586(±910)
Oocytes collected		13(9-20.5)
Type of treatment	*IVF*	31(40.8)
	ICSI	34(44.7)
	FER	7(9.2)
Day of embryo transfer	*Day 2*	5(6.6)
	Day 3	1(1.3)
	Day 5	58(76.3)
Freeze-all due to risk of OHSS		5(6.6)
Number of embryos transferred	*SET*	41(53.9)
	DET	23(30.3)
Good quality embryos		62/87
Clinical pregnancy rate		42(55.3)

Table 24. Endoscratch biopsy group characteristics.

Data presented as mean (±SD), median (interquartile range) or number (%) as appropriate; AMH- anti-Mullerian hormone; FER- frozen embryo replacement; OHSS- ovarian hyperstimulation syndrome; SET- single embryo transfer; DET- double embryo transfer.

8.3.3.1. ROC curve analysis

The uNK cell levels were unable to predict reliably the outcome of treatment, with the highest AUC of 0.545 for clinical pregnancy with a sensitivity of 69.2% and specificity of 42.1% for a cut-off NK cell count of >4.81%. Respective values for biochemical pregnancies were AUC of

0.512 with a sensitivity 69.0%, specificity 43.7% for cut-off value >4.81%; and for miscarriage rates AUC of 0.51 (sensitivity 66.7%, specificity 44.2% for a cut-off value of >2.0%) (see Figure 62).

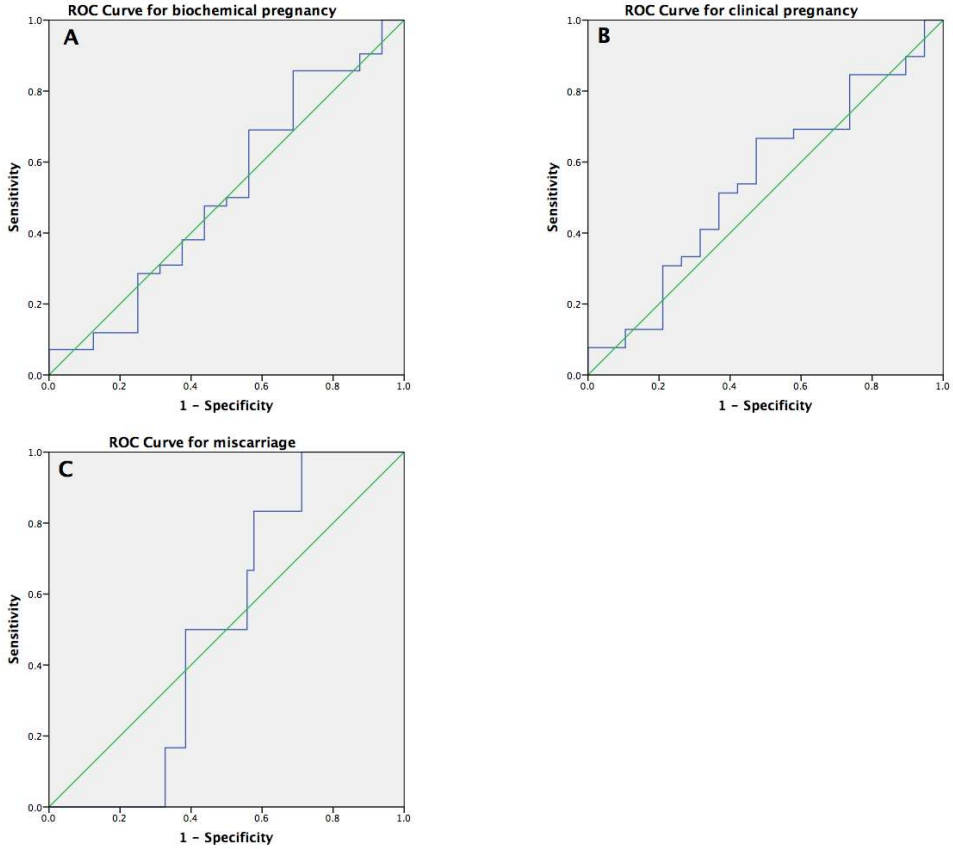


Figure 62. ROC analysis curves for NK cell count and biochemical (A), clinical (B) and miscarriage rates (C).

8.3.3.2. Subgroup analysis based on treatment cycle number

Due to low numbers of multiple failed cycles, the population was divided into first cycle patients (n=40) and second and higher order cycles (n=18). Sensitivity and specificity for predicting a biochemical and clinical pregnancy, as well as first trimester miscarriage were low in these subpopulations and similar to the overall Endoscratch study population (see Table 25).

	First cycle	*Repeated cycle*	*General population*
AUC	0.56	0.538	0.545
Sensitivity (%)	69.2	69.2	69.2
Specificity (%)	42.9	40.0	42.1
Level of uNK cells (%)	4.81	5.57	4.81

Table 25. Sensitivity and specificity for clinical pregnancy rate prediction based on uNK cell levels.

Data presented as mean (±SD), median (interquartile range) or number (%) as appropriate.

8.3.4. **uNK cells and sonographic appearance of the endometrium**

Endometrial thickness (r=0.125; P>0.05), pattern (r=0.183; P>0.05) and volume (r=0.183; P>0.05) were not associated with uNK cell count. 3DPD and HD vascularity indices were similarly not related. UA vascularity indices, apart from S/D, were not correlated with uNK cell count. Correlation of the uNK cell count with S/D was positive (r=0.583; R^2=0.340; P<0.001) (see Figure 63), but this relationship became non-significant, when the two very high S/D values were removed from analysis. Manual maximum STIC VFI (r=0.638; P<0.001) and average values (r=0.661; P<0.001) were positively correlated with uNK cell levels. Manual STIC VFI derived vPI of the endometrium was in a similar way associated with uNK cell count suggesting that higher resistance was associated with higher uNK cell number (r=0.420; R^2= 0.176; P=0.002). Spherical STIC VI derived vPI was negatively associated with uNK cell count, indicating that higher endometrial (and associated subendometrial) vascular resistance was associated with lower uNK cell counts (r=0.253; R^2= 0.064; P=0.048) (see Figure 64).

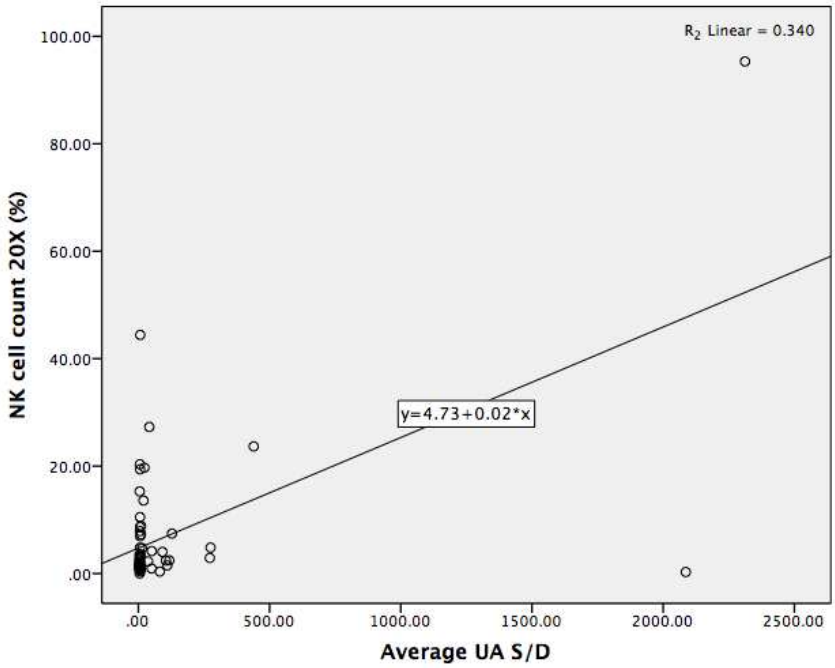
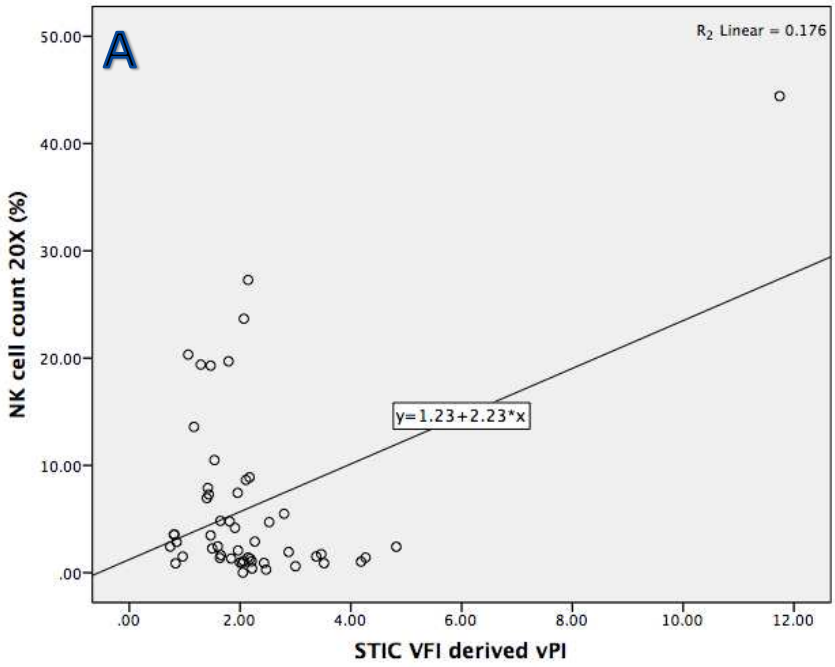


Figure 63. UA S/D in correlation with uNK cell numbers.



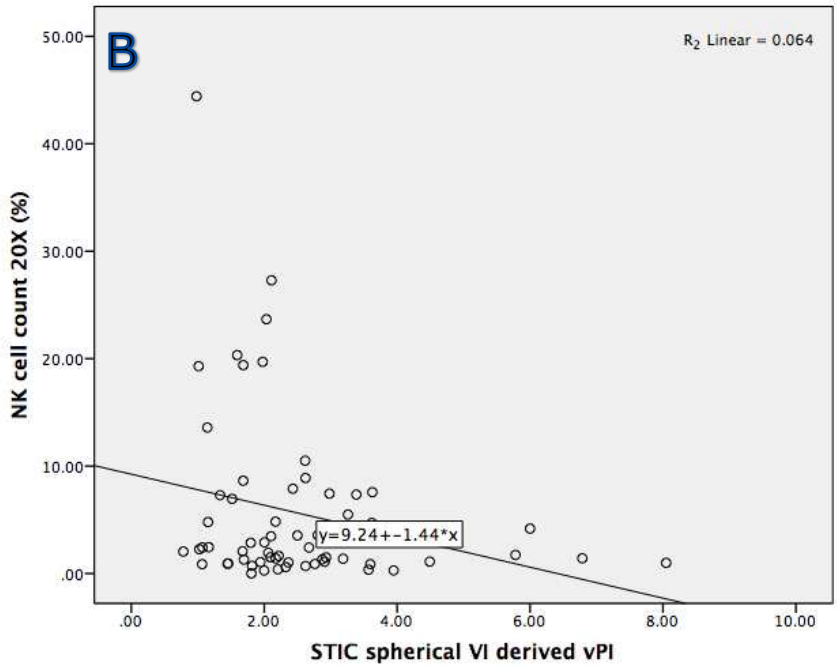


Figure 64. Correlation of STIC vPI and uNK cell count (A- manual analysis, B- spherical analysis).

8.3.5. **uNK cell levels in subsequent menstrual cycles**

The baseline uNK cell level in the first cycle was 2.4% of ESC surface area (0.78- 4.8%). The levels in the second cycle were significantly decreased at 0.7% (0.4-0.91%; P<0.01; see Figure 65 and Figure 66). When reporting the results as clinically significant (>5% of ESC), a positive result was present in 2.2% (4/18) of women in the first cycle and 1.3% (2/16) in the second cycle. In three cases, where the initial NK cell count was <1%, an increase in the NK cell count was observed in the second biopsy (Figure 67; see Table 4 in the Appendix for individual participant data). All of these women were infertile. In two women with an initial NK cell count <1% the NK cell count decreased further in the second sample. One of these women had 7 miscarriages, two terminations and one live birth; the other, had one termination only.

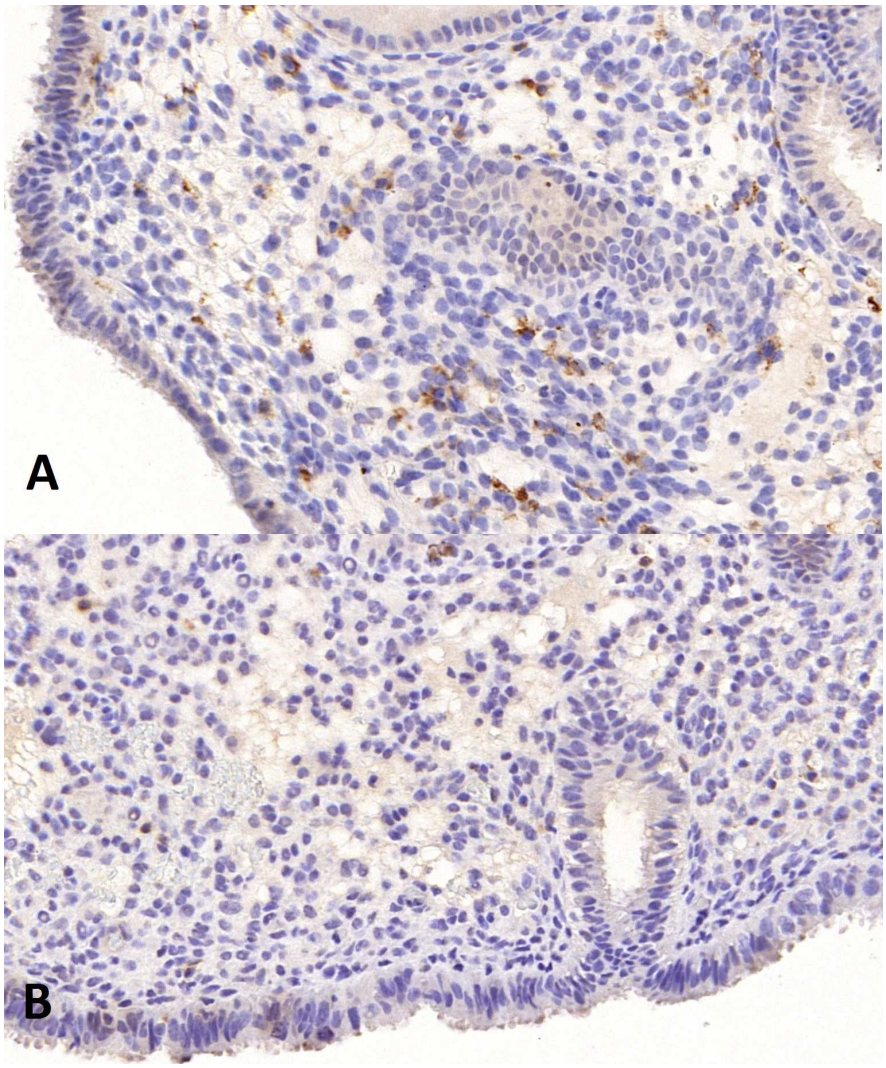


Figure 65. Endometrial appearance in the first cycle (A) and second cycle (B). Note significantly less uNK cells (DAB stained cells).

The endometrial thickness, pattern, volume, 3DPD vascularity indices, and UAPWD indices did not differ in the two biopsy cycles (P>0.05) (see Table 26).

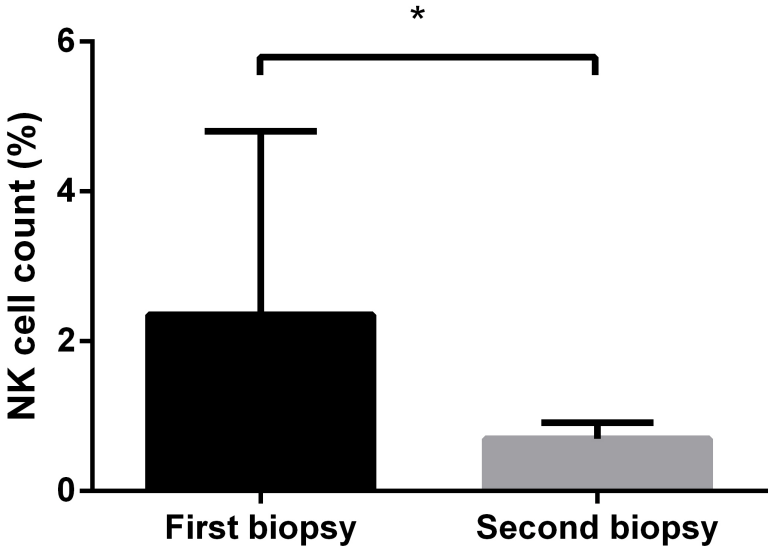


Figure 66. NK cell count in repeated samples obtained from the same patient.

**P<0.01 assessed using Mann-Whitney test.*

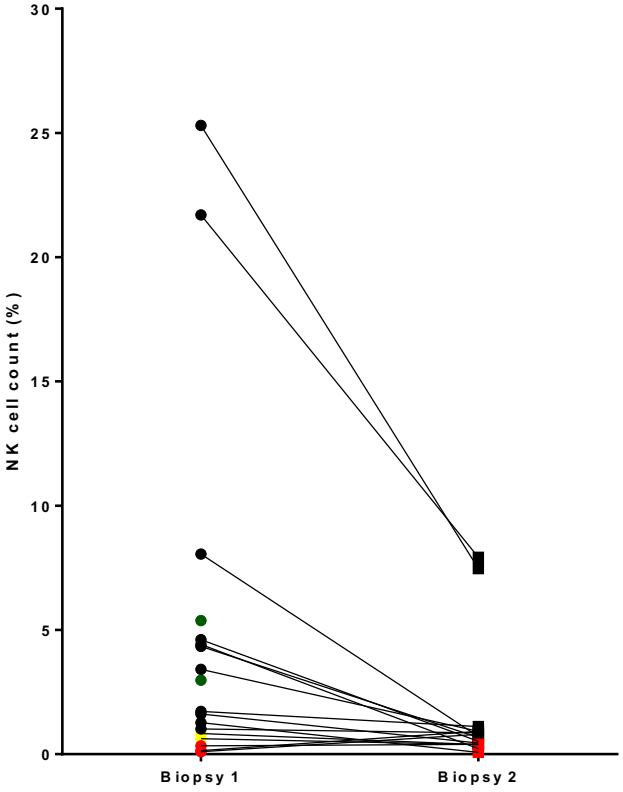


Figure 67. Individual uNK cell values in repeat endometrial samples from same participant.

Ultrasound variable	***Biopsy cycle 1 (N=18)***	***Biopsy cycle 2 (N=16)***
Endometrial thickness (mm)	8.1(6.8-10.5)	9.37 (7.85-11.55)
Endometrial volume (cm3)	2.78 (±1.27)	2.89 (±1.23)
3DPD VI	2.07 (0.78-4.1)	3.61 (0.4-3.43)
3DPD FI	27.05 (±3.3)	28.16 (±3.28)
3DPD VFI	0.6 (0.19-1.13)	0.42 (0.1-1.02)
UA PI	2.2 (1.83-2.65)	2.18 (1.95-2.46)
UA RI	0.83 (±0.07)	0.82 (±0.05)
UA S/D	12.44 (±20.4)	6.89 (±3.44)

Table 26. Sonographic variables in the first and second biopsy cycle.

Data presented as mean (±SD), median (interquartile range) or number (%) as appropriate.

8.4. Discussion

It has been demonstrated in this study that the prevalence of elevated uNK cells in the endometrial samples varies across populations with various manifestations of reproductive problems. The highest values of 4.25% (1.8-7.6%) were found in women with RIF and RM. Women undergoing their first IVF cycle tended to have a lower level of positive uNK cell results, (28.3%) compared to women with RIF, however statistical significance was not achieved (42.2%; $P>0.05$). uNK cell levels were unable to effectively predict clinical pregnancy with an AUC of 0.545, sensitivity 69.2% and specificity 42.1%. A similar result was obtained for subgroup analysis based on treatment cycle number. Finally, this work has also demonstrated for the first time a decrease in the uNK cell count in the mid-luteal phase of a menstrual cycle following an endometrial biopsy in the cycle before. In a repeated biopsy specimen, the uNK cell levels were on average, significantly lower compared to the initial biopsy. This might therefore, be one of the explanations for the beneficial effect of endometrial injury on the outcome of ART in women with RIF.

As there is no universally agreed method of counting uNK cell levels, we decided to use computer aided image analysis. Historically, these levels have been expressed as mean numbers of uNK cells (Clifford et al. 1999), percentage of total stromal cells (Quenby et al. 1999, Tuckerman et al. 2004) and as percentage of CD45+ cells (Michimata et al. 2002). One study indicates, that manual cell counting using computer software should be used as the gold standard, against which other counting methods should be assessed (Drury et al. 2011). However, a close correlation between the total NK cell numbers and percentage of total cells has been confirmed by Tuckerman et al. (Tuckerman et al. 2007), allowing for interchangeable use of manual and automated counting methods. Mariee et al. (2012) has compared manual counting of uNK cells with digital image analysis and has concluded that an excellent correlation exists between the numbers of cells obtained using both methods (Mariee et al. 2012b).

The findings of our study indicate that intentional endometrial injury has a significant effect on the uNK cell population and that women with RIF and RM are more likely to have an immune causative component to their infertility. High uNK cell levels observed in our subjects decrease significantly, hinting at a normal upper level of uNK cells, which at present is commonly quoted as 5% of ESCs (Quenby et al. 2005, Tuckerman et al. 2007). Based on the very limited observations of increasing uNK cell levels in three women having repeated biopsies (initial <0.34%, rising to <0.92%), one can speculate that endometrial biopsy may revert a yet undetermined endometrial pathology to a 'normal' state of immune homeostasis, and may indicate the existence of a lower cut-off value for normal uNK cell level. Further research is however required to validate this preliminary observation. In a study by Mariee et al. (2012b) 10 women had repeat endometrial biopsies with a minimum gap of two months between sampling. Using manual counting method, the results indicate no correlation between the uNK cell results in sample one and two (Mariee et al. 2012b). When an endometrial biopsy was performed in oocyte donors randomized to having an endometrial injury in a natural or stimulated cycle (four arm study), the authors observed a lower uNK cell count in the COH cycle compared to a natural one. An earlier endometrial injury did not alter the uNK cell number (Junovich et al. 2011). Flow cytometry was used to assess the uNK cell levels and approximately one week elapsed between the endometrial injury and the analysed biopsy. These major methodological differences could have contributed to the discrepancies between the study by Junovich et al. (2011) and the current work.

Current assumptions of normality related to uNK cell levels are based on very small numbers of patients, with varying definition of normality. uNK cell levels in 15 healthy women were 5% (2.1-19.2%) in one study (Tuckerman et al. 2010). Another study reports uNK cell levels in 10 control women with no history of miscarriages at 6.2±1.4% (Tuckerman et al. 2007). The authors of the abovementioned study suggest an upper normal uNK cell level of 13.9% as this was above the 90th percentile of the healthy controls (Tuckerman et al. 2007, Mariee et al. 2012b). The

results in the current study were lower at 2.7% (1.5-7.4%), which might be related to the fact that the control population in this study consisted of infertile women seeking ART or possibly due to the analysis methodology.

In agreement with previous studies, the present study has found elevated uNK cell levels in women with RIF or RM, however the results were not statistically significant. Past studies assessing women with RM, in concordance with the present study, have failed to find uNK cells as a useful predictor of subsequent pregnancy or miscarriage (Tuckerman et al. 2007). These findings are consistent with a systematic review of the literature where uNK cell density failed to predict pregnancy outcome (n=72; OR 1.33, 95% CI 0.16-11.11) (Tang et al. 2011). In our study, the reproductive failure population was defined as two or more first trimester miscarriages or two or more failed ART cycles, which differs from definitions used in other studies, and hence the possible observed uNK cell level differences (4.25%; 1.8-7.6%) compared to historical levels of 11.2±0.9% (Tuckerman et al. 2007) or 14.5% (1.5-71.4%) (Tuckerman et al. 2010). Other authors have failed to find such an association, and reported uNK cell levels in RIF women as comparable to controls (Matteo et al. 2007). In the study in question, participants had a diagnostic hysteroscopy in the proliferative phase of the cycle, followed by luteal phase hysteroscopic biopsies in the next cycle. This may have confounded the observed result, bearing in mind the potential effect of endometrial injury on uNK cell levels in repeated biopsies, as shown in this study (see Figure 66).

It is unlikely however, that the biopsy directly affects the uNK cell numbers, but rather alters the intricate local regulatory pathways. Evidence of a link between the metabolic pathways and uNK cells has been demonstrated with the assessment of endometrial cortisol metabolism. Affecting the rate of cortisone to cortisol conversion, 11β-hydroxysteroid dehydrogenase type 1 (11βHSD1) forms a gradient of active cortisol, which is thought to govern the uNK cell numbers and distribution within the endometrial stroma (Kuroda et al. 2013a). An attempt to verify this correlation in our population has been made as part of this thesis (see Chapter 9).

Some of the data observed in this work, suggests a relationship of uNK cells with endometrial vascularity. The most significant of these is related to the increased endometrial and subendometrial resistance associated with low uNK cell numbers when analysing spherical samples using STIC. This was not observed when whole endometrial STIC was employed. UA PI or RI were not correlated with uNK cell levels, however S/D ratio was. Knowing that uNK cells play an important role in neoangiogenesis within the endometrium, it is not surprising that low uNK cell levels will be associated with increased vascular resistance in the target organ. A similar observation was noted when UA blood flow characteristics were measured in a population of women with reproductive failures, in which low uNK cell numbers were associated with elevated PI>3 (Quenby et al. 2009). The observed discrepancy between the endometrium and subendometrium, as highlighted by the different correlation of manual STIC analysis and spherical analysis with uNK cell numbers, once again suggests that these two organs should be perceived as separate entities in the context of reproduction. Future studies should assess the spatial distribution of uNK cells not just in the *zona functionalis*, but also in the *zona basalis* and potentially even deeper, at the base of the spiral arteries.

The limitations of this study could be related to the definition of RIF used, and hence the obvious discrepancies with available literature regarding the uNK cell levels. Utilized methodology for uNK cell counting could also be a source of bias. The low numbers of patients recruited to the repeat biopsy arm of the study, means that this component is severely underpowered. Similarly, observed trends of decreasing and rising uNK cell levels in the second biopsy may be related to the 'reversion to the mean' phenomenon. Larger, appropriately powered study should eliminate these limitations.

Overall, the findings of this study indicate that the limited clinical benefit of uNK cell testing in women prior to the first ART cycle does not warrant performing this expensive and invasive test routinely. The population of women with reproductive failure could however benefit from this test in two ways. First- a possible diagnosis of the cause of infertility may be

identified, secondly- the endometrial biopsy might have a beneficial effect on the subsequent ART cycle by reverting the endometrium to a receptive, 'wild' state, provided this is performed shortly after the diagnostic biopsy, as demonstrated in the literature (Nastri et al. 2012). Though uNK cells appear to be an important component of the feto-maternal unit, many uncertainties exist. Further work is required to assess the effect of endometrial biopsy on the uNK cell population characteristics, especially in the RIF/ RM population, and what effect this might have on pregnancy outcomes.

CHAPTER 9. Selected molecular markers of successful pregnancy

9.1. Introduction

Multiple factors are involved in the complex process of pregnancy establishment. Deregulation of these systems can lead to various forms of reproductive failure. Appropriate decidualisation is necessary to allow absorption of uterine fluid, apposition and stable adherence of the blastocyst to the endometrial surface; penetration through the luminal epithelium and basal lamina, and final invasion of the stroma (Kimber et al. 2000, Norwitz et al. 2001, Wang et al. 2006). The receptive endometrial phenotype is expressed in the mid-luteal phase (day 20-24), or 6- 10 days after the pre-ovulatory luteinising hormone (LH) surge (Bergh et al. 1992, Achache et al. 2006).

Prolactin (PRL) and insulin-like growth factor binding protein 1 (IGFBP-1) are well established markers of human endometrial stromal cells (HESC) decidual transformation in *in vitro* culture. PRL production by HESC has been demonstrated to commence around day 22 of the menstrual cycle (Daly et al. 1983) and is positively correlated with decidual cell size (Wu et al. 1995). PRL seems to stimulate trophoblast growth and invasion, promotes angiogenesis, modulates uterine natural killer (uNK) cell survival, and regulates water transport through the amnion towards the maternal tissues (Jabbour et al. 2001, Corbacho et al. 2002, Stefanoska et al. 2013). Decidualising HESC secrete IGFBP-1 upon transition to a secretory phenotype during the window of implantation (WOI) with levels peaking around week 16 of pregnancy (Wathen et al. 1993). IGFBP-1 regulates the bio-availability of insulin-like growth factor (IGF)-I and stimulates trophoblast invasion (Gleeson et al. 2001). High levels of this protein seem to be necessary for pregnancy establishment; however raised levels in the latter half of pregnancy are associated with pregnancy complications such as intra-uterine growth restriction (IUGR) and pre-eclamptic toxaemia (PET) (Giudice 2002, Carter et al. 2004, Fazleabas et al. 2004).

As the glucocorticoid homeostasis is important in maintaining immune tolerance to the fetal allograft, 11-β hydroxysteroid dehydrogenase (HSD) is an integral enzyme responsible for this state. The bidirectional

11-β HSD1 predominantly converts the inactive cortisone to the bioactive cortisole leading to an increase in levels of active glucocorticoids (Courtney et al. 2008). 11-β HSD2 on the other hand, converts cortisole to cortisone (Ferrari et al. 1996). Isoform 1 of the enzyme is highly up regulated during the transition from undifferentiated HESC to decidual cells *in vitro* (Takano et al. 2007). 11-β HSD1 isoform expression is highest in the decidua and menstrual endometrium (McDonald et al. 2006, Michael et al. 2008). Type 2 isoform, is expressed mainly in the glandular epithelium throughout the entire menstrual cycle (Thompson et al. 2002, McDonald et al. 2006). Presence of 11-β HSD1 has also been confirmed on uNK cells suggesting that the action of these cells is modulated by the bioavailability and metabolism of glucocorticoids (McDonald et al. 2006). Based on these observations, some authors speculate that the increased activity of the bidirectional isoform of 11-β HSD upon decidualisation leads to modulation of the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) dependant pathways through the increased bioavailability of cortisole (Kuroda et al. 2013c). Formation of a corticosteroid gradient within the feto-maternal interface might be, at least partially, responsible for the immune tolerance between the maternal immune system and the fetus. Recent evidence seems to suggest that elevated subluminal uNK cells are a marker of relative cortisol deficiency putting these patients at risk of reproductive failure (Kuroda et al. 2013c).

The aim of this study was to assess the expression of selected markers of endometrial receptivity in a population of infertile women undergoing ART and correlate these levels with treatment outcome. As a secondary objective, we have explored the correlation of the molecular and sonographic markers of endometrial receptivity.

9.2. Materials and Methods

9.2.1. **Study design and participants**

The study was designed as a prospective cohort study. Study participants were selected from infertile women enrolled to the Endoscratch study. Twenty-four consecutive samples were selected based on the clinical pregnancy result- eleven positive and thirteen negative. No other variables were taken into account when selecting the samples. REC approval was granted for the study.

9.2.2. **Timed endometrial biopsies**

Endometrial biopsies were obtained using Pipelle endometrial sampler (CCD, Paris) as described on page 86. Endometrial biopsy was divided into two parts if possible- one put into 10% DCC for fresh ESC culture and the other into 4% PFA for immunostaining.

9.2.3. **ESC culture, decidualization and CD56+ staining**

Endometrial samples were processed immediately as described on page 88. Once confluent, ESCs were frozen in liquid nitrogen and transported to Clinical Sciences Research Laboratories, University Hospitals Coventry and Warwickshire, Clifford Bridge Road, Coventry, where culture, decidualization and analysis of the samples took place. ESC decidualization was carried out as described on page 90. uNK cell staining and analysis was carried out as described on page 95.

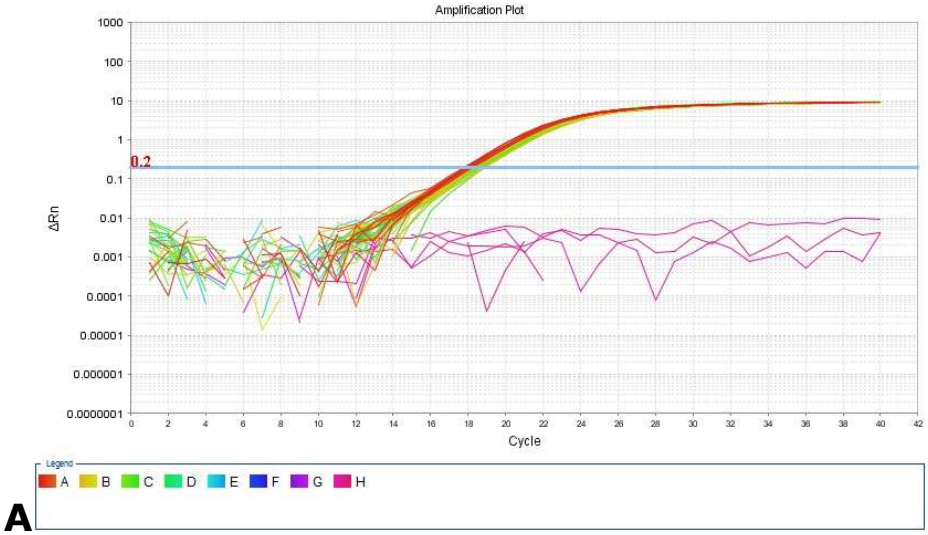
9.2.4. **PCR analysis**

Gene expression was carried out using real time quantitative PCR as described on page 92. PRL and IGFBP-1 were used as markers of decidualisation in order to confirm the transformation of cells into the secretory type. L-19, a ribosomal house-keeping gene, was used as a reference gene. 11-β HSD1 was the selected endometrial receptivity gene due to its important potential role in uNK cells homeostasis and endometrial decidualization. PCR primer sequences are presented in Table 27. Analysis of the data was carried out using the ΔCt with

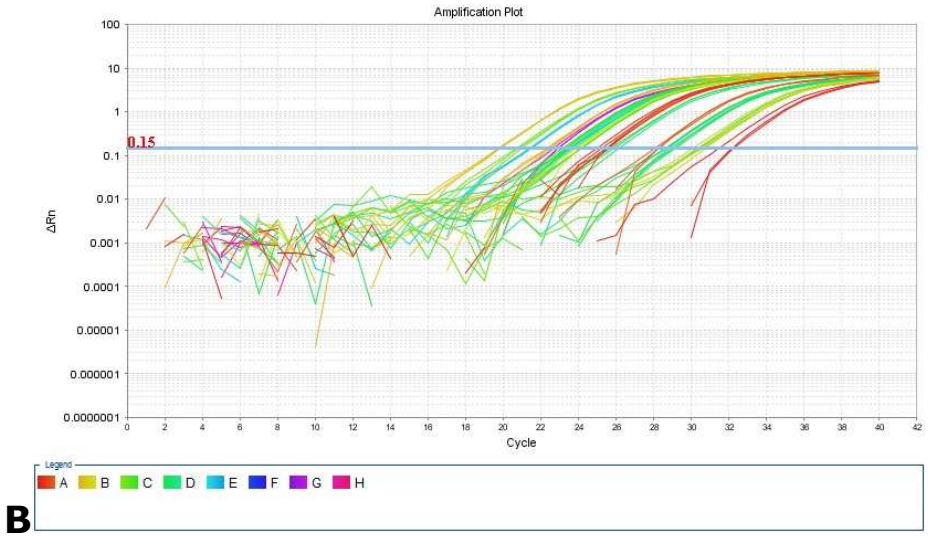
efficiency correction method and was presented as relative expression of the gene in question compared to the reference gene.

Gene	*Forward primer sequence (5'-3')*	*Reverse primer sequence (3'-5')*
L19	GCGGAAGGGTACAGCCAAT	GCAGCCGGCGCAAA
PRL	AAGCTGTAGAGATTGAGGAGCAAAC	TCAGGATGAACCTGGCTGACTA
IGFBP-1	CGAAGGCTCTCCATGTCACCA	TGTCTCCTGTGCCTTGGCTAAAC
11-Beta HSD1	AGCAAGTTTGCTTTGGATGG	AGAGCTCCCCCTTTGATGAT

Table 27. Primer sequences used in study.



A



B

Figure 68. PCR amplification plots of L-19 (A) and PRL (B).

9.2.5. **Ovarian stimulation protocols**

All women underwent an ART cycle as described on page 140. A urinary pregnancy test was carried out 14 days after embryo transfer. If this was positive, a transvaginal ultrasound (TVUS) scan was scheduled for approximately 4 weeks later to confirm presence or absence of clinical pregnancy.

9.2.6. **Outcome variables**

The data collected included basic demographic details, past obstetric history, cause and duration of infertility, sonographic data including two-dimensional (2D), three-dimensional (3D), three-dimensional power Doppler (3DPD) and pulse-wave Doppler (PWD) data, ART outcome data including duration of stimulation, number and quality of embryos, and clinical pregnancy outcomes. Clinical pregnancy was defined as presence of a fetal heart action on a TVUS scan at approximately 6 week's gestation. Molecular outcomes included relative gene expressions and levels of $CD56^+$ uNK cells.

9.2.7. **Statistical analysis**

Statistical analysis was performed using the Statistical Package for the Social Sciences (version 21; SPSS, Chicago, IL, USA). Distribution of data was assessed using Shapiro-Wilk test. For parametric un-paired data student's t test was used and for non-parametric data, Mann-Whitney U test was carried out. For paired samples, student's t test was used for parametric data and Wilcox signed rank test was used for non-parametric data. A P value of <0.05 was considered as statistically significant.

Changes over time were analysed using repeated-measures ANOVA, reported as *F*-ratio with corrected degrees of freedom if data sphericity was violated. Data sphericity was assessed using Mauchly's test, with $p<0.05$ signifying lack of sphericity. Greenhouse-Geisser correction (ε) was automatically applied for data with minimal violation of sphericity (ε approaching 1). When significant violation of sphericity was confirmed, MANOVA was performed and reported as *F*-ratio with degrees of freedom.

Linear regression analysis was used to assess the effect of different variables for prediction of the outcome of assisted reproduction treatment. The effect of the same variable on treatment outcome was assessed using binary logistic regression analysis. ROC curve analysis was performed to quantify the ability of any significant factors to discriminate between pregnant and non-pregnant study participants.

9.3. Results

9.3.1. **Participants**

Twenty-four women have been enrolled in this study. Following ART, eleven were found to have a clinical pregnancy at approximately 6 weeks gestation, two had a biochemical pregnancy only and eleven had a negative pregnancy test. The demographic details are given in Table 28. Twenty-two women were of Caucasian origin, one was Indian and one was of mixed race. The cause of infertility did not differ between groups and included male factor infertility in 9 cases (37.5%), anovulation in 3 cases (12.5%), tubal factor in 1 case (4%), unexplained in 6 cases (25%), and mixed infertility in 5 cases (21%). None of the women were smokers.

Variable	**Positive group (n=11)**	**Negative group (n=13)**
Age, years	34.9(±3.1)	35.3(±3.3)
Duration of infertility, months	38.0(±14.6)	38.6(±12.3)
Previous live births (range)	0.5(0-2)	0.1(0-1)
Undergoing ART cycle number (range)	1.8(1-4)	1.4(1-4)
BMI (kg/m2)	23.9(±2.8)	24.5(±2.0)
AMH (pmol/L)	11.03(6.09-16.72)	15.13(6.96-36.29)
AFC	24.00(12.00-32.00)	18.00(11.75-30.50)
Dose of gonadotropin (IU)	2956.3(±1032.1)	2417(±834.4)
Duration of stimulation (days)	11.6(±1.4)	11.4(±1.3)
Number of oocytes collected	13.3(±6.2)	15.0(±8.5)

Table 28. Demographic details of study participants.

*Data presented as mean (±SD), median (interquartile range) or number (%) as appropriate; BMI- body mass index; AMH- anti-Mullerian hormone; AFC- antral follicle count; * P<0.05.*

One patient underwent a short antagonist protocol and one had a natural cycle frozen embryo transfer. Remaining patients had a long agonist protocol. Twelve patients had IVF, day 2 embryo transfer was carried out in two cases, and double embryo transfer was carried out in 9 cases. Embryo quality was similar between the patients and embryos were of good quality as judged by visual assessment by duty embryologist on the day of embryo transfer in 70% (23 of 33 embryos).

9.3.2. **Baseline sonographic data**

The uterine cavity shape was normal in 17 (71%) cases and arcuate in 7 cases. Adenomyosis was present in 5 cases (21%). ET, EV, MGS, 3DPD and PWPD values are presented in Table 5 in the Appendix. Factors that were found to be significantly different between groups were further examined to assess the effect on the outcome using linear regression.

Linear regression of significantly different sonographic factors demonstrated that none of the two (MGS and UAVmax at TVOR) were significantly associated with the final ART outcome (R^2=0.183 for step one, ΔR^2=0.153 for step two; P=0.08).

9.3.3. **Expression of PRL, IGFBP-1 and 11β-HSD1**

The average time to achieve confluence by ESC cultures before decidualization was commenced was 5.00 (3.25-7.00) days. Analysis of PRL and IGFBP-1 was possible in all cases. There was a significant increase in the relative PRL, IGFBP-1 and 11β-HSD1 expression between days 0, 2 and 8 of hormone treatment in all analysed samples (P<0.05) (see Figure 69).

Expression of IGFBP-1 on day 2 of decidualization was higher in the pregnant group compared to the negative population (P=0.02; see Figure 70). Similarly, expression of PRL and IGFBP-1 on day 2 versus day 0 was higher in the pregnant population (P=0.02 and P=0.04, respectively; see Figure 71). Removal of data from women with biochemical pregnancy loss did not alter the analysis result.

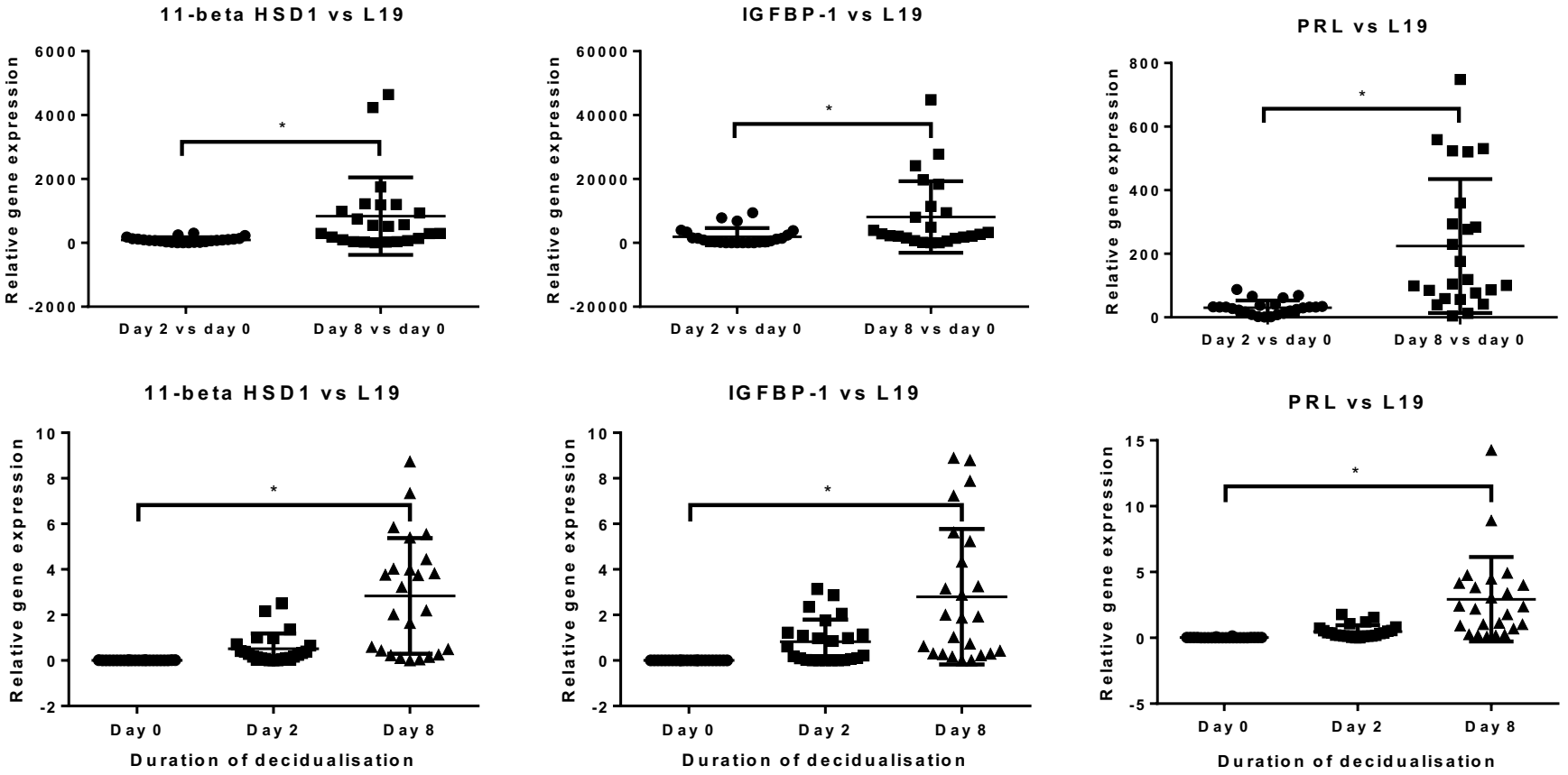
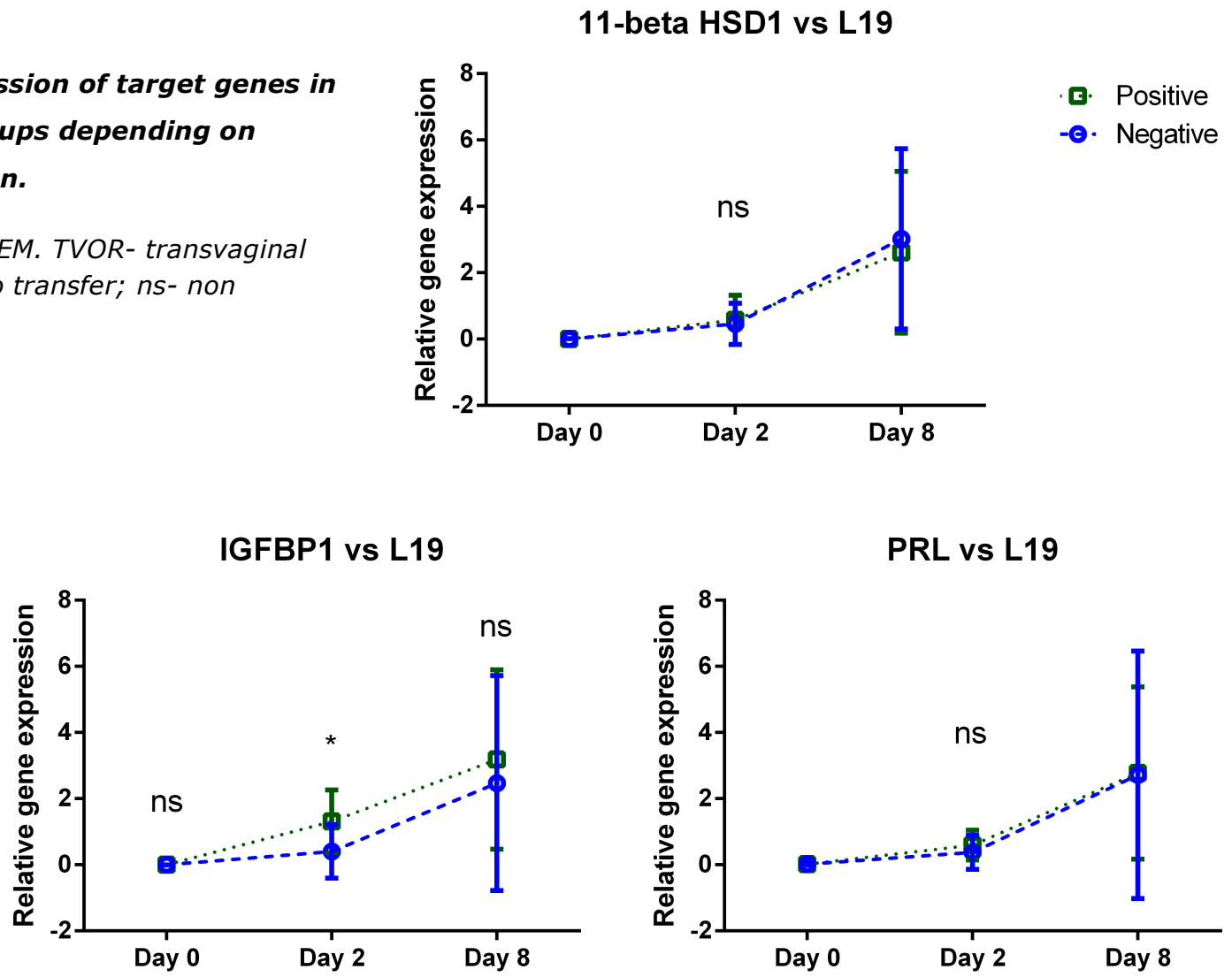


Figure 69. Relative expression of target genes depending on the duration of decidualization in all samples.

*P<0.05

Figure 70. Relative expression of target genes in positive and negative groups depending on duration of decidualization.

*Data presented as mean ±SEM. TVOR- transvaginal oocyte retrieval; ET- embryo transfer; ns- non significant; * P<0.05.*



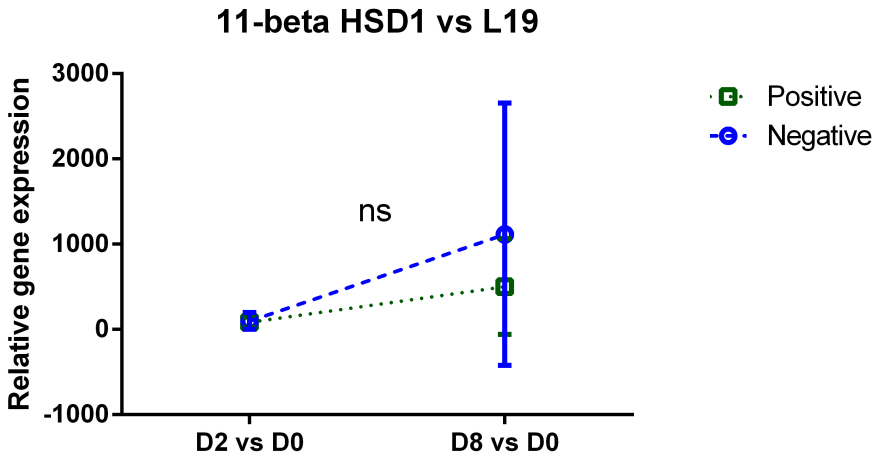
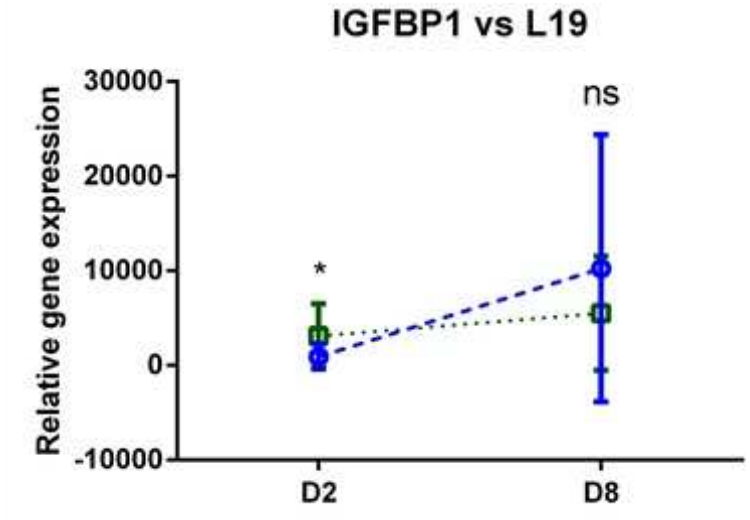
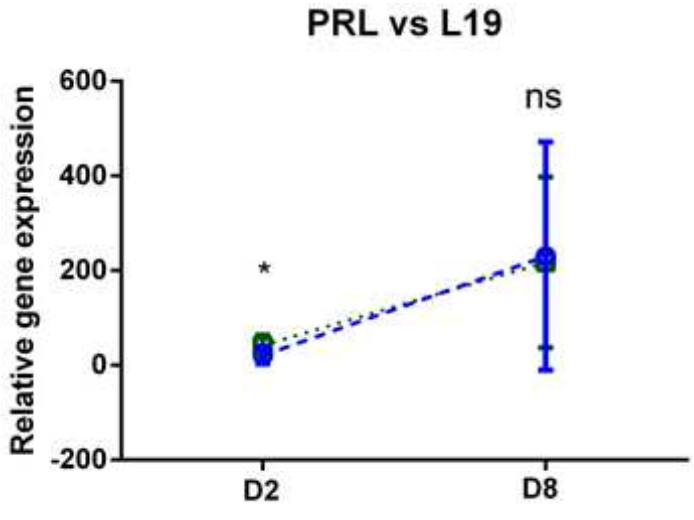


Figure 71. Relative expression of target genes in positive and negative groups on day 2 and 8 versus control.

*Data presented as mean ±SEM. TVOR- transvaginal oocyte retrieval; ET- embryo transfer; ns- non significant; * P<0.05.*





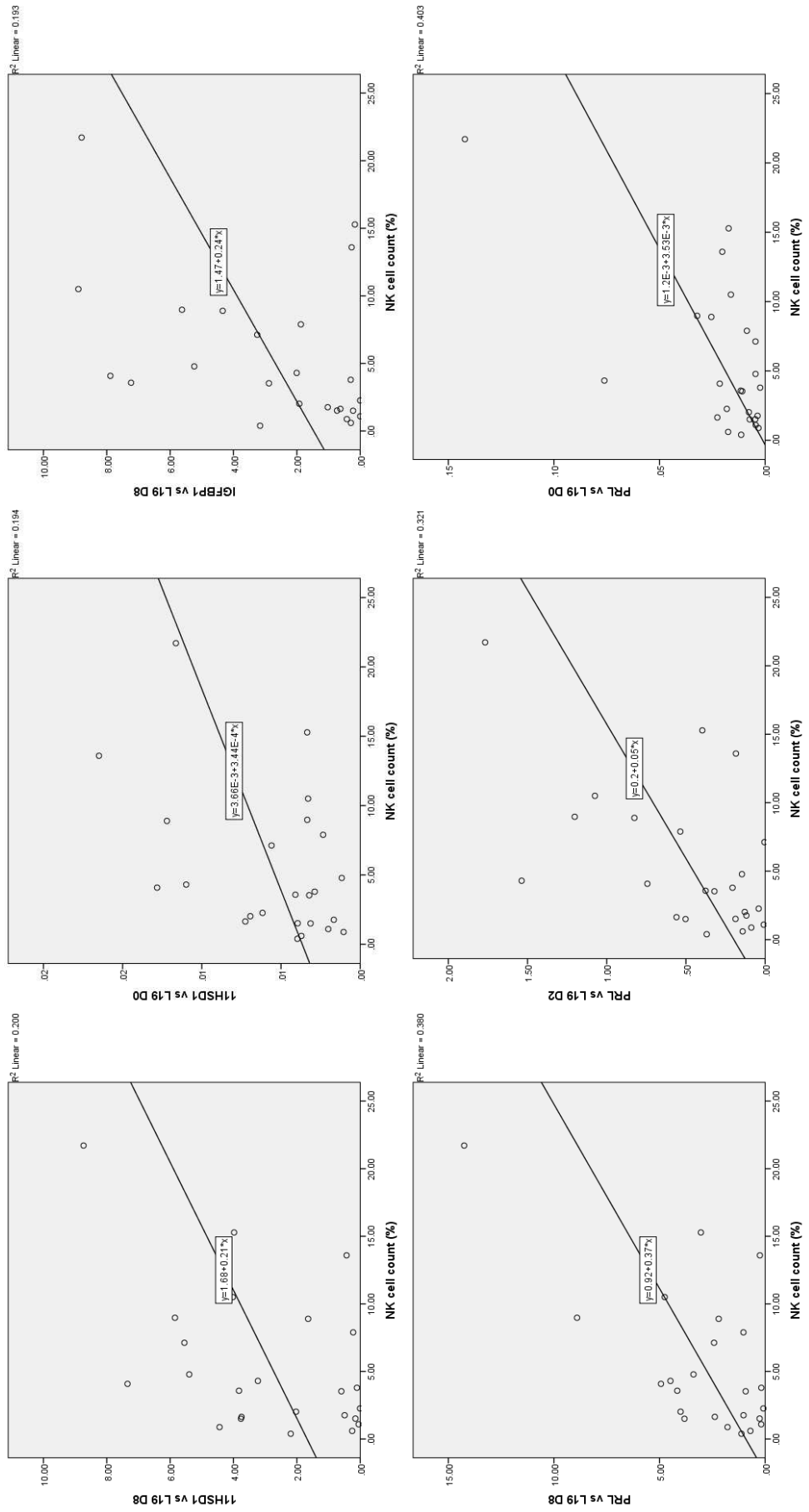


Figure 72. Correlation between uNK cell levels and markers of decidualization.

9.3.4. **uNK cell correlation with markers of decidualization**

uNK cell levels expressed as % of sample area were positively correlated with PRL expression on day 0, day 2 and day 8 of decidualization (R^2=0.403; R^2=0.567; R^2=616 respectively; P=0.004) and with IGFBP1 expression on day 8 of decidualization (R^2=0.193; P=0.032). uNK cell number was correlated with 11-β HSD1 levels on day 0 (R^2=0.194; P=0.03) and day 8 (R^2=0.200; P=0.028; see Figure 71). When uNK cells were categorised into clinically significant (>5%) or non-significant (<5%), then statistical significance was lost (P>0.05).

uNK cell levels were higher, though not significantly, in the population that did not achieve a pregnancy (7.16±7.2%) versus hCG positive population (4.07±2.8%; P=0.167) (see Figure 73).

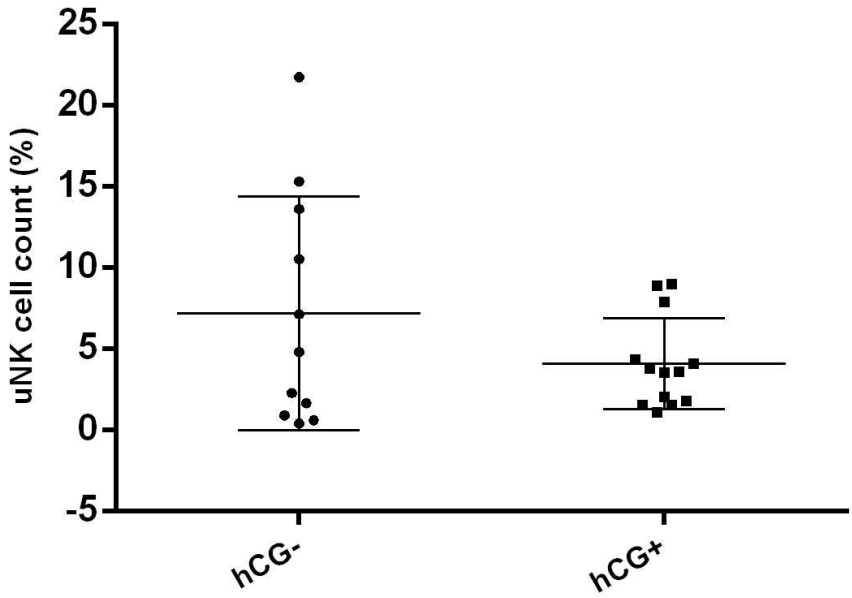


Figure 73. uNK cell distribution in positive and negative samples.

9.3.5. **Sonographic data and molecular markers of endometrial receptivity**

Mid-luteal phase of the cycle

PRL expression on day 0 of decidualization was negatively associated with UA Vmin (r=-0.411; P=0.046) and UA RI (r=-0.441; P=0.031). Day 8 of decidualization PRL levels were associated with UA RI (r=-0.466; P=0.022). IGFBP1 expression levels on day 0 of decidualization were negatively correlated with UA Vmin (r=-0.497; P=0.014). Day 8 levels were correlated with UA RI (r=-0.434, P=0.034). 11β HSD1 expression levels on day 0 were associated with UA Vmin (r=-0.429; P=0.036) and UA RI (r=-0.494; P=0.014) (see Figure 74). Oestrogen and progesterone levels were not associated with expression of any of the markers (P>0.05).

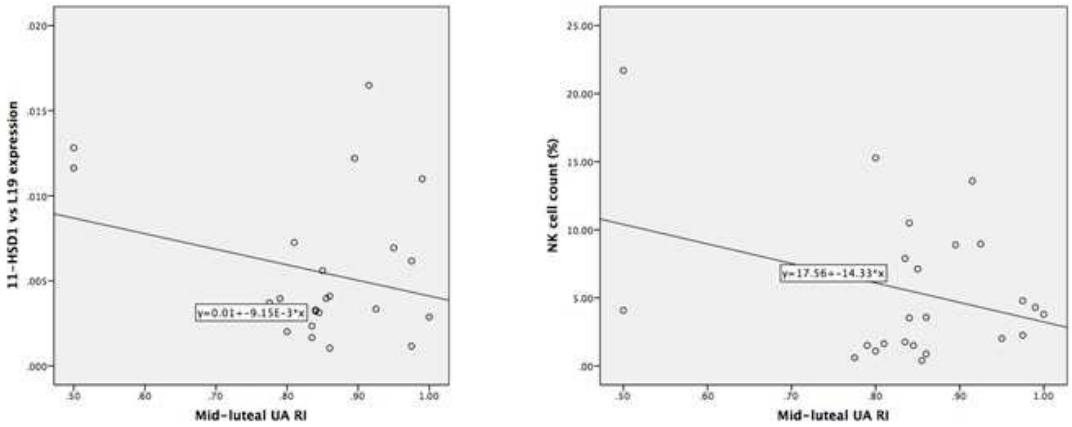


Figure 74. 11-β HSD1 expression and uNK cell levels in the mid-luteal phase correlated with UA RI.

Transvaginal oocyte retrieval

PRL expression on day 0 was associated with endometrial VI (r=0.525; P=0.018), VFI (r=0.489; P=0.029) and HDVI (r=0.548; P=0.012). Day 2 expression was associated with endometrial VI (r=0.645; P=0.002), VFI (r=0.626; P=0.003) and HDVI (r=0.510; P=0.022). Day 8 was associated with VI (r=0.614; P=0.004), VFI (r=0.578; P=0.008) and HDVI (r=0.532; P=0.016).

IGFBP1 day 0 expression levels were associated with VI (r=0.473; P=0.035) and VFI (r=0.451; P=0.046). Day 2 levels were associated with endometrial thickness (r=-0.438, P=0.047) (see Figure 75) and UA Vmax (r=-0.607; P=0.008). Levels on day 8 of decidualization were correlated with VI (r=0.5; P=0.025), VFI (r=0.468; P=0.038) and HDVI (r=0.492; P=0.028).

11-β HSD1 expression levels on day 2 were associated with endometrial VI (r=0.543; P=0.013), VFI (r=0.528; P=0.017) and HDVI (r=0.507, P=0.023). Day 8 levels were associated with VI (r=0.673; P=0.001), VFI (r=0.649; P=0.002) and HDVI (r=0.656; p=0.002).

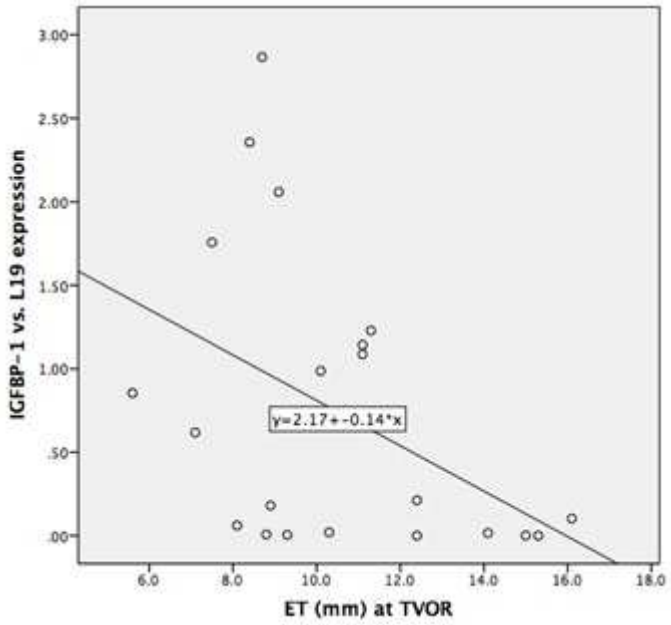


Figure 75. Correlation of IGFBP-1 expression and endometrial thickness (ET) at transvaginal oocyte retrieval (TVOR).

Embryo transfer

PRL and IGFBP1 expression levels were not associated with any of the sonographic markers of decidualization at embryo transfer. 11β HSD1 expression levels on day 0 were negatively correlated with UA Vmax (r=-0.504, P=0.017). Expression on day 8 was correlated with UA Vmin (r=-0.427, P=0.047), UA PI (r=0.554, P=0.017) and UA RI (r=0.563; P=0.006) (see Figure 76).

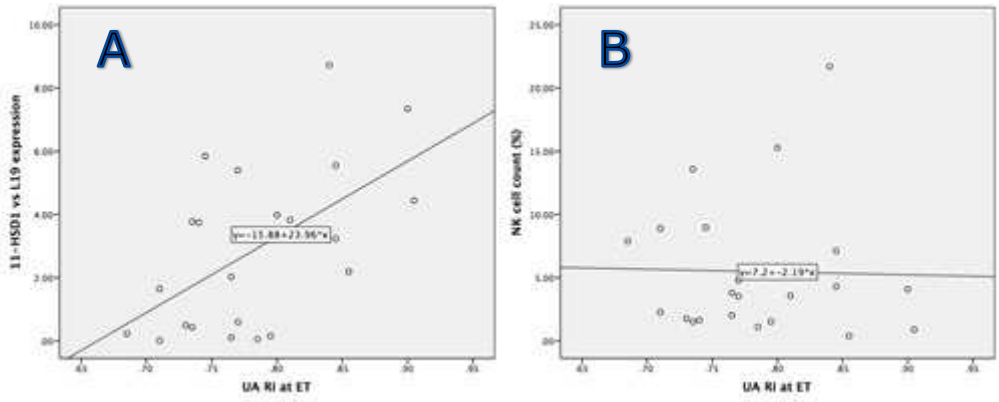


Figure 76. 11-β HSD1 expression (A) and uNK cell levels (B) at embryo transfer correlated with UA RI.

9.3.6. **Predictive value of selected molecular markers for clinical pregnancy**

The best predictive value for clinical pregnancy was achieved by relative PRL mRNA expression on day 2 versus day 0 of decidualization (AUC 0.769, sensitivity 76.9% and specificity 72.7% for value of 28.43). IGFBP-1 mRNA expression on day 2 versus day 0 demonstrated an AUC of 0.720 (sensitivity 76.9%, specificity 54.5% for value of 315.51). The highest predictive value for 11-β HSD1 mRNA levels was achieved on day 8 of decidualization (AUC 0.65, sensitivity 76.9%, specificity 54.4% for value of 3.9) (see Figure 77).

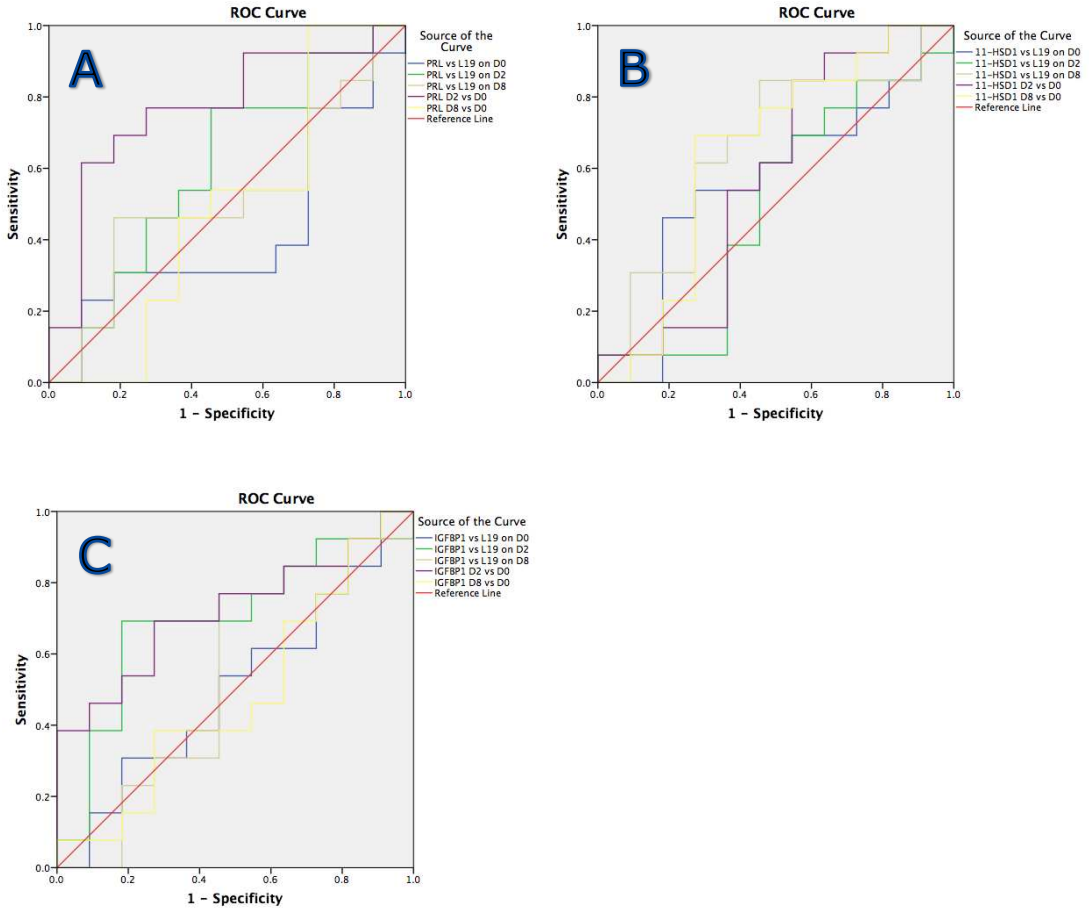


Figure 77. Expression of PRL (A), 11-β HSD1 (B) and IGFBP-1 (C) and prediction of clinical pregnancy (ROC diagrams).

9.4. Discussion

The study has shown that primary cell cultures obtained from infertile women undergo appropriate response to MPA and 8-Br-cAMP stimulation *in vitro* mirroring *in vivo* changes. The elicited response was monitored using established genes of decidual transformation (PRL and IGFBP-1) (Bell et al. 1991, Cloke et al. 2008, Lynch et al. 2008), expression of which has significantly increased with duration of decidualization. Expression of 11-β HSD1- a marker of glucocorticoid metabolism was not able to differentiate a phenotype predictive of subsequent pregnancy, but is expression was correlated with uNK cell levels on day 0 and 8 of decidualization (P=0.031).

In a study by Salker et al. (2010), where samples from RM and control patients were subjected to *in vitro* decidualization, no difference in PRL expression was noted on day 0 and day 2, however a significant decrease in the PRL expression was apparent on day 8 (Salker et al. 2010). A similar behaviour of PRL and IGFBP-1 protein levels in women with primary infertility was noted in a study by Karpovich et al (Karpovich et al. 2005). Fresh endometrial tissue gene expression analysis in women suffering from unexplained infertility (n=4) also demonstrates a decreased down-regulation of IGFBP-1 in samples obtained on day LH+7 compared to healthy controls (n=5) (Altmae et al. 2010). Our samples have not demonstrated such behaviour. At TVOR, the IGFBP1 expression on day 2 of decidualization has been found to be negatively correlated with endometrial thickness (Pearson r -0.438, P=0.047). This might indicate that the expression of this protein is responsible at this time point for the bioavailability of IGF-1 and related expansion of ESC resulting in increased endometrial thickness. This finding has not been observed at the later stages of decidualization, which finds biological plausibility in the fact that when decidualization sets in, proliferation ceases (Jabbour et al. 2006).

In the available literature, uNK cells have been found to be associated with reproductive failure, especially with RM (Clifford et al. 1999, Quenby et al. 2009). Subsequent treatment with prednisolone has shown a

decline in the numbers of these cells and a more favourable pregnancy outcome (Quenby et al. 2005). In a study by Kuroda et al. (2013a), a negative correlation has been identified between 11β HSD1 and uNK cell numbers (Kuroda et al. 2013a), which was not demonstrated in our study population. This is likely related to the differences in the study population characteristics between our study (mainly infertile women undergoing first ART cycle) and the study by Kuroda et al. (2013a) (women with RM or RIF). The authors of the mentioned study infer that elevated uNK cell levels within the endometrium can be an indirect indicator of steroid deficiency at the tissue level, thus contributing to disturbed fetal allograft tolerance and subsequent negative pregnancy outcome (Kuroda et al. 2013a).

In our study, the expression of 11-β HSD1 has been associated with the UA and endometrial blood flow to a certain extent. The day 0 expression levels were negatively associated with UA RI, suggesting that the higher the resistance to blood flow through the uterus, the lower the expression of 11-β HSD1. A similar trend was observed for uNK cell levels. This would be in keeping with deranged neo-angiogenesis and increased vascular resistance when the steroid and uNK cell homeostasis is distorted. A reversal of the association was noted at embryo transfer, when low 11-β HSD1 expression was associated with low UA resistance. uNK cells did not follow this trend indicating that potential vasoactive factors, other than uNK cell and their cytokine products, have a stronger influence on endometrial vascularity at this stage.

The study does have some inherent limitations, with low numbers of participants (24), retrospective sample selection, long-term cryogenic storage, and only three analysed genes, being the most obvious ones. The correlation of *in vitro* gene expression and uNK cells with sonographic markers of endometrial appearance at selected ART time-points forms a potential source of bias, as this correlation was carried out under the assumption that HESCs would behave in a similar way *in vivo* and *in vitro*, and so, sonographic sampling of the endometrium in the mid-luteal phase would correlate with day 8 of cAMP and MPA exposure *in vitro*; TVOR would approximate day 2/3, and embryo transfer would

depend on the stage at which the embryo was replaced. Validation of this would require multiple biopsies at different time points in a natural and stimulated cycle, with contemporaneous correlation of molecular and sonographic data.

The use of frozen frozen-thawed cell samples could be perceived as a potential source of errors. We have decided to use frozen ESC in order to pool the samples and perform a contemporaneous analysis of all samples. This could potentially alter the observed response of frozen ESC when compared to their fresh counterparts. Human fibroblast cells when exposed to cryopreservation demonstrate an aberrant expression profile of VEGF and platelet derived growth factor (PDGF) compared to the fresh cultures. Heat shock protein expression was also increased following thawing and re-establishment of cell cultures. This response was however temporary and has been spontaneously restored to baseline after a maximal period of 120 hours in culture (Liu et al. 2000). Based on the observations reported in the literature and the fact that all our cell samples included in this study have been cultured for a period of time exceeding the stated timeframe of 120 hours before decidualization, we can assume that potential detrimental effects of cryopreservation were reversed, minimising this factor as a potential source of bias.

In conclusion, the selected markers of endometrial receptivity were only able to demonstrate the decidual response to hormonal stimulation. Receptive and non-receptive endometrial phenotype was not depicted with the analysed factors. Combination of clinical data (ultrasound, ART) with gene analysis and immunohistochemistry, thought extensive, did not yield the anticipated answers aiming to identify markers predictive of a receptive phenotype and subsequent ART success. Analysis of more complex interactions on the gene and protein levels is necessary to understand and possibly alter abnormal endometrial receptivity.

CHAPTER 10. Conclusions and discussion

In this project, the focus has been on the endometrium as the organ responsible for pregnancy establishment and development. An extensive literature search was performed to identify the molecular pathways governing the decidualization of human endometrial stromal cells (HESCs), diagnosis of endometrial (dis)function and means of altering the non-receptive endometrial phenotype. An attempt to define an iatrogenic condition, recurrent implantation failure, has also been carried out in order to accurately and consistently select populations for subsequent studies. In the main part of the study, the effectiveness of performing an endometrial biopsy in an unselected population of infertile women in the cycle directly preceding assisted reproductive treatment (ART) in order to improve the outcome of treatment has been assessed. Collected material (tissue samples and sonographic data) has been used to identify potential markers of endometrial receptivity, which in the context of ART, could be used as predictors of success or failure of treatment.

10.1. Summary of research findings

The research conducted within the remits of this study, however broad, has focused on the endometrium. The initial project design was supposed to focus on women with reproductive failure as the most interesting population, hence the attempt to standardise the definition of recurrent implantation failure (RIF). The low numbers of such patients in the hosting unit and the limited time frame for the conduct of the study has forced a redesign with inclusion of an unselected population of infertile women. This approach could be criticized due to the potential and acknowledged differences between the subjects, but at the same time, allows for generalizability of obtained results to the infertile populations attending fertility centres. In the context of endometrial biopsy and ART outcome, the study design used in this work has not been reported elsewhere.

In Chapter 3, the uncertainty of the definition of RIF has been addressed. As the population of RIF women is relatively small, varying definitions used in the literature prevent meaningful compilation of data from different studies. Thus we attempted to identify used definitions, highlight

the most frequently used and, with expert help, provide an 'evidence based' definition that would allow for standardisation of future prospective studies dealing with RIF. As such, we propose that RIF should define a population of women where implantation did not occur after two consecutive cycles of *in vitro* fertilisation (IVF), intra-cytoplasmic sperm injection (ICSI) or frozen embryo transfer (FER) where the cumulative number of transferred embryos was no less than four cleavage stage embryos or two blastocyst. Due to geographical differences related to access to expensive ART, our definition allows for early testing and identification of possible causative factors limiting costs incurred by couples or the healthcare provider. The proposed definition is in contrast to a recent paper attempting to define RIF, where at least three fresh or frozen unsuccessful cycles with a cumulative transfer of at least four embryos were necessary to occur before a woman was categorised as having RIF. No clear rationale for this definition was however produced (Li 2012). Due to lack of rationale, our study has superiority over the mentioned opinion paper. The acceptance of either definition will however depend on the scientific community and only future works will show which definition, if either, became widely accepted.

The main clinical question that the research process aimed to answer was whether an endometrial biopsy could improve chances of pregnancy in an unselected population of infertile women undergoing IVF or ICSI. The choice of the study populations, apart from the number of available patients for recruitment and time frame, was guided by the lack of evidence of the effect of endometrial biopsy and subsequent ART outcome in these women. At the time of the study design, all prior studies have focused on women with failed ART cycles (Karimzadeh et al. 2009, Francisco et al. 2011, Inal et al. 2012, Faghih et al. 2013). Before the current study has been completed, Nastri et al. (2013) and Yeung et al. (2014) have published trials where women undergoing first ART cycles were included. Results of these trials contradict each other, with Nastri et al. (2013) showing benefit of the procedure.

The results of the endometrial biopsy study presented in Chapter 5, indicate that there is no benefit in performing this procedure routinely in

all women undergoing ART. A moderate, however not statistically significant, difference in clinical pregnancy rates has been observed when subgroup analysis based on cycle number has been performed, with higher pregnancy rates in women with previous failed cycles compared to first cycle controls. Due to the limited number of patients with RIF (n=8), further research is required to appropriately power the observed results. In the analysis of the study data, we have considered important factors related to ART, such as the number of oocytes collected, embryo quality, and ease of embryo transfer, and these did not differ between groups. In view of this, the study and control groups were as homogenous as possible, which adds credibility to the reported results. To some limited extents, the findings of the current study are in concordance with the most recent update of the Cochrane review of endometrial biopsy and ART outcome, where the authors conclude that a beneficial effect on clinical pregnancy and live birth rates can be observed in women that have had at least two failed embryo transfers (Nastri et al. 2015). The quality of evidence included in the review was moderate at best.

Data collected as part of the study of endometrial biopsy prior to controlled ovarian hyperstimulation (COH), needed to be as comprehensive as possible in order to identify any confounding factors. As such, the use of endometrial samples and sonographic data has been embedded in the protocol. Extensive application of ultrasound aimed to identify any non-invasive markers of endometrial receptivity, as well as macroscopic changes related to the biopsy. Obtained endometrial tissue was used to assess uterine natural killer (uNK) cell levels and establish primary cell cultures in order to identify cellular and molecular differences between the pregnant and non-pregnant populations.

Analysis of collected endometrial sonographic data presented in Chapter 6 has, in accordance with the available evidence, failed to detect single or multiple markers able to predict a successful or unsuccessful ART cycle outcome (Kupesic et al. 2001, Jarvela et al. 2005, Merce et al. 2008). In agreement with the available literature, a triple layer appearance on day of transvaginal ultrasound guided oocyte retrieval (TVOR) has been found to be correlated with a higher chance of pregnancy compared to other

endometrial patterns (Rashidi et al. 2005, Singh et al. 2011b). Uterine artery (UA) blood flow studies failed to identify vascularity index values predictive of pregnancy in our study population, which is in agreement with previous studies assessing this parameter at the same time points (Schild et al. 2001, Zollner et al. 2012). Similarly, three-dimensional power Doppler (3DPD) vascularity indices obtained from our participants were unreliable as non-invasive markers of endometrial receptivity. This is in contrast to a study by Kim et al. (2014) that has found endometrial vascularisation index (VI) of 0.95 to predict pregnancy with 71.7% sensitivity and 68.9% specificity. Merce et al. (2008) has provided evidence that 3D flow index (FI)>26.1 can predict pregnancy with a sensitivity of 85.6% and specificity of 72.4%. As the timing of the ultrasound examinations in the mentioned studies differed from ours, this can be responsible for the observed discrepancies. At this point it is also worth mentioning, that not all fertility units have access to 3D ultrasound equipment, and most likely none would delegate work force for the timely off-line analysis of datasets with conflicting usefulness as a marker guiding embryo transfer.

An extensive analysis was conducted to evaluate Spatio-Temporal Image Correlation (STIC) as a non-invasive marker of endometrial receptive phenotype (Chapter 7). This decision was based on the novelty of the technology, the analysis of the entire cardiac cycle within the entire endometrium and supposed high resolution and sensitivity to low blood flow, as well as derivation of vascularity indices resembling those of spectral PWPD. In this work, we have demonstrated that it is possible to acquire and measure the STIC dataset from an entire endometrium reliably and reproducibly with high ICC values. The obtained vascularity index values demonstrated however high biological variation (CV>20%). The analysis of STIC datasets obtained in the mid-luteal phase, TVOR and embryo transfer was only possible in 46.1% and 59.2% for the manual and spherical methods, respectively. This was due to failure to obtain datasets related to anatomical differences and COH related alterations of uterine flexion and version, as well as expansion of the endometrial volume related to stimulation with gonadotropins, precluding inclusion of

the entirety of the endometrium. The analysis was also very time-consuming irrespective of the sampling method (1478.9 seconds for the manual analysis method versus 266.8 seconds for the spherical analysis; $P<0.05$). The values of average STIC vascularity indices (VI and FI) were found to be significantly lower ($P<0.05$) compared to static 3DPD indices. Static 3DPD averages the vascularity over a certain number of cardiac cycles, whereas STIC takes into account one cycle when producing results (Martins et al. 2011). As STIC vascularity indices differ between cycles by approximately 20%, it is not surprising, that averaging the data over few cycles that it takes to acquire a 3DPD dataset will produce different results. One promising aspect of the technology was a relatively high predictive value for first trimester miscarriage when manual analysis method was applied [AUC=0.8 for the minimal manual VI at TVOR (cut-off value ≥0.7; sensitivity 80.0% and specificity 68.1%)]. However, due to the low numbers of datasets suitable for analysis (n=11) further validation of the finding is required in a larger population. Predictive values for other ART outcomes did not achieve clinically relevant results. Reported different vascularity progression when manual and spherical STIC datasets were analysed, could be explained by the volume of interest (VOI) selection. Spherical analysis included the fundus of the endometrial cavity (presumed site of embryo implantation) and, in some cases, a small amount of the surrounding subendometrium. The on-average higher STIC vascularity indices obtained using spherical sampling could be interpreted in two ways. The subendometrial component, however small, contributes significantly to the observed values, or the endometrial vascularity within the fundus is significantly higher than in the lower part of the endometrial cavity. The first statement finds some support in the literature, where static 3DPD vascularity indices were higher within the subendometrium versus the endometrium (Raine-Fenning et al. 2004c, Kim et al. 2010). Observations graphically reported in Figure 54, indicate that the blood flow characteristics as observed using STIC, behave in a discordant way. Considering subendometrial vascular component as significant, we could speculate that in the context of endometrial receptivity, the endometrium and subendometrium act as separate entities with distinct blood flow and possibly immune (see

conclusions in Chapter 8) characteristics. Further targeted research is however required to validate these conclusions.

Analysis of uNK cell populations as described in Chapter 8, has highlighted some discrepancies compared to the available literature. The main differences are in the lower uNK cell levels in the populations assessed. This finding might be explained by the different definitions of reproductive complications (RIF defined according to findings in Chapter 4) and analysis method used (computer aided NK cell counting). Irrespective of this, women with RIF or recurrent miscarriage (RM) had, on average, a higher uNK cell count expressed as percentage of sample area occupied by ESCs compared to women with no reproductive issues other than infertility. It is possible, that aberrant uNK cell numbers are not the actual cause of reproductive complications, but rather a symptom of deeper underlying pathology. Recent evidence indicates that uNK cells are involved in the scar free healing of tissues in combination with senescent cells (Krizhanovsky et al. 2008). Deregulation of the patomechanisms related to decidualization and menstruation, and possible abnormal senescence-related inflammatory response, might lead to reproductive failure. Considering every menstrual cycle as a separate entity, we can only get a snippet of the entire menstrual history of the individual. It is possible, that the endometrial environmental differences between cycles can be an inherent evolutionary feature assuring endometrial homeostasis enabling deep trophoblast invasion during pregnancy (Brosens et al. 2009c, Gellersen et al. 2014). As such, selection of normal control populations for studies assessing endometrial receptivity can be a challenging feat. Should women with no previous pregnancies serve as controls, or should women with proven fertility be considered normal? Opinions in this matter differ, as supposedly pregnancy alters the endometrial environment, so that it differs to the one prior to conception (Gellersen et al. 2007, Brosens et al. 2009c). For our study, the ideal control women should comprise of female partners of couples where male factor is the only identified cause for infertility. The numbers of such patients were low (n=52) and as such, properly powered subgroup analyses were not possible. uNK cell levels by

themselves, proved also to be poor predictors of pregnancy outcome following ART.

Lack of full understanding of the exact mechanism of the endometrial biopsy effect on the endometrial environment has led to conception of the second study of repeated biopsies in two subsequent menstrual cycles. No subsequent ART cycles were conducted following the second biopsy hence no pregnancy outcomes were available. The analysis of uNK cell levels has demonstrated that, when analysing the sample using the same methodology, second cycle uNK cell levels were significantly less compared to the first biopsy specimen (2.4% versus 0.7%; $P<0.05$). This might be the one of the most significant findings explaining the beneficial effect of endometrial biopsy on ART outcome. However, the higher the initial uNK cell level, the more pronounced the drop in the second cycle. This can indicate that women with 'unexplained' infertility might have an immune component in the form of elevated uNK cell levels. More work is required to describe and diagnose a new and potentially very prevalent cause of infertility- 'immune infertility'. Decreasing exceptionally high uNK cell levels may have a beneficial effect on subsequent pregnancy outcome and this population might benefit most from having an endometrial biopsy performed prior to embarking on COH, compared to patients with normal uNK cell levels. The data from the endoscratch study, does not seem to fully support this notion as a poor correlation between COH outcome and NK cell levels was identified (AUC=0.545, cut-off value >4.81%, sensitivity 69.2%, specificity 42.1%), indicating a role for additional factors responsible for pregnancy establishment.

Analysis of molecular markers as described in Chapter 9, has failed to identify mRNA 11-βHSD expression as a marker of endometrial receptivity. We were also not able to demonstrate a significant correlation between the uNK cell levels and this factor, which previous works have demonstrated (Kuroda et al. 2013a). This might be related to the differences in included populations between these two studies, with the current work assessing infertile women. PRL and IGFBP-1 expression levels also failed to identify the receptive phenotype. It is however noteworthy, that culture and exposure to MPA and cAMP in *in vitro*

environment was able to elicit decidual transformation in the samples collected from our patients. The limited number of samples and the retrospective selection of these based on the outcome of treatment might be partially responsible for the lack of statistical significance. Potential selection of samples based on past reproductive history (RM or RIF versus first COH cycle patients) could have produced a more significant and clinically relevant result.

10.2. New findings in relation to markers of endometrial receptivity

In this thesis, several new findings related to endometrial receptivity and subsequent ART outcome have been identified. An attempt has been made to unify the definitions used to describe recurrent implantation failure. Based on the available literature and with expert opinion, we suggest that recurrent implantation failure should be defined as the absence of implantation, itself defined by a negative serum hCG 14 days after oocyte collection, after two consecutive cycles of IVF, ICSI or frozen embryo replacement where the cumulative number of transferred embryos was no less than 4 for cleavage stage embryos and no less than 2 for blastocysts, with all embryos being of 'good quality' and of appropriate developmental stage. Thus we also suggest, that patients in the oocyte donation scheme failing to achieve pregnancy and meeting the above criteria, should also be treated as being affected by recurrent implantation failure (Polanski et al. 2014).

The beneficial effect of endometrial biopsy in the cycle directly preceding COH is likely to be observed in the population of women with previous failed embryo transfer cycles (though statistical significance has not been reached). In the context of evidence obtained in the current study, routine endometrial biopsy for all women undergoing ART is not indicated. Potentially more information can be obtained from this study, once live birth data becomes available.

The use of the novel ultrasound modality- STIC, as a non-invasive endometrial receptivity marker only appeared beneficial for prediction of

first trimester miscarriage. The time-consuming analysis and limited availability of the technology currently prevent the generalizability of STIC in an IVF setting. Assessment of the endometrial vascularity also confirmed that relative hypoperfusion around the time of TVOR as demonstrated by lower manual STIC minimal VI, average VI and minimal FI, increases the chances of and on-going pregnancy. This suggests that oxidative stress related to abundant vascular perfusion at the time of implantation has a negative effect on chances of pregnancy following COH. Findings of different STIC vascularity index progression when performing the manual and spherical analysis and converse relationships between uNK cell levels with corresponding STIC indices implies a functional difference between the endometrium and subendometrium.

Endometrial biopsy performed in the mid-luteal phase of a cycle has been shown to decrease the levels of uNK cells in the subsequent cycle. This might be one of the most important mechanisms responsible for the beneficial effect of the endometrial biopsy on ART outcome, however only in the selected population of women with 'immune infertility'. As such, this is potentially the most important finding reported in this thesis. Though statistical significance was reported, in order to achieve appropriate power of 0.8, 82 participants should be included in a follow up study to fully confirm these findings.

10.3. Study limitations

Every effort has been made to perform this work in a standardised and reproducible way, however potential sources of bias could be identified. Patient selection for the study aimed to include the representative population of infertile women attending fertility clinics. Though 750 women were approached, only 151 expressed will to participate in the study. This might form selection bias, as only motivated, educated and economically well-off couples were interested in participation. Insufficient numbers of participants did not allow for appropriate power and significance to be achieved. Subgroup analyses were similarly not conclusive due to low numbers.

Inability to acquire and analyse all sonographic and molecular data represents selection bias. Due to the anatomical factors and ultrasound technology limitations, correction of this confounding factor was not possible. Analysis method used for NK cell counting, though reproducible and comparable to the manual counting (Mariee et al. 2012b), can be perceived as a source of methodological bias. Differences in tissue staining between samples could confound the obtained results, and as such lead to derivation of false conclusions. The use of same reagents and sample preparation protocols as well as single site analysis, should limit these confounders. High costs associated with molecular work only permitted retrospective selection and analysis of twenty-four samples. Knowledge of the outcome precludes blinding and is a significant limitation.

This study has focused only on human subjects and changes within the human endometrium. This is related to the clinical setting in which the studies were conducted. Animal models of reproduction have not been utilized in the works conducted in this thesis. This may be considered as a limitation, but resources and expertise did not allow for inclusion of such models in the thesis. However informative animal models are, inherent interspecies differences exist, which may confound the results reported here and that are otherwise could not be extrapolated onto human subjects. Genetically engineered animal models however could aid in our understanding of human reproduction, especially when ethical dilemmas prohibit work on early pregnancy in human subjects.

10.4. Suggestions for future scientific work

As the initial design of the endometrial biopsy study was a pilot feasibility project, a fully powered study with women undergoing first cycles only, could answer if this procedure has truly no effect on outcome in that population. Small numbers of women undergoing frozen embryo cycles warrant a fully powered randomised controlled trial aiming to assess the efficacy of the procedure in this population of women. Assuming a 10% difference in live birth rates, power of 80% and α=0.05, the total sample

size for each of the studies would be 779 women in order to unequivocally assess the effect of endometrial injury on ART outcomes.

Though biologically variable, STIC vascularity indices may prove to be beneficial in prediction of ART outcome. More baseline work is however required to validate the technology. Furthermore, if US equipment developers could automate the process of volume analysis, future research could usefully focus on the description of the endometrial vascularity throughout a natural menstrual cycle, thus forming normograms of endometrial vascularity. Similar studies could be then carried out in women undergoing COH with various stimulation protocols. Larger data obtained during these studies could be used to validate the findings of the work reported in this study.

Endometrial stromal cell samples could be used in order to identify additional markers of endometrial receptivity. Studies on the secretome of cell cultures stratified by the outcome might have the potential to shed more light on the endometrial environment favourable of pregnancy. Analysis and correlation of senescence markers within the HESCs, uNK cell levels and pregnancy outcomes could provide useful insights into pregnancy development and gestational complications. As possibilities to screen the entire genome and secretome are becoming more accessible and affordable, these scientific methods should be utilized to identify changes during decidual transformation in normal, fertile women. This baseline data should be compared with the corresponding data obtained from women with reproductive failure in order to identify the level at which reproductive dysfunction occurs and the possible targets for modification. Epigenetic changes within the genome of couples with reproductive failure should also be explored. Identification of potential factors limiting or favouring implantation could therefore be a starting point for development of targeted treatments aiming to induce a pregnancy favouring endometrial phenotype.

As endometrial biopsy appears to alter the levels of uNK cells, it is vital to explore whether this is an effect of the biopsy *per se*, or are there other pathways, which are directly altered by the physical injury to the

endometrium and thus influence the uNK cell numbers. Secretome work could shed more light on this hypothesis. Once identified, follow up studies with pregnancy outcomes should be designed to confirm the role of the factors involved in pregnancy development, ideally with the correction for other confounding factors such as age, cause of infertility, oocyte and embryo quality.

To conclude, in this work it was not possible to clearly demonstrate a benefit of routine endometrial biopsy in all women undergoing ART on treatment outcomes, though clues, as to which population might benefit from the procedure, were identified. Extensive sonographic analysis of endometrial factors did not produce results allowing for unequivocal non-invasive identification of a receptive endometrial milieu. Triple layer endometrial pattern at TVOR was correlated with positive outcome and endometrial biopsy. Spatio-Temporal Image Correlation did not fulfil expectations as a non-invasive marker of endometrial receptivity and was not able to identify women that would go on to have a successful ART outcome. In a small number of patients, STIC indices were able to predict first trimester miscarriage with relatively high sensitivity and specificity. Currently, the technology is not widely available and extremely time consuming for routine implementation. uNK cell numbers were not associated nor predictive of ART outcome, and as such not useful as a routine diagnostic tests prior to ART. An observed significant decrease in uNK cell levels following endometrial biopsy indicates a possible mechanism of action of this intervention. Limited (3) molecular cues were not able to differentiate between a receptive and non-receptive endometrium. This work, however extensive, indicates that the endometrium is a complex microenvironment requiring further investigation in order to understand and influence the mechanisms related to pregnancy establishment and development.

CHAPTER 11. Bibliography

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CHAPTER 12. Appendix

Author	Year of publication	ART modality in previous cycles	Number of embryos transferred	Quality of embryos used	Additional comments
De Placido G. et al. (De Placido et al. 1991)	1991	embryo transfers	-	-	-
Carp H.J.A. et al. (Carp et al. 1994)	1994	IVF	3 or more each time	-	-
Stein M. et al. (Stein et al. 1995)	1995	IVF	3 or 4 each time	good quality	-
Creus M. et al. (Creus et al. 1998)	1998	IVF	2 or more each time	-	-
Safran A. et al. (Safran et al. 2000)	2000	-	-	-	-
Rubio C. et al. (Rubio et al. 2001)	2001	ICSI	-	poor quality embryos in previous attempts	-
Levran D. et al. (Levran et al. 2002)	2002	IVF, fresh cycles	-	-	-
Ng S.C. et al. (Ng et al. 2002)	2002	-	-	-	-
Loutradis D. et al. (Loutradis et al. 2004)	2003	IVF or ICSI	-	good quality	-
Rufas-Sapir O. et al. (Rufas-Sapir et al. 2004)	2003	IVF	-	-	consecutive failed cycles
Pehlivan T. et al. (Pehlivan et al. 2003)	2003	IVF	10 or more	good quality	-
Ledee-Bataille N. et al. (Ledee-Bataille et al. 2004b)	2004	IVF	-	-	good ovarian reserve (FHS<8 IU/L)

Ledee-Bataille N. et al. (Ledee-Bataille et al. 2004a)	2004	IVF	-	good quality	good ovarian reserve (FSH< 8 IU/L) and good response in previous cycle
Primi M.P. et al. (Primi et al. 2004)	2004	fresh treatment cycles	2 or more each time	good quality	-
Pantos K. et al. (Pantos et al. 2004)	2004	IVF	-	-	-
Ledee-Bataille N. et al. (Ledee-Bataille et al. 2005)	2005	IVF	-	good quality	-
Taranissi M. et al. (Taranissi et al. 2005)	2005	IVF	10 or more	-	-
Ghobara T. et al. (Ghobara et al. 2006)	2006	IVF or ICSI	-	-	-
Platteau P. et al.(Platteau et al. 2006)	2006	IVF or ICSI	-	-	-
Qublan H.S. et al. (Qublan et al. 2006)	2006	IVF	-	-	-
Yakin K. et al. (Yakin et al. 2007)	2006	-	-	-	-
Mantzouratou A. et al. (Mantzouratou et al. 2007)	2007	IVF	-	-	-
Quenby S. et al. (Quenby et al. 2007)	2007	IVF or ICSI	9 or more	good quality	-
Matsubayashi H. et al. (Matsubayashi et al. 2007)	2007	IVF	-	-	-
Weissman A. et al. (Weissman et al. 2007)	2007	IVF, fresh cycles	-	-	-
van den Heuvel M. et al. (van den Heuvel et al. 2007)	2007	IVF	-	-	consecutive failed cycles

Matteo M. et al. (Matteo et al. 2007)	2007	IVF, fresh cycles	2 or more each time	good quality	good ovarian reserve (FSH< 8 IU/L) and good response in previous cycle
Kling C. et al. (Kling et al. 2008b)	2008	IVF, fresh cycles	-	-	-
Blockeel C. et al. (Blockeel et al. 2008)	2008	IVF	10 or more	good quality	-
Prakash A. et al. (Prakash et al. 2008)	2008	IVF, fresh or frozen	-	good quality	-
Thum M.Y. et al. (Thum et al. 2008)	2008	IVF	2 to 3 each time	good quality	-
Yakin K. et al. (Yakin et al. 2008)	2008	fresh treatment cycles	-	-	-
Simur A. et al. (Simur et al. 2009)	2008	IVF	3 or more	good quality	-
Kling C. et al. (Kling et al. 2008a)	2008	IVF	-	-	consecutive failed cycles
Pagidas K. et al. (Pagidas et al. 2008)	2008	IVF	-	-	-
Jee B.C. et al. (Jee et al. 2009)	2009	IVF	-	-	good number of oocytes, embryos and adequate transfer
Koler M. et al. (Koler et al. 2009)	2009	IVF	10 or more	high quality	-
Brosens J. et al. (Brosens et al. 2009b)	2009	IVF	-	good quality	-
Debrock S. et al. (Debrock et al. 2009)	2009	IVF, fresh cycles	-	-	-
Varla-Leftherioti M. et al. (Varla-Leftherioti et al. 2010)	2010	-	-	-	-
Fragouli E. et al. (Fragouli et al. 2010)	2010	IVF	10 or more	good quality	-

Germeyer A. et al. (Germeyer et al. 2010)	2010	embryo transfers	-	good quality	-
Achache H. et al. (Achache et al. 2010b)	2010	IVF, fresh cycles	-	-	-
Heilman L. et al. (Heilmann et al. 2010)	2010	-	-	-	-
Achache H. et al. (Achache et al. 2010a)	2010	IVF	-	good quality	good ovarian reserve and age under 40
Chernyshov V. et al. (Chernyshov et al. 2010)	2010	IVF	2 or more each time	good quality	-
Li G.G. et al. (Rajaei et al. 2011)	2011	IVF	-	-	-
Kim C.H. et al. (Kim et al. 2011)	2011	IVF or ICSI	-	grade 1 or 2	-
Martnez-Zamora M. et al. (Maritnez-Zamora et al. 2011)	2011	fresh or frozen treatment cycles	1 or more each time	grade 1 or 2	-
Choi YS. Et al. (Choi et al. 2011)	2011	IVF	-	-	-
Tiboni GM. et al. (Tiboni et al. 2011)	2011	embryo transfers	-	-	-
Rajaei S. et al. (Rajaei et al. 2011)	2011	IVF or ICSI	10 or more	-	
Sudoma I. et al. (Sudoma et al. 2011)	2011	-	2 or more each time	good quality	-
Sacks et al. (Sacks et al. 2012)	2012	fresh or frozen treatment cycles	-	-	-
Scarpellini et al. (Scarpellini et al. 2012)	2012	IVF	7 or more	Good quality	Age <39, no systemic illness

Table 1. Studies using 'three or more' failed ART treatment cycles and number of embryos replaced as definition of recurrent implantation failure.

Author	Year of publication	ART modality in previous cycles	Number of embryo transferred	Quality of embryos used	Additional comments
Gianaroli L. et al. (Gianaroli et al. 1997)	1997	IVF	-	-	-
Kahraman S. et al. (Kahraman et al. 2000)	2000	-	-	-	-
Stephenson M. et al. (Stephenson et al. 2000)	2000	fresh or frozen cycles (modality not specified)	-	-	-
Matsubayashi H. et al. (Matsubayashi et al. 2001a)	2001	IVF	-	Good visually	-
Spandorfer S. et al. (Spandorfer et al. 2002b)	2002	IVF	-	-	-
Kwak-Kim JYH. et al. (Kwak-Kim et al. 2003)	2003	IVF	2 or more each time	-	-
Kahraman S. et al. (Kahraman et al. 2004)	2004	-	3 or more	good quality	-
Martinuzzo M. et al. (Martinuzzo et al. 2005)	2005	IVF	-	Good quality	-
Petersen C. et al. (Petersen et al. 2005)	2005	fresh or frozen cycles (modality not specified)	-	-	-
Fukui A. et al. (Fukui et al. 2006)	2006	IVF	-	Good quality	-
Koscinski I. et al. (Koscinski et al. 2006)	2006	IVF	2 or more	good quality	-
Vaquero E. et al. (Vaquero et al. 2006)	2006	IVF	-	-	-
Foulk RA. et al. (Foulk et al. 2007)	2007	-	-	Top quality	-
Arefi S. et al. (Arefi et al. 2008)	2008	IVF or ICSI	3 or more	good quality	-

Fukui A. et al. (Fukui et al. 2008)	2008	IVF	4 or more	-	-
Hiraoka K. et al. (Hiraoka et al. 2008)	2008	fresh or frozen cycles (modality not specified)	-	-	Cleavage stage embryos replaced
Kalu E. et al. (Kalu et al. 2008)	2008	IVF	-	Good quality	-
Valojerdi MR. et al. (Valojerdi et al. 2008)	2008	-	-	-	-
Urman B. et al. (Urman et al. 2009)	2009	fresh cycles (modality not specified)	-	-	-
Aletebi F. (Aletebi 2010)	2010	-	3 or more	good quality	-
Berker B. et al. (Berker et al. 2011)	2010	ICSI	-	-	-
Eyheremendy V. et al. (Eyheremendy et al. 2010)	2010	IVF or ICSI, fresh or frozen cycles	-	-	-
Johnston-MacAnanny EB. et al. (Johnston-MacAnanny et al. 2010)	2010	IVF	1 or more each time	good quality	-
Schoolcraft WB. et al. (Schoolcraft et al. 2010)	2010	-	-	-	-
Sharif KW. et al. (Sharif et al. 2010)	2010	IVF or ICSI, fresh cycles	6 or more	good quality (G1 or G2)	-
Tsoumpou I. et al. (Tsoumpou et al. 2010)	2010	-	-	Good quality	-
Yang KM. et al. (Yang et al. 2010)	2010	IVF	2 or more each time	-	-
Chou PY. et al. (Chou et al. 2011)	2011	IVF	-	Good quality	-
Huang SY. et al. (Huang et al. 2011)	2011	IVF	-	Good quality	-
Lodigiani C. et al. (Lodigiani et al. 2011)	2011	IVF or ICSI	-	-	-

Oliveira JBA et al. (Oliveira et al. 2011)	2011	ICSI	-	Morphologicall y good	-
Takahashi K. et al. (Takahashi et al. 2011)	**2011**	**fresh or frozen cycles (modality not specified)**	**-**	**-**	**-**
Ivanov et al. (Ivanov 2012)	2012	IVF	-	-	-
Sermondade et al. (Sermondade et al. 2012)	2012	IVF	-	-	-

Table 2. Studies using 'two or more' failed ART treatment cycles and number of embryos replaced as definition of recurrent implantation failure.

Authors	Year of publication	Number of previous failed cycles	ART modality used in previous cycles	Number of embryos transferred in previous cycles	Quality of transferred embryos	Additional comments
Huang C.C. et al. (Huang et al. 1999)	1999	5 or more	IVF or ICSI	3 or more each time	-	-
Raziel A. et al. (Raziel et al. 2002)	2002	6 or more	-	cumulative of 15 or more	-	-
Voullaire L. et al. (Voullaire et al. 2002a)	2002	multiple failed cycles	IVF	cumulative of 10 or more	-	-
Elram T. et al. (Elram et al. 2005)	2005	7 or more	fresh cycles	at least 2 embryos each time	good quality	-
Varla-Leftherioti M. et al. (Varla-Leftherioti et al. 2007)	2007	5 or more	IVF	more than 2 each time		less than 38 years old
Ledee N. et al. (Ledee et al. 2008)	2008	several failed cycles	fresh or frozen cycles	cumulative of 10 or more	fragmentation <20% and at least 4-cell stage by day 2	unexplained failure of treatment cycles
Brinsden P.R. et al. (Brinsden et al. 2009)	2009	2 or more or 3 or more	ART cycles, fresh	2 or more or 1 or more each time, respectively	grade A or B	-
Karimzadeh et al. (Karimzadeh et al. 2009)	2009	2 to 6 cycles	IVF	cumulative of 10 or more	good quality	Less than 40 years old, good responders (>4 follicles at hCG), good ovarian reserve (FSH <10mIU/mL), no uterine anomalies, endometriomas, hydrosalpinges and no coagulation disorders

Tuckerman E. et al. (Tuckerman et al. 2010)	2010	3 fresh or more or 2 fresh and 2 frozen cycles	IVF, fresh or frozen	-	good quality	-
Dos Santos et al. (Dos Santos et al. 2012)	2012	several failed cycles	IVF, fresh or frozen	cumulative of 10 or more	fragmentation <20% and at least 4-cell stage by day 2	Good hormonal reserve (FSH <10mIU/mL) and good response to previous treatment

Table 3. Studies using less common inclusion criteria into study populations.

Patient ID	NK cell count in first sample (%)	NK cell count in second sample (%)	Previous CPs	Previous miscarriages/ TOPs/EPs	Previous LBs
1	2.98	<	1	0/1/0	0
2	0.83	0.39†	10	7/2/0	1
3	4.61	0.52	0	0	0
4	0.62	0.39†	1	0/1/0	0
5	21.7	7.92	1	0/1/0	0
6	5.38	↓	2	2/0/0	0
7	3.41	0.92	3	2/0/1	0
8	1.62	0.45	2	1/0/1	0
9	4.34	0.68	3	3/0/0	0
10	8.05	0.71	0	0	0
11	4.42	0.22	0	0	0
12	1.27	0.07	2	1/0/0	1
13	0.1	0.8*	1	1/0/0	0
14	0.34	0.4*	0	0	0
15	0.14	0.92*	0	0	0
16	1.02	0.86	1	0	1
17	25.3	7.47	0	0	0
18	1.72	1.11	0	0	0

Table 4. Individual data for patients with repeated endometrial biopsies.

CPs- clinical pregnancies; TOPs- terminations of pregnancy; Eps- ectopic pregnancies; LBs- live births; < - sample too small to be processed; †- initial NK cell count <1% and a further drop in NK cell count in second sample; ↓- vasovagal reaction following first biopsy; *- initial NK cell count <1% and an increase in NK cell count in second sample.

Timing of examination	Variable	Positive group (n=11)	Negative group (n=13)
At biopsy	*ET (±SD) mm*	9.3(±3.0)	9.5(±2.4)
	EV (± SD) mm^3	3.1(±1.9)	3.4(±1.5)
	MGS (±SD)	48.6(±8.5)	46.3(±8.6)
	VI (±SD)	3.91(±6.01)	3.1(±2.45)
	FI (±SD)	28.3(±4.99)	27.96(±3.14)
	VFI (±SD)	1.31(±2.36)	0.91(±0.78)
	UAVmax(±SD) cm/s	30.12(±13.0)	33.5(±8.5)
	UAVmin(±SD) cm/s	3.7(±3.2)	4.6(±2.77)
	RI (±SD)	0.86(±0.14)	0.83(±0.12)
	PI (±SD)	2.25(±0.47)	2.4(±0.69)
At TVOR	*ET (±SD) mm*	9.8(±2.6)	11.2(±3.0)
	EV (± SD) mm^3	3.26(±1.5)	4.4(±1.6)
	MGS (±SD)	**46.8(±6.3)***	**39.4(±5.5)***
	VI (±SD)	2.3(±2.7)	2.8(±2.1)
	FI (±SD)	26.8(±2.7)	27.0(±3.6)
	VFI (±SD)	0.69(±0.87)	0.82(±0.62
	UAVmax(±SD) cm/s	**25.5(±12.3)***	**40.1(±10.6)***
	UAVmin(±SD) cm/s	3.42(±2.7)	5.46(±3.6)
	RI (±SD)	0.87(±0.1)	0.88(±0.07)
	PI (±SD)	2.23(±0.24)	2.48(±0.5)
At embryo transfer	*ET (±SD) mm*	10.9(±3.1)	11.3(±1.6)
	EV (± SD) mm^3	3.96(±2.15)	5.25(±1.78)
	MGS (±SD)	41.16(±9.92)	47.35(±5.68)
	VI (±SD)	1.56(±1.49)	1.85(±1.7)
	FI (±SD)	22.78(±9.0)	27.34(±3.6)
	VFI (±SD)	0.43(±0.43)	0.55(±0.56)
	UAVmax(±SD) cm/s	35.2(±8.74)	43.0(±11.8)
	UAVmin(±SD) cm/s	8.26(±3.1)	9.1(±4.1)
	RI (±SD)	0.78(±0.06)	0.8(±0.06)
	PI (±SD)	1.66(±0.32)	1.95(±0.5)

Table 5. Sonographic details of study participants.

*Data presented as mean (±SD), median (interquartile range) or number (%) as appropriate; * P<0.05; P-values assessed by unpaired t test.*