Angiogenesis in endometriosis:

The role of circulating angiogenic cells and the endometrium

Katie Webster

Wolfson College, University of Oxford

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Contents

Abstract

Endometriosis is a common cause of subfertility and pelvic pain, affecting up to 10% of women of reproductive age. It is characterised by the presence of endometrial-like tissue outside the uterus. The development of the disease is still poorly understood and, currently, the diagnosis relies on visualisation of typical lesions during surgery. There is great interest in identifying biomarkers to assist in diagnosis and disease management.

Blood vessel development is known to be a crucial feature of endometriosis, but the mechanisms involved in angiogenesis are not well described for this disease. Most vessel development relies on the proliferation and migration of pre-existing endothelial cells. However, there may also be roles for cells derived from peripheral blood (circulating angiogenic cells) and surrounding stromal cells. In this thesis, the contribution of these different cell types to vessel development in endometriosis is assessed.

In chapter 2, a robust protocol was optimised to identify circulating angiogenic cells (CACs) with flow cytometry. The reliability of the protocol was verified, and the level of these cells was found not to fluctuate with the menstrual cycle in healthy women (P=0.279, F=1.359, 3 d.f.). In chapter 3, levels of CACs in women with and without endometriosis were found to be equivalent (0.0835% ±0.0422 compared to 0.0724% ±0.0414), demonstrating that they have no use as a disease biomarker.

In chapter 4, isolation and culture of endothelial cells from the endometrium was attempted. However, a pure culture of endometrial endothelial cells could not be obtained, which may be due to contamination by other cell types or cellular transdifferentiation.

Finally, in chapter 5, the contribution of endometrial stromal cells to vessel development was considered. Stromal cells were found not to differentiate towards an endothelial cell phenotype, but were able to participate in tube formation assays. However, the presence of endometriosis did not influence this behaviour.

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Abbreviations

AcLDL	Acetylated Low Density Lipoprotein
BMI	Body Mass Index
BSA	Bovine Serum Albumin
CAC	Circulating Angiogenic Cell
CD	Cluster of Differentiation
CFU	Colony Forming Unit
CV	Coefficient of Variation
DAPI	4', 6-diamidino-2-phenylindole
DiI	1,1'-Dioctadecyl-3,3,3’,3'-tetramethylindocarbocyanine perchlorate
DMEM	Dulbecco’s Modified Eagle’s Medium
DMSO	Dimethylsulphoxide
ECFC	Endothelial Colony Forming Cell
ECGM MV2	Endothelial Cell Growth Medium Microvascular 2
EDTA	Ethylenediaminetetraacetic acid
EEC	Endometrial Endothelial Cell
EPC	Endothelial Progenitor Cell

FBS	Fetal Bovine Serum
FMO	Fluorescence Minus One
GnRH	Gonadotrophin Releasing Hormone
HUVEC	Human Umbilical Vein Endothelial Cells
HMVEC	Human Microvascular Endothelial Cells
IUCD	Intrauterine Contraceptive Device
MSC	Mesenchymal Stem Cells
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
SD	Standard Deviation
SE	Standard Error
VEGF	Vascular Endothelial Growth Factor
VEGFR-2	Vascular Endothelial Growth Factor Receptor 2
vWF	von Willebrand Factor

Background to the study and participants

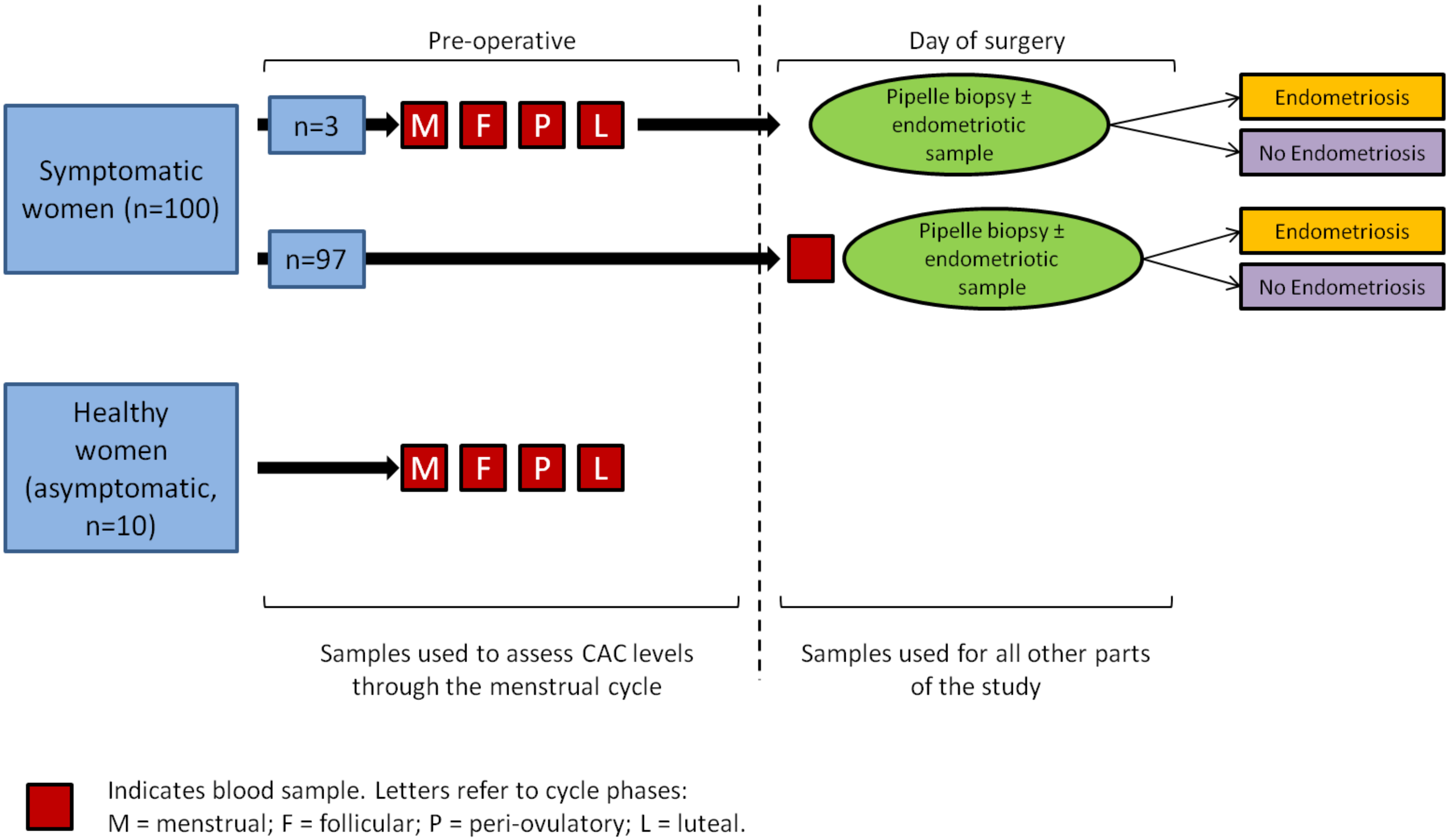
Data presented in this thesis are the first results from a study conducted by the Nuffield Department of Obstetrics and Gynaecology, University of Oxford. The study was approved by the local ethics committee (Oxfordshire REC A 09/H0604/58) and all women provided written, informed consent prior to participation. The primary goal of the study was to investigate potential biomarkers of endometriosis.

Initially we aimed to assess the fluctuation of biomarkers throughout the menstrual cycle (over four time points) in women with symptoms suggestive of endometriosis. However, insufficient numbers of women were recruited for meaningful analysis. In view of this an amendment to the study ethical approval was sought. This permitted recruitment of symptomatic women at only one time point, and allowed recruitment of healthy, asymptomatic women to donate samples across a menstrual cycle.

Consequently, the vast majority of participants were women scheduled for laparoscopy to investigate symptoms or signs of endometriosis (including pelvic pain, subfertility and ovarian cysts). These women segregated into cases (women with endometriosis) and controls (those with no endometriosis) depending on the operative findings. Women donated blood and/or endometrial pipelle biopsies on the day of surgery, as well as endometriotic tissue where appropriate.

A small number of participants donated blood on four occasions to assess fluctuations in biomarker levels with the menstrual cycle. Stages of the menstrual cycle were based on cycle length and last menstrual period, and confirmed with serum estradiol and progesterone levels. These women comprised both symptomatic women, awaiting laparoscopy (as above), and asymptomatic women with no known gynaecological complaints, recruited as healthy controls through advertisements.

Figure I shows an overview of the subjects recruited and sample collection.



🟥 Indicates blood sample. Letters refer to cycle phases:
M = menstrual; F = follicular; P = peri-ovulatory; L = luteal.

Figure I: Outline of subjects recruited to the study and samples used

Chapter 1: Introduction

1.1 Endometriosis

Endometriosis is a common cause of subfertility and pelvic pain, predominantly affecting women of reproductive age (Giudice, 2010). It is characterised by the "presence of endometrial-like tissue outside the uterus, which induces a chronic, inflammatory reaction" (Kennedy et al., 2005). Typically, this tissue is found within the pelvis where it forms lesions on peritoneal surfaces. These lesions can be superficial or deeply infiltrating and can have a red, brown, black or white appearance. The disease can also present with adhesions, ovarian cysts (termed endometriomas), or as nodules in the uterosacral ligaments or rectovaginal septum.

1.1.1 Presentation and prevalence of endometriosis

The main symptom associated with endometriosis is that of pelvic or lower abdominal pain. The pain may be related to specific phases of the menstrual cycle or persist throughout. Other features include painful intercourse (dyspareunia), heavy periods and fatigue. Pain with urination (dysuria), pain with bowel movements (dyschezia) and cyclical rectal bleeding sometimes occur, and are more suggestive of deep infiltrating endometriosis (Fauconnier et al., 2002). However, severity of symptoms generally correlates poorly with the extent of disease (Gruppo Italiano per lo Studio dell Endometriosi, 2001; Vercellini et al., 2007).

Endometriosis is also a cause of subfertility, although the mechanism for this remains unclear and is probably multifactorial. Subfertility may result from damaging effects of endometriomas on ovarian reserve, the effects of abnormal peritoneal fluid on the oocyte, or abnormalities of the eutopic endometrium (de Ziegler et al., 2010). Adhesions and endometriotic nodules may distort the pelvic anatomy, further decreasing fertility.

Endometriosis is thought to affect around 10% of women of reproductive age (Buck Louis et al., 2011). However, calculating the true prevalence of the disease is difficult as surgery is required to make the diagnosis. The severity of symptoms varies widely and some women may be affected by endometriosis without ever consulting a doctor, or being fully investigated and diagnosed. A recent study assessed the prevalence of endometriosis in premenopausal women attending their General Practitioner for non-gynaecological complaints (Ferrero et al., 2010). Although these women had not requested a consultation for symptoms suggestive of endometriosis, 3.6% of them were eventually identified as having the condition. Furthermore, around 12 - 22% of asymptomatic women were found to have evidence of the disease when undergoing surgery for laparoscopic sterilisation (Moen and Muss, 1991; Chu et al., 1995).

Prevalence of endometriosis in symptomatic women is much higher. Studies have reported endometriosis in up to 47% of women undergoing laparoscopy for subfertility (Mahmood and Templeton, 1991; Meuleman et al., 2009). A prevalence of 15-28% has been found in women undergoing laparoscopy for pelvic pain (Mahmood and Templeton, 1991; Stanford et al., 2005).

1.1.2 Diagnosis and staging

Currently, endometriosis remains a surgical diagnosis in the vast majority of cases, requiring laparoscopy (or laparotomy) to visualise typical lesions. Ideally, this should be supported with histological verification of the disease, although this is not necessary to make the diagnosis (Kennedy et al., 2005).

The American Society for Reproductive Medicine has developed a scoring system to quantify the extent of endometriosis seen at surgery (American Society for Reproductive Medicine, 1997). This classifies the disease into four "stages", dependent on the quantity, location and size of endometriotic lesions. Stages are referred to by number (1, 2, 3 or 4) or severity of disease (minimal, mild, moderate or severe). This system allows some estimate of disease progression (or regression) between operations, as well as permitting comparisons between studies.

There is currently great interest in identifying biomarkers of endometriosis to permit non-invasive diagnosis of the disease (D'Hooghe et al., 2006; Rogers et al., 2009). Whilst laparoscopy is relatively minor surgery, it carries risks to the patient, including infection, bleeding and anaesthetic complications (Härkki-Sirén and Kurki, 1997; Li et al., 1997; Lamont et al., 2011). There is even a small mortality rate associated with the procedure (Jansen et al., 1997; Chapron et al., 1998). It would, therefore, be of benefit to identify alternative means of diagnosing the disease.

Despite this interest, no biomarker has been shown to have sufficiently high sensitivity and specificity to be used for the diagnosis of endometriosis (May et al., 2010). The most common marker in clinical use is CA125 - a glycoprotein better known for detecting ovarian cancer. A meta-analysis calculated that for a specificity of 90%, the sensitivity of CA125 as a diagnostic test was only 28% (Mol et al., 1998). An increase in the sensitivity to 50% resulted in a decline in specificity to 72%. This poor diagnostic performance means that CA125 is not a useful alternative to laparoscopy, although it is still occasionally used as an adjunct to diagnosis.

Non-invasive diagnosis of endometriosis appears to be more accurate for advanced stages of disease. Ultrasound has been shown to have high sensitivity and specificity to diagnose endometriomas (Moore et al., 2002) and MRI is useful for the assessment of rectovaginal disease (Bazot et al., 2012). Furthermore, the use of CA125 was shown to have more diagnostic accuracy when limited to women with moderate or severe disease (sensitivity 60%, specificity 81% for revised American Society for Reproductive Medicine score 3-4 disease)(Mol et al., 1998).

1.1.3 Treatment

Treatment options for endometriosis range from simple analgesia for pain relief, through various hormonal therapies to surgical management. Non-steroidal anti-inflammatory drugs are widely used despite a lack of evidence for their efficacy in endometriosis (Allen et al., 2010). However, these are the only medical treatments available to women who wish to conceive. The combined oral contraceptive pill, progestagen treatment and GnRH analogues are all useful alternatives for women in whom fertility is not the current priority.

Surgical options are also available, including laparoscopic ablation or excision of endometriotic lesions, ovarian cystectomy, and potentially hysterectomy and oophorectomy. Operative laparoscopy has been demonstrated to be effective at reducing pain from endometriosis (Jacobson et al., 2009) but the benefits of surgery to improve fertility are less clear. A recent Cochrane review suggested that women with minimal/mild endometriosis may have a slight increase in fertility following operative laparoscopy, compared to diagnostic laparoscopy alone (Jacobson et al., 2010). However, the two articles included in this review paper actually came to opposing conclusions (Marcoux et al., 1997; Gruppo Italiano per lo Studio dell Endometriosi, 1999). Similarly, whilst removing endometriomas may facilitate oocyte collection in women undergoing IVF, it is not certain that it has a beneficial effect on pregnancy rates (Tsoumpou et al., 2009; Benschop et al., 2010).

1.1.4 Pathogenesis

How endometriosis develops is still unclear. The most accepted theory is that of Sampson, who proposed retrograde menstruation as the cause (Sampson, 1927a). This hypothesis states that endometrial tissue is shed at menstruation, refluxes through the Fallopian tubes and enters the peritoneal cavity. The tissue is then able to adhere to peritoneal surfaces where it locally invades and grows to form endometriotic lesions.

This theory has a number of attractive features. Firstly, it accounts for the occurrence of the disease predominantly in women of reproductive age, especially those who experience regular menses. Secondly, it explains why the overwhelming majority of endometriotic lesions are found within the pelvis, as these sites are anatomically close to the refluxed tissue. Thirdly, it accounts for the increased occurrence of endometriosis in women with outflow obstruction to the uterus - as they are likely to experience increased amounts of retrograde flow (Olive and Henderson, 1987). Finally, it explains the ability to induce endometriosis in women or animals by intra-peritoneal injection of endometrial tissue (Ridley and Edwards, 1958; Somigliana et al., 1999).

However, retrograde menstruation has been shown to occur in 90% of healthy women – demonstrated by the presence of blood in the peritoneal cavity at the time of menses (Halme et al., 1984) – despite a disease prevalence of only 10%. Retrograde menstruation alone is, therefore, not sufficient for the development of the disease: there must be other contributing factors.

These other factors may include changes in the peritoneal environment in women with endometriosis. Peritoneal fluid from women with the disease is recognised to contain increased amounts of pro-inflammatory cytokines, which may promote the development of endometriotic lesions (reviewed in Wu and Ho, 2003). Similarly, there are increasing data showing subtle differences in the eutopic endometrium of women with the disease (reviewed in May et al., 2011), which may change the ability of sloughed tissue to adhere and invade peritoneum, permitting the development of lesions. Significant changes in the immune system of women with endometriosis have also been identified (reviewed in Berkkanoglu and Arici, 2003; Dmowski and Braun, 2004). These may affect peritoneal immunological surveillance, allowing prolonged survival of menstrual tissue.

Other theories have been proposed for the development of endometriosis. These include the spread of endometrial cells to extra-uterine locations by lymphatic or haematogenous routes (Sampson, 1927b). This possibility better explains the rare occurrence of endometriosis in extra-peritoneal locations, such as the brain or lung. However, several case reports exist describing the occurrence of endometriosis in men (Martin and Hauck, 1985). This would imply that endometriosis can also develop from the differentiation of another cell type, such as a stem cell population, potentially derived from bone marrow or other sources (Sasson and Taylor, 2008). Other theories propose the metaplasia of mesothelial peritoneal cells into endometrial like tissue, in response to stimulation by estrogen (Ferguson et al., 1969). It is possible that these different pathogenic mechanisms may all cause endometriosis under certain circumstances. However, the prevailing consensus is that the majority of cases are likely to be the result of retrograde menstruation.

1.1.5 Development of a vascular supply in endometriotic lesions

One aspect of endometriosis, which has recently been the focus of research, is the development of a vascular supply. Tissues larger than approximately 1-2mm^3 require a blood supply to provide nutrients and oxygen (Folkman, 1990). The chick chorioallantoic membrane model of endometriosis has been used to show that only endometrial fragments >1mm^3 are able to establish disease (Nap et al., 2003). This implies that endometriotic lesions require an immediate blood supply to ensure their development and continued survival. Furthermore, analysis of endometriotic lesions at both a macro- and microscopic level demonstrates profuse vascularity (Nisolle et al., 1993; Taylor et al., 2001).

The prospect of interrupting this blood supply has become an attractive therapeutic option in endometriosis, and has shown some promise in murine models (Nap et al., 2004; Becker et al., 2005; Becker et al., 2008). As yet, however, this approach has not been extended to clinical trials in humans. This is likely due to valid concerns regarding the use of anti-angiogenic compounds in women of reproductive age, with particular regard to teratogenicity. A better understanding of vascular development in endometriosis may lead to new insights regarding the pathogenesis of the disease, as well as offering a chance to interrupt vessel formation and prevent lesions from enlarging.

1.2 Mechanisms of angiogenesis

Angiogenesis is the process of blood vessel development from pre-existing vessels. This contrasts with vasculogenesis, which is predominantly an embryonic process – the development of vessels *de novo*, rather than by extension of existing vasculature. Angiogenesis is a frequent event in the female reproductive system – occurring monthly with the menstrual cycle as part of endometrial development and the formation of the corpus luteum. By contrast, the remainder of the human body sees little physiological angiogenesis in adult life. Instead, most vessel development occurs as part of pathological states – wound healing, tumour growth or inflammatory processes such as rheumatoid arthritis.

1.2.1 Classical mechanisms of angiogenesis – the role of the endothelium

Different patterns of vessel growth have been described, all dependent on the proliferation and migration of pre-existing endothelial cells. The mechanisms described include:

- Sprouting angiogenesis – the formation of a new branch point by sprouting from the side of a pre-existing vessel.
- Intussusception – alignment of endothelial cells in the centre of a vessel lumen, to form two vessels running in parallel. This process generates a network of interconnected vessels.
- Pruning – modification of existing vessels to generate an optimised vascular network. This may include elongation or widening of vessels, or apoptosis of endothelial cells (Risau, 1997).

1.2.2 New concepts in angiogenesis – the role of endothelial progenitors and circulating angiogenic cells

Over the past 15 years there has been great interest in the possibility of a second cell type contributing to angiogenesis (and potentially vasculogenesis) in adults. These cells were first identified in 1997, and termed endothelial progenitor cells (EPCs)(Asahara et al., 1997). Inoculation of EPCs into mice and rabbits with hind limb ischaemia resulted in incorporation of these cells into capillary walls of the affected limb (Asahara et al., 1997). This suggested the existence of a population of cells in peripheral blood which could directly differentiate into endothelial cells.

The identification of these cells was of interest to many researchers, but especially to those in the fields of cardiovascular medicine and tumour biology. Great potential was seen for EPCs as biomarkers of diseases involving angiogenesis or vessel damage. There was also interest regarding their therapeutic potential. The infusion of EPCs or insertion of stents designed to capture these cells could be used to promote revascularisation of ischaemic tissue (Alev et al., 2011). By contrast, in oncology, the use of modified EPCs could provide a mechanism to target anti-angiogenic drugs specifically to tumours (Dome et al., 2009).

A major challenge in this area has been determining the precise phenotype of EPCs. Early studies made use of cells expressing CD34, a haematopoietic stem cell marker (Holyoake and Alcorn, 1994), or Vascular Endothelial Growth Factor Receptor 2 (VEGFR-2), typically found on endothelial cells (Holmes et al., 2007). However, both these markers are found on a variety of cell types and are not specific to EPCs. To refine the phenotype more recent studies have incorporated other cell surface markers.

Subsequent studies have incorporated other markers, such as CD133, CD45, CD144 and CD31 to identify EPCs (for review see Timmermans et al., 2009). Other authors have preferred cell culture based assays to identify the cells, including short (5 days), medium (10-14 days) or long term (14-21 days) culture strategies (reviewed in Fadini et al., 2012).

The use of such diverse methods to identify EPCs casts doubt on whether all these techniques identify the same cell populations. It now appears clear that cells identified by each of the three cell culture assays have very different properties (Hirschi et al., 2008). Colonies of “EPCs” in culture have also been shown to encompass a variety of cell types, including T cells and macrophages, rather than consisting of a specific progenitor cell (Rehman et al., 2003; Hur et al., 2007; Desai et al., 2009). Furthermore, evidence now suggests that cells identified with flow cytometry do not correlate with those identified using cell culture assays (George et al., 2006; Povsic et al., 2009; Robb et al., 2009).

The use of CD133 has been the subject of recent controversy. This cell surface glycoprotein of unknown function has been widely used to identify EPCs. However, CD133+ cells are unable to generate colonies of EPCs in culture based assays (Timmermans et al., 2007). Despite this finding, recent data confirms that CD133 expressing cells have pro-angiogenic potential (Barcelos et al., 2009; Sanchez-Guijo et al., 2010). Consequently this marker may be of relevance for identifying pro-angiogenic cells, although not of use when isolating cells with the potential to differentiate into true endothelial cells. CD133 may help to distinguish pro-angiogenic cells from non-angiogenic cells. Murine data indicates that CD133+ EPCs are able to promote vessel growth in tumours, whilst CD133- EPCs are unable to do this (Estes et al., 2010b).

Despite controversy in the published literature, it remains certain that there exists a population of circulating cells that contribute to angiogenesis – either by directly participating in vessel growth or by the local production of pro-angiogenic factors. The lack of defining features has led some authors to use the term “circulating angiogenic cells" (CACs), which more appropriately describes the role of these cells and accounts for the heterogeneity of the population (Rehman et al., 2003; Alev et al., 2011).

1.2.3 Facilitating vessel growth – the role of surrounding tissue and stroma

The process of angiogenesis must be preceded by stimuli that indicate a need for new vessel growth. These may arise directly from the endothelium in an autocrine manner, but may also be derived from surrounding cells.

In tumour biology, the stroma is increasingly recognised as contributing significantly to angiogenesis. Fibroblasts in tumour connective tissue have been identified as a major source of Vascular Endothelial Growth Factor (VEGF, a potent pro-angiogenic agent), and have the ability to promote angiogenesis (Fukumura et al., 1998; Orimo et al., 2005). *In vitro* assays have shown that endothelial cells and fibroblasts can co-operate to form stable vessels (Nakatsu et al., 2003).

In addition, recent work has identified the ability of some mesenchymal stem cells (MSC) to secrete pro-angiogenic cytokines and differentiate into endothelial cells *in vitro* (Oswald et al., 2004; Mirshahi et al., 2009). Exposure of these cells to high concentrations of vasculogenic substances such as VEGF resulted in the expression of endothelial-like markers in these cells, as well as an increased ability to form tubes in an angiogenesis assay (Oswald et al., 2004; Liu et al., 2007). MSC have now been identified in a number of tissues, including bone, cartilage and muscle (reviewed in

Minguell et al., 2001) therefore may act to support angiogenesis within these locations.

Therefore, as well as requiring functional endothelial cells and CACs, angiogenesis *in vivo* is likely to require close co-operation between endothelial cells and tissue stroma to produce mature, functional vessels.

1.3 Endometrial angiogenesis

The endometrium is one of the most regenerative tissues of the human body, undergoing monthly shedding, repair and growth during the reproductive years. The maintenance of this tissue requires a rich vascular supply which is also capable of repair and remodelling. Vessels in the endometrium include a sub-epithelial capillary network, as well as the spiral arterioles which coil upwards through the endometrium (Rogers, 1996).

Sprouting angiogenesis does not appear to make a major contribution to endometrial angiogenesis. The $\alpha_v\beta_3$ integrin (a marker of sprouting angiogenesis) has only been shown within pre-existing vessels, rather than at the site of sprouts (Hii and Rogers, 1998). It has, therefore, been proposed that vessel elongation and intussusception are more common mechanisms of angiogenesis in the endometrium (Gargett and Rogers, 2001). Some authors have shown fluctuations in CAC levels with the menstrual cycle suggesting that they may participate in endometrial regeneration (Fadini et al., 2008; Lemieux et al., 2009; Robb et al., 2009). The role of endometrial stroma has not been well studied, although preliminary data suggest that it may also promote angiogenesis (Print et al., 2004).

1.4 Angiogenesis in endometriosis

The retrograde menstruation hypothesis indicates that ectopic endometrium arises directly from eutopic endometrium in women with endometriosis. Therefore, mechanisms of angiogenesis that are important in the eutopic endometrium may also be involved in promoting vasculature for endometriotic lesions. Factors affecting angiogenesis have been assessed in women with and without endometriosis. A variety of subtle changes have been reported that may result in a pro-angiogenic environment, contributing to the neovascularisation seen with the disease. These are discussed below.

1.4.1 Peripheral blood

Many studies have assessed levels of pro-angiogenic substances in the peripheral blood of women with endometriosis. VEGF is perhaps the best studied of these substances, although results have been conflicting. Two studies demonstrated a significant difference in peripheral VEGF levels in women with endometriosis (Matalliotakis et al., 2003; Xavier et al., 2006). However, a further five studies showed identical levels to those in women without the condition (Pellicer et al., 1998; Gagne et al., 2003; Bourlev et al., 2006b; Pupo-Nogueira et al., 2007; Othman et al., 2008). One recent study has indicated that high serum VEGF levels may be a more sensitive and specific biomarker of moderate/severe endometriosis than CA125 (Mohamed et al., 2013).

TNFα is best known as a pro-inflammatory cytokine, but may also act to stimulate angiogenesis *in vivo* (Fràter-Schröder et al., 1987). Many studies have identified raised levels of TNFα in the serum of women with endometriosis (Matalliotakis et al., 1997; Markham and Fraser, 1997a; Markham and Fraser, 1997b; Pizzo et al., 2002; Darai et al., 2003; Xavier et al., 2006; Cho et al., 2007). However, other authors have failed to identify a difference in TNFα levels between patients and controls (Vercellini et al., 1993; Kalu et al., 2007; Othman et al., 2008; Seeber et al., 2008).

A small number of other factors involved in angiogenesis have been shown to be elevated in the periphery. These include angiogenin, fibroblast growth factor 2 (FGF-2) and gremlin-1 (Steff et al., 2004; Bourlev et al., 2006a; Sha et al., 2009). Hepatocyte growth factor (HGF) was also found to be elevated in one study, but unchanged in another (Zong et al., 2003; Khan et al., 2006).

These conflicting results are difficult to interpret and may be due to different patient groups, stages of disease and control groups being studied. On balance, however, it appears that levels of some pro-angiogenic factors are affected by the presence of endometriosis. Whether this is a consequence or cause of the disease remains to be determined.

1.4.2 Peritoneal environment

Many studies have suggested that the peritoneal fluid encourages vessel growth in endometriosis (Oosterlynck et al., 1993). Levels of pro-angiogenic factors have been shown to be elevated in the peritoneal fluid of women with the disease. These include: VEGF (McLaren et al., 1996); soluble forms of VEGF receptors (VEGFR-1 and-2), angiogenin and angiopoietin 2 (Bourlev et al., 2010) and interleukin-8 (Ryan et al., 1995).

As well as promoting vessel growth directly, the peritoneal fluid of affected women may be able to stimulate the production of pro-angiogenic factors from nearby cells. Na and colleagues were able to demonstrate that peritoneal fluid from women with endometriosis could stimulate production of VEGF from cultured neutrophils, as well as having higher basal VEGF levels than control peritoneal fluid (Na et al., 2006). Peritoneal fluid from women with and without endometriosis could also stimulate the production of factors involved in angiogenesis from cultured endometrial stromal cells (VEGF, urokinase plasminogen activator and matrix metalloproteinase 3)(Cosín et al., 2010). The levels of these factors were highest in cultures where both stromal cells and peritoneal fluid were obtained from women with endometriosis.

1.4.3 Eutopic endometrium

Interestingly, it appears that vessel formation in the eutopic endometrium of women with endometriosis may also differ from that of healthy women. Three papers have suggested that microvascular density in the eutopic endometrium may have subtle alterations in endometriosis. Khan and colleagues found an overall increase in microvascular density in women with the disease (Khan et al., 2003). Two other papers suggested that the variation over the cycle was changed in endometriosis: healthy controls tended to have consistent microvessel density during the menstrual cycle, whilst that of endometriosis patients significantly increased during the secretory phase (Burlev et al., 2005; Bourlev et al., 2006b). However, one earlier study failed to find any change in microvessel density (Liu et al., 2003).

Concurrently, pro-angiogenic factors are enriched in the endometrium of women with endometriosis: several reports indicate that levels of VEGF are higher, particularly during the secretory phase of the cycle (Donnez et al., 1998; Tan et al., 2002; Khan et al., 2003; Takehara et al., 2004; Burlev et al., 2005; Bourlev et al., 2006b; Gilabert-Estelles et al., 2007; Cosin et al., 2009; Di Carlo et al., 2009). Conversely, one study identified an increase in VEGF levels only in the proliferative phase (Novella-Maestre et al., 2010) and yet another failed to identify any difference in VEGF levels (Kyama et al., 2006).

1.4.4 Endothelial cells

Recent data have also suggested alterations in endometrial endothelial cells (EECs) in women with endometriosis. The first of three papers assessed the production and secretion of interleukin-8 from EECs of women with and without endometriosis (Luk et al., 2005). They were able to demonstrate that estrogen and progesterone cause a reduction in IL-8 production by endothelial cells from healthy women. However, cells from women with endometriosis show a dramatic increase in IL-8 production in response to the same stimuli. A similar experimental procedure was used for the group's second paper, where the secretion of monocyte chemotactic protein 1 (MCP-1) in response to steroid hormones was investigated (Luk et al., 2010). Estrogen and progesterone did not affect the secretion of MCP-1 by cells derived from healthy women, but stimulated MCP-1 production by cells obtained from women with endometriosis. Both IL-8 and MCP-1 have previously been shown to promote angiogenesis (Salcedo et al., 2000; Li et al., 2003).

The second group to isolate EECs from women with endometriosis used microarray analysis to identify changes in gene expression (Sha et al., 2007). The authors found a total of 288 differentially regulated genes in samples from women with endometriosis. These were predominantly found in pathways associated with extracellular matrix and focal adhesion. There was also an up-regulation of genes involved in TGF-β and GREM-1 pathways, which are likely to affect angiogenesis.

1.4.5 Circulating angiogenic cells

Finally, and most recently, the role of CACs in endometriosis has been assessed. Only two papers have considered the possible involvement of CACs in endometriosis, and both have used a murine model of disease, rather than human studies (Becker et al., 2011; Laschke et al., 2011). This model requires uterine explants to be sutured into the peritoneal cavity of mice to mimic endometriotic lesions. Whilst it is a commonly used animal model for endometriosis there are clear limitations to the technique, as the lesions are induced rather than spontaneous.

The first paper created a mouse model that expressed green fluorescent protein (GFP) in bone marrow derived cells, under the control of the *Tie-2* promoter (Laschke et al., 2011), which resulted in the inclusion of GFP expressing (Tie2+) cells into the vessels of the “endometriotic” lesions. However, no change in peripheral CAC levels was identified.

In the second paper, GFP expressing bone marrow was transplanted into wild type host mice (Becker et al., 2011). Surgical induction of endometriosis in these mice also led to GFP expressing cells being identified in the vasculature of the endometriotic lesions. Furthermore, peripheral levels of CACs were found to be elevated in some mice with endometriosis.

1.5 Outstanding questions

The data described above indicate that angiogenesis is an important aspect of endometriosis. Development of lesions clearly depends on new vessel formation and levels of pro-angiogenic factors appear to be increased in pelvic tissues and peripheral blood of women with the disease. Whether this alteration contributes to the development of the disease or is a consequence of the neovascularisation is still unknown.

The levels of CACs have not yet been determined in women with endometriosis, only in mouse models of disease. It is possible that these cells contribute to the vasculature of endometriotic lesions. As such they may represent both a potential therapeutic target in endometriosis, as well as a putative disease biomarker.

Similarly, the differences in behaviour of endothelial cells from women with and without endometriosis have not been thoroughly assessed. Gene expression appears different, as does the response to sex steroids, but whether these cells have different abilities to form vessels is not clear. This may relate to the ability of vessels to develop in shed endometrium and newly forming endometriotic lesions.

Finally, the role of endometrial stroma in facilitating vessel growth is unknown. The recent discovery of stem cells in the endometrium (Chan et al., 2004) may mean that endometrial stroma can differentiate into endothelial cells, directly contributing to vessel growth. Alternatively, the stromal cells may facilitate blood vessel growth through other means.

1.6 Hypothesis

My hypothesis is that pro-angiogenic factors are altered in women with endometriosis, and that this contributes to the development of the disease.

1.7 Aims of this thesis

To identify changes in pro-angiogenic factors in women with endometriosis I aimed:

- To optimise a reliable protocol to identify CACs with multicolour flow cytometry.
- To assess the levels of CACs in women with and without endometriosis.
- To isolate, culture and compare endometrial endothelial cells from women with and without endometriosis.
- To assess the contribution of endometrial stromal cells to vessel development.

Chapter 2: Identification of circulating angiogenic cells by flow cytometry

2.1 Introduction

Although more than a decade has passed since the first description of CACs there is still no universally accepted method for their identification. A major challenge has been the lack of a cell surface marker expressed uniquely by CACs. Instead, researchers have largely relied on functional assays or a combination of phenotypic markers to identify these cells.

2.1.1 Phenotypic markers of CACs

The underlying principle for the phenotypic identification of CACs is that they should express markers typical of both endothelial cells and progenitor/stem cells, reflecting their dual capacity for differentiation into endothelium and self-renewal. Hence, a combination of these markers is frequently used. The first report of CACs identified these cells using the cell surface markers CD34 and VEGFR-2 (Asahara et al., 1997). These authors injected CD34$^+$ or VEGFR-2$^+$ cells into a mouse model of hind limb ischaemia, and noted that the inoculated cells participated in new vessel development. CD34 is a cell surface glycoprotein of unknown function, expressed predominantly by haematopoietic stem cells and vascular endothelial cells (Krause et al., 1996). VEGFR-2 (also known as kinase insert domain receptor or KDR) is a transmembrane receptor tyrosine kinase, strongly expressed by vascular endothelial cells. VEGFR-2 binds VEGF-A and mediates many of its pro-angiogenic effects (Neufeld et al., 1999). Both these markers are thus expressed by other cell types and are not specific to CACs. However, many subsequent papers have used these two markers either in isolation or in combination with others to detect CACs (Asahara et al., 1997; Timmermans et al., 2009).

CD133 (also known as Prominin-1) is commonly used in addition to CD34 and VEGFR-2 to identify CACs. The function of this transmembrane glycoprotein remains unclear, but it has been found on a variety of stem cells (Shmelkov et al., 2005). Many authors exclude cells that highly express CD45 (leucocyte common antigen) from the CAC population. CD45 is found on all haematopoietic cells and their precursors, except red blood cells (Clark and Ledbetter, 1989). Therefore, excluding CD45 positive cells removes potential contaminating haematopoietic cells from the CAC gates. More recently, CD31 (known as Platelet Endothelial Cell Adhesion Molecule-1 or PECAM-1) has also been used. This is an endothelial cell marker which also identifies cells with pro-angiogenic ability (Kim et al., 2010).

2.1.2 Identifying CACs with flow cytometry

Protocols for identifying CACs using flow cytometry have now been published, the first of which was presented by Duda and colleagues (Duda et al., 2007). These authors described the use of whole blood or isolated mononuclear cells to identify both circulating endothelial cells (CECs, described as $CD34^{dim}CD31^{bright}CD45^{-}CD133^{-}$) and circulating progenitor cells (CPCs, described as $CD34^{bright}CD133^{+}CD45^{dim}CD31^{+}$).

The CAC identification procedure has been refined by recent work (Estes et al., 2010a; Estes et al., 2010b). These authors have separated circulating progenitor cells into two subsets – pro-angiogenic and non-angiogenic cells, distinguished by the respective presence or absence of the cell surface marker CD133. Pro-angiogenic progenitor cells ($CD34^{bright}CD31^{+}CD45^{dim}CD133^{+}$) promote neovascularisation of tumours in mice, as compared to non-angiogenic progenitors ($CD34^{bright}CD31^{+}CD45^{dim}CD133^{-}$), which do not have this capacity (Estes et al., 2010b). Furthermore, the proportion of these cells in the circulation is affected by disease, such that the pro-angiogenic: non-angiogenic ratio is decreased in individuals with peripheral arterial disease (Estes et al., 2010b) and increased in children with solid tumours (Pradhan et al., 2011).

These studies were taken as the basis for CAC identification in this thesis. There is broad consensus on the use of CD34 as a marker of CACs. This was the first marker used to isolate EPCs by Asahara and colleagues (Asahara et al., 1997) and has been widely used in the majority of CAC studies since that time. Many authors have also used VEGFR-2 as a marker of endothelial features. However, the reproducibility of results obtained when staining with VEGFR-2 has been questioned (Masouleh et al., 2010), and the specificity of commercially available antibodies has also been reported as poor (Estes et al., 2010a). In view of this, and in combination with results from preliminary experiments (described in the appendix), CD31 was chosen as a more appropriate endothelial marker. CD31 has been shown to identify cells which promote new vessel growth in hindlimb ischaemia models, analogous to the initial experiments which identified circulating angiogenic cells (Kim et al., 2010). CD45 was used to remove contaminating haematopoietic cells from the cell population. Finally, CD133 was incorporated into the panel, as it is known to identify pro-angiogenic cells (Barcelos et al., 2009; Sanchez-Guijo et al., 2010). Although the presence of CD133 on cells which differentiate into endothelial cells has been disputed (Timmermans et al., 2010), CD133 positive cells have been shown to promote wound healing by stimulating angiogenesis (Barcelos et al., 2009), as well as enhance recovery in hindlimb ischaemia experiments (Sanchez-Guijo et al., 2010).

2.1.3 The effect of the menstrual cycle

Previous data indicate that CAC levels are increased in pre-menopausal women, compared to age-matched men or post-menopausal women (Bulut et al., 2007; Rousseau et al., 2010). This suggests that CACs may participate in angiogenesis in the female reproductive tract. As such, levels of these cells in women may fluctuate in accordance with the menstrual cycle. Earlier studies have concluded that this is indeed the case for CACs defined using CD34, CD133 and VEGFR-2 (Lemieux et al., 2009; Robb et al., 2009; Foresta et al., 2010). However, there is no consensus as to when this fluctuation occurs, and at which time point the peak CAC level is found. In addition, the use of a different flow cytometry protocol warrants further assessment to confirm if the menstrual cycle affects CAC levels.

2.2 Aims

- To establish a flow cytometric protocol to identify CACs.
- To determine the optimal gating strategy to identify CACs.
- To confirm the reproducibility of the protocol.
- To assess fluctuation of CAC levels with the menstrual cycle in healthy women.
- To assess whether estradiol and CAC levels are correlated.

2.3 Materials and methods

2.3.1 Isolation of peripheral blood mononuclear cells (PBMCs)

Approximately 15ml peripheral blood was collected with a syringe and 21G needle. Immediately after collection, blood was transferred to Vacutainer tubes (BD Bioscience, Oxford, UK) containing EDTA as an anticoagulant.

On arrival at the laboratory, 1ml whole blood was reserved for a lymphocyte count (see below). The remaining sample was diluted 1:1 with PBS and mixed well. The resulting solution was carefully layered over 10ml Ficoll Paque PLUS (GE Healthcare, Amersham, UK) as per the manufacturer's instructions. The sample was then subjected to density gradient centrifugation at 18^{o}C for 30 minutes, in a Beckman J-6B centrifuge (1400rpm [400 x *g*] with no brake). This separates the sample into a layer of dense granulocytes and erythrocytes at the bottom of the tube. Above this is a layer of Ficoll, then a thin layer of mononuclear cells, which are covered with a layer of plasma. The resulting plasma layer was aspirated carefully and discarded. The mononuclear cell layer was collected and re-suspended in 2% FBS (in PBS) followed by centrifugation (10 minutes, 18^{o}C, at 2000rpm [800 x *g*]). The supernatant was aspirated, and mononuclear cells were re-suspended in 50ml 2% FBS before a final centrifugation (10 minutes, 18^{o}C) at 1200rpm (300 x *g*). The supernatant was again discarded, and the resulting cell pellet was re-suspended in 500µl 2% FBS before counting with a haemocytometer.

2.3.2 Antibodies used to identify CACs

Antibodies were titrated prior to use to determine their optimum concentration for labelling (see appendix). Details of antibodies used are shown in the appendix.

2.3.3 Antibody labelling

Prior to incubation with antibodies, PBMCs were incubated with Fc Receptor blocking agent (FcR block, Miltenyi Biotec, Bergisch Gladbach, Germany) for 10 minutes at 4°C. 10μl FcR block was used for 90μl cell suspension, as per the manufacturer's protocol. $2x10^6$ PBMCs were incubated with each antibody combination described below (Table 2-A) for 30 minutes at 4°C. Sample tubes contained all four antibodies and the viability dye. "Fluorescence minus one" (FMO) control tubes were also analysed for each sample. These included all the antibodies (and viability dye) bar one. This enabled the extent of background staining for each fluorophore to be seen, and therefore defined positive staining boundaries. Combinations of antibodies and buffer used for each sample and control tube are shown in Table 2-A.

Table 2-A: Antibody and buffer combinations for CAC identification.

Tube	**CD45**	**CD34**	**CD133**	**CD31**	**YO-PRO -1**	**Buffer (2% FCS)**
Blank	-	-	-	-	-	26μl
CACs	✓	✓	✓	✓	✓	-
CD34 FMO	✓	-	✓	✓	✓	5μl
CD133 FMO	✓	✓	-	✓	✓	10μl
CD31 FMO	✓	✓	✓	-	✓	5μl
YO-PRO 1 FMO	✓	✓	✓	✓	-	1μl

Isotype control antibodies were incorporated into FMO tubes for some experiments to assess background staining (see results). Combinations of antibodies and buffer used for each sample and control tube are detailed in Table 2-B.

Table 2-B: Antibody and buffer combinations for CAC experiments with isotype controls.

Tube	**CD45**	**CD34**	**CD133**	**CD31**	**YO-PRO 1**	**Isotype control**	**Buffer (2% FCS)**
Blank	-	-	-	-	-	-	26µl
CACs	✓	✓	✓	✓	✓	-	-
YO-PRO-1 FMO	✓	✓	✓	✓	-	-	1µl
CD34 FMO + Isotype	✓	-	✓	✓	✓	Mouse IgG1 PE-Cy7 Isotype Control	2.5µl
CD133 FMO + Isotype	✓	✓	-	✓	✓	Mouse IgG1 PE Isotype Control	8.75µl
CD31 FMO + Isotype	✓	✓	✓	-	✓	Mouse IgG1 APC-Cy7 Isotype Control	-

Following incubation, cells were washed three times by mixing with 2% FCS, followed by centrifugation at 4000rpm for 2 minutes in a microcentrifuge. Final resuspension of cells was in 500µl 2% FCS.

2.3.4 Flow cytometry: data acquisition

All events were acquired using a BD LSR II flow cytometer equipped with a 488nm and 633nm laser. The machine was routinely calibrated prior to each experiment using SPHERO™ calibration particles (Spherotech, Lake Forest, USA). Compensation settings for multicolour experiments were calculated using single-stained bead controls for antibodies (BD Bioscience, Oxford, UK) and single-stained apoptotic PBMCs for the viability dye. Forward and side scatter voltages and forward scatter threshold were set to exclude debris. $1x10^6$ events were collected for all sample and control tubes. Event rates were maintained at less than 2000 events per second.

2.3.5 Flow cytometry: quantifying absolute counts of cells

Perfect Count beads were used to quantify the absolute numbers of CACs per ml of blood. 50μl whole blood was transferred into a flow cytometry tube with the reverse pipette technique (for improved accuracy). 5μl CD45 PerCP-Cy5.5 was added, and incubated in the dark at 4^oC for 15 minutes. 450μl deionised H_2O and 50μl red cell lysis buffer (made with 8.26 g NH_4Cl, 1 g $KHCO_3$, 0.037 g EDTA and 1 litre deionised H_2O) was then added. The sample was kept at room temperature in the dark for a further 15 minutes. 50μl well mixed Perfect Count bead solution was added immediately prior to data acquisition on the flow cytometer (reverse pipette technique). 100,000 events were collected for each sample.

2.3.6 Gating of samples

2.3.6.1 Preliminary gating

The strategy is shown in Figure 2.1. Initial gating was based on a PBMC gate, identified by characteristic forward and side scatter (Salzman et al., 1975; Hoffman et al., 1980). Debris was excluded using threshold gating for forward scatter – this was adjusted as required for individual samples. Mononuclear cells were then shown on a plot of side scatter against YO-PRO 1 FITC (viability dye). The viability dye FMO tube was used to define the positive staining boundary for this fluorophore. Viable cells were subsequently displayed on a scatter plot of CD31-APC-Cy7 against CD34-PE-Cy7. FMO tubes for each antibody were used to define positive staining boundaries. Finally, viable CD31^{+}CD34^{+} mononuclear cells were shown on a scatter plot of CD45-PerCP-Cy5.5 against CD133-PE. An FMO tube was used to identify CD133 positive cells. Two populations of CD45^{+} cells are clearly visible in the four decade log-scale of this scatter plot. The CD45$^{\text{bright}}$ population has high fluorescence intensity, falling in the fourth log decade. The CD45$^{\text{dim}}$ population has low-moderate fluorescence intensity, falling in the third log decade. CD45$^{\text{bright}}$ cells were identified and excluded. FMO tubes were not required for CD45 as both negative and dim events were of interest. Gating from FMO tubes can only define positive versus negative staining, not delineate bright and dim cells. The final gate comprised viable CD31^{+}CD34^{+}CD133^{+}CD45$^{-/\text{dim}}$ mononuclear cells.

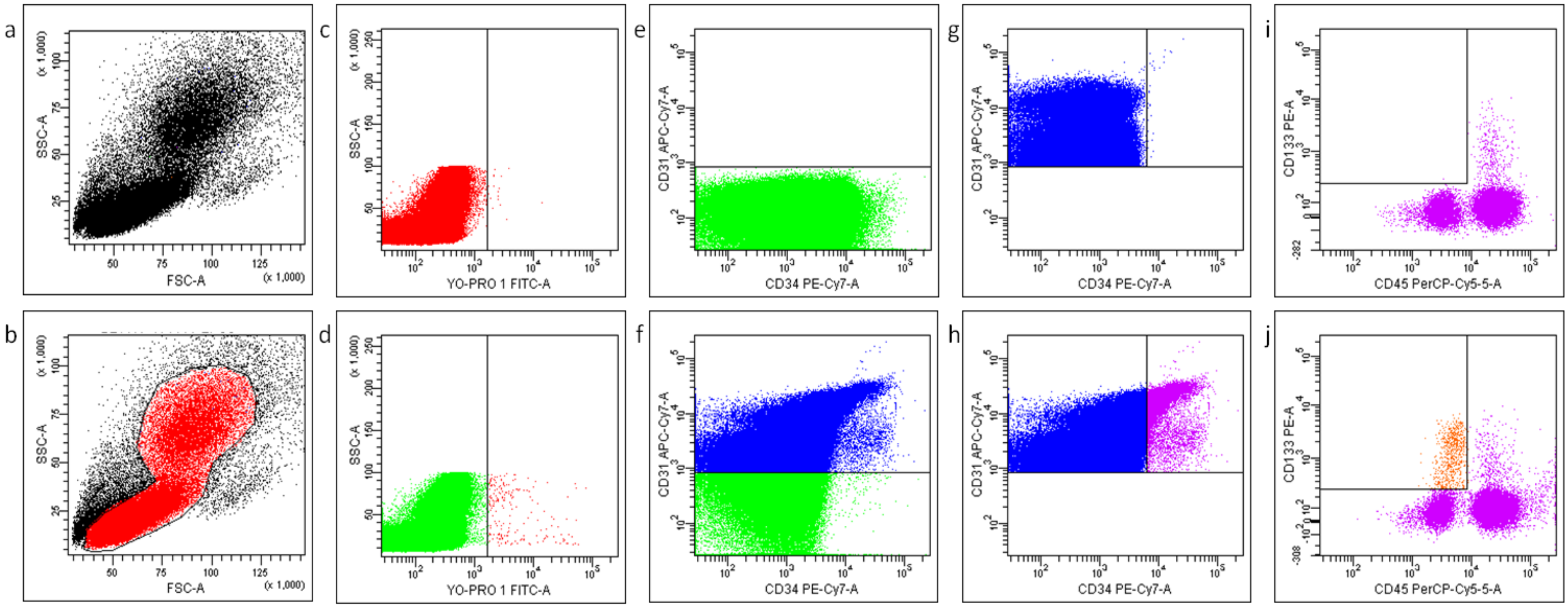


Figure 2.1: Preliminary gating strategy for flow cytometry. Upper panels show representative plots from FMO controls. Lower panels show plots from a fully stained sample. (a) typical forward and side scatter plot of PBMCs; (b) selected PBMC gate, shown in red; (c) identification of positive staining from the viability dye FMO tube; (d) corresponding cells in the sample tube (viable cells in green); (e) CD31 positive gate from FMO tube; (f) CD31 positive cells in blue; (g) CD34^{+} cells identified from the CD34 FMO tube; (h) CD34^{+} cells in purple; (i) CD133^{+}CD45$^{-/dim}$ gate, from the CD133 FMO tube; (j) final population of viable CD34^{+}CD31^{+}CD45$^{/dim}$CD133^{+} PBMCs (orange).

2.3.6.2 Second gating strategy

The revised gating strategy is shown in Figure 2.2. This used an initial gate of low forward and low side scatter cells – a typical lymphocyte gate, instead of a mononuclear cell gate (Salzman et al., 1975; Hoffman et al., 1980). Low forward and side scatter cells were then shown on a scatter plot of CD34-PE-Cy7 against YO-PRO1-FITC. The YO-PRO1 FMO sample tube was used to identify a positive staining boundary for viability. This was followed by identifying CD34 positive cells using the CD34 FMO. Resulting cells (low forward/side scatter, viable $CD34^+$) were shown on a scatter plot of CD31-APC-Cy7 against CD45-PerCP-Cy5.5. The CD31 FMO sample was again used to identify $CD31^+$ cells, and the CD45 bright cells were excluded, as described previously. Finally, the resulting cell population (low forward and side scatter, viable $CD34^+CD31^+CD45^{-/dim}$) was shown on a plot of CD133-PE against CD31-APC-Cy7. The positive staining boundary for CD133 was determined from a CD133 FMO sample. The final population was low forward/side scatter viable $CD34^+CD31^+CD45^{-/dim}CD133^+$ cells.

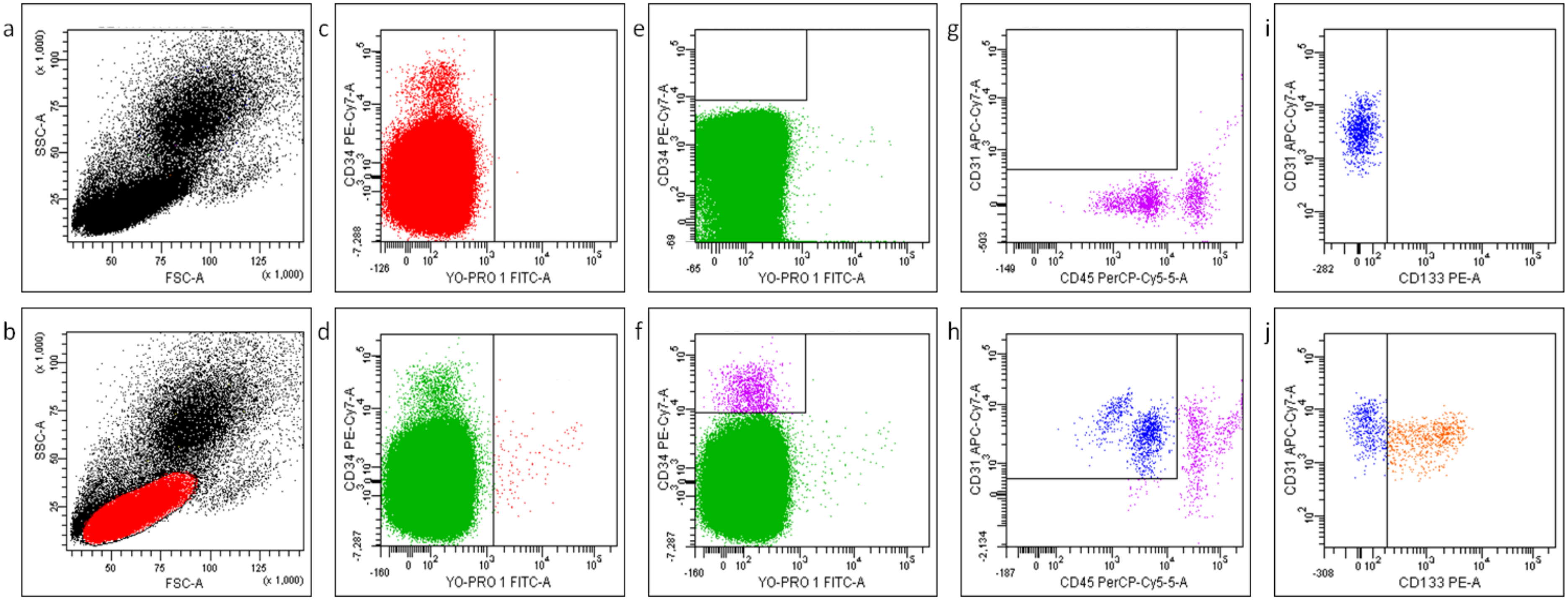


Figure 2.2: Revised gating strategy for flow cytometry. Upper panels show representative plots from FMO controls. Lower panels show plots from a fully stained sample. (a) typical forward and side scatter plot of PBMCs; (b) selected low forward and side scatter gate, in red; (c) identification of positive staining from the viability dye FMO tube; (d) corresponding cells in the sample tube (viable cells in green); (e) CD34 positive gate from FMO tube; (f) CD34 positive cells in purple; (g) CD31^{+} CD45$^{-/dim}$ cells, identified from the CD31 FMO tube; (h) sample tube with CD31^{+}CD45$^{-/dim}$ cells in blue; (i) CD133^{+} gate, from the FMO tube; (j) sample tube with low forward/side scatter, viable CD34^{+}CD31^{+}CD45$^{-/dim}$CD133^{+} cells in orange.

2.3.6.3 Gating for absolute count

Lymphocytes were identified based on characteristic forward and side scatter parameters, and confirmed with CD45 expression. The two populations of fluorescent beads were also identified based on forward and side scatter, and confirmed with levels of fluorescence in the PE channel. The number of beads in each population was determined and the ratio of one bead population to the other was confirmed as 1:1 (Figure 2.3).

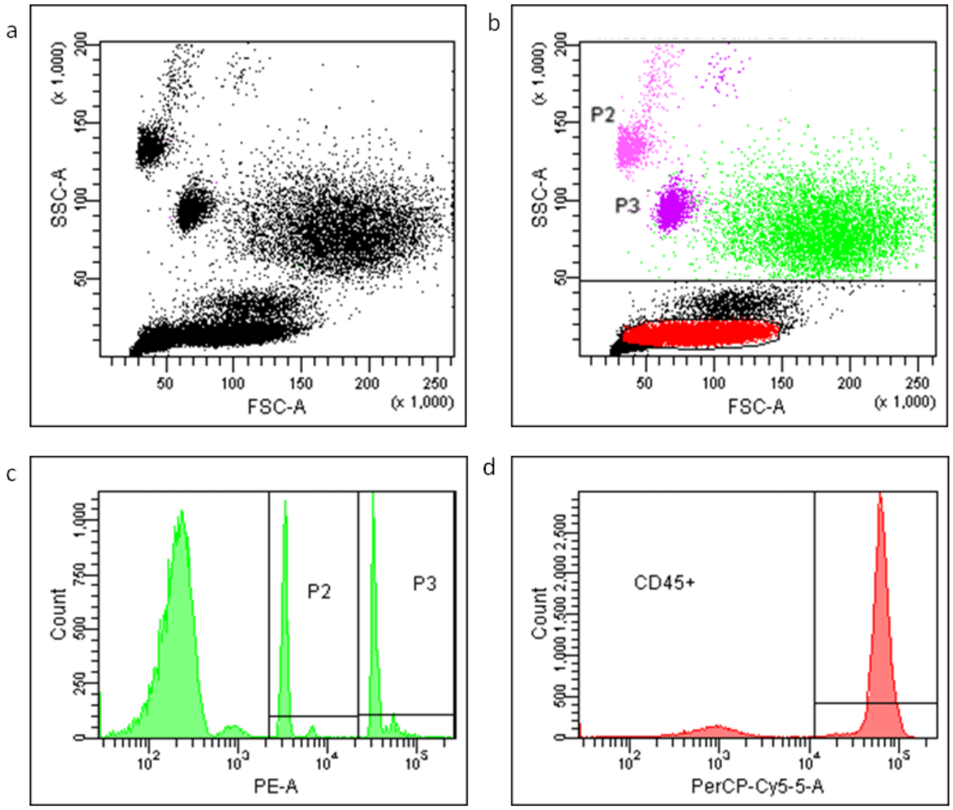


Figure 2.3: Gating for Perfect Count beads, to calculate total lymphocyte count.(a) forward and side scatter profile of whole blood and Perfect Count beads; (b) lymphocyte populations (in red). Two populations of "Perfect Count" beads are shown in pink and purple (P2 and P3). These have high and moderate fluorescence in the PE channel, shown in (c). CD45+ lymphocytes are identified from the histogram shown in (d).

To calculate lymphocyte count per millilitre whole blood for each subject

The total number of beads in the acquired sample was calculated, as the sum of the two bead populations (P2+P3). The amount of “Perfect Count” solution analysed could then be calculated:

$$\frac{\text{Number of beads in sample}}{\text{Number of beads per µl “Perfect Count” solution}} = \text{µl “Perfect Count” solution analysed}$$

As Perfect Count solution was added in a 1:1 ratio to whole blood, the equivalent volume of blood analysed was therefore known. The number of lymphocytes (low forward/side scatter, CD45+ cells) was also known by gating (as described above). Therefore the number of lymphocytes per ml of blood could be calculated.

To calculate number of CACs per millilitre of blood

The number of lymphocytes analysed in each CAC sample could also be calculated by gating (low forward/side scatter, CD45+ cells). The number of lymphocytes in the CAC sample was then compared to the known lymphocyte count per ml of blood. This allowed the approximate amount of blood processed by the flow cytometer to be calculated for the CAC sample. Finally, the number of CACs was compared to this blood volume to estimate the number of CACs per ml of whole blood.

2.3.7 Independent verification of lymphocyte count

For five samples, a lymphocyte count was also requested from an independent laboratory, determined with a Sysmex XE-2100 analyser.

2.3.8 Quantification of CACs

Levels of CACs are expressed as absolute number of cells per ml of blood (as described above), and as a percentage of viable, low forward/side scatter cells.

2.3.9 Variation of CAC count through the menstrual cycle: healthy controls

Healthy, asymptomatic women were invited to donate blood at four time points to assess the variability of CAC count through a ‘natural’ menstrual cycle. All women were free of exogenous hormones for at least three months prior to participation, and throughout the study. All were non-smokers. Blood was collected and processed as described above. An additional 5ml blood was collected into a serum separating tube (Becton Dickinson, Oxford, UK) for estradiol measurement. Menstrual cycle phase was determined based on the date of the last menstrual period and confirmed with the presence of an ovulatory rise in serum progesterone. Samples were collected during the menstrual (day 1-4), follicular (day 6-9), peri-ovulatory (day 13-16) and luteal (day 18-23) phases of the cycle. These were normalised to a 28 day cycle where required.

2.3.9.1 Estradiol and progesterone measurement

5ml blood was collected from the 10 asymptomatic controls into a serum separating tube and centrifuged at 3000rpm (1800 x *g*) for 10 minutes at 18^{o}C. Aliquots of serum were stored at -80^{o}C. Estradiol and progesterone concentrations were measured using a chemiluminescence immunoassay on a Siemens ADVIA Centaur analyser (Siemens Healthcare Diagnostics Limited, Frimley, UK).

2.3.10 Variation of CAC count through the menstrual cycle: symptomatic women

Women awaiting laparoscopy for symptoms suggestive of endometriosis were also invited to participate in the study. All women were free of exogenous hormones for at least three months prior to participation, and throughout the study. All were non-smokers. Blood was collected at four time points through a natural menstrual cycle as described above (2.3.9).

2.3.11 Statistical analysis

2.3.11.1 Reproducibility of results

Reproducibility for different aspects of the CAC protocol was confirmed by calculating the coefficient of variation (CV) between two samples, and expressed as a percentage. This was performed for the comparison of FMO controls with FMO + isotype controls, lymphocyte count with an independent laboratory count, sample variability, and intra- and inter-observer variability.

2.3.11.2 Fluctuation in CAC levels across the menstrual cycle, and association with estradiol and progesterone

CAC levels were confirmed to be normally distributed using the Kolmogorov-Smirnov test. Fluctuation in levels across the menstrual cycle was established using repeated measures ANOVA. Multiple regression was used to identify any association between CAC and serum estradiol/progesterone levels.

2.3.11.3 Variation in CAC levels in individual women

For each woman, the means and standard deviations of CAC percentage, CAC count per ml and pro-angiogenic: non-angiogenic cell ratio at the four time points were calculated. The CV for each woman across the different time points was then ascertained, followed by the mean CV.

2.4 Results

2.4.1 CAC identification

CACs were identified as CD34$^+$CD133$^+$CD31$^+$YO-PRO$^-$CD45$^{-/\mathrm{dim}}$ cells. The panel was found to identify a distinct population of cells in every sample stained.

2.4.2 Gating of samples

Reliable identification of a cell population is critically dependent upon the accurate determination of positive staining, to identify cells which are positive or negative for any antigen. Careful analysis of the cell populations was conducted to identify the optimal gating strategy, allowing clear distinction between cell populations.

The initial gating strategy is shown in Figure 2.1. A parent population was selected from a forward/side scatter plot to include all mononuclear cells (lymphocytes and monocytes). This gate excluded debris and residual granulocytes based on typical light scattering characteristics, with the use of a forward scatter threshold. However, back-gating of the resulting “CAC” population onto a CD31/CD34 scatter plot shows two distinct cell populations (see Figure 2.4). The predominant population is CD34$^+$ and expresses CD31 at a moderate level. A second, smaller population of cells is CD34$^+$ and CD31$^{\mathrm{bright}}$. Furthermore, back-gating these two subpopulations of cells onto the original FSC/SSC plot reveals that all CD31$^{\mathrm{bright}}$ cells are found dispersed within the monocyte region (moderate forward and side scatter). Conversely, the cells expressing moderate CD31 form a reasonably homogenous population within the lymphocyte region (low forward and side scatter). CD31$^{\mathrm{bright}}$ cells are, therefore, likely to represent contaminating monocytes that bind antibodies non-specifically with a high affinity. Whilst these cells are relatively few in number they lead to a continued over-estimation of the CAC count, and should be excluded.

The gating strategy was, therefore, revised to ensure that contaminating monocytes were excluded from the CAC population. Instead of using a PBMC gate to identify CACs, a low forward and side scatter gate was chosen, typical of lymphocytes (Figure 2.2).

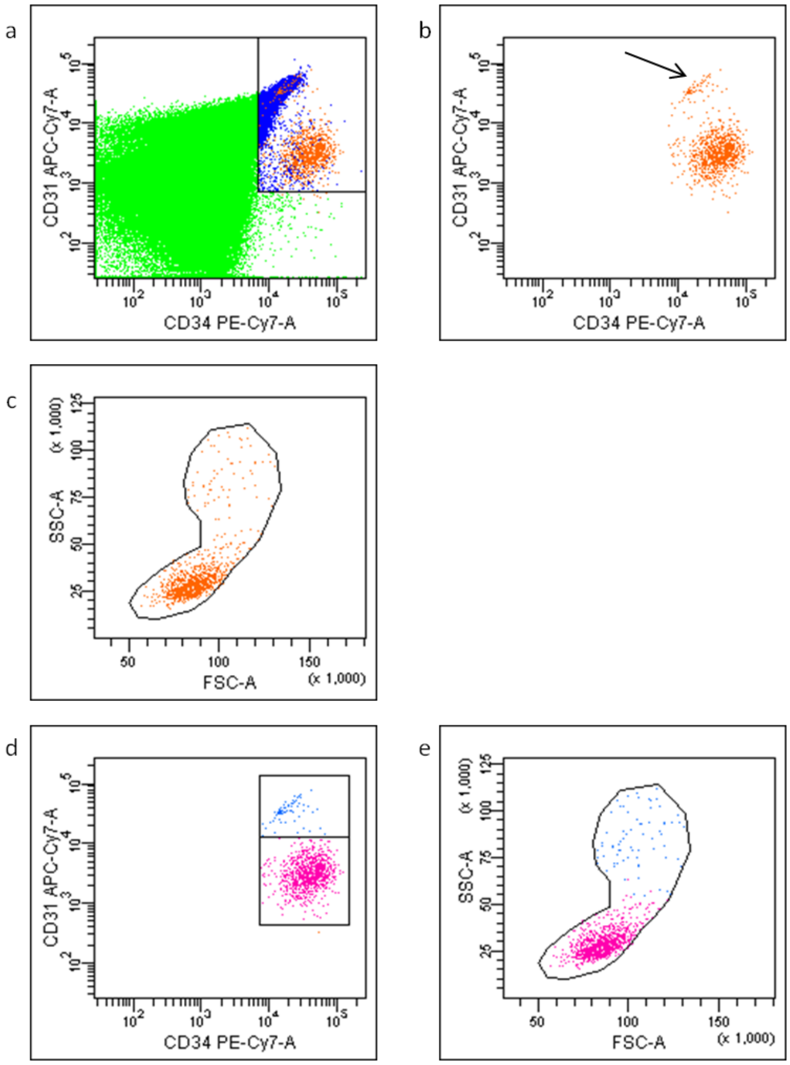


Figure 2.4: Accurate identification of CACs requires an initial lymphocyte gate. (a) Back-gating of the CAC population (orange) onto the CD31/CD34 scatter plot. The population comprises a majority of CD34$^+$CD31$^+$ cells, with a small population of CD34$^+$CD31^{bright} cells (b, arrow); (c) CAC population back-gated onto a forward/side scatter plot. The majority of CACs fall into the low forward/side scatter population (lymphocyte gate). A minority of cells are scattered through the moderate forward/side scatter area; (d) The CAC population can be separated into CD31^{bright} (blue) and CD31$^+$ (pink) cells; (e) Back-gating of these populations onto a forward/side scatter plot shows CD31$^+$ cells with forward and side scatter characteristics typical of lymphocytes, and CD31^{bright} cells with characteristics typical of monocytes.

2.4.3 Comparison of FMO controls with "FMO plus isotype" controls

The use of FMO controls is increasingly accepted as essential for multicolour phenotyping with flow cytometry. However, to ensure that non-specific binding was not affecting the results, a comparison of pure FMO tubes with "FMO + isotype" tubes was made. The latter tubes included all antibodies bar one, with the final antibody substituted by an isotype control for that fluorophore. No isotype control was used for the viability dye. YO-PRO 1 is a fluorescent dye that crosses disrupted cell membranes (apoptotic or necrotic cells). Therefore, use of an antibody to imitate "non-specific" binding of this dye is inappropriate.

Inclusion of isotype control antibody in the FMO tubes made very little difference to the overall numbers of cells identified (n=4). This is demonstrated in Figure 2.5, showing a Bland-Altman plot to compare the difference between the tubes.

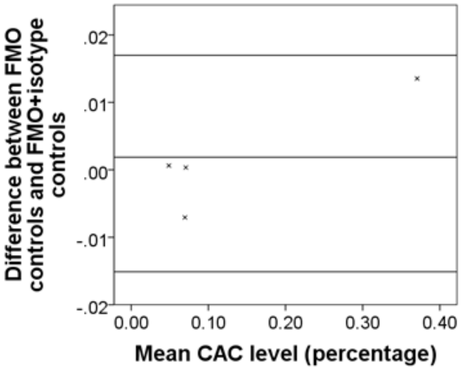


Figure 2.5: Bland-Altman plot to demonstrate minimal difference between CAC percentage calculated using FMO tubes and FMO+ isotype tubes (CV=3.86%). Y axis reference lines show the mean difference between the tubes and two standard deviations above and below the mean difference.

As the CV was 3.86%, standard FMO controls (without additional isotype control antibody) were thought to represent an accurate and reliable method of gating the CAC population, and were used for all experiments.

2.4.4 Confirmation of CD45 staining

Preliminary experiments included the use of a CD45 FMO control tube. However, as events of interest were CD45 dim or negative, a CD45 FMO did not assist with the gating strategy. After confirming that the CD45 positive cells were not seen in the CD45 FMO tube, these tubes were excluded from the data analysis.

2.4.5 Confirmation of lymphocyte count analysis

"Perfect Count" beads were used to determine the lymphocyte count for each CAC sample. Good correlation was seen between the independent laboratory count and the estimated lymphocyte count from flow cytometry (CV = 12.05%) (data not shown).

2.4.6 Reproducibility of results

2.4.6.1 Sample variability

Sample variability was initially assessed by staining two replicates per sample and comparing the results from each tube (n=10). A Bland-Altman plot is shown to demonstrate this (Figure 2.6). Good agreement was seen (CV = 12.97%). However, to improve the accuracy of the measurements, two samples were routinely measured in all experiments, and an average of the two tubes used as the CAC count.

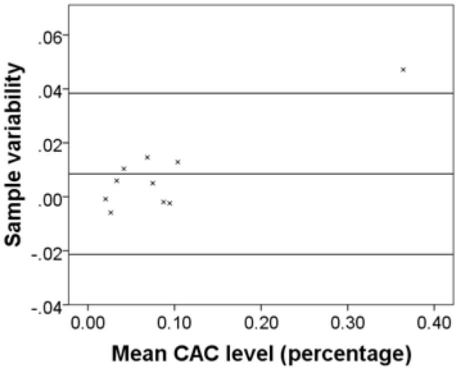


Figure 2.6: Bland-Altman plot to demonstrate difference in CAC percentage between two replicates of the same sample. Good agreement is demonstrated, with a CV of 12.97%.

2.4.6.2 Intra-observer variation

Gating strategies for flow cytometry can bring a degree of subjectivity into data analysis. To minimize this bias, all samples were analysed for CAC counts in a blinded fashion, before the primary outcome (endometriosis or no endometriosis) was known.

To confirm the reliability of measurements from one observer, results for 10 samples were gated on two separate occasions, to assess consistency in the results. A Bland-Altman plot demonstrating this is shown (Figure 2.7). Excellent correlation was seen (CV = 5.15%).

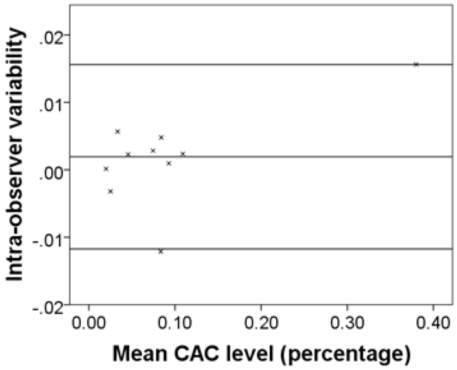


Figure 2.7: Bland-Altman plot of intra-observer variability for gating of samples, showing excellent correlation with a CV of 5.15%.

2.4.6.3 Inter-observer variation

The agreement in CAC levels was also assessed between two observers. The gating strategy was followed by two observers independently, and the CAC level was compared (Figure 2.8). Good agreement was seen between two separate observers, blind to disease status, demonstrating that the protocol is reproducible (CV=15.51%).

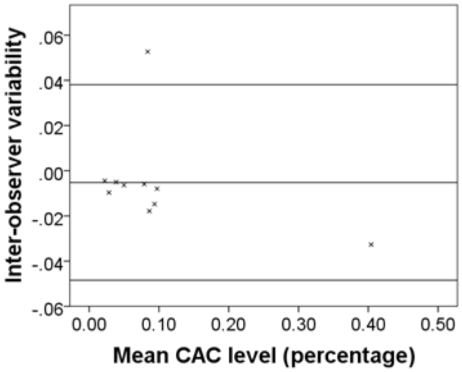


Figure 2.8: Bland-Altman plot of inter-observer variability for gating of samples. Good agreement is seen with a CV of 15.51%.

2.4.7 Variation with the menstrual cycle

Samples were collected from 10 women for this part of the study. The average age of volunteers was 32.60 (±5.52) years. All women were non-smokers and were having regular menstrual cycles (26-35 days). Serum estradiol and progesterone levels collected at each time point confirmed a normal hormonal profile for nine women. One woman was excluded from the final analysis as the luteal sample failed to demonstrate a post-ovulatory progesterone rise. Whilst CAC levels varied considerably between individual women there was no consistent fluctuation in CAC percentage across the menstrual cycle (P=0.279, F=1.359, 3 d.f., Figure 2.9). Similarly, there was no fluctuation in absolute CAC numbers across the cycle (P=0.226, F=1.556, 3 d.f.).

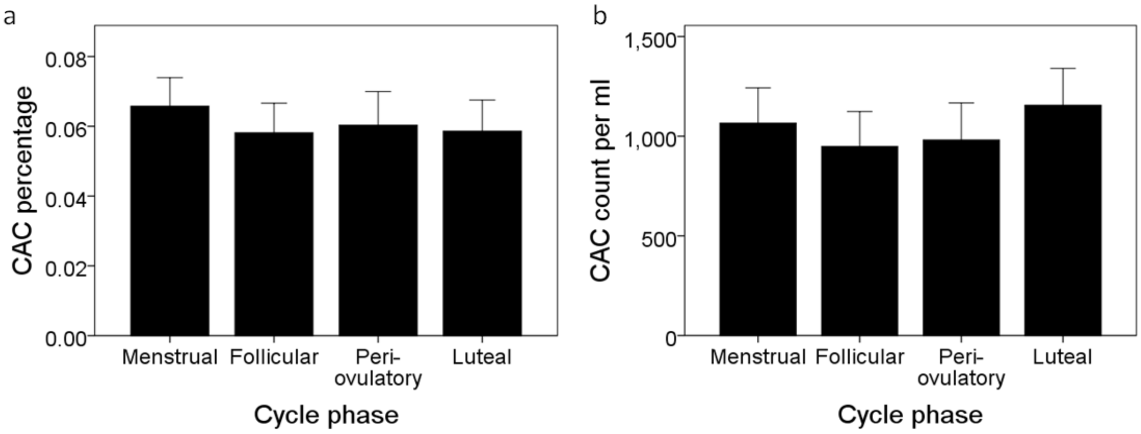


Figure 2.9: No fluctuation in CAC levels was noted across the menstrual cycle. (a) Mean (and SE) CAC percentage in four phases of the menstrual cycle (n=9, P = 0.279); (b) Mean (and SE) number of CAC per ml of blood in four phases of the menstrual cycle (n=9, P =0.226). (Menstrual = day 1-4; Follicular = day 6-9; Peri-ovulatory = day 13-16; and Luteal = day 18-23).

No correlation was found between serum estradiol level and CAC percentage (P=0.322) or CAC number per ml of blood (P=0.881)(Figure 2.10). Serum progesterone level was also not correlated with either CAC percentage (P=0.704) or CAC number (P=0.055, data not shown).

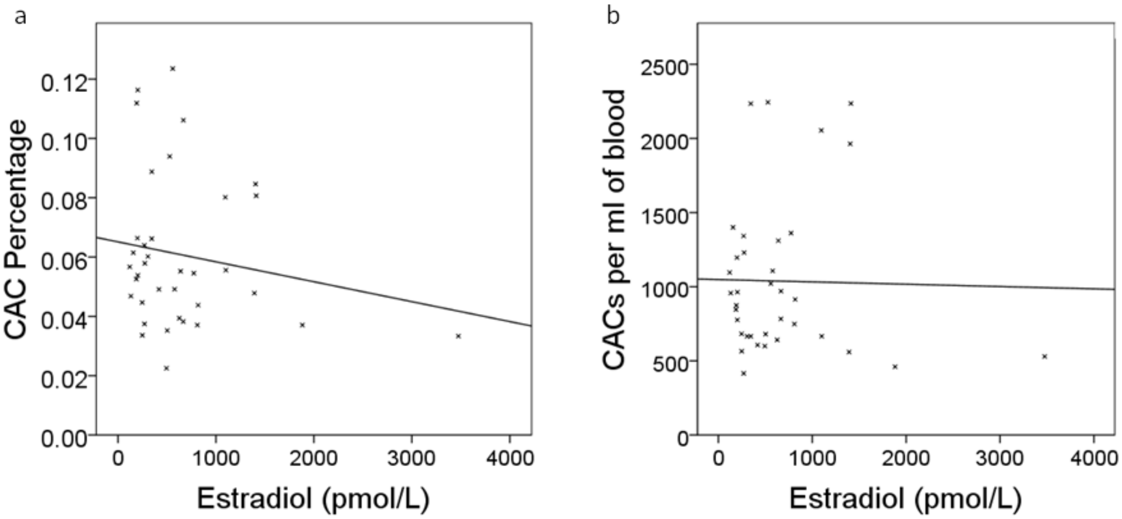


Figure 2.10: No correlation was identified between estradiol level and CAC count. (a) Correlation of estradiol level with CAC percentage (P =0.322); (b) Correlation of estradiol level with absolute CAC number per ml of blood (P =0.881).

2.4.8 Ratio of pro-angiogenic to non-angiogenic cells in healthy women

The ratio of these cells was assessed through the menstrual cycle in healthy women (Figure 2.11). An apparent trend was seen towards a reduced ratio over the course of the cycle, but this was not statistically significant (P=0.132, F=2.059, 3d.f.).

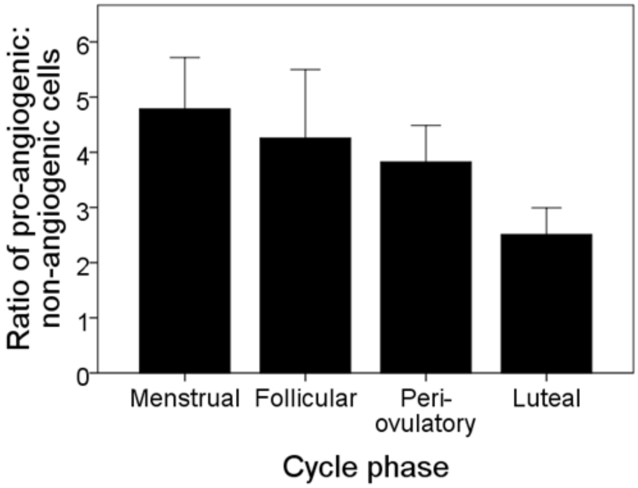


Figure 2.11: Average pro-angiogenic: non-angiogenic cell ratio across the menstrual cycle in healthy women (n=9). No change in the ratio was identified (P = 0.132)

2.4.9 Variation in CAC levels with time

Although no variation in CAC level was attributable to the menstrual cycle, there was considerable variation in CAC levels within an individual woman over time. This is demonstrated in Figure 2.12.

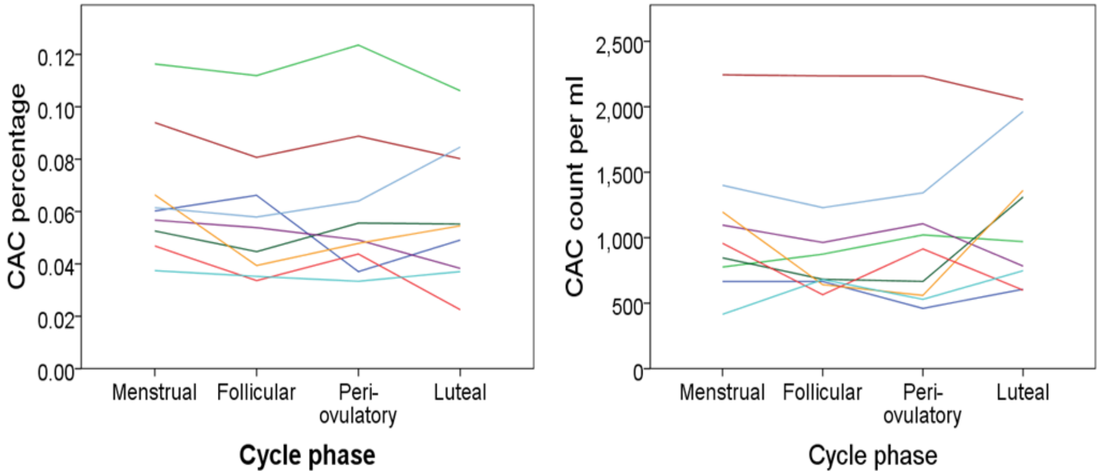


Figure 2.12: CAC levels (percentage and count per ml of blood) do fluctuate over the menstrual cycle, although not according to cycle phase. Each line represents levels in one individual over time (n=9).

For the four samples in each woman, the mean CV for the four time points was 15.5% (CAC percentage) and 22.1% (CAC count per ml of blood). Therefore, despite the lack of consistent variation over the menstrual cycle, levels of CACs do fluctuate considerably in an individual woman over time.

The CV for the pro-angiogenic to non-angiogenic cell ratio was considerably higher (50.0%), indicating that this ratio may not be consistent in an individual woman over time (shown in Figure 2.13).

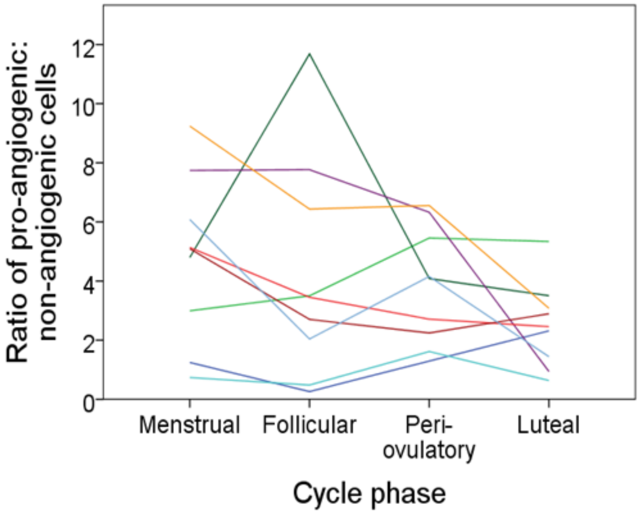


Figure 2.13: Ratio of pro-angiogenic: non-angiogenic cells vary across the menstrual cycle, but not according to cycle phase. Each line represents levels in one individual over time (n=9).

2.4.10 Fluctuation in CAC counts in women with symptoms of endometriosis

Women with symptoms suggestive of endometriosis awaiting laparoscopy were also recruited to the study to monitor CAC levels over a menstrual cycle. All women were free of exogenous hormones and having regular menstrual cycles. Unfortunately only 3 women participated in this part of the study due to difficulties with subject recruitment. Data for these three women are shown below (Figure 2.14, Figure 2.15). These preliminary data show CAC levels similar to those seen in healthy women but, clearly, no other comments can be made given the small numbers involved.

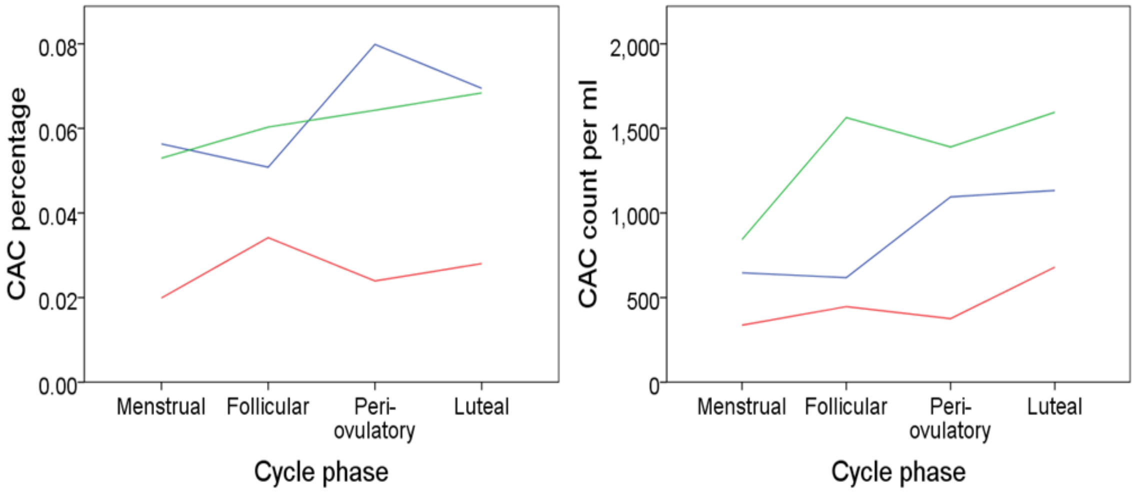


Figure 2.14: CAC levels (percentage and count per ml of blood) across the menstrual cycle in 3 women with symptoms of endometriosis. Each line represents levels in one individual over time.

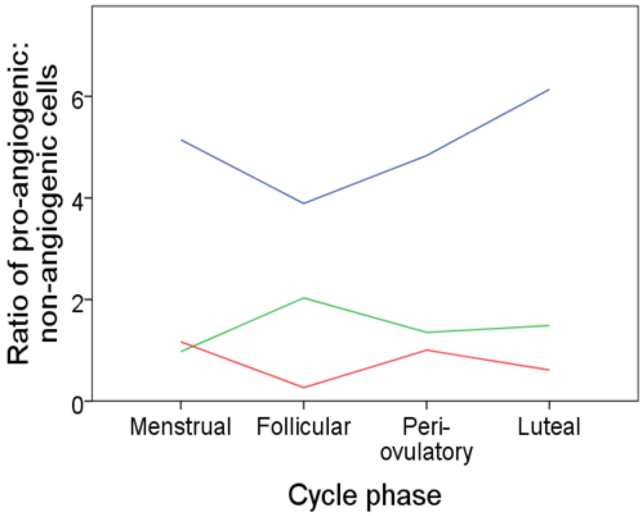


Figure 2.15: Ratio of pro-angiogenic: non-angiogenic cells across the menstrual cycle in 3 women with symptoms of endometriosis. Each line represents levels in one individual over time.

2.5 Discussion

The quantification of small populations of cells in blood can be challenging and subject to error. Minimising this error requires time and effort to ensure that a highly reproducible and rigorous protocol is in place.

2.5.1 FMO controls

It is now increasingly recognized that FMO controls provide the optimum gating strategy for multicolour flow cytometry experiments, by correctly compensating for fluorescence spillover between fluorophores (Roederer, 2001). This technique is replacing the use of isotype control antibodies for identifying positive populations. Whilst isotype control antibodies may account for non-specific antibody staining, they are unable to correct for fluorescence spillover between channels, which represents a major source of background staining in multicolour experiments.

There is great variety in the number and type of control tubes run in such experiments, but FMO controls are still not used by all researchers. The use of single-stained isotype controls persists and can result in inappropriate gates being set to define positive cell populations. A major criticism of this technique is the poor correlation between the level of non-specific binding obtained with the isotype control and that of a highly specific monoclonal antibody. Furthermore, differences may exist between the conjugation of fluorophores to the antibody of interest and the isotype control. These differences may be minimised by obtaining both antibodies from the same supplier, but still may cause discrepancies when assessing non-specific binding.

A criticism of the FMO technique is that it may fail to account for non-specific antibody binding. In the CAC panel used, I have demonstrated that non-specific antibody binding (as represented by isotype control antibodies) makes little difference to the gating strategy, and that FMO controls are sufficient and appropriate to determine boundaries for positive cells. It is likely that the high total concentration of antibody in each control tube (containing 4 different antibodies) means than non-specific binding by an isotype control antibody is rendered irrelevant.

Nonetheless, placing of gates to identify positive cells may introduce some subjectivity into the gating strategy. Ideally, FMO tubes should result in a clear and absolute boundary between positive and negative cell populations. However, when collecting large numbers of events (typically 1,000,000 for each control tube) it is almost inevitable that a small number will appear positive in FMO tubes, perhaps due to cellular auto-fluorescence. This produces a degree of subjectivity when placing gates, which could potentially lead to discrepancies in cell number. Repeating the gating of 10 samples on separate occasions demonstrated good intra-observer reliability for this technique.

2.5.2 Sequential gating strategy

Many published gating strategies for CACs utilise a generic PBMC gate as the first step in defining the cell population. However, back gating of the CAC population clearly demonstrates that these cells have forward and side scatter characteristics that are more typical of lymphocytes. Furthermore, these experiments show that the inclusion of a large population of monocytes in the initial gating step may lead to the erroneous inclusion of non-specifically stained cells, despite the use of FcR blocking agent, and repeated washes of stained cells. Therefore using a low forward/side scatter gate to identify CACs is probably best practice for future studies. It is notable that a previous protocol based on ISHAGE guidelines uses a preliminary gate from PBMCs, but later refines this gate to include only low forward and side scatter lymphocytes (Schmidt-Lucke et al., 2010). Other protocols utilise a specific marker of monocytes (CD14) to exclude these contaminating cells from the CAC population (Estes et al., 2010a; Estes et al., 2010b).

2.5.3 Choice of antibodies and methods of staining

The use of VEGFR-2 in identifying CACs is widespread. However, preliminary experiments revealed a significant degree of dose-dependent staining with some commercially available VEGFR-2 antibodies (see appendix). This feature has been commented on by previous authors (Estes et al., 2010a). Masouleh and colleagues also reported poor reproducibility of CAC numbers when using a gating strategy incorporating VEGFR-2 (Masouleh et al., 2010) and instead advocated the use of CD31 as a more reliable endothelial marker.

The use of Fc Receptor block is not ubiquitous for flow cytometry. This may lead to increased antibody staining, mistakenly labelling cells as positive for any given antigen. Therefore, exposure of cells to FcR block before antibody incubation is advocated, to reduce the number of false positive events caused by non-specific binding through Fc receptors.

2.5.4 Rare event analysis

Finally, the number of events acquired for flow cytometry analysis is critical when assessing rare populations of cells to ensure reproducibility of the data. Previous studies have acquired lower numbers of events (Robb et al., 2009) or have relatively high CVs in their data (Fadini et al., 2006; Fadini et al., 2008; Smythe et al., 2008), suggesting limited reproducibility of the protocol. To improve reproducibility, samples were routinely stained in duplicate and the mean CAC count was used for analysis. $1x10^6$ events were acquired for each sample and control tube to improve the precision of the counts, which is directly related to the number of positive events acquired (Roederer, 2008).

2.5.5 Lack of association with the menstrual cycle

There were no significant fluctuations in CACs across the menstrual cycle. These findings contrast with the work of other groups, which have shown alterations in CAC levels throughout the cycle (Matsubara et al., 2006; Farha et al., 2007; Fadini et al., 2008; Lemieux et al., 2009; Robb et al., 2009; Foresta et al., 2010).

Notably, most studies assessing the effect of the menstrual cycle have used VEGFR-2 to identify CACs; in the present study CD31 was included. This may have resulted in the identification of slightly different cell populations, accounting for the difference in menstrual cycle variation. In addition, the present study is the first to utilise FMO controls when assessing CAC levels through the menstrual cycle.

One of the earliest studies to assess CAC levels through the menstrual cycle was carried out by Farha (Farha et al., 2007). These authors identified CACs as dual positive cells for CD34 and CD133 – without any endothelial marker. Furthermore, four of the ten women included were taking hormonal contraception throughout the study. However, despite this, a significant change in CAC levels was noted through the "cycle", with a peak at the time of menses (or withdrawal bleed) (Farha et al., 2007).

The study by Fadini and colleagues included only 5 women with natural menstrual cycles (and a further 5 women taking oral contraceptives) (Fadini et al., 2008). The authors demonstrated a change in CAC levels during the cycle in hormone-free women, with an increase around the time of ovulation. This fluctuation was not seen in women who taking contraception, but only $CD34^+VEGFR\text{-}2^+$ CACs were assessed through the cycle. CD133 was included in other aspects of the study, but $CD34^+VEGFR\text{-}2^+CD133^+$ cells were not described over the cycle. No difference was seen in levels of $CD34^+VEGFR\text{-}2^+CD133^+$ cells between fertile women and age matched men.

Conversely, $CD34^+VEGFR\text{-}2^+CD133^+$ cells were one of the few CAC subsets found to change with the cycle by Lemieux and colleagues (Lemieux et al., 2009). No change in $CD34^+VEGFR\text{-}2^+$ was found, contrary to Fadini et al. However, the peak in CACs was also seen around ovulation. This study used isotype control antibodies instead of FMO controls to gate positive cells, and also used a two-step labeling system for VEGFR-2 (biotin/streptavidin), which may have affected the results.

Three other papers have identified menstrual cycle changes in $CD34^+VEGFR\text{-}2^+CD133^+$ cells through the cycle (Matsubara et al., 2006; Robb et al., 2009; Foresta et al., 2010). However, the timing of this fluctuation varied. Robb showed a peak in CAC levels at the mid-follicular phase (day 6-8), with the lowest level around ovulation (day 13-15) (Robb et al., 2009). Conversely, Foresta showed the highest level of CACs in the ovulatory phase (day 13-16) (Foresta et al., 2010). Finally, Matsubara found elevated levels in the luteal phase, although this was a cross-sectional rather than longitudinal study (Matsubara et al., 2006).

Clearly there is a lack of consensus about how CACs fluctuate during the cycle. Although most studies have identified some fluctuation, the pattern is inconsistent. This may be largely due to different protocols for sample preparation and analysis. However, a contributing factor is likely to be the small numbers of women participating in these studies. It can be challenging to recruit women to participate in a longitudinal study which requires considerable commitment, over such a short period (one menstrual cycle). Ideally, a larger study should be conducted, or alternatively protocols for identifying CACs should be standardised so that results from different laboratories can be combined in a meta-analysis.

2.5.6 No correlation with estradiol

The present study did not show a correlation between CAC and serum estradiol levels, in keeping with the lack of effect of the menstrual cycle. Three previous studies have also shown that CAC and estradiol levels do not correlate (Lemieux et al., 2009; Robb et al., 2009; Rousseau et al., 2010). Two studies have shown that higher CAC levels are associated with increased estradiol levels, although one of these studies assessed post-menopausal women taking hormone replacement therapy, which is not representative of normal reproductive physiology (Bulut et al., 2007; Foresta et al., 2010).

It is interesting to note that no cyclical changes in endometrial endothelial cell proliferation have been identified either, despite the obvious changes in vessel growth across the cycle (Goodger and Rogers, 1994; Wingfield et al., 1995). Therefore, these observations suggest that there is not a direct hormonal link to vessel development in the endometrium.

2.5.7 Fluctuation over time

Despite the lack of association in CAC levels with the menstrual cycle in this study, individual women did experience fluctuations in CAC levels over time. Of note, the most stable parameter appeared to be the percentage of CACs (number of CACs expressed as percentage of low forward/side scatter cells). The CV for this value was only 15.5%, compared to 22.1% for CAC count and 50% for pro-angiogenic: non-angiogenic ratio. This may imply that expressing CACs as a percentage is a more repeatable measure over time than using absolute cell counts.

2.5.8 Summary

The CAC protocol described above includes a variety of features which enhance its reliability and reproducibility:

1. FcR blocking agent was used to minimize non-specific binding of antibodies.

2. Individual FMO tubes were analysed for all appropriate stains, and for each individual sample to set correct staining boundaries.
3. All samples were tested in duplicate, and an average CAC level taken for each individual.
4. Test-retest reproducibility was confirmed both for individual samples, and across the menstrual cycle in healthy women.

This ensures a reliable, reproducible method of quantification of CACs from peripheral blood.

Chapter 3: Circulating angiogenic cells in endometriosis

3.1 Introduction

Our understanding of the mechanisms involved in blood vessel development is constantly improving, principally because they play such an important role in tumour growth. However, despite the equally important role of angiogenesis in endometriosis, we currently have very limited knowledge of precisely how blood vessels develop in this disease.

Murine data suggest that new vessels are likely to be recruited from the surrounding vasculature, rather than generated by cells derived from the lesions themselves. Fragments of human endometrium sutured into immunodeficient mice become infiltrated with murine endothelial cells after 2 days, and human endothelium becomes undetectable in the lesions after 12 days (Grümmer et al., 2001). However, murine derived cells may also originate from CACs as well as fully differentiated endothelial cells.

If CACs are found to contribute to the vasculature of endometriotic lesions then this may help us to understand the development of the disease. Blocking the action of CACs may also be of therapeutic benefit in preventing lesion growth by inhibiting angiogenesis, or they may be used as carriers to target drugs to endometriotic lesions. Furthermore, as CACs are biomarkers in diseases such as breast cancer (Jain et al.,

2012) and multiple myeloma (Bhaskar et al., 2012) they may be used in the same way in the management of endometriosis.

3.1.1 Previous work

To date, the only studies examining the role of CACs in endometriosis have been conducted in mice (Becker et al., 2011; Laschke et al., 2011). This approach can yield interesting results but it is important to note that the current mouse models of endometriosis are very limited. Three rodent models exist. The first involves autologous transplantation of rodent endometrium into the peritoneal cavity, which then forms lesions similar to human endometriosis. The second model involves the transplantation of human endometrium into the peritoneal cavity of immunocompromised rodents. The third (most recently described) model involves genetically modified mice with a mutation of the oncogene K-ras, expressed in ovarian surface epithelium. These mice spontaneously develop endometriotic-like lesions. Whilst these models resemble the human disease to some degree, they clearly cannot mimic the actual formation of endometriotic deposits in humans. The only non-human species that develop spontaneous endometriosis are primates (Grümmer, 2006), which are little studied for obvious financial and ethical reasons.

Two groups have assessed the involvement of CACs in rodent models of endometriosis. The first study used a bone marrow transplantation model to demonstrate the involvement of CACs in endometriotic lesions (Laschke et al., 2011). Bone marrow from mice in which GFP was expressed under the endothelial specific Tie-2 promoter was transplanted into wild type mice. Endometriotic lesions were then surgically induced. After 7 days, cells expressing GFP were noted in the vasculature of endometriotic lesions. Whilst the overall number of these cells was relatively small, they represented up to 18% of all endothelial cells within the lesion. This indicates a significant contribution to vascularisation. However, despite the apparent involvement of CACs in endometriotic vessel development, the authors were unable to identify changes in peripheral CAC levels. Using the markers VEGFR-2 and Sca-1 (a haematopoietic stem cell marker), they showed no significant change in CAC levels between days 3 and 14 after the induction of endometriosis (Laschke et al., 2011).

The second study also identified incorporation of bone marrow derived cells into endometriotic lesions (Becker et al., 2011). Co-staining with CD31 and VEGFR-2 identified these cells as putative bone marrow derived endothelial cells. Notably, this study also identified increased CAC levels in mice with endometriotic lesions, as compared to sham operated mice, although different CAC markers were used (VEGFR-2^+CD31^+CD133^+CD45^-). The elevation in CAC levels was particularly marked in a mouse strain known to have a pro-angiogenic phenotype (129SvJ mice), as compared to C57BL/6 mice. 129SvJ mice have previously been shown to have exaggerated vessel development in response to pro-angiogenic stimuli such as FGF-2 (Zhu et al., 2003).

These studies, therefore, provide some evidence of CAC involvement in vessel development in endometriosis, as well as altered peripheral CAC levels.

3.1.2 Cell culture based assays for CACs

In addition to phenotypic enumeration of CACs, many authors have used cell culture assays to quantify these cells. It now appears probable that these methods identify different cell populations, all of which contribute to angiogenesis in some way. The best known of the cell culture methods is that used by Hill (Hill et al., 2003), which involves culture of isolated PBMCs on fibronectin coated dishes. An initial 48 hour adherence depletion step is followed by cell culture for 72 hours, after which typical colony forming units (CFUs) can be identified. These are found to comprise a central core of round cells, surrounded by spindle shaped cells, radiating outwards. Enumeration of these colonies was found to correlate inversely with the Framingham risk score for individuals – suggesting that higher numbers of CFUs are associated with better cardiovascular health and endothelial regeneration (Hill et al., 2003). Elevated CFU numbers have also been shown in diabetic retinopathy – a common disease involving profuse angiogenesis (Liu et al., 2010). To date, no studies have investigated the effect of endometriosis on CFU number.

More recent work has identified a distinct, pro-angiogenic cell type in peripheral blood, termed endothelial colony forming cells (ECFCs)(Ingram et al., 2004). These cells have been shown to express markers consistent with an endothelial phenotype; have endothelial-like properties in culture (cobblestone morphology), and are able to form tubes in angiogenesis assays. However, their levels in peripheral blood are extremely low – the average number of ECFC colonies obtained from one million PBMCs was 0.017 (Yoder et al., 2007). Nevertheless, these cells may also contribute to new vessel development.

3.2 Aims

- To compare numbers of Colony Forming Units in women with and without endometriosis.
- To isolate ECFCs from women with and without endometriosis.
- To compare levels of CACs in women with and without endometriosis.
- To compare the pro-angiogenic: non-angiogenic ratio of cells in women with and without endometriosis.
- To assess the effect of stage on CFU numbers, CAC levels and the pro-angiogenic: non-angiogenic cell ratio.

3.3 Materials and methods

3.3.1 Subject recruitment

Women scheduled for laparoscopy for symptoms suggestive of endometriosis, including pelvic pain or subfertility, were approached to participate in the study. Inclusion criteria for this part of the study were aged 18 years or over, and having regular menstrual cycles (26-35 days). No women had taken exogenous hormones (including oral contraceptives, clomiphene, GnRH analogues or hormone releasing IUCD) for at least 3 months prior to participation.

Blood samples were collected on the day of surgery, before the start of the operation. All women underwent diagnostic/operative laparoscopy as clinically indicated. Presence or absence of endometriosis (and other pathology) was determined by the operating surgeon, who was unaware of the laboratory results. Stage of disease was assessed according to the revised American Society for Reproductive Medicine (ASRM) classification, using the operation note (American Society for Reproductive Medicine, 1997).

Women without evidence of endometriosis at laparoscopy were designated as controls for this part of the study. Any control patient with a prior history of endometriosis (diagnosed at a previous laparoscopy) was excluded.

3.3.2 CFU assay

Blood was collected with a 21G needle and syringe, and immediately transferred to vacutainer tubes (BD Bioscience, Oxford, UK) containing EDTA as an anticoagulant. On arrival at the laboratory PBMCs were isolated using density gradient centrifugation as described previously (Chapter 2).

Cells were cultured according to the manufacturer's instructions, using a commercially available medium optimised for the growth of CFUs (Stemcell Technologies, Grenoble, France). This medium is referred to as CFU medium. 5×10^6 PBMCs per well were cultured in duplicate in a 6 well fibronectin coated plate (BD Bioscience, Oxford, UK). Cells were then cultured at 37^oC in 5% CO_2. After 48 hours, non-adherent cells were gently collected and counted again. 1×10^6 cells per well were re-cultured in a fibronectin coated 24 well plate in CFU medium, also in duplicate (thus a total of four wells were plated at this stage). After a further 72 hours in culture, cells were washed once with PBS and fixed with 3% paraformaldehyde (in PBS), before colonies were counted. CFU were identified by their typical morphology, according to the descriptions provided by the manufacturer and as previously described (Hill et al., 2003). The number of colonies in each of the four wells was counted using a light microscope (x40 magnification), and the mean colony count for each individual was calculated. An example of two typical CFUs is shown in Figure 3.1, below. A central colony of round cells is surrounded by spindle-shaped cells radiating outwards.

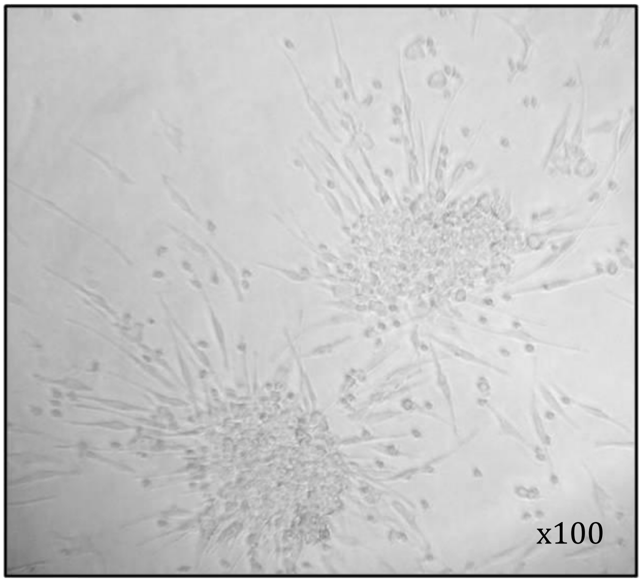


Figure 3.1: Representative example of Colony Forming Units (CFU)

3.3.3 Culture of Endothelial Colony Forming Cells

PBMCs were isolated from whole blood as described above. Cells were resuspended in ECFC medium, comprising Endothelial Basal Medium (EBM-2) with 10% FCS and Singlequots Bullet Kit (containing hEGF, hydrocortisone, gentamicin, amphotericin-B, VEGF, FGF-2, IGF-1, Ascorbic Acid and heparin) (Lonza, Slough, UK). Cells were cultured in 6 well plates, pre-coated with 1% gelatin. Medium was changed on alternate days and cultures were checked daily after day 7 for the emergence of prospective ECFC colonies. Cells were cultured for a minimum of 25 days.

3.3.4 Flow cytometry

Blood collection, PBMC isolation and flow cytometry were carried out as described in Chapter 2.

3.3.5 Identification of CACs

Gating of samples and control tubes was carried out as described in Chapter 2. As before, all samples were analysed in duplicate, and FMO control tubes were used for each sample analysed. CACs were defined as viable $CD34^{+}CD31^{+}CD133^{+}CD45^{-/dim}$ low forward/side scatter cells.

3.3.6 Pro-angiogenic: Non-angiogenic ratio

The ability to promote new blood vessel growth may not rely on the absolute number of pro-angiogenic cells in peripheral blood. Instead, the balance between pro-angiogenic and non-angiogenic cells may be more important. Previous authors have used the expression of CD133 to distinguish pro-angiogenic cells from non-angiogenic cells (Estes et al., 2010b). To assess this, the numbers of pro-angiogenic CACs ($CD34^{+}CD31^{+}CD133^{+}CD45^{-/dim}$ low forward/side scatter, viable cells) and non-angiogenic cells ($CD34^{+}CD31^{+}CD133^{-}CD45^{-/dim}$ low forward/side scatter, viable cells) were compared and a ratio was calculated.

3.3.7 Statistical analysis

Results in the text are expressed as mean values ± SD to demonstrate the range of values. Bar charts show mean values ± SE to indicate the precision of the estimate of the population mean. Box plots show the median value, 25th and 75th percentiles (whiskers represent minimum and maximum values, with outliers as filled circles). Numbers of CFU colonies, CAC levels, and the ratio of pro-angiogenic to non-angiogenic cells were confirmed to be normally distributed using the Kolmogorov-Smirnov test. Comparisons between two groups (endometriosis and controls) were performed with a Student T-test. Comparisons between the three groups (controls, minimal/mild and moderate/severe endometriosis) were made using one way analysis of variance (ANOVA).

Demographics for the continuous variables were compared using one-way ANOVA after confirmation of normally distributed data. Nominal variables were compared using a Chi^2 test for association.

Correlation between CAC or CFU number with other variables (age and BMI) was assessed using Pearson's correlation. Association between CAC level or CFU number and smoking status was assessed with a Student T-test.

3.3.8 Power calculation

A power calculation was performed to identify the number of women required for the study, based on the possibility of detecting a difference of at least one standard deviation between the women with and without endometriosis. As the primary goal was to explore the potential use of CACs as a biomarker of endometriosis, differences of less than one standard deviation from the mean would lead to a test with insufficient sensitivity and specificity to be of clinical use.

For the calculation, the formula used was:

$$(\mu_1 - \mu_2)^2 = \int(\alpha,P)\sigma^2(1/n_1 + 1/n_2)$$

where μ_1 = mean CAC level of group 1

μ_2 = mean CAC level of group 2

$\int(\alpha,P)$ = F statistic for a given alpha value and power level

σ = standard deviation

n_1 = size of group 1

n_2 = size of group 2

We took an α value of 5% and power level of 90% (i.e. 90% power to identify a difference at the 0.05 significance level) which gave the F statistic of 10.5 (Bland, 2000). To identify a difference of at least one standard deviation between the two groups:

$\mu_1 - \mu_2 = \sigma$

Therefore:

$(\mu_1 - \mu_2)^2 = \int(\alpha,P)\sigma^2(1/n_1 + 1/n_2)$

$\sigma^2 = 10.5\ \sigma^2(1/n_1 + 1/n_2)$

$1 = 10.5(1/n_1 + 1/n_2)$

The size of groups n_1 and n_2 was assumed to be equal, therefore:

$1 = 10.5\ (2/n)$

$1 = 21/n$

i.e. $n = 21$ for each group.

Twenty one subjects in each arm of the study were required to detect a difference of one standard deviation or greater, with 90% power at the 5% level.

3.4 Results

3.4.1 CFU Assay

Thirty women donated blood for this study. Of these, 10 women had stage 1-2 endometriosis, 10 had stage 3-4 endometriosis, and 10 had no apparent endometriosis (laparoscopically confirmed controls). The menstrual cycle was not accounted for, as previous data has indicated that the number of CFUs are not affected by the menstrual cycle (Robb et al., 2009; Elsheikh et al., 2011). Demographics for the included women are shown in Table 3-A. No significant differences in age, BMI, smoking status or clinical presentation were noted between the groups. Findings in the control group included: a normal pelvis (n = 3); adhesions (n = 2); ovarian cysts (n = 3) and polycystic ovarian syndrome (n = 2).

Table 3-A: Demographics of women included in CFU analysis.

Variable	Controls (n=10)	Stage 1-2 Endometriosis (n=10)	Stage 3-4 Endometriosis (n=10)	P value
Age in years Mean (± SD)	33.6 (±7.0)	33.2 (±6.7)	35.3 (±5.6)	0.746
BMI Mean (± SD)	25.0 (±3.5)	23.6 (±2.5)	26.0 (±4.6)	0.351
Number of smokers	3 (30%)	4 (40%)	4 (40%)	0.866
Presenting symptoms[1]				
Pain	3 (30%)	7 (70%)	6 (60%)	0.272
Subfertility	8 (80%)	5 (50%)	5 (50%)	0.452
Ovarian cyst	2 (20%)	2 (20%)	3 (30%)	1.000

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[1] Some women presented with more than one symptom, e.g. pain and subfertility, or pain and ovarian cyst.

3.4.1.1 *CFU in endometriosis*

The mean number of CFUs in the control and endometriosis (all disease stages) groups were 11.5 (±9.6) and 10.3 (±11.7), respectively. For the stage 1-2 and 3-4 groups, the mean numbers were 15.4 (±14.0) and 5.1 (±5.6).

There was no significant difference in the number of CFUs between women with and without endometriosis (P=0.776, Figure 3.2). Although fewer CFUs were noted in women with more advanced disease, this did not reach statistical significance when the three groups of women were compared (P= 0.083, F=2.736, 2 d.f. Figure 3.2).

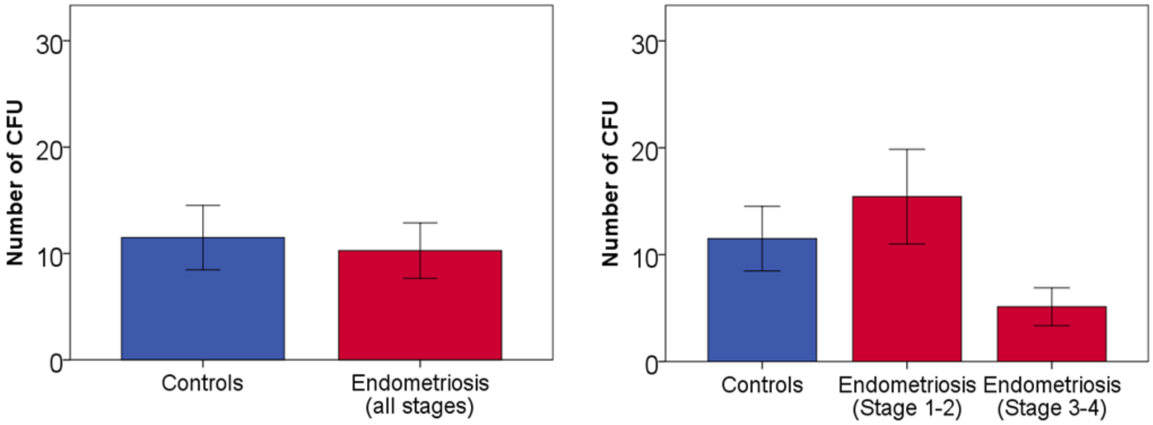

Figure 3.2: Number of CFUs (mean ± SE) are equivalent in women with and without endometriosis (P = 0.776), and in different stages of disease (P = 0.083).

3.4.1.2 Correlation between CFU number and age, BMI and smoking status

The correlation between the number of CFU and a variety of known cardiovascular risk factors was calculated, as these have previously been shown to affect CAC levels (Hill et al., 2003). Figure 3.3 shows a scatter plot of subject age and number of CFUs, with the corresponding regression line. No correlation was seen (P=0.196, Pearson's correlation -0.243).

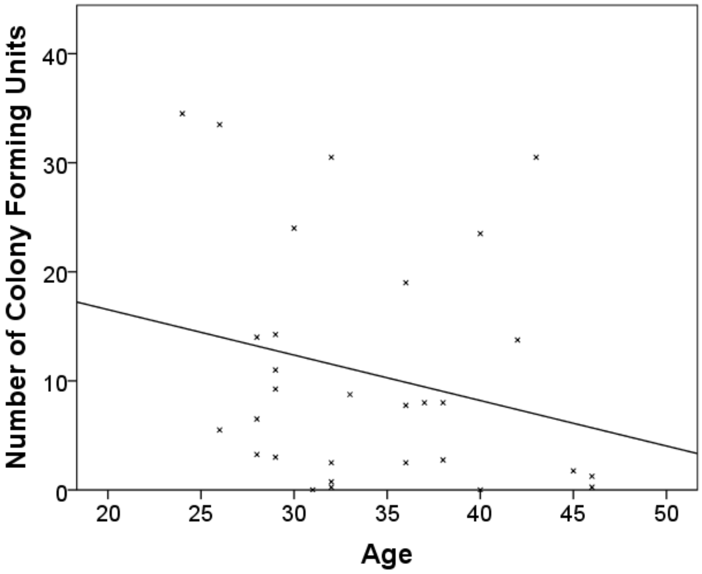

Figure 3.3: No correlation was identified between subject age and CFU number (P=0.196).

Similarly, no correlation was found for BMI and CFU numbers (P=0.826, Pearson's correlation 0.042)(Figure 3.4).

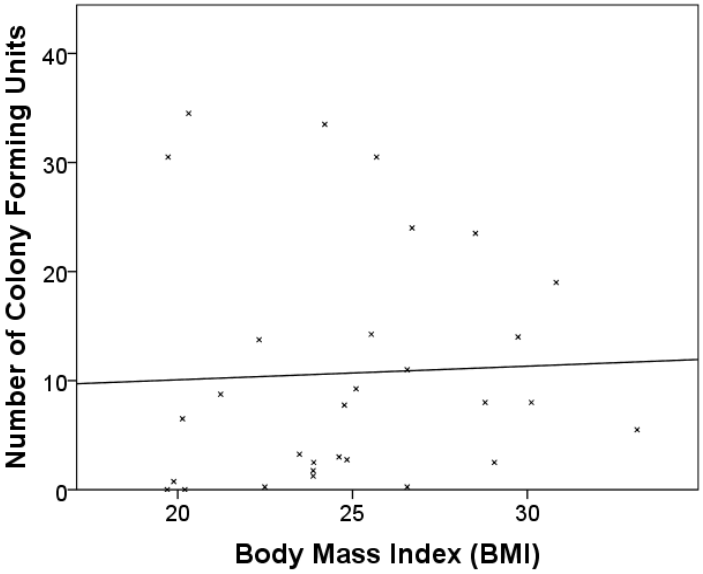

Figure 3.4: No correlation was identified between BMI and CFU number (P=0.826).

The mean number of CFUs in non-smokers and smokers were 11.4 (±12.7) and 9.5 (±7.1) respectively. This difference was not statistically significant (P=0.539).

3.4.2 Culture of Endothelial Colony Forming Cells

Eleven blood samples were used for ECFC culture. Of these 11 samples, only one ECFC colony was identified, arising on day 13 of culture (Figure 3.5). However, this colony started to disintegrate on day 27 of culture, and cells did not survive after passage. Given the scarcity of ECFC colonies, and the requirement for a large amount of blood to grow these cells, culture of ECFCs was abandoned.

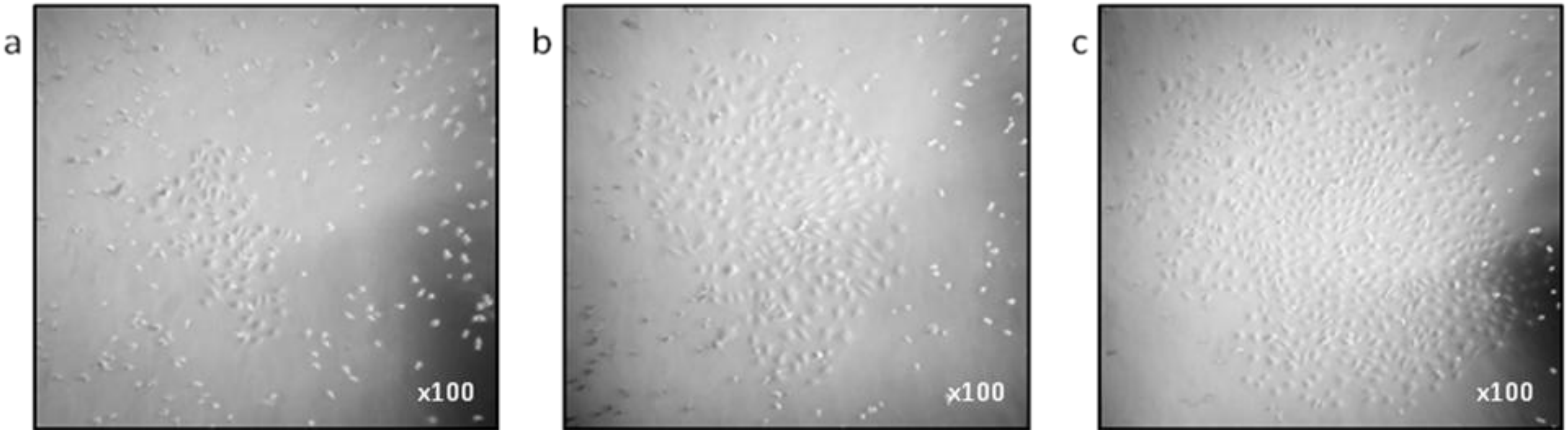

Figure 3.5: Endothelial Colony Forming Cells. One colony of ECFCs was identified in culture, from a total of 11 samples. (a) Colony on day 13 of culture; (b) day 15; (c) day 17. Note the homogenous morphology and rapid increase in colony size.

3.4.3 Quantification of CACs by flow cytometry

Sixty-five women were recruited for these experiments (n=22 controls, n=21 minimal/mild endometriosis, n=22 stage 3-4 endometriosis). However, one endometriosis subject (stage 3-4) was excluded due to a concomitant diagnosis of endometrioid adenocarcinoma of the ovary. Demographics are shown in Table 3-B. No significant differences in age, BMI, smoking status, clinical presentation or cycle phase were present between the groups. As CAC levels are unaffected by cycle phase (Chapter 2) this was not accounted for in the analysis. Findings in the control group included: a normal pelvis (n = 11); adhesions (n = 5); ovarian cysts (n = 2); polycystic ovarian syndrome (n = 1); pelvic inflammatory disease (n = 2) and hydrosalpinx (n = 1).

Table 3-B: Demographics for women included in CAC analysis by flow cytometry.

Demographics	Controls (n = 22)	Endometriosis stage 1-2 (n = 21)	Endometriosis stage 3-4 (n=21)	P value
Age in years Mean (± SD)	32.91 (±7.25)	35.33 (±5.36)	35.76 (±4.65)	0.236
BMI Mean (± SD)	22.80 (±4.40)	25.59 (±3.71)	26.04 (±3.71)	0.584
Number of smokers	8 (36%)	9 (43%)	4 (19%)	0.236
Presenting symptoms[2]				
Pain	13 (59%)	14 (67%)	17 (81%)	0.293
Subfertility	14 (64%)	11 (52%)	11 (52%)	0.690
Ovarian cyst	5 (23%)	2 (10%)	8 (38%)	0.091
Phase of cycle				
Menstrual	3	2	4	0.824
Follicular	8	6	7	
Peri-ovulatory	3	2	3	
Luteal	8	9	5	
Not available	0	2	2	

3.4.3.1 CACs in endometriosis

CAC levels were compared both as a percentage of viable low forward/side scatter PBMCs, and as an absolute count of cell numbers per ml of blood. The mean percentage of CACs in women without endometriosis was 0.0724% (±0.0414%) and the mean number of cells per ml of blood was 1015 (±620). The mean percentage of CACs in women with endometriosis was 0.0835% (±0.0422%) and the mean cell number per ml of blood was 1252 (±664). There were no significant differences in

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[2] Some women presented with more than one symptom, e.g. pain and subfertility, or pain and ovarian cyst.

CAC percentage (P=0.320) or CAC number (P=0.171) between women with and without endometriosis (Figure 3.6).

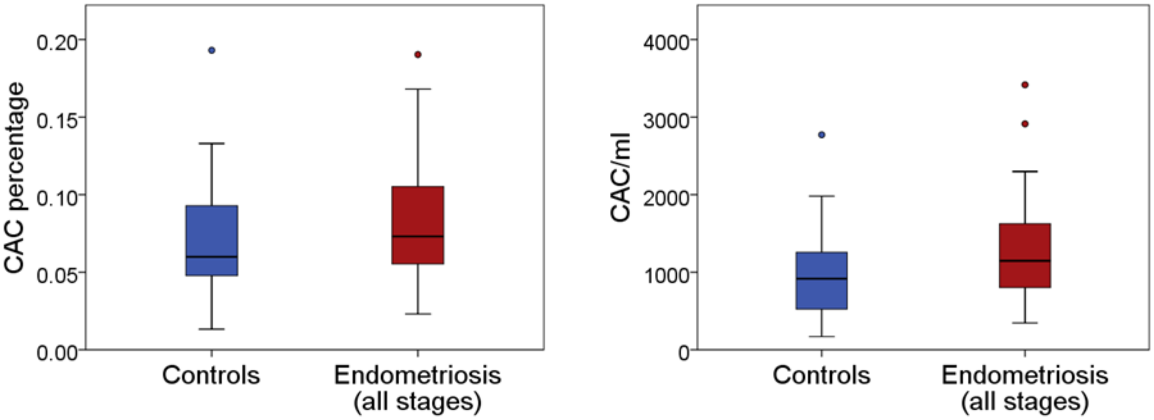


Figure 3.6: CAC levels were not significantly different in women with and without endometriosis, either when compared as a percentage of low forward/side scatter cells (P = 0.320), or as the number of CACs per ml of blood (P = 0.171).

The mean percentage of CACs in women with stage 1-2 endometriosis was 0.0897% (±0.0413%) and the mean number of CACs per ml of blood was 1231 (±508). In women with stage 3-4 disease, the mean percentage of CACs was 0.0774% (±0.0430%) and the mean number per ml of blood was 1273 (±803). Comparison across the three groups (no endometriosis, stage 1-2 and stage 3-4 disease) showed no significant difference in CAC percentage (P=0.390, F=0.956, 2d.f.) or CAC number (P=0.386, F=0.968, 2d.f.)(Figure 3.7).

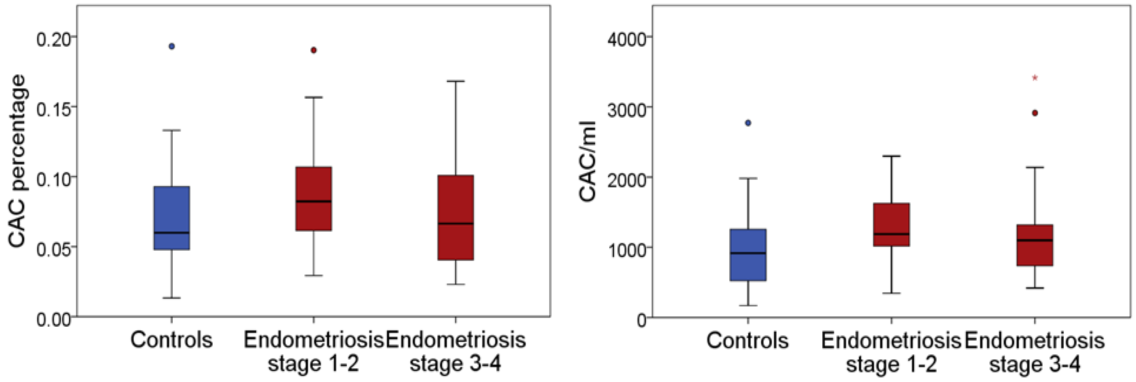


Figure 3.7: CAC levels in women with endometriosis (by stage) and controls. Levels were not significantly different between the three groups of women, either by comparing percentage of CACs (P = 0.390) or CAC number (P = 0.386).

3.4.3.2 Correlation of CACs with age, BMI and smoking status

There was no correlation between subject age and CAC levels, either by percentage or absolute cell number (P=0.668, Pearson's correlation 0.055 for percentage; P=0.698, Pearson's correlation -0.049 for absolute count) (Figure 3.8).

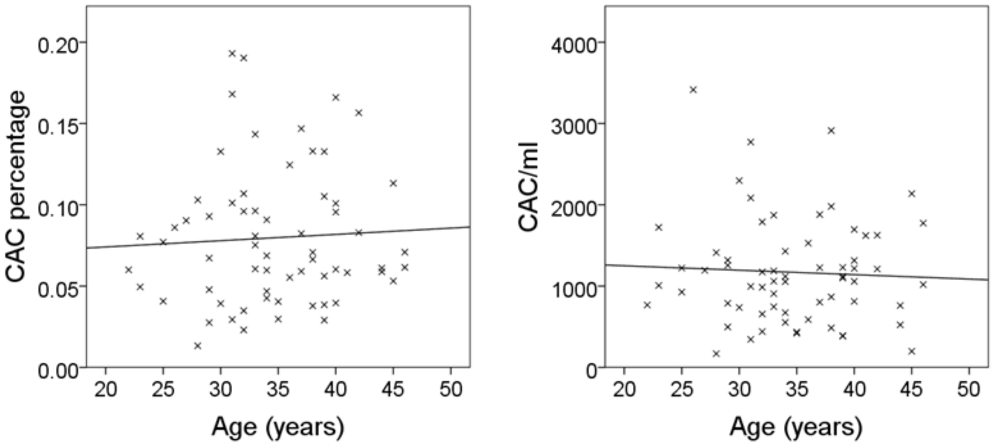


Figure 3.8: No correlation was identified between age and CAC levels.(P=0.668 for CAC percentage, P=0.698 for absolute count)

Similarly, no correlation was noted between BMI and CAC levels (P=0.726, Pearson's correlation 0.045 for percentage; P=0.581, Pearson's correlation 0.070 for absolute count)(Figure 3.9).

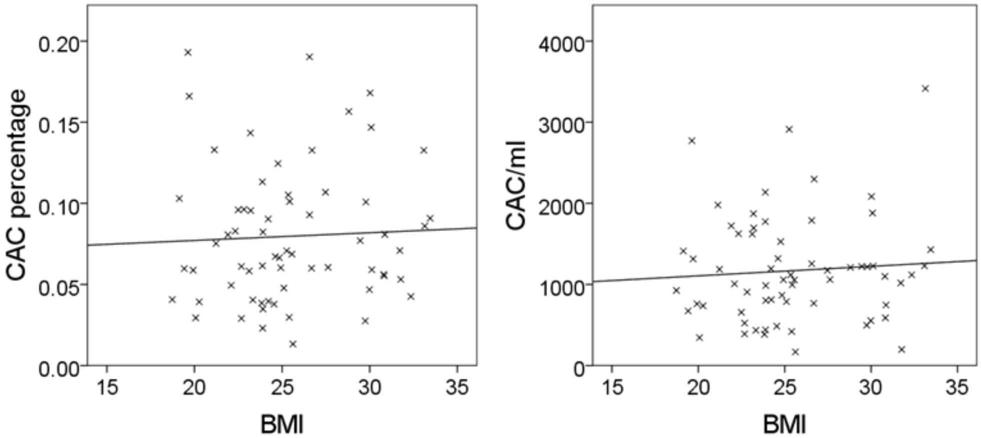


Figure 3.9: No correlation was identified between BMI and CAC levels. (P=0.726 for percentage, P=0.070 for absolute count)

CAC levels were similar in smokers and non-smokers, by percentage of low/forward side scatter PBMCs (P=0.543) and as an absolute count (P=0.886)(Figure 3.10).

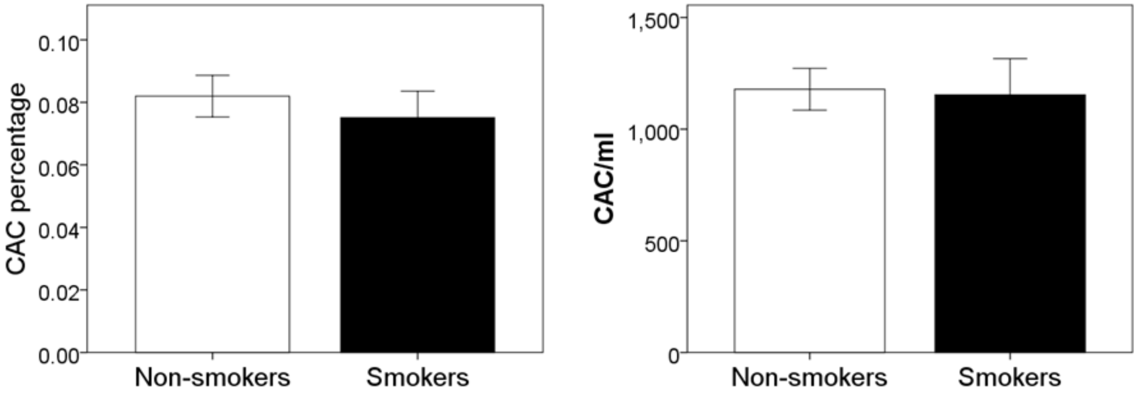


Figure 3.10: CAC levels in smokers compared to non-smokers. No significant difference was identified between the two groups.

3.4.3.3 Pro-angiogenic: Non-angiogenic ratio in endometriosis

The ratio of pro-angiogenic to non-angiogenic cells in controls was 4.94 (±3.46) compared to 4.10 (±2.49) in women with endometriosis. Women with stage 1-2 disease had a mean ratio of 4.32 (±2.30) and those with stage 3-4 disease had a mean ratio of 3.88 (±2.71). There was no significant difference in the ratio of pro-angiogenic to non-angiogenic cells when comparing controls to women with all disease stages (P=0.266) or to women with stage 1-2 and stage 3-4 disease (P=0.482, F=0.739, 2d.f.)(Figure 3.11).

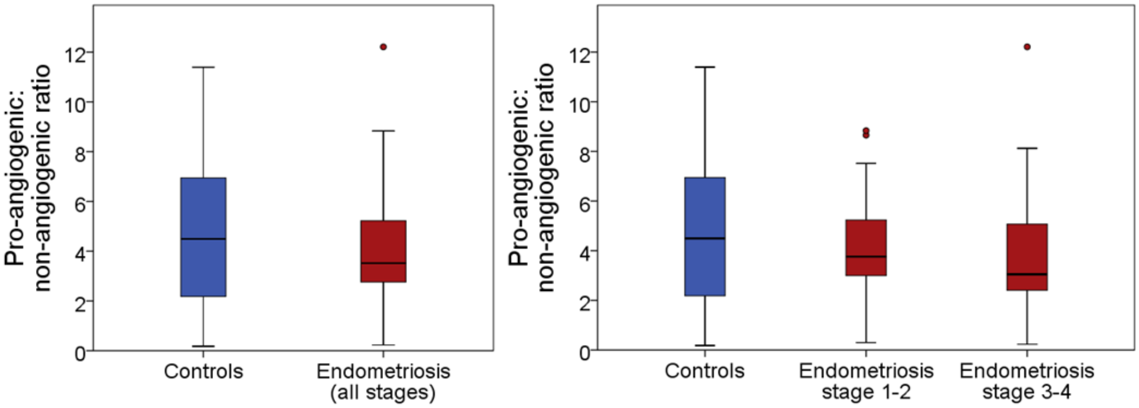


Figure 3.11: Ratio of pro-angiogenic to non-angiogenic cells in women with and without endometriosis. No significant difference was identified between women with and without endometriosis (P = 0.266) or between women with different stages of disease (P = 0.482).

3.5 Discussion

These data demonstrate that CAC levels in peripheral blood are not significantly affected by the presence of endometriosis, or the stage of disease. The possible reasons for the lack of effect are outlined below.

3.5.1 CACs are not be involved in angiogenesis in endometriosis

The first reason could simply be that CACs are not involved in angiogenesis occurring in endometriotic lesions. Hence, perhaps only the endothelial cells located in endometrial fragments or surrounding endothelial cells from the peritoneum are responsible for the local vessel growth. Certainly the peritoneal fluid of women with endometriosis is known to contain high levels of pro-angiogenic factors (Oosterlynck et al., 1993), and these may stimulate new vessel growth without any contribution from CACs.

Although data from murine models suggest a role for CACs in endometriotic vessel development this may not be the case in humans. Suturing endometrial fragments to the peritoneum in mice may stimulate different types of vessel growth to that seen with spontaneous adhesion and invasion of sloughed endometrial tissue in humans.

Of note, the paper by Laschke and co-workers identified CACs using GFP expressed under the control of the Tie2 promoter (Laschke et al., 2011). Tie2 is known to be expressed in cells of the macrophage lineage as well as endothelial cells, and is therefore not a CAC-specific marker (Venneri et al., 2007). Tie2^{+} macrophages appear to enhance angiogenesis in endometriotic lesions and have been identified in perivascular locations (Capobianco et al., 2011). Therefore, the “progenitor cells” identified by Laschke and colleagues may actually represent pro-angiogenic macrophages temporarily associated with the vessel wall. It is also possible that bone marrow derived cells other than CACs contribute to vessel development in endometriosis, accounting for the unchanged peripheral CAC levels seen in the disease in the present study.

3.5.2 CACs contribute in a minor way to angiogenesis in endometriosis

Another possibility is that CACs do contribute to endometriotic vessel development but only in a minor way compared to their involvement in physiological angiogenesis. Although CAC levels do not fluctuate with the menstrual cycle (as shown in Chapter 2), they are significantly higher in reproductive age women than in either age-matched men or post-menopausal women (Fadini et al., 2008; Lemieux et al., 2009). This suggests that CACs do contribute to physiological angiogenesis in the female reproductive tract. The higher levels of these cells in menstruating women may mask any small increases due to endometriosis.

The primary goal of this study was to assess the possible utility of CACs as a biomarker of endometriosis, and the power calculation was performed so as to identify relatively large differences between the groups of women. It is possible that small alterations in CAC levels exist between women with and without endometriosis but that the study was underpowered to detect such minor changes.

Furthermore, pro-angiogenic cells may be mobilised locally and contribute to lesion development, rather than being recruited from the circulation (Naito et al., 2012). This would result in the inclusion of bone marrow derived cells in the vasculature, but would not affect the levels of these cells in peripheral blood.

3.5.3 CACs are affected at specific time points in lesion development, or by different subtypes of endometriosis

It is also possible that altered CAC levels only occur at certain phases in the development of endometriotic lesions, or in certain types of lesions. It is likely that blood vessel development is at its most prolific as the endometriosis lesions are beginning to establish, early in the course of the disease. As such, CAC levels may be increased in this early phase, but they are not elevated after the lesions have become established.

This is in keeping with the murine data: CAC levels were elevated until day 10 after lesions were established, and were then equivalent to those in sham operated mice (Becker et al., 2011). Similarly, the number of CACs incorporated into lesions was found to be highest at 7 days after surgery, and then gradually declined over the following three weeks (Laschke et al., 2011).

The lack of an effect of stage of disease on CAC levels is not consistent with these findings. However, it is not clear that disease stage correlates with duration of endometriosis, and some women with minimal/mild disease (stage 1-2) may have these lesions for a protracted time without progressing to more advanced stages.

It is also well recognised that endometriotic lesions have a variety of macroscopic appearances, including red, white and black lesions (Nisolle and Donnez, 1997). It is possible that angiogenic activity varies with different lesion types, such that alterations in CAC levels may only be seen in women with more “active” lesions. Certainly, microvessel density and VEGF levels have previously been shown to vary between the different lesions (Nisolle et al., 1993; Donnez et al., 1998). A possible limitation of the present study is that data on lesion type (red/white/black) were not collected and it was, therefore, not possible to assess whether altered CAC levels were associated with specific lesions.

3.5.4 Choice of control group

The choice of control group in endometriosis studies has been debated. To understand the pathogenesis of the disease it may be relevant to compare endometriosis subjects with asymptomatic controls, who are known to have no endometriosis. Typically this group has included women undergoing laparoscopic sterilisation. However, the focus of this work was to assess the potential use of CACs as a biomarker for endometriosis. For biomarker studies the control group must also be from the population in whom a biomarker would be clinically useful, i.e. women with symptoms suggestive of endometriosis. This is the only way to demonstrate appropriate sensitivity and specificity in the target population. All women participating in this study had symptoms that would be consistent with endometriosis. However, the presence of symptoms meant that some women in the control group had other pathology identified at the time of surgery, including polycystic ovarian syndrome (PCOS), adhesions or ovarian cysts, which may affect

CAC levels. One recent study identified reduced CAC levels (defined as $CD34^+CD133^+$ cells) in women with PCOS (Dessapt-Baradez et al., 2011).

Therefore, we cannot conclude that endometriosis does not affect CAC levels, but only that their levels are not of use as a biomarker of disease. It is still possible that, when compared to asymptomatic women without endometriosis, CAC levels are affected by the disease.

3.5.5 Endothelial Colony Forming Cells

Endothelial Colony Forming Cells are another member of the family of circulating pro-angiogenic cells. Of all cell types suggested as putative "endothelial progenitors" these demonstrate the most similar phenotype to genuine endothelial cells. Some authors have even speculated that they may represent sloughed circulating endothelial cells, which can participate in vessel construction or repair (Yoder, 2010). However, these cells are found at very low density in circulating blood. Furthermore, the proliferation of ECFCs derived from adult blood is much more limited than those derived from neonatal blood (Ingram et al., 2004). This may explain the disintegration of the single colony grown in our experiments. This study was limited by the amount of blood that could be obtained from the subjects, and therefore it was decided not to pursue the ECFC culture from women with endometriosis. Previous authors have required 50-100ml blood in attempts to grow ECFCs (Yoder et al., 2007). However, it would be interesting to assess the numbers and behaviour of ECFCs from women with endometriosis in the future.

3.5.6 CACs do not correlate with age, BMI or smoking

Contrary to previously published data, there was no correlation between CAC levels and age, smoking status or BMI, either by flow cytometry or CFU analysis.

The subjects were a relatively homogeneous group, which may have made it difficult to detect changes in CAC levels with BMI or age. The majority of women recruited were aged from 25 to 40, with a normal BMI. Very few women were obese or significantly underweight, which is relevant because obesity has been associated with decreased CAC levels (MacEneaney et al., 2008; Müller-Ehmsen et al., 2008).

Other small studies have also failed to identify a correlation between age and CAC levels (Chen et al., 2006; Rousseau et al., 2010). However, larger studies including patients with cardiovascular risk factors have identified a significant inverse correlation (Schmidt-Lucke et al., 2005).

One study reported reduced numbers of $CD34^+$ cells in female smokers (>15 cigarettes per day), but no significant change in $CD34^+CD133^+$ cells or CFU numbers (Ludwig et al., 2010). In the present study there were very few heavy smokers (i.e. > 15 cigarettes per day) which may have affected the results.

Therefore it is possible that the lack of correlation with CACs and traditional cardiovascular risk factors is due to the small sample size, and the homogeneous nature of the study group.

3.5.7 Summary

Overall, it appears that CAC levels are not a prospective biomarker of endometriosis, although it remains possible that these cells do contribute in some way to the development of vessels in endometriosis. Low level, persistent recruitment of these cells to endometriotic lesions could stimulate vessel development. Further work is needed to investigate whether these cells could be a therapeutic target in endometriosis, despite their lack of potential as a biomarker.

Chapter 4: Isolation of endothelial cells from endometrium and endometriotic tissue

4.1 Introduction

The endometrial vasculature changes greatly over the course of a menstrual cycle. Vessels increase in number during the proliferative phase of the cycle and undergo complex remodelling to form spiral arterioles during the luteal phase (Rogers and Gargett, 1998). Menstruation causes shedding of the functional layer of the endometrium and disruption of the vessels, which must be repaired at the start of the next cycle. These processes are dependent on the activity of endometrial endothelial cells.

Entry of endometrial tissue into the peritoneal cavity is thought to be a critical step in the development of endometriosis. This tissue carries with it endometrial endothelial cells which could contribute to the developing vasculature. As retrograde menstruation is a common event in most menstruating women, differing abilities of endothelial cells to proliferate and form vessels may be part of the reason that the disease develops in some, but not all, women. Consequently, it is possible that differences in angiogenic potential between women with and without endometriosis may be due to inherent differences in endometrial endothelial cells.

Isolating endometrial endothelial cells would be one way to identify possible differences in the behaviour of these cells in women with endometriosis – with particular regard to their response to hormonal stimulation, ability to proliferate and angiogenic activity.

Previous authors have described the successful isolation and culture of endometrial endothelial cells (Iruela-Arispe et al., 1999; Nikitenko et al., 2000; Schatz et al., 2000). Since then, other researchers have sought to identify differences in behaviour of endometrial endothelial cells from women with endometriosis (Luk et al., 2005; Sha et al., 2007; Luk et al., 2010). Two of these papers reported altered responses to estradiol and progesterone in endothelial cells derived from women with endometriosis (Luk et al., 2005; Luk et al., 2010). These papers identified significantly increased production of IL-8 and MCP-1 by endometrial endothelial cells in response to these sex steroids. The third paper showed altered gene expression in endothelial cells derived from women with endometriosis (Sha et al., 2007).

This chapter describes the attempted isolation of a pure population of endometrial endothelial cells from women with and without endometriosis.

4.2 Aims

- To isolate and culture endometrial endothelial cells.
- To isolate and culture endothelial cells from endometriotic lesions.
- To identify endometrial endothelial cells by flow cytometry.

4.3 Materials and methods

4.3.1 Tissue biopsies

Endometrial pipelle biopsies were collected at the time of laparoscopy and placed immediately into approximately 10ml of medium (DMEM containing 10% FCS, 100U/ml penicillin and 100mg/ml streptomycin).

Where possible, samples of endometriosis tissue (peritoneal lesions, rectovaginal nodules and endometriomas) were also collected during operative laparoscopy. These samples were also transferred directly into medium, as above.

All samples were kept at 4^{o}C until being processed in the laboratory within 4 hours of collection.

4.3.2 Isolation of endometrial cells

All procedures were carried out in a class II laminar flow biosafety cabinet. Samples were rinsed in sterile PBS to remove excess blood, mucus and debris. The tissue sample was finely minced using sterile scalpel blades, into approximately $1mm^3$ pieces. These endometrial fragments were then transferred to a fresh container with 9ml serum free DMEM, containing 100U/ml penicillin and 100mg/ml streptomycin. 1mg/ml collagenase II (Invitrogen, Paisley, UK) was added, along with 2.5μg/ml DNase I solution (Sigma-Aldrich, Poole, UK). The solution was digested at 37^{o}C for 1 hour using a sterile stirring rod and magnetic stirrer. Inspection of the solution at this point revealed partially digested tissue, with visible glandular structures intact, but the majority of the endometrial stroma dissociated into a single cell suspension.

10ml medium (DMEM with 10% FCS and 100U/ml penicillin and 100mg/ml streptomycin) was added to the cell suspension before filtering through a 70µm cell strainer to remove intact glands and undigested tissue. The filtrate was then centrifuged in a Heraeus Sepatech centrifuge at 1500rpm (470 x *g*) for 3 minutes. The resulting cell pellet was resuspended in 1ml ECGM MV2 (microvascular medium).

4.3.3 Endometriotic tissue samples

On arrival at the laboratory, samples were washed free of blood and debris with sterile PBS, and cut finely into approximately 1mm^3 pieces with two scalpel blades. For endometriomas, fibrotic cyst wall tissue was first carefully separated from endometrial-like tissue with a scalpel, and discarded. Endometriotic tissue was then enzymatically digested in an identical way to endometrial samples (as described above).

4.3.4 Endothelial cell isolation

4.3.4.1 Immediate selection

Preliminary experiments involved immediate isolation of endothelial cells, directly from the primary cell suspension, before the cells had been cultured (n=12).

After enzymatic digestion of biopsies, endometrial cells (eutopic and ectopic) were resuspended in 1ml ECGM MV2 medium. 5µl CD31 Dynabeads were added to the cell suspension and incubated with frequent agitation for 10 minutes at 4^{o}C. These conditions were chosen to minimise non-specific binding of CD31 antibodies to non-endothelial cells. This occurs more commonly at increased temperatures, with longer duration of incubation and higher antibody concentration.

After incubation, the mixed cell and bead suspension was placed into a magnetic particle concentrator for 2 minutes to separate out bead-bound cells. Supernatant was aspirated, taking care to leave the bead-bound cells undisturbed. The remaining cells (bound to magnetic beads) were washed with 0.1% BSA in PBS and exposed to the magnet again for 2 minutes, before removing the supernatant. A total of five washes were carried out in this manner to remove all non bead-bound cells.

Finally, the resulting bead-bound cells were resuspended in ECGM MV2 medium and plated into tissue culture flasks (T25), pre-coated with 1% gelatin.

4.3.4.2 Delayed selection

The majority of endometrial (n=31) and endometriotic (n=10) biopsies were used for delayed selection– isolation of endothelial cells after a short period of time in culture, rather than on the day of sample collection. After enzymatic digestion of the biopsy, the cell suspension was plated into tissue culture flasks (T175), pre-coated with 1% gelatin, in ECGM MV2 medium. These cultures, therefore, contained a heterogeneous population of endometrial cells, including endometrial stroma, endothelial cells, epithelial cells and leucocytes.

When these primary cultures reached confluence (usually within 1-3 days), the cells were harvested with trypsin, resuspended in 1ml ECGM MV2 and then incubated with

5μl CD31 Dynabeads for 10 minutes at 4^{o}C with frequent agitation. CD31 positive cells were isolated as described above (4.3.4.1) and the resulting bead-bound cells were plated into a T25 tissue culture flask, pre-coated with 1% gelatin.

4.3.5 Reselection of endothelial cultures

To remove possible contaminating cells from the cultures, flasks were “reselected” using CD31 Dynabeads after reaching confluence. Cells were washed with sterile PBS and harvested with trypsin. After centrifugation, the cultures were resuspended in 1ml ECGM MV2 and incubated with 5μl CD31 Dynabeads as described above. Cells were exposed to a magnetic field and washed to isolate bead-bound cells as before. Bead bound cells were cultured in a T25 tissue culture flask, pre-coated with 1% gelatin.

4.3.6 Flow cytometry of endothelial cultures

On reaching confluence, endothelial cell cultures were analysed by flow cytometry to assess their purity by examining the expression of endothelial cell surface markers. Cultures were assessed after one selection (n=4), 2 selections (n=5) and 3 selections (n=5). Cells were harvested with trypsin and resuspended in 2% FCS. The antibodies and concentrations used are shown in appendix 3. Cells were dual-stained with CD90 FITC and CD31 APC-Cy7, and corresponding isotype control antibodies. Cells were also single-stained with CD144 FITC and a corresponding isotype control.

After incubation with antibodies for 30 minutes on ice, cells were washed three times with 2% FCS prior to analysis on a BD LSR II flow cytometer; 20,000 events were collected for each sample. Positive staining boundaries were determined by comparison to isotype controls. Data were analysed using FlowJo software.

4.3.7 Immunocytochemistry

Culture purity was also assessed with immunocytochemistry. Cells were cultured on glass cover slips in four-well plates. At confluence, medium was removed and cells were washed once in PBS. 400μl of 3% paraformaldehyde (PFA) was added to each cover slip to fix the cells. After 10 minutes, PFA was removed and the cover slips were washed twice with PBS.

PBS was aspirated from the cover slips and 400μl of 0.2% Tween (in PBS) was added for 30 minutes to permeabilise cell membranes. After removal, cells were washed once in PBS and 500μl blocking solution (3% BSA) was added for 1 hour to block non-specific binding sites.

The primary antibody was mouse anti-human vWF (AbCam, Cambridge, UK). This was diluted in 1% BSA to the required concentration (1:800, 0.5μg/ml). Blocking solution was removed and 50μl primary antibody solution was added to each cover slip. Incubation was for 1 hour at room temperature.

After incubation, cover slips were washed three times (for 3 minutes each) with 0.1% BSA. The secondary antibody was goat anti-mouse Alexa-Fluor 488 (Invitrogen, Paisley, UK). This was diluted to the required concentration (1:400, 5μg/ml) in 1% BSA. 50μl secondary antibody solution was added to each cover slip. Incubation was for 50 minutes at room temperature in the dark.

Following secondary antibody incubation, cover slips were washed three times (for 3 minutes each) with PBS, in the dark. Cover slips were carefully lifted from their wells and mounted onto glass slides using approximately 7µl mounting medium with DAPI (for nuclear staining).

Slides were inspected using a Leica DMRBE fluorescence microscope. Images were acquired with a Hamamatsu camera. Original magnification was recorded for each image.

4.3.8 Uptake of DiI Acetylated-LDL

For some cultures, cells were exposed to DiI labeled, acetylated LDL (DiI AcLDL) prior to fixation and immunocytochemistry. Cells were washed once in PBS. DiI AcLDL was diluted in medium (ECGM MV2) to a final concentration of 1µg/ml. 500µl medium containing DiI AcLDL was then added to each cover slip for 4 hours. Cover slips were washed twice in PBS before cells were fixed as described above, and used for immunocytochemistry.

4.3.9 Culture of Human Microvascular Endothelial Cells (HMVEC)

HMVEC were obtained from Promocell (Heidelberg, Germany). Cells were maintained in culture with microvascular endothelial cell media, ECGM MV2 (Promocell, Heidelberg, Germany) and grown in flasks pre-coated with 1% gelatin (Sigma-Aldrich, Poole, UK). When confluent, cells were split 1:3 to passage.

4.3.10 Culture of Human Umbilical Vein Endothelial Cells (HUVEC)

HUVEC were kindly donated by Dr. Dionne Tanetta, Nuffield Department of Obstetrics and Gynaecology, Oxford. Cells were maintained in culture with ECGM MV2 and grown in flasks pre-coated with 1% gelatin. When confluent, cells were split 1:3 to passage.

4.3.11 Seeding of stromal cells into HUVEC and HMVEC cultures

HUVEC and HMVEC were grown to confluence, harvested and counted. Endometrial stromal cells (isolated and cultured as described in Chapter 5) were harvested and counted. Stromal cells were then artificially seeded into HUVEC or HMVEC at four different ratios (1.5%, 5%, 10% and 25% stromal cells). Cells were maintained in culture for six passages, and assessed for expression of CD31 (HUVEC and HMVEC) and CD90 (stroma) at each passage by flow cytometry. Antibodies and isotype controls were identical to those described in 4.3.6. Positive staining boundaries were defined using isotype control antibodies. Data were analysed using FlowJo software.

4.3.12 Selection of HUVEC and HMVEC from stromal cultures

HUVEC and HMVEC were artificially seeded into a stromal cell culture at a ratio of 1 endothelial cell to 99 stromal cells. These mixed cultures were then resuspended in 1ml ECGM MV2 medium and 5µl CD31 Dynabeads were added. Cells were incubated at 4^{o}C for 10 minutes, and bead-bound cells were isolated in a manner identical to that used with primary cell cultures. The resulting cell culture was grown to confluence and maintained in culture for six passages. Antibodies and isotype controls were identical to those described in 4.3.6. At each passage the expression of CD31 and

CD90 was quantified with flow cytometry. Positive staining boundaries were determined using isotype controls, set with a gate at 1%. The experiment was carried out in duplicate for HUVEC and HMVEC. Data were analysed using FlowJo software.

4.3.13 Culture of endothelial cells at increased density

After delayed selection with CD31 Dynabeads, endothelial cells (n=12) were cultured in a single well of a 4 well plate (1.9cm^2 surface area), instead of a T25 tissue culture flask (25cm^2 surface area). At confluence, five cultures were fixed and stained for vWF expression, as described previously (4.3.7).

The remaining cultures (n=7) were harvested and placed in a magnetic particle concentrator before culturing in a single well of a 6 well plate (9.6cm^2 surface area). This ensured that only cells that remained bound to CD31 beads after the first passage were included in the ongoing culture, which was done to try to reduce contamination by non-endothelial cells. These were more likely to have bound only one bead initially (non-specific binding); therefore, daughter cells should have had no beads attached to them. Conversely, endothelial cells usually bound at least three or four beads during primary incubation, leading to beads being passed on to daughter cells at cell division. At the next passage this process was repeated with cells exposed to the magnetic particle concentrator before re-plating in a T25 flask.

4.3.14 Flow cytometry of endometrial biopsies

Pipelle biopsies (n=9) were collected and enzymatically digested as described above (4.3.2). After digestion, cells were resuspended in 2% FCS.

Three colour antibody staining was used throughout, and cells were incubated and washed as described in 4.3.6. Preliminary experiments assessed expression of CD31, CD45 and CD90 (n=3). A second set of experiments assessed expression of CD31 and CD45 with CD34, CD14 or CD105 (n=3). Final experiments assessed expression of CD31 and CD45 with CD90, CD10 and CD144 (n=3). Positive staining boundaries were identified using FMO controls. Single antibody stained compensation beads were used to define compensation settings. The antibodies used are shown in appendix 3.

4.3.15 Numbers of endothelial cells

For each biopsy (n=9), the number of CD31+CD45- events was recorded and used to calculate the percentage of endothelial cells within the moderate forward/side scatter gate. The proportion of each pipelle sample used for flow cytometry was also recorded to estimate the total number of endothelial cells in the whole biopsy.

4.3.16 Statistical analysis

Results in the text are expressed as mean values ± SD. All graphs show mean values ± SE. Comparison of cell surface marker expression on cells was carried out using a one-way ANOVA.

4.4 Results

4.4.1 Immediate selection of endothelial cells

Ten endometrial biopsies and two endometrioma samples were used for immediate selection of endothelial cells (CD31 bead selection from freshly digested biopsies, before cell culture). Two cultures were infected and discarded after overnight culture in the laboratory. One poor quality endometrial biopsy contained very few cells after selection, another contained mucus only - these were discarded. Demographics for the remaining 8 cultures, obtained from 7 different women are shown below (Table 4-A) (N.B. one pipelle and endometrioma were from the same subject).

Table 4-A: Demographics for samples used for immediate selection of endothelial cells.

Variable	
Age in years, mean (± SD)	31.57 ± 7.00
BMI, mean (± SD)	26.25 ± 4.41
Number of smokers (%)	1 (14%)
Follicular phase (day 1-14)	4 (57%)
Luteal phase (day 15-28)	3 (43%)
Endometriosis	3 (43%)

The cultures (n=8) took between 5 and 17 days to reach confluence. At this stage, small patches of cells with a typical endothelial morphology (round, cobblestone appearance) were seen in all cultures. However, cultures were also seen to contain variable amounts of another cell type with a spindly, fibroblast-like appearance, resembling that of endometrial stromal cells. Representative examples are shown below (Figure 4.1).

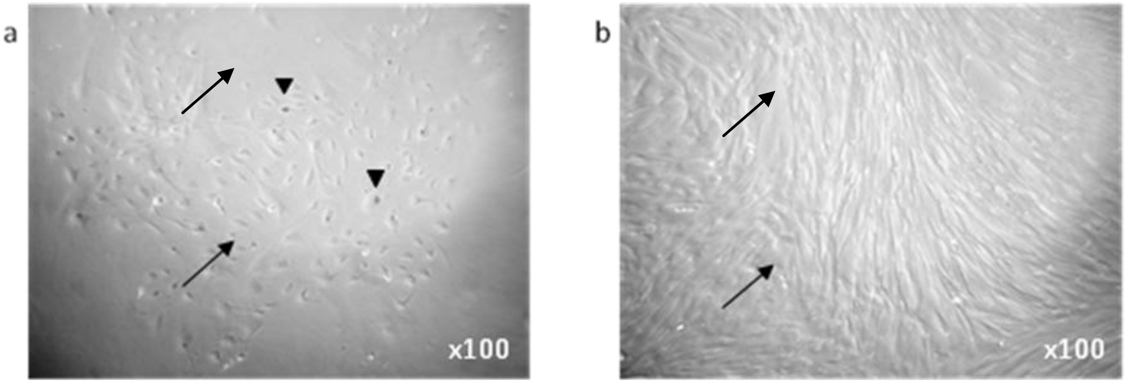


Figure 4.1: First passage culture after immediate selection with CD31 Dynabeads. (a) Endothelial-like morphology with cobblestone appearance (arrow). Note the presence of occasional beads (seen as dark circles, marked with arrowheads) within the cells; (b) Fibroblast-like morphology of overlapping, densely packed spindle shaped cells (arrow). No beads are visible.

4.4.2 Reselection of cultures

The presence of cells with fibroblast-like morphology suggested that endothelial cell cultures may have been contaminated by the presence of endometrial stromal cells. To try to remove these cells, cultures were exposed to CD31 Dynabeads for repeated selections (2-3 times). However, cultures continued to retain a heterogeneous appearance. Some cells exhibited a cobblestone-like pattern but contained fine projections from the cell membrane. Other areas contained spindle-shaped, fibroblast-like cells. Examples are shown below (Figure 4.2).

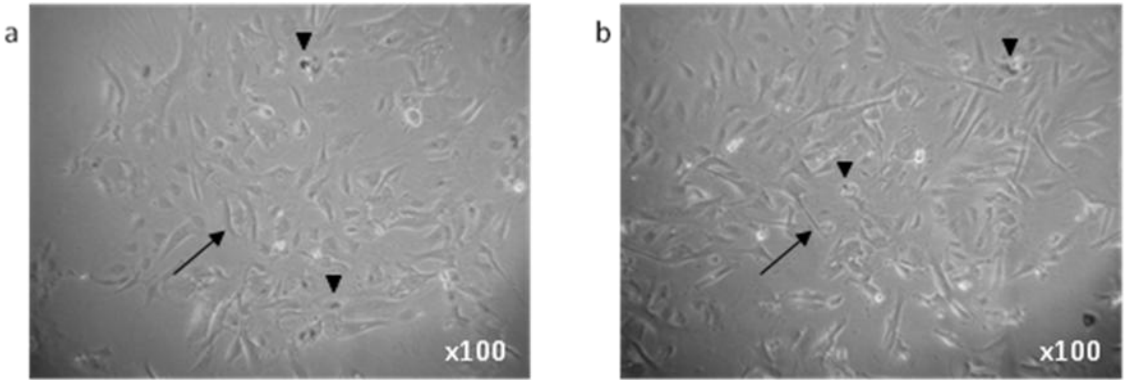


Figure 4.2: Reselected cultures after immediate CD31 Dynabead selection. (a)One reselection; (b)Two reselections. Note the continued presence of scattered Dynabeads (arrowheads), but also the increasingly uneven cobblestone pattern, with mixed spindle-shaped cells and membranous projections (arrows).

4.4.3 Delayed selection of endothelial cells from endometrium

Due to the difficulty obtaining pure cultures of endothelial cells, the isolation method was modified. Instead of selecting the endothelial cells from freshly digested tissue, primary cultures were plated and allowed to grow to confluence before isolating and removing endothelial cells (n=31). This approach was based on previous reports which describe selecting endothelial cells after a short length of time in culture (Sha et al., 2007; Luk et al., 2010), and the principle that this would allow the endothelial cell population to increase in number before isolation. Of these 31 samples, some biopsies contained no endometrial tissue (mucus/blood only) and were unsuitable for digestion (n=6). Other samples were discarded as they became infected after overnight culture (n=5). Demographics for the remaining samples (n=20) are shown below (Table 4-B): 19 women were taking no hormones at the time of sample collection, although two had recently taken clomiphene (6-8 weeks previously). One woman was taking the combined oral contraceptive pill (Logynon) at the time of sample collection.

Table 4-B: Demographics for samples used for delayed selection of endothelial cells.

Variable	
Age in years, mean (±SD)	34.65 ± 7.07
BMI, mean (±SD)	25.25 ± 3.96
Number of smokers (%)	6 (30%)
Follicular phase (day 1-14)	6 (30%)
Luteal phase (day 15-28)	11 (55%)
Uncertain of cycle phase	3 (15%)
Endometriosis	9 (45%)

The number of bead-bound cells obtained from these cultures was counted in some cultures. This varied widely, from 3,000 to 296,000 cells. The mean number of beaded cells was 64,714 ± 75,658 (n=14).

Unfortunately, cultures became overgrown with spindle-shaped, fibroblast-like cells. Even by the end of the first passage, many cultures had very mixed and varied morphology. Often flasks contained some areas with typical endothelial morphology, but other areas with a more spindle-shaped, abnormal cell growth. Representative examples are shown in Figure 4.3.

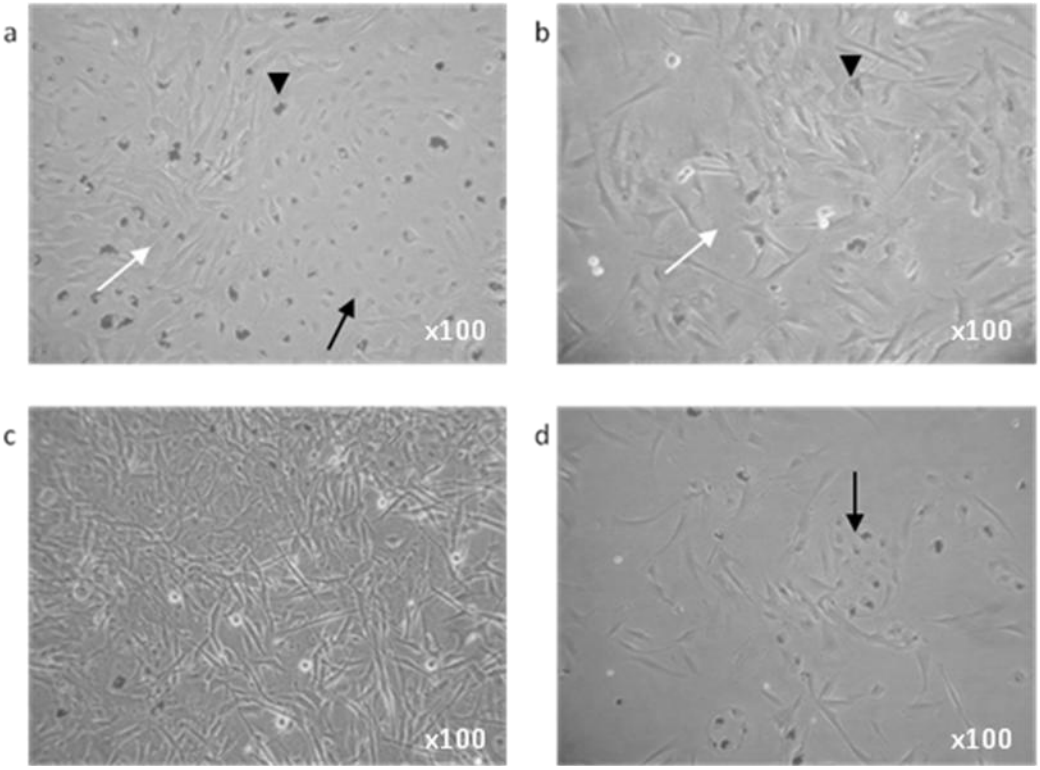


Figure 4.3: Examples of endothelial cultures after delayed selection with Dynabeads. All cultures are at first passage. (a) Note a large area of endothelial-like growth to the right of the image (black arrow), and more spindle-shaped cells to the left (white arrow); (b) Shows many spindle-shaped cells, despite the clear presence of beads (arrowheads), indicating CD31 positive cells; (c) Very overgrown area of culture, with lack of contact inhibition; (d) Mixed culture with visible "islands" of endothelial cell growth (arrow), and surrounding spindle-shaped cells.

These cultures were also repeatedly mixed with Dynabeads at each passage to try to improve their purity. However, even after two or three selections with Dynabeads, fibroblast-like cells were still found in all cultures. Whilst some areas of the flask appeared to contain confluent endothelial cells, others showed heavy contamination with different cellular morphologies (Figure 4.4, Figure 4.5).

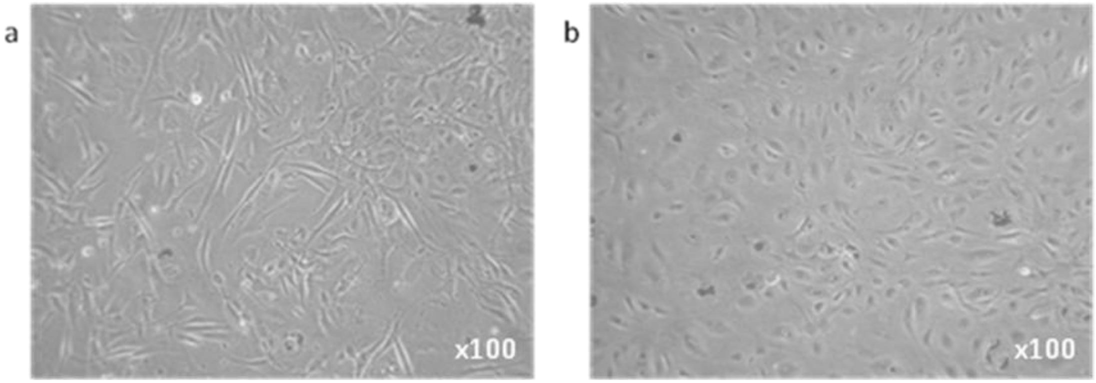


Figure 4.4: Representative images of cells after two selections with Dynabeads. (a) Typical spindle-shaped/fibroblast-like morphology. (b) Endothelial-like appearance.

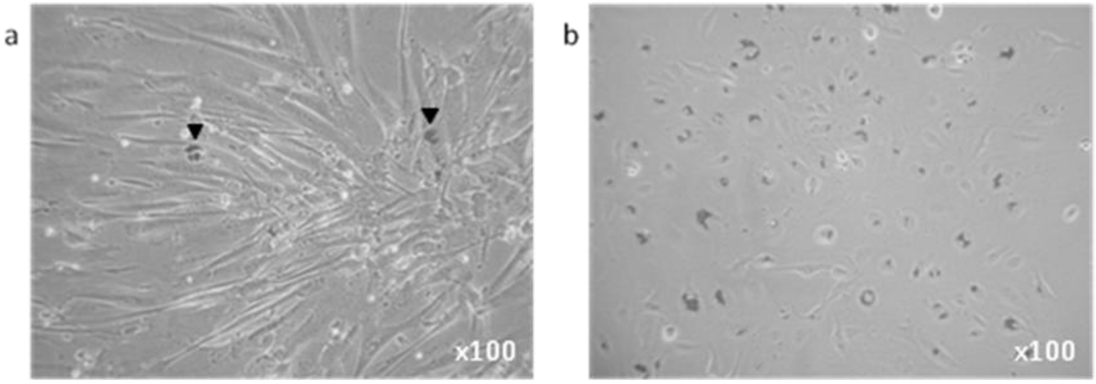


Figure 4.5: Representative images of cells after three selections with Dynabeads. (a) Very abnormal fibroblastic appearance – note the presence of beads within the spindle-shaped cells (arrowheads); (b) Endothelial-like appearance.

The expression of cell surface markers was quantified by flow cytometry to assess culture purity more precisely than by cell morphology. Typical endothelial markers (CD31, CD144) and a characteristic stromal cell marker (CD90) were used. Repeated selections of endothelial cells showed a strong trend to increasing expression of CD31, although this was not statistically significant (P=0.056, F= 3.786, 2d.f.). Similarly, expression of CD144 tended to increase with repeated selections, but this also did not reach statistical significance (P=0.136, F= 2.400, 2d.f.). Even after three selections, only 47% of cells were positive for CD31 and 50% positive for CD144 (Figure 4.6). This indicated the presence of a large number of non-endothelial cells in the cultures.

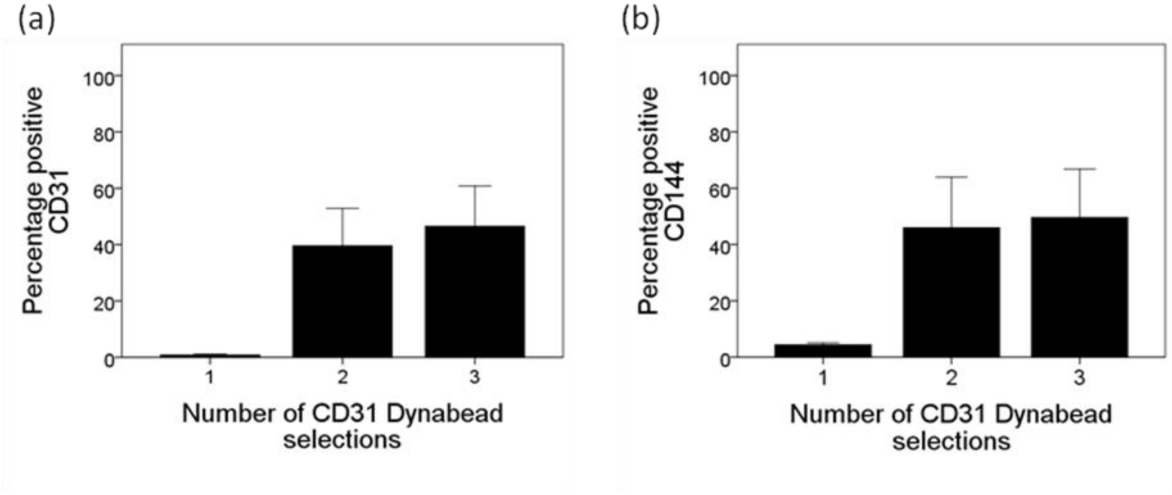


Figure 4.6: Expression of cell surface markers after repeated selection for endothelial cells with Dynabeads. (a) CD31$^+$ cells with repeated selections; (b) CD144$^+$ cells with repeated selections. A trend to increasing expression was seen for both markers, but this was not statistically significant.

The number of CD90 positive cells significantly decreased with repeated selection (P=0.034, F=4.652, 2d.f.). However, many cells expressing CD90 remained in the cultures, despite repeated exposure to CD31+ beads. Interestingly, a small number of cells were found to be positive for both CD90 and CD31, although the number of these cells did not appear to change with increasing selections (P=0.205, F=1.835, 2d.f.) (Figure 4.7).

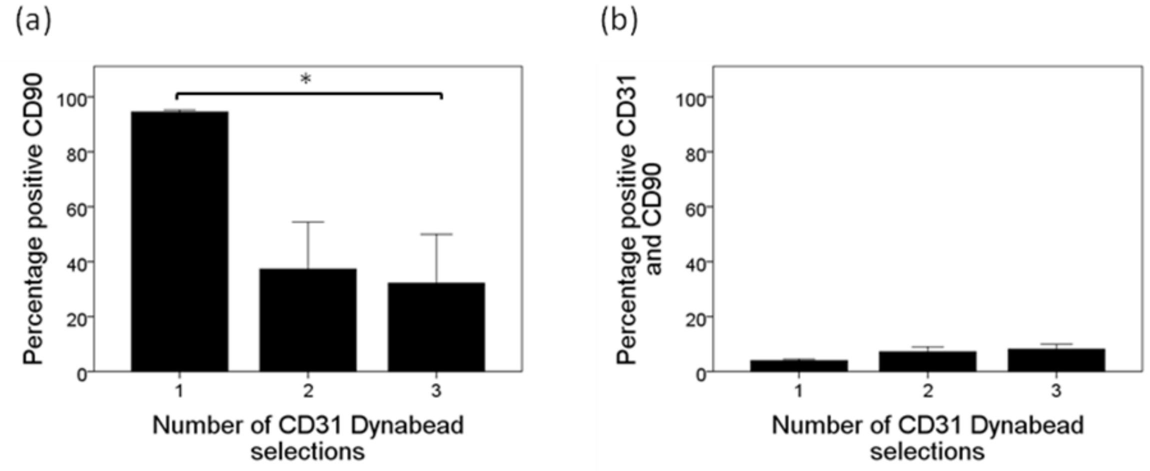


Figure 4.7: Expression of cell surface markers after repeated selection for endothelial cells with Dynabeads. (a) The number of CD90+ cells shows a reduction with increasing number of Dynabead selections * p<0.05;(b) The number of CD31+CD90+ cells does not change with repeated selections.

4.4.4 Endothelial cells from endometriotic lesions

Peritoneal lesions were found to contain too few cells from which adequately to digest and recover any endothelial cells (n=2). Digestion of endometriomas (n=8) and one rectovaginal nodule was successful. Preliminary cultures of endothelial cells could be established, although spindle-like morphology was seen with increasing time in culture. Two very pure endothelial cultures were generated from endometriomas: both exhibited good cobblestone morphology and over 90% CD31+CD90- phenotype by flow cytometry (Figure 4.8).

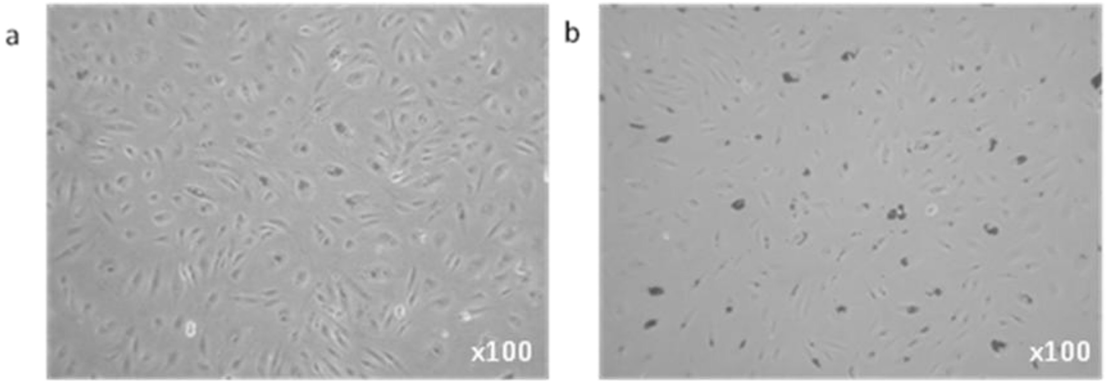


Figure 4.8: Examples of endothelial cell cultures generated from endometriomas, showing confluent cultures of apparently pure endothelial cells.

4.4.5 Artificial contamination of endothelial cultures

The overgrowth of fibroblast-like cells in endothelial cultures may result from small numbers of contaminating stromal cells. These could then exhibit a growth advantage over endothelial cells and gradually increase in number. To assess this, flasks of endothelial cells (HUVEC) were artificially seeded with stromal cells. The resulting cultures were maintained for 6 passages and the number of HUVEC and stromal cells were assessed at each passage (using CD31 and CD90).

Figures 4.9 and 4.10 show the percentage of positive cells at each passage for the different ratios of cells. Even when cultures were contaminated with 25% stromal cells, these rapidly decreased in number with time in passage and an almost pure flask of endothelial cells resulted.

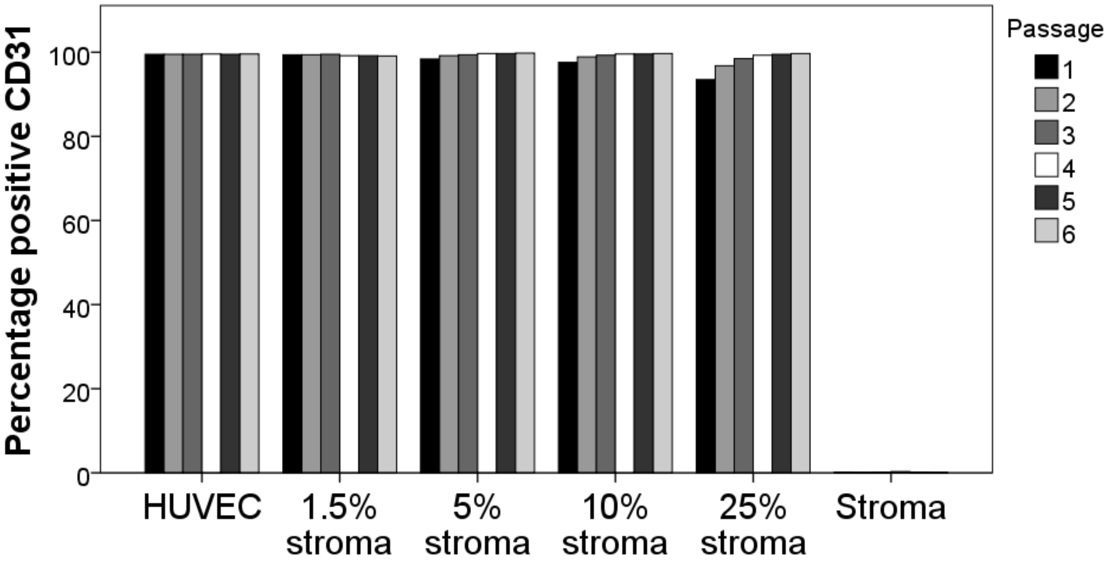


Figure 4.9: CD31 expression in HUVEC cultures artificially contaminated with stromal cells at different ratios. Pure HUVEC and stromal cells are shown for comparison. Even when contaminated with 25% stromal cells, HUVEC cultures rapidly become almost entirely pure with increasing passage in culture.

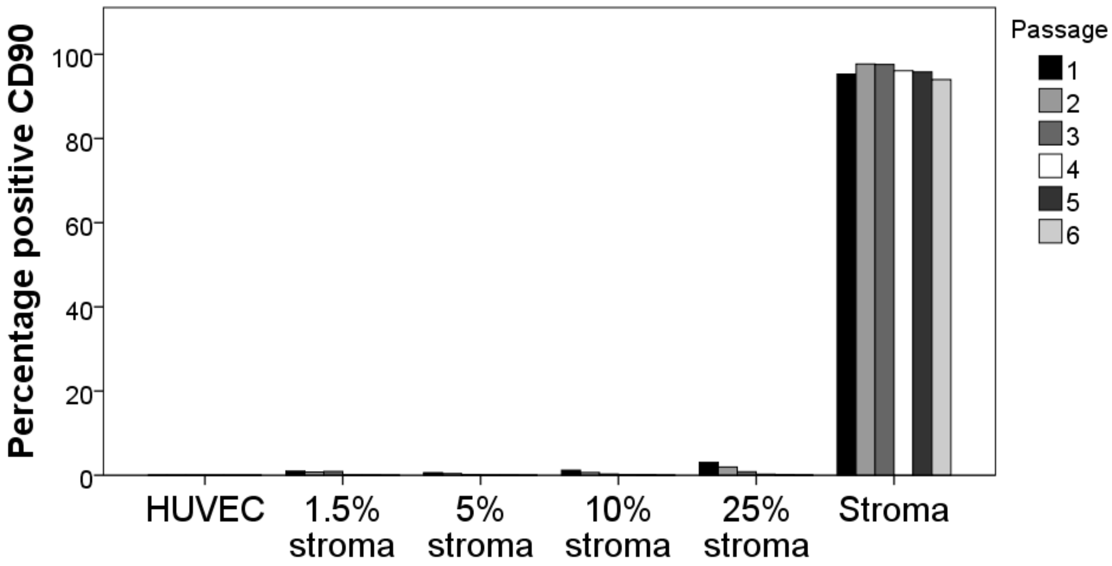


Figure 4.10: CD90 expression in HUVEC cultures artificially contaminated with stromal cells. Pure HUVEC and stromal cells are shown for comparison. Even when contaminated with 25% stromal cells, HUVEC cultures rapidly become almost entirely pure with increasing passage in culture.

As endometrial endothelial cells are derived from small vessels (microvascular endothelial cells), this experiment was also conducted using HMVEC, in order to assess different behaviour of the two cell types.

Contrary to the data obtained from HUVEC, stromal cells did persist in the HMVEC cultures. However, their levels remained static over the course of the experiment (6 passages), suggesting that stromal cell growth did not out-compete the growth of HMVEC and overgrow the flask (Figures 4.11 and 4.12).

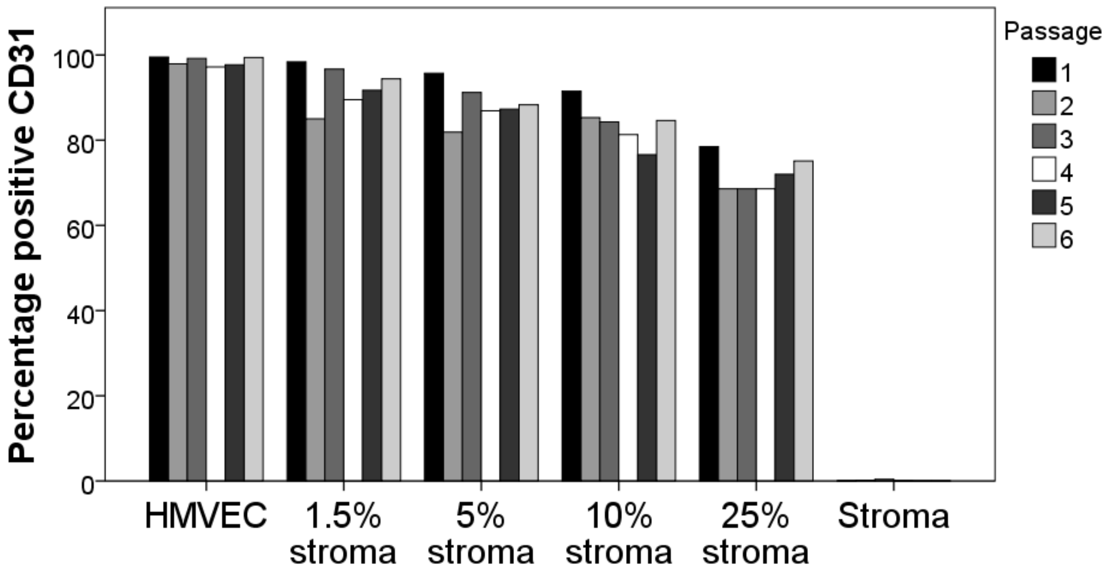


Figure 4.11: CD31 expression in HMVEC cultures artificially contaminated with stromal cells at different ratios. Pure HMVEC and stromal cells are shown for comparison. Contaminating stromal cells persist in the HMVEC cultures, although do not overgrow the endothelial cells with increasing passage in culture.

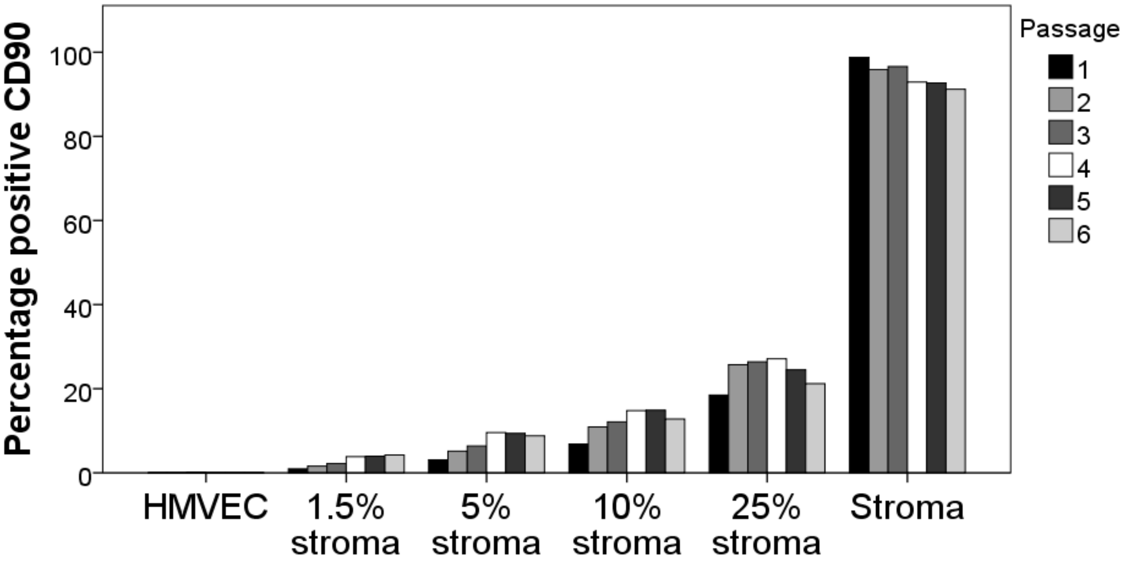


Figure 4.12: CD90 expression in HMVEC cultures artificially contaminated with stromal cells. Pure HMVEC and stromal cells are shown for comparison. Contaminating stromal cells persist in the HMVEC cultures, although do not overgrow the endothelial cells with increasing passage in culture.

4.4.6 Assessment of specificity of CD31 beads

A second possibility for the overgrown cultures was that non-specific binding of CD31 Dynabeads resulted in a large number of stromal cells being erroneously selected by the beads. Therefore, stromal cell cultures were seeded with 1% HUVEC to mimic a newly digested endometrial biopsy (n=2). The mixed suspension of cells was then exposed to CD31 Dynabeads in an identical manner to new cultures. The bead bound cells were grown to confluence and maintained in culture for 6 passages. At each passage the expression of CD31 and CD90 was assessed. Again, an almost perfectly pure culture of CD31 positive cells was found at first and subsequent passages, with minimal to no contamination by CD90 positive cells (Figure 4.13). This indicated that the CD31 beads showed good specificity for HUVEC and minimal non-specific binding to stroma.

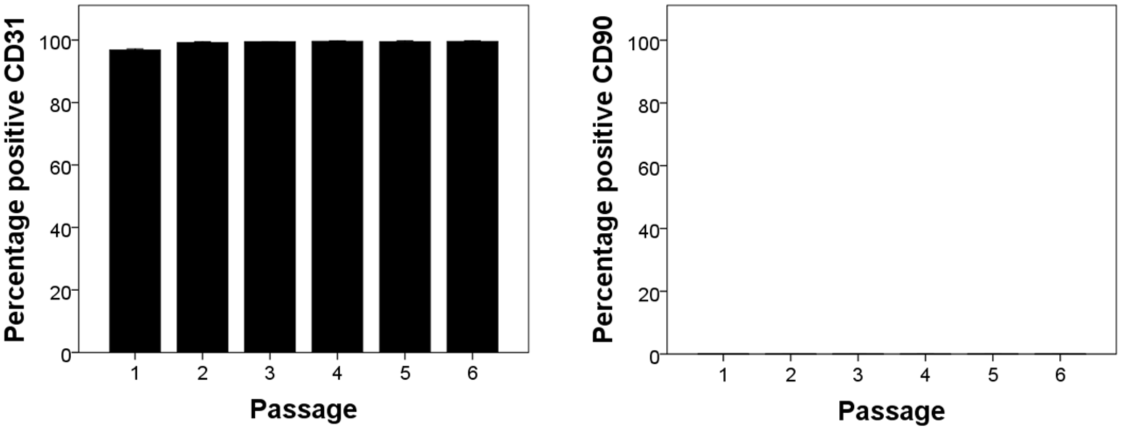


Figure 4.13: CD31 and CD90 expression in cultured HUVEC isolated using CD31 Dynabeads. Even at first passage the culture is almost completely pure for HUVECs, with little contamination by CD90+ stromal cells.

Again, this experiment was repeated using HMVEC, to better model the interaction between stroma and microvascular endothelial cells. In these cultures contamination with CD90 positive cells was higher than that seen with HUVEC – accounting for approximately 20% of cells. Only approximately 75% of cells expressed CD31. However, the ratio of CD31 to CD90 positive cells remained similar throughout the passages, again indicating that stromal cells did not progressively overgrow the cultures (Figure 4.14).

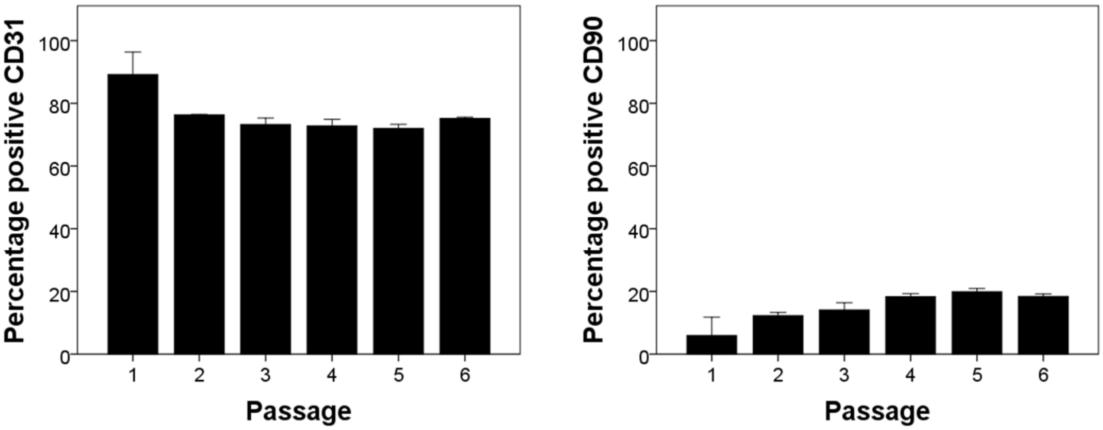


Figure 4.14: CD31 and CD90 expression in cultured HMVEC isolated using CD31 Dynabeads. Despite selection by CD31+ beads, a number of CD90+ stromal cells persist in the culture, and proliferate with increasing passage, although the culture does not become overgrown with stroma.

4.4.7 Culture in smaller flasks

A further possibility for the abnormal morphology seen in the cell cultures was that endothelial cells were cultured too sparsely by plating in T25 flasks. Potentially the cells may require closer contact with neighbouring cells to maintain their typical characteristics. To overcome this, samples were cultured at higher cell density (n=12). After delayed selection with Dynabeads, cells were plated into 15.6mm wells (1.9cm^2 surface area). Five samples were fixed and stained for vWF at this stage: two showed good vWF positivity but the other three were seen to contain bead bound cells without clear vWF staining. The beads were seen to autofluoresce. However, the staining pattern of vWF is clearly distinguished at high magnification (see Figure 4.15).

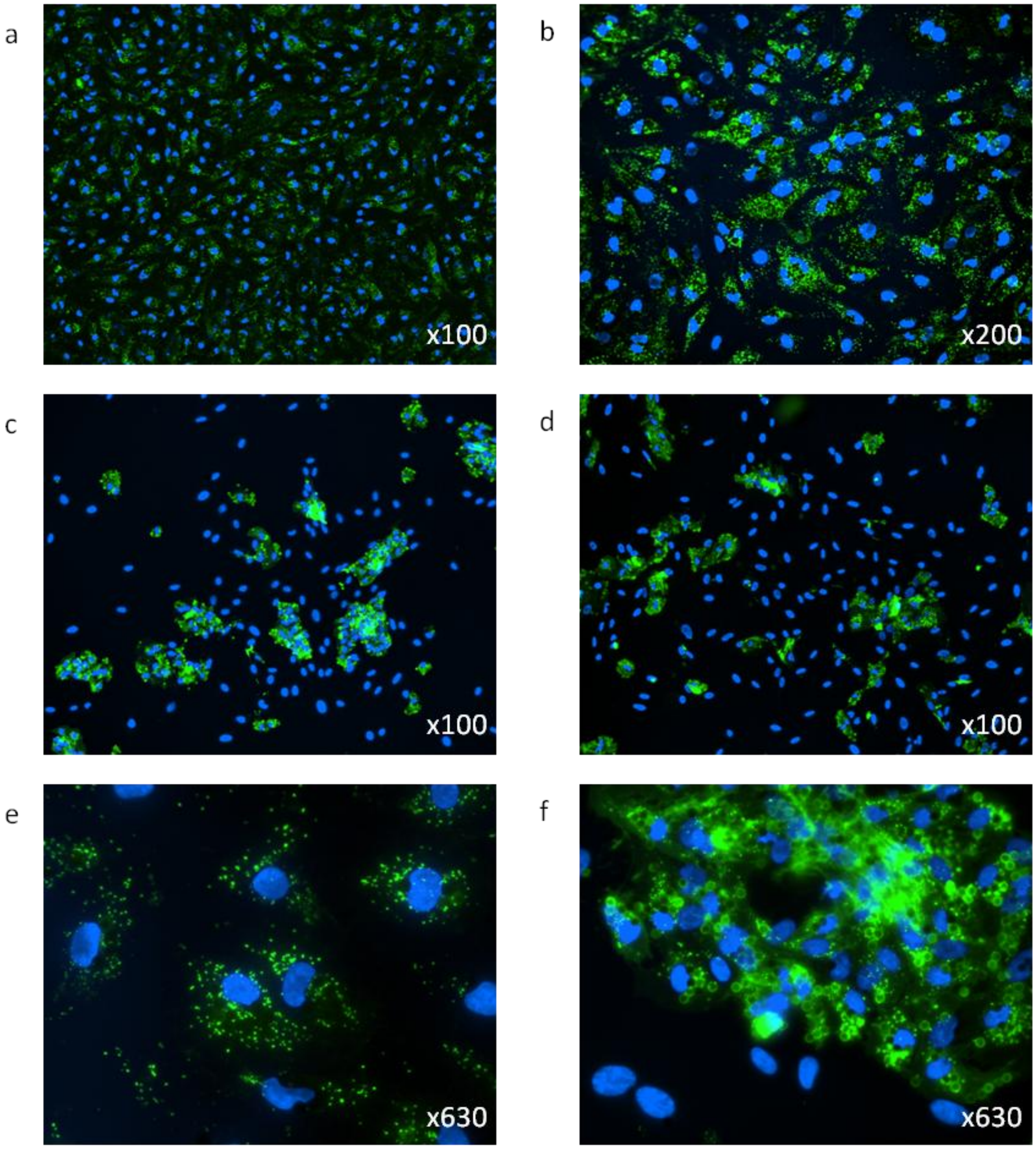


Figure 4.15: vWF staining (green) in first passage cultures, nuclei counterstained with DAPI (blue). (a) vWF staining in almost all cells of primary culture; (b) same culture at higher magnification; (c) and (d) examples of autofluoresence of beads only – note many cells with no surrounding green stain, showing no beads and no vWF staining; (e) high power magnification of endothelial cells, showing typical punctate vWF staining; (f) high power magnification of bead-bound cells, showing large circular staining of beads, with occasional punctate vWF staining.

To try to produce purified cultures containing only CD31 positive cells, cells were exposed to the magnetic field at each passage to isolate the remaining bead-bound cells. This resulted in the loss of some cultures as the magnetic beads had been diluted out prior to passage (n=3). However, four cultures continued to grow at each passage.

Despite this approach, pure endothelial morphology was not seen in the four successful cultures. Although cells generally retained a more rounded morphology, the presence of spindle-like cells was still noted and cells became closely packed (Figure 4.16).

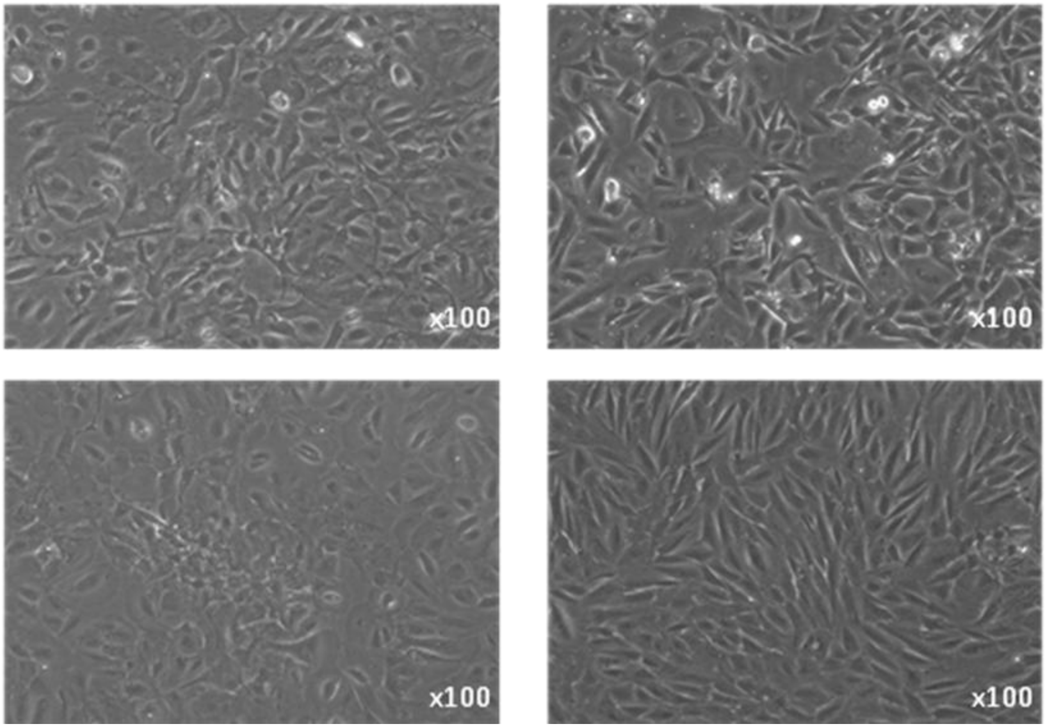
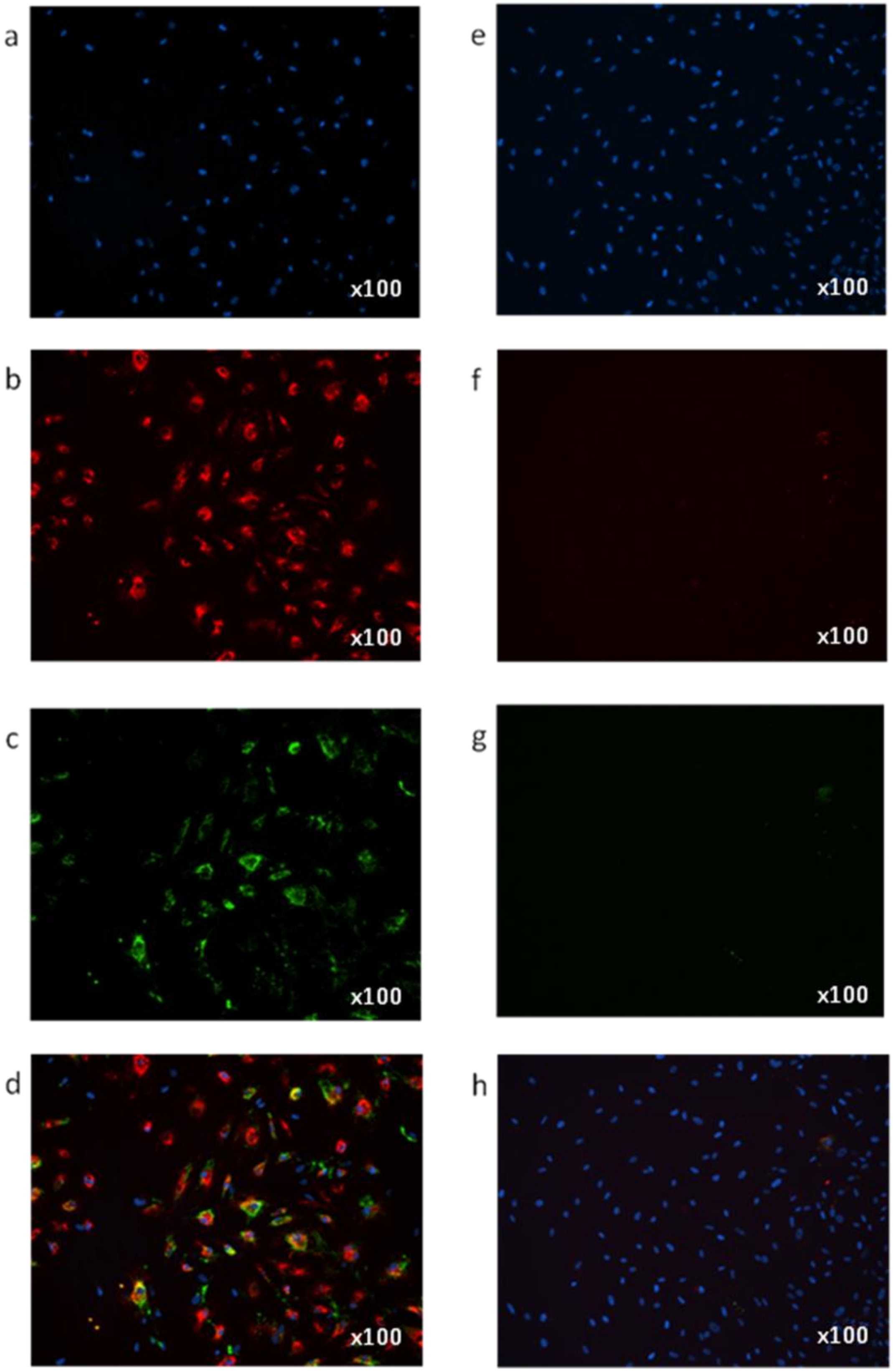


Figure 4.16: Representative images of 4 cell cultures generated by repeated selections of bead-bound cells. All cultures are shown at passage 3.

Immunocytochemistry confirmed the heterogeneous phenotype of the cells. Some were unable to take up acetylated LDL and did not express vWF, suggestive of a non-endothelial phenotype. Others took up acetylated LDL, but did not express vWF: these may represent endothelial cells that have lost some characteristic features in culture. This may suggest that some endothelial features are preserved longer in culture that others. The phenotype of cells also appeared to change with passage, with a reduction in vWF staining and AcLDL uptake with time (Figure 4.17).


a
x100
e
x100
b
x100
f
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c
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g
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Figure 4.17: Assessing endothelial phenotype in cultured cells - representative example of one culture. Blue = DAPI, red = DiI labeled acetylated LDL, green = vWF. Images (d) and (h) are merged pictures. Pictures (a)-(d) show passage 3 cells; pictures (e) to (h) show the same cells at passage 4. Note that cells at passage 3 are almost universally able to take up AcLDL and many express vWF. However, by passage 4 there is almost no vWF expression or AcLDL uptake.

4.4.8 Identification of endothelial cells by flow cytometry

As it proved impossible to generate a pure population of endothelial cells using CD31 Dynabeads, an attempt was made to identify the endothelial population using flow cytometry. The aim of these experiments was to provide preliminary data towards isolating endothelial cells using fluorescence activated cell sorting (FACS).

Freshly isolated endometrial pipelle biopsies were used for enzymatic digestion, to generate single cell suspensions (as described previously) for immediate flow cytometry instead of cell culture.

Showing all events on a typical forward/side scatter profile demonstrated two clear cell populations – a large population with moderate forward and side scatter, and a smaller one with low forward and side scatter (Figure 4.18). The larger population comprised mainly CD90 positive cells, likely to include endometrial stroma, whilst the small one comprised mainly CD45 positive cells, likely to represent endometrial leucocytes (n=3).

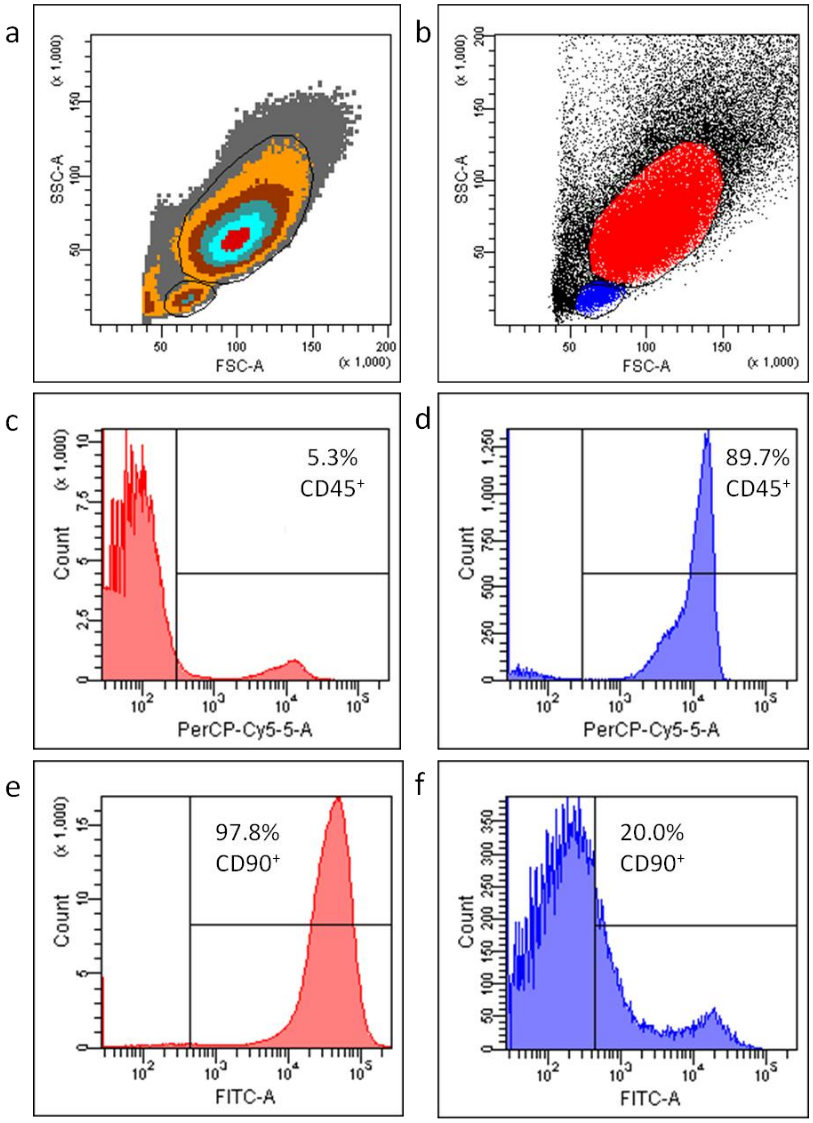

Figure 4.18: Example of flow cytometry analysis of endometrial cells. (a) Two populations revealed on a contour plot; (b) Moderate forward/side scatter population shown in red, and the low forward/side scatter population in blue. These populations are then shown on a histogram plot of CD45 expression; (c) The majority of moderate forward/side scatter cells are CD45 negative; (d) the majority of low forward/side scatter cells are CD45 positive; (e) The majority of moderate forward/side scatter cells are CD90 positive; (f) The majority of low forward/side scatter cells are CD90 negative.

Further analysis of CD31 expression showed no cells in the leucocyte region that expressed CD31 but not CD45. However, a discrete population of cells was identified within the larger, moderate forward/side scatter population that expressed CD31, but not CD45, potentially representing endometrial endothelial cells (Figure 4.19).

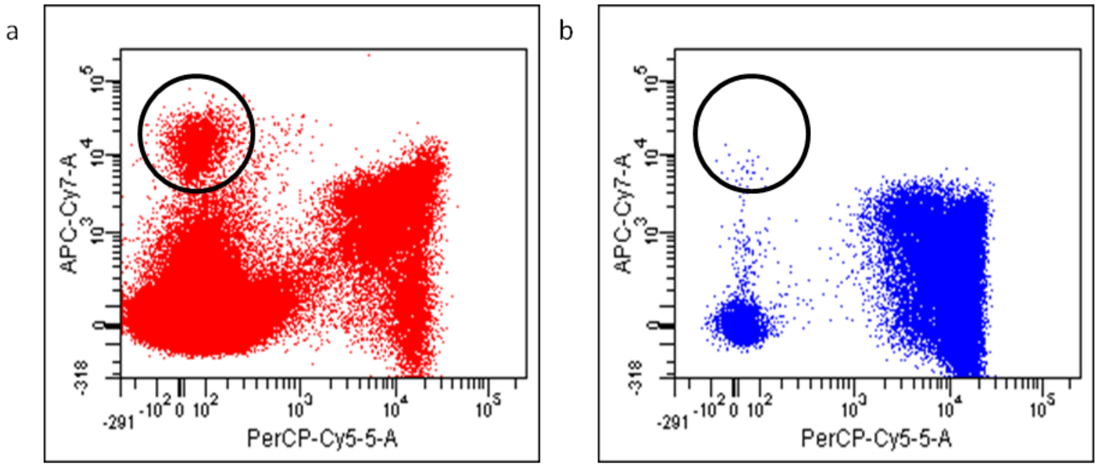

Figure 4.19: CD31 and CD45 expression in "endometrial" and "leucocyte" regions. (a) CD31-APC-Cy7 and CD45-PerCPCy5-5 expression in moderate forward/side scatter cells. A population of CD31+CD45- cells can be seen in the top left corner of the plot (circled); (b) CD31-APC-Cy7 and CD45-PerCPCy5-5 expression in low forward/side scatter cells. The majority of cells are CD45+. No clear population of CD31+CD45- cells is seen.

CD45-CD31+ cells were then further characterised by assessing expression of a variety of cell surface markers (n=3 for each marker, Figure 4.20). These cells were found to express markers consistent with an endothelial cell phenotype, including CD144, CD105 and CD34. Expression of CD14 (macrophage marker) was consistently low. Expression of CD10 was more variable – this marker is present on both endometrial stromal cells and endothelial cells. Interestingly, cells were also strongly positive for CD90 – which has been previously reported to be present on activated endothelial cells.

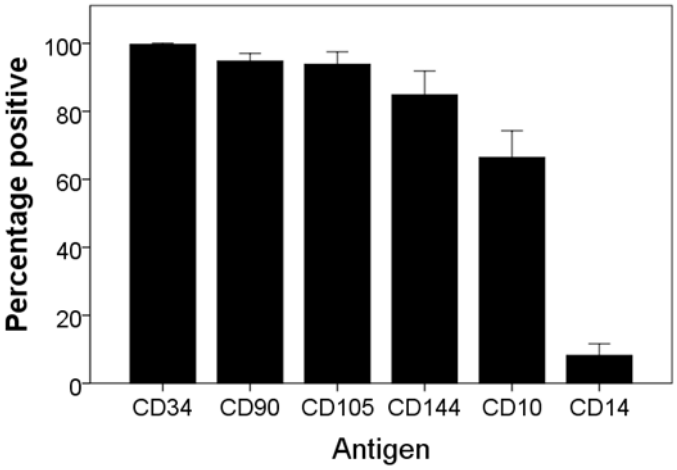

Figure 4.20: Expression of cell surface markers on CD31+CD45- cells. High expression is seen for CD34, CD90, CD105 and CD144. Moderate expression of CD10 is also seen, with low levels of CD14.

4.4.9 Numbers of endothelial cells possible to obtain

From the samples analysed (n=9), CD31+CD45- endothelial cells comprised 0.48±0.31% of the moderate forward/side scatter population. This represents a potential yield of approximately 7,287 ± 4,621 endothelial cells per pipelle sample.

4.5 Discussion

This chapter describes the attempts to isolate a pure population of endothelial cells from eutopic endometrium and endometriotic lesions. The experiments were unsuccessful because the cell culture did not have sufficient endothelial cell marker purity.

A major difficulty in isolating endothelial cells was the heterogeneity of starting cultures. Different phases of the menstrual cycle as well as individual variation between patients resulted in highly variable biopsies. In addition, it was not always possible to obtain adequate samples. For example, some biopsies comprised scanty mucus and blood only, with no visible endometrial tissue. Even those samples containing tissue often included large areas of blood clot, cervical mucus or debris. Furthermore, the size of the biopsies was often extremely small. This made it challenging to compare different tissues, and standardise a method for their culture.

The varying number of cells acquired with each biopsy also meant that standardising the growth and number of passages of cells was impossible. A confluent flask might be reached within a day, or not for up to one week.

However, the main problem encountered was the varied morphology of endothelial cultures. This is suggestive of either contamination with another cell type, or a change in the morphology of cultured endothelial cells with time.

4.5.1 Possible contamination with different cell types

Potentially, selection of bead bound cells may include contaminating cell types. This may be due to difficulty in isolating a single cell suspension – clumping of cells sometimes resulted in bead bound cells being seen attached to non-bead bound cells in early cultures. This would have enabled contamination of endothelial cultures with other cell types. However, every effort was made to ensure a single cell suspension from the primary tissue, including lengthy digestion of tissue (1 hour) and filtering through a 70μm strainer to remove cell clumps. Repeated selections should also have reduced this problem, as they allow more opportunities to remove non-endothelial cells.

Non-specific binding of Dynabeads to other cell types may have been a problem. Some cell types may directly phagocytose the Dynabeads - to reduce this possibility bead incubations were carried out at 4°C. The incubation time was also reduced to ten minutes to minimise non-specific binding to other cell types.

Isolating HUVEC or HMVEC from cultures of stromal cells gave mixed results. Resulting HUVEC cultures were devoid of contaminating stromal cells, whilst HMVEC cultures contained around 20% CD90 positive cells. This suggests that non-specific binding to stromal cells may be a problem, although it is unclear why this was the case with microvascular and not macrovascular cells. Different proliferation rates of these endothelial cells in culture may have contributed – more rapid proliferation of HUVEC has been reported, and this may have limited the growth of any stromal contaminants (Beekhuizen and van Furth, 1994).

CD31 is also expressed by some haematopoietic cells, notably monocytes/ macrophages and, therefore, these cells may also have been isolated by the beads. However, cultured activated monocytes or macrophages are known to require specific conditions for cell culture to prevent them becoming firmly adherent to tissue culture flasks, preventing their removal with trypsin (Andreesen et al., 1983). Furthermore, maintenance of monocytes requires liquid suspension culture, without which the cells terminally differentiate into non-replicating macrophages (Salahuddin et al., 1982). It is, therefore, unlikely that any contaminating macrophages would have remained in culture for longer than the first passage.

Alternatively, it is possible that the stromal cells have a marked growth advantage in culture over endothelial cells. If this is the case then even a small amount of stromal contamination in the cultures could result in the endothelial cell flasks being gradually overgrown with stroma. This appeared not to be so with macrovascular endothelial cells (HUVEC), but persistent growth of CD90 positive cells was seen in cultures of microvascular cells (HMVEC). However, despite the presence of CD90 positive cells they did not appear to dramatically overgrow the HMVEC cultures. Endometrial endothelial cells may have different growth characteristics to either HUVEC or HMVEC, however, and have the potential to become overgrown by contaminating stroma. Ideally these experiments would be conducted with endometrial endothelial cells, but these cells are not commercially available.

These endothelial and stromal mixing experiments also cannot recapitulate the possible adhesion of endothelial cells to other cell types as a consequence of poor tissue digestion. Whilst every effort was made to ensure a single cell suspension was produced, it is possible that some endothelial cells remained bound to other cell types, leading to a contaminated culture.

A particular problem was assessing the purity of cultures at early passage. Bead bound endothelial cells display autofluoresence with both fluorescence microscopy and flow cytometry, making assessment of antigen expression difficult. It was necessary to wait until sufficient numbers of cells were generated to assess them by flow cytometry, by which time the appearance of cell cultures was often greatly changed from their initial appearance.

4.5.2 Possible change in endothelial cell morphology

If contaminating cells were not responsible for the abnormal morphology and cellular phenotypes seen, then these must be due to a change in the appearance of endothelial cells in culture.

Previous work has demonstrated that endothelial cells can transdifferentiate in culture, i.e. change their phenotype to show characteristics of other cell types. This has been shown for dermal (Tuder et al., 1990), aortic valve (Paranya et al., 2001) and glomerular endothelial cells (Sommer et al., 2005). Transdifferentiated endothelial cells become elongated, and spindle-shaped in culture, forming sheets of close packed cells (Lipton et al., 1991). Furthermore expression of characteristic endothelial markers, such as vWF, is also lost (Lipton et al., 1991). This would be consistent with the cellular phenotype found in the cultures described above.

The inability to verify a 100% pure endothelial cell population meant that the conversion of endothelial cells into a different phenotype could not be confirmed – the possibility of contaminating cells remains. However, it is plausible that transdifferentiation is occurring *in vitro*, either as a consequence of the culture conditions or the nature of the endothelial cells. Of interest, one previous paper, identifying endometrial endothelial cells, refers to the occasional presence of densely packed endothelial cells with different morphology in later passages (Sha et al., 2007). The appearance of these cells resembles that of cells seen in the experiments above.

4.5.3 Previous work

Previous reports have described successful isolation of apparently pure cultures of endometrial endothelial cells, with the use of magnetic-bead conjugated antibodies. The first report of successful isolation came from Iruela-Arispe and colleagues (Iruela-Arispe et al., 1999), who utilised growth medium promoting endothelial cell growth and fibronectin coated culture plates to select endothelial cells. This was followed by exposure of the cells to magnetic beads conjugated to CD34. The use of these beads gave resulting cultures of cells that could take up DiI labelled acetylated LDL, expressed vWF and CD31 and had the morphology of endothelial cells. Similar results were found by other authors with beads conjugated to *Ulex europaeus* agglutinin-1 (UEA) (Nikitenko et al., 2000) or CD105 (Schatz et al., 2000).

Given the difficulties described in the present study in obtaining a pure culture of endothelial cells, these results are perhaps a little surprising. However, a critical difference is that all the previous studies used endometrial samples obtained from hysterectomy specimens or endometrial curettage. The present study is the first to attempt to isolate endothelial cells from pipelle biopsies. The advantage of pipelle biopsies is that they may be obtained without the need for surgery, making them a possible source of tissue for biomarkers. However, pipelle samples tend to result in much smaller quantities of endometrium from which to isolate cells. Secondly, endometrial tissue scraped from the uterus (*in situ* or *ex vivo*) may have important differences to that obtained by pipelle biopsy. In particular, pipelle samples generally obtain tissue from the upper, functionalis layer of the endometrium, and are unable to sample the basalis (Taylor et al., 2001; Talbi et al., 2006; Williams et al., 2007). Immunohistochemistry has previously identified a significantly higher proliferation rate in endothelial cells from the functionalis, compared to the basalis (Rogers et al., 1998). This may mean that isolated endothelial cells from the functionalis have already undergone multiple cell divisions *in vivo*, and are unable to continue to divide and grow in culture.

Other studies have also used different antibodies to isolate endothelial cells. In particular, some authors have used CD105 (Kayisli et al., 2004; Luk et al., 2005) and CD34 (Iruela-Arispe et al., 1999; Sha et al., 2007). However, the present data (see Chapter 5), and those of others (Schwab and Gargett, 2007) show CD105 to be expressed on endometrial stromal cells and stem cells, making this unsuitable for isolating endothelial cells. Furthermore, although CD34 does not appear to be present at high levels on cultured endometrial stromal cells or stem cells (Chapter 5, and Schwab and Gargett, 2007) expression of CD34 has been shown on freshly isolated stromal cells (see appendix, Figure 7.3), again making this an unsuitable antibody to identify pure endothelial cells.

Two papers have previously reported the use of CD31 Dynabeads to isolate endometrial endothelial cells from hysterectomy specimens (Koolwijk et al., 2001; Bredhult et al., 2007). Koolwijk and colleagues were able to isolate endothelial cells from only 13 out of 33 endometrial samples (Koolwijk et al., 2001). The authors of the second paper did not describe their success rate, and showed no data to verify the endothelial phenotype of the cultured cells (Bredhult et al., 2007). The use of UEA may be a suitable alternative to CD31 (Iruela-Arispe et al., 1999; Nikitenko et al., 2000).

4.5.4 Summary

Given the challenges of generating a reliable and pure population using magnetic beads, future work will concentrate on the use of FACS to isolate a pure population of endothelial cells. However, work presented here indicates that a pure endothelial cell population could be isolated with the use of just two antibodies (CD45 and CD31). These cells could then be maintained in culture and assessed for phenotypic and functional differences that may be of relevance to endometriosis.

Chapter 5: Endothelial-like features of endometrial stromal cells

5.1 Introduction

The endometrium is one of the most regenerative tissues in the body. Continual cyclical stimulation by estrogen and progesterone during the reproductive years promotes proliferation of endometrial glands and stroma, preparing the uterine environment for embryo implantation. In the absence of implantation this lining disintegrates and is shed at the time of menses, only to be reconstructed again with the next cycle.

The remarkable proliferative ability of cultured endometrial stromal cells has been recognised for some time (Dormon et al., 1982; Fleming and Gurpide, 1982). However, it is only recently that the endometrium has been recognised to contain a highly clonogenic stem cell population. The first paper to describe the existence of endometrial stem cells was published by Caroline Gargett's group (Chan et al., 2004). These authors demonstrated that plating endometrial stromal (and epithelial) cells at clonal density resulted in the formation of colonies of cells derived from a single precursor. Whilst the overall cloning efficiency was relatively low (only 1.25% for stromal cells, 0.22% for epithelial cells), after 10-12 days in culture, the growth of some colonies increased dramatically, leading to large numbers of daughter cells. This strongly suggests that a proportion of endometrial stromal cells can self-renew profusely. This is a feature often associated with stem cells, which have been described as cells that "have the capacity both to self-renew and to generate differentiated progeny" (Morrison et al., 1997).

The production of differentiated progeny from these clonogenic, endometrial stromal cells has been demonstrated by several groups. Differentiation towards a variety of cell fates has been shown, including chondrocytes, adipocytes and neurons, implying that these cells are pluripotent (Schwab and Gargett, 2007; Dimitrov et al., 2008; Wolff et al., 2011).

One well studied and widely available stem cell population are mesenchymal stem cells, or MSC. These are multipotent cells, originally discovered in bone marrow, but subsequently shown to be present in a wide variety of tissues (Pittenger et al., 1999; Dominici et al., 2006). Similarities have been noted between MSC and endometrial stem cells (Schwab and Gargett, 2007). In particular, cell surface markers present on endometrial stem cells are in keeping with those recommended for identifying MSC – CD105 (endoglin), CD90 (Thy-1) and CD73 (Dominici et al., 2006; Dimitrov et al., 2008; Gargett et al., 2009). MSC have previously been recognised as playing a role in angiogenesis, demonstrated *in vitro* by their ability to form tubes on basement membrane extract (Kim et al., 2012). MSC also support endothelial cells in tube formation assays (Tögel et al., 2007), and have been shown to differentiate into endothelial cells *in vitro* (Oswald et al., 2004; Alviano et al., 2007).

Given the similarities between MSC and endometrial stromal stem cells, it was appropriate to investigate whether these cells could also be stimulated to differentiate towards an endothelial cell like phenotype with the use of appropriate media. Further, endometrial stromal cells were assessed for the ability to participate in tube-formation assays, in a manner analogous to MSC. Finally, the effects of endometriosis on stromal cell behaviour was studied in these assays – using cells derived from women with and without the disease.

5.2 Aims

- To characterise surface marker expression of cultured endometrial stromal cells.
- To assess the effects of endothelial differentiation medium on endometrial stromal cells.
- To assess the participation of endometrial stromal cells in tube formation assays.
- To compare the pro-angiogenic effects of stromal cells derived from women with and without endometriosis.

5.3 Materials and methods

5.3.1 Endometrial stromal cell isolation

Endometrial biopsies were collected at the time of laparoscopy, and enzymatically digested as described in Chapter 4.

After digestion, endometrial cells were resuspended in stromal cell medium (DMEM containing 10% FCS, 100U/ml penicillin and 100mg/ml streptomycin) and cells were cultured in a 175cm^2 flask until confluent. Further purification steps were not necessary – epithelial cells, endothelial cells and contaminating leucocytes either failed to grow in stromal cell medium, or did not survive the first passage *in vitro*.

For some cultures, endothelial cells were removed from the primary cultures with the use of CD31 conjugated Dynabeads (as described in Chapter 4). After removal of bead bound cells, remaining cells were maintained in stromal cell medium, as above.

5.3.2 Endometrial stromal cell culture

Cells were maintained in culture using stromal cell medium. When confluent, cells were harvested by washing with sterile PBS and adding trypsin-EDTA (Sigma-Aldrich, Poole, UK) at 37^{o}C for approximately 3 minutes to detach cells from the flask. Trypsin solution was then neutralised with the addition of an equal quantity of stromal cell medium. Cells were pelleted by centrifugation at 1500rpm (470 x *g*) for 3 minutes. The cell pellet was then resuspended in stromal cell medium and cells were replated at a ratio of 1:3, unless otherwise stated.

5.3.3 Flow cytometric analysis of cell surface markers

Flow cytometry was used to identify surface marker expression on stromal cells. All experiments were carried out using a BD LSR II flow cytometer as described previously (Chapter 2). Where necessary (for multicolour experiments), colour compensation was calculated using single antibody stained compensation beads. Cells were harvested as described (5.3.2). The resulting cell pellet was resuspended in PBS containing 2% FCS in preparation for flow cytometry. The antibodies used are shown in appendix 7.5.

FACS DIVA was used to acquire flow cytometry events. The data were imported to FlowJo software for analysis. Colour compensation was applied where appropriate (for multicolour experiments). Percentage positivity was determined using the Overton method of histogram subtraction (Overton, 1988) which compares a fully stained sample with the background staining seen in an isotype control tube. The percentage of stained cells can be calculated by comparing the two histograms.

5.3.4 Differentiation of stromal cells

Two separate culture media were used to stimulate endothelial differentiation of stromal cells. The first was a standard microvascular endothelial cell growth medium - ECGM MV2 (Promocell, Heidelberg, Germany) which includes a variety of growth factors and supplements to encourage microvascular endothelial cell growth (see appendix). The other (called VEGF medium) contained low serum and a high concentration of VEGF: basal endothelial growth medium (ECGM, Promocell, Heidelberg, Germany) with 2% FCS and 50ng/ml recombinant human VEGF-121 (Promocell, Heidelberg, Germany).

Stromal cell cultures derived from 3 subjects were used. After the first passage, cells were split and seeded at 12,000 cells/cm^2 into three 25cm^2 flasks. One flask contained standard stromal cell medium, one contained ECGM MV2 and the third contained VEGF medium. For cells in VEGF medium, flasks were pre-coated with 1% gelatin for 30 minutes before plating. Further passages maintained the cells in the same medium and conditions. Cells were cultured for a total of 21 days.

5.3.5 Analysis of differentiated stromal cells

Photographs of each cell culture were taken at each time point with a Leica DMIRB microscope and Orbit Improvision camera for morphological assessment. Cells were then harvested and flow cytometric analysis of cell surface markers was carried out on days 3, 7, 14 and 21. The antibodies used are shown in appendix 7.5.

At the final time point, cells were plated onto glass cover slips in a four-well plate for immunocytochemistry and used for a tubule formation assay (see below).

5.3.6 Immunocytochemistry

Immunocytochemistry and identification of AcLDL uptake was carried out as described in Chapter 4. The primary antibodies used are shown below (Table 5-A).

Table 5-A: Primary antibodies for immunocytochemistry.

Antigen	**Manufacturer**	**Dilution**	**Final concentration**
von Willebrand Factor (vWF)	AbCam	1:800	0.5µg/ml
CD31	BioLegend	1:500	1µg/ml

5.3.7 Tubule formation assay

For each assay, 35µl basement membrane extract (RnD systems, Minneapolis, USA) was added to each well of a 96 well, flat-bottomed plate. Plates and basement membrane extract were kept on ice throughout the procedure. When all wells were filled, the plate was placed at 37^{o}C for at least 30 minutes to allow the extract to gel.

After the extract had set, cells were lifted from flasks with trypsin, harvested and counted; 15,000 cells were used for each assay. Cells were diluted in serum free DMEM medium (unless otherwise stated) to give a final concentration of 15,000 cells in 100µl medium. 100µl of cell suspension was transferred to each well and incubated at 37^{o}C. Plates were then inspected at 4 hours to assess tubule formation using a Leica DMIRB phase contrast microscope.

5.3.7.1 Tubule formation assay for cells exposed to different media

After 21 days culture in each type of medium, cells were used for a tube formation assay. Each experiment was carried out in triplicate (i.e. three wells for each cell line in different media).

5.3.7.2 Tubule formation assay comparing endometrial stromal cells derived from women with and without endometriosis

Cells derived from women with (n=10) and without (n=10) endometriosis were compared. All women had been free of exogenous hormones for at least 3 months prior to participating. Each experiment was carried out in duplicate.

5.3.7.3 Tubule formation assay combining HMVEC and endometrial stromal cells

Endometrial stromal cells and HMVEC were co-cultured to assess whether they were able to co-operate in a tubule formation assay. Preliminary experiments indicated that the optimum ratio to plate was 1 stromal cell: 3 HMVEC (data not shown). Therefore, cells were mixed in this ratio before plating at 15,000 cells per well. HMVEC alone were also plated. Stromal cells derived from women with (n=6) and without (n=6) endometriosis were used. All experiments were conducted in triplicate.

5.3.7.4 Tubule formation in conditioned media

The effect of conditioned medium from stromal cells on endothelial cell tube formation was assessed. Endometrial stromal cells from women with (n=6) and without (n=6) endometriosis were cultured to confluence. At confluence, cells were washed once with PBS and the medium was changed to serum free DMEM. After 72 hours, conditioned medium was collected. The medium was centrifuged at 1300rpm for 3 minutes to remove debris and dead cells, and the supernatant was aspirated and retained.

HMVEC were used for tubule formation assays. Cells were harvested and counted as above; 15,000 cells were used for each assay. However, conditioned medium from control stromal cells (non-endometriosis) or endometriosis stromal cells was used in place of serum-free DMEM for the duration of the assay. The tubes formed in conditioned medium were compared to those formed in serum-free DMEM (n=4) or ECGM MV2 (n=4). All experiments were conducted in triplicate.

5.3.8 Image analysis for tubule formation assays

One photograph of each tubule formation assay was taken at low magnification (x40) using a Leica DMIRB phase contrast microscope with an Orbit Improvision camera. All images were centred on the middle of the well to ensure consistency. Images were uploaded to an online analysis system (Wimasis), which processes individual images for a variety of parameters including total tubule length, mean tubule length, number of tubules, number of branch points and number of loops formed. An example is shown in Figure 5.1. Mean (± SD) values for all parameters were calculated for each replicate experiment.

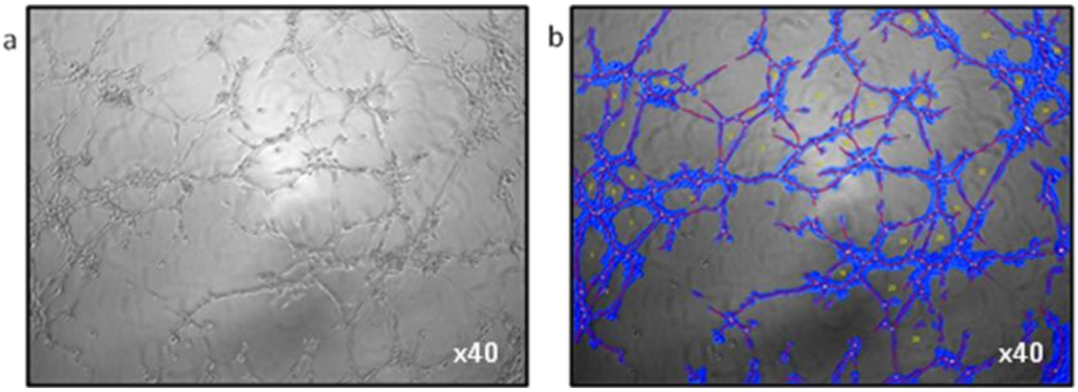

Figure 5.1: Example of tubule analysis. (a) original image; (b) processed image after analysis.

5.3.9 Statistical analysis

Results in the text are expressed as mean values ± SD. All graphs show mean values ± SE.

5.3.9.1 Cell surface markers

Small numbers of cell lines (n=3) were used to assess the effect of different media on stromal cells, as well as to compare cell surface markers between cells exposed and not exposed to Dynabeads. The results are therefore displayed graphically without statistical analysis.

5.3.9.2 Tube formation assays

Tubule formation parameters were confirmed to be normally distributed using the Kolmogorov-Smirnov test. Five parameters were measured for each tubule assay and, therefore, a P value of <0.01 was taken as statistically significant (to correct for multiple comparisons).

5.3.9.2.1 Stromal cells exposed to different media

Comparison of tube formation in cell lines grown in different media was performed with repeated measures ANOVA, as the same three cell lines were grown in all three media types. Post-hoc analysis was conducted with a Bonferroni correction.

5.3.9.2.2 Tube formation by stromal cells of women with and without endometriosis

Comparison of tube formation parameters between cells derived from women with and without endometriosis was conducted with a Student T-test.

5.3.9.2.3 Tube formation by HMVEC - in combination with stromal cells and in conditioned medium

Comparison of tube formation for HMVEC with and without stromal cells (3 groups) and HMVEC in conditioned medium (4 groups) was conducted with one-way ANOVA. Post-hoc analysis of the ANOVA results was conducted with Bonferroni correction for multiple comparisons.

5.3.9.2.4 Demographic data

All continuous demographic data were confirmed to be normally distributed (age and BMI). Data from women with and without endometriosis were compared with a Student T-test. Ordinal data were compared with Fisher's exact test.

5.4 Results

5.4.1 Confirmation of stromal cell phenotype and characterisation

Cultured stromal cells demonstrated typical morphological features and growth patterns. Specifically, cells exhibited an elongated, spindle-shaped morphology in culture and grew abundantly in DMEM with 10% serum. To confirm the purity of stromal cell cultures, 6 separate cell lines were assessed by flow cytometry at the second passage. Endothelial cells had been removed from three of these cell lines using CD31 Dynabeads (see Chapter 4) at the first passage. The remaining cell lines had not been exposed to Dynabeads (n=3). The phenotypic markers used included:

- CD90 (Thy-1, characteristic stromal cell marker)
- CD10 (membrane metallopeptidase found on endometrial stroma)
- CD45 (pan-leucocytic marker)
- CD31 (PECAM-1, endothelial marker)
- CD105 (Endoglin, endothelial marker, also expressed by endometrial stromal stem cells)
- CD34 (endothelial marker and haematopoietic stem cell marker)
- CD146 (MUC18, perivascular and endothelial marker, expressed by endometrial stromal stem cells)
- PDGFRβ (pericyte receptor, also expressed by endometrial stromal stem cells)

The percentage of positive cells for each antigen is shown below (Figure 5.2). All cell lines studied (n=6) showed high expression of CD90 (99.10±0.79% positive).

Similarly, and as expected, the majority of cells were positive for CD10 (85.4±11.85%).

Consistently low amounts of staining were seen with the leucocyte marker CD45 (4.59±3.98%) and endothelial marker CD31 (0.57±0.58%), demonstrating minimal contamination with haematopoietic and endothelial cells.

Of interest, a large number of cells in every culture were also positive for CD105 (88.00±7.73%), CD146 (81.07±12.05%) and PDGFR-β (64.58±19.78%) – markers previously used to isolate and characterise endometrial stromal stem cells. This indicates that the majority of isolated and cultured stromal cells may represent endometrial stromal stem cells, likely due to the growth advantage in culture for cells with self-renewing properties. The expression of CD34 (10.91±9.25%) was more variable, although for most cell lines was <15%.

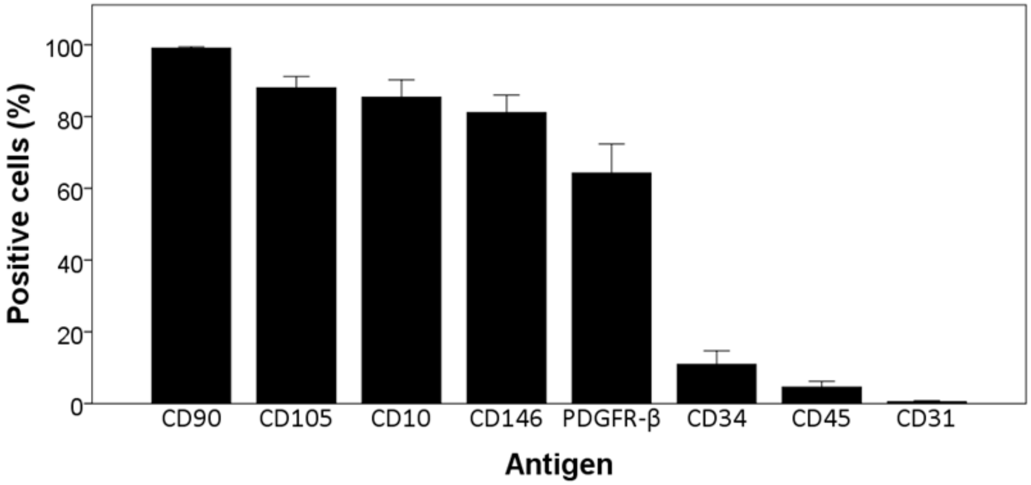

Figure 5.2: Expression of cell surface antigens in endometrial stromal cells.

Expression of cell surface markers was similar between cultures that had been exposed to CD31 Dynabeads and those that had not (Figure 5.3 below, Table 7-E, appendix). It appeared that levels of CD45 expression were slightly reduced in cultures that had been exposed to Dynabeads, perhaps due to the removal of contaminating macrophages by the CD31 beads.

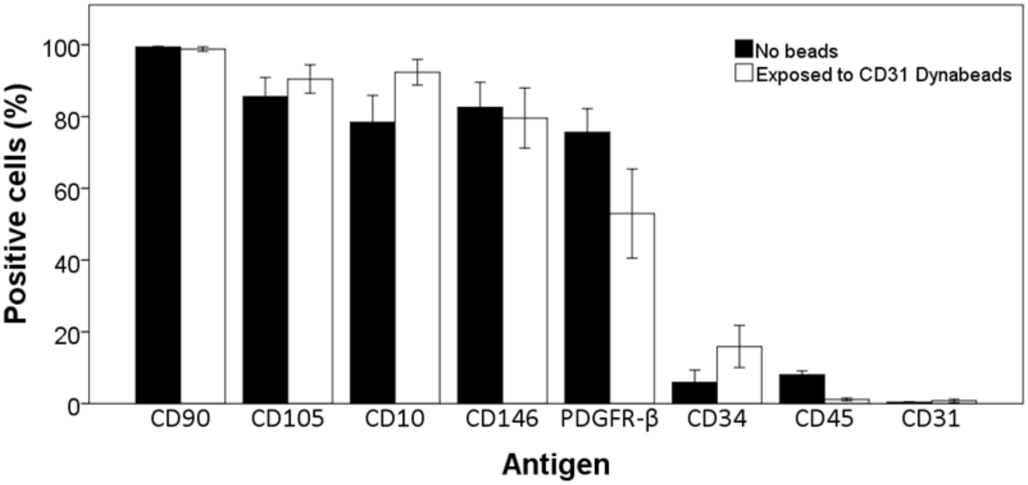

Figure 5.3: Comparison of cultures exposed and not exposed to CD31 Dynabeads.

5.4.2 Morphology of endometrial stromal cells in different media

The high expression of "stem cell" markers (CD146 and PDGFR-β) in stromal cell cultures indicates that a significant proportion of cultured endometrial stromal cells were stromal stem cells. Hence, these cells were assessed to determine if they could be stimulated to differentiate towards an endothelial-like phenotype. Cultures of stromal cells (n=3) were assessed at baseline and four time points (3, 7, 14 and 21 days).

The morphology of the cells did not change with time, nor with different media. Specifically, stromal cells retained their characteristic elongated spindle shape. Cells maintained in endothelial growth medium grew more rapidly than those maintained in DMEM, and became closely packed. However, they did not form cobblestone colonies resembling endothelial cells. Representative images from one cell line are shown below (Figure 5.4).

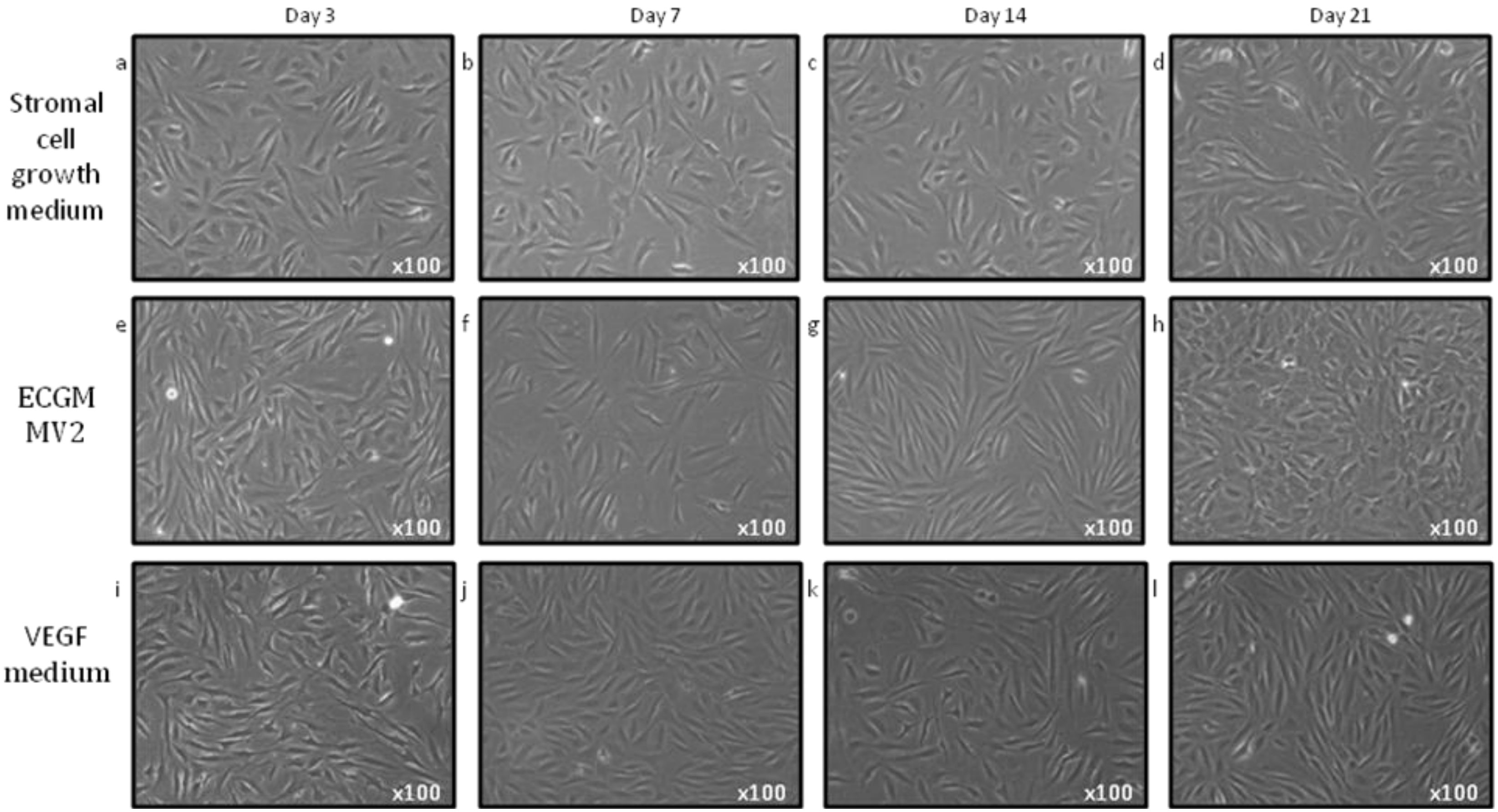

Figure 5.4: Representative images of one stromal cell line grown in three different types of media over 21 days. Images (a)-(d) show cells grown in standard DMEM; Images (e)-(h) show cells grown in ECGM MV2; Images (i)-(l) show cells grown in VEGF medium.

5.4.3 Cell surface phenotype of endometrial stromal cells in different media

Expression of a variety of cell surface markers was assessed at baseline and each time point as the cells grew in different media (3, 7, 14 and 21 days) (Figure 5.5). In all types of media, expression of endothelial markers CD31 and CD144 remained extremely low, and did not appear to change with duration of exposure. Expression of CD45 was also low in all cell cultures. However, the percentage of cells positive for CD45 tended to increase marginally with duration in culture. This appeared to be the case for all media types.

Expression of CD90 was consistently high for all cell lines in all media types. However, expression levels also tended to reduce slightly with increasing time in culture.

Expression of CD105 showed the most marked change with time in each medium. Initial cultures showed high levels of CD105 expression; however, expression noticeably reduced over the course of 21 days for all three media types.

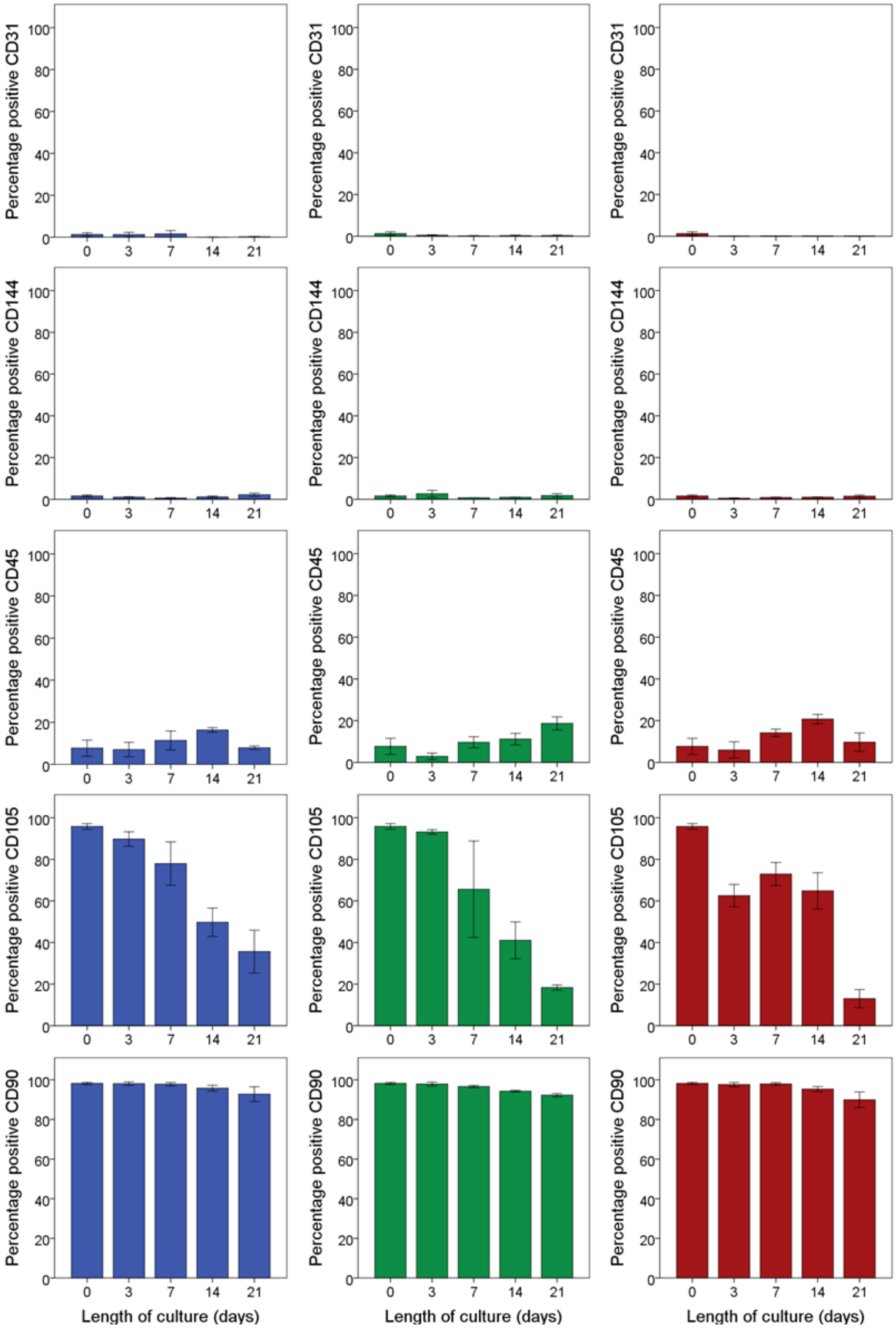

Figure 5.5: Change in expression of surface markers with time in different media. Blue bars show cells maintained in stromal growth medium, green bars show cells in ECGM MV2, red bars show cells in VEGF medium.

After 21 days exposure to the different media, cells were incubated with DiI AcLDL to identify the presence or absence of scavenger receptors (typically expressed by endothelial cells). Immunocytochemistry was then performed to identify the expression of typical endothelial markers (CD31 and vWF). Immunocytochemistry confirmed the lack of expression of CD31, consistent with the flow cytometry results. Furthermore, expression of vWF and uptake of DiI AcLDL were absent in all cultures, regardless of the medium. Representative images are shown of one cell line, with HMVEC as a positive control (Figure 5.6).

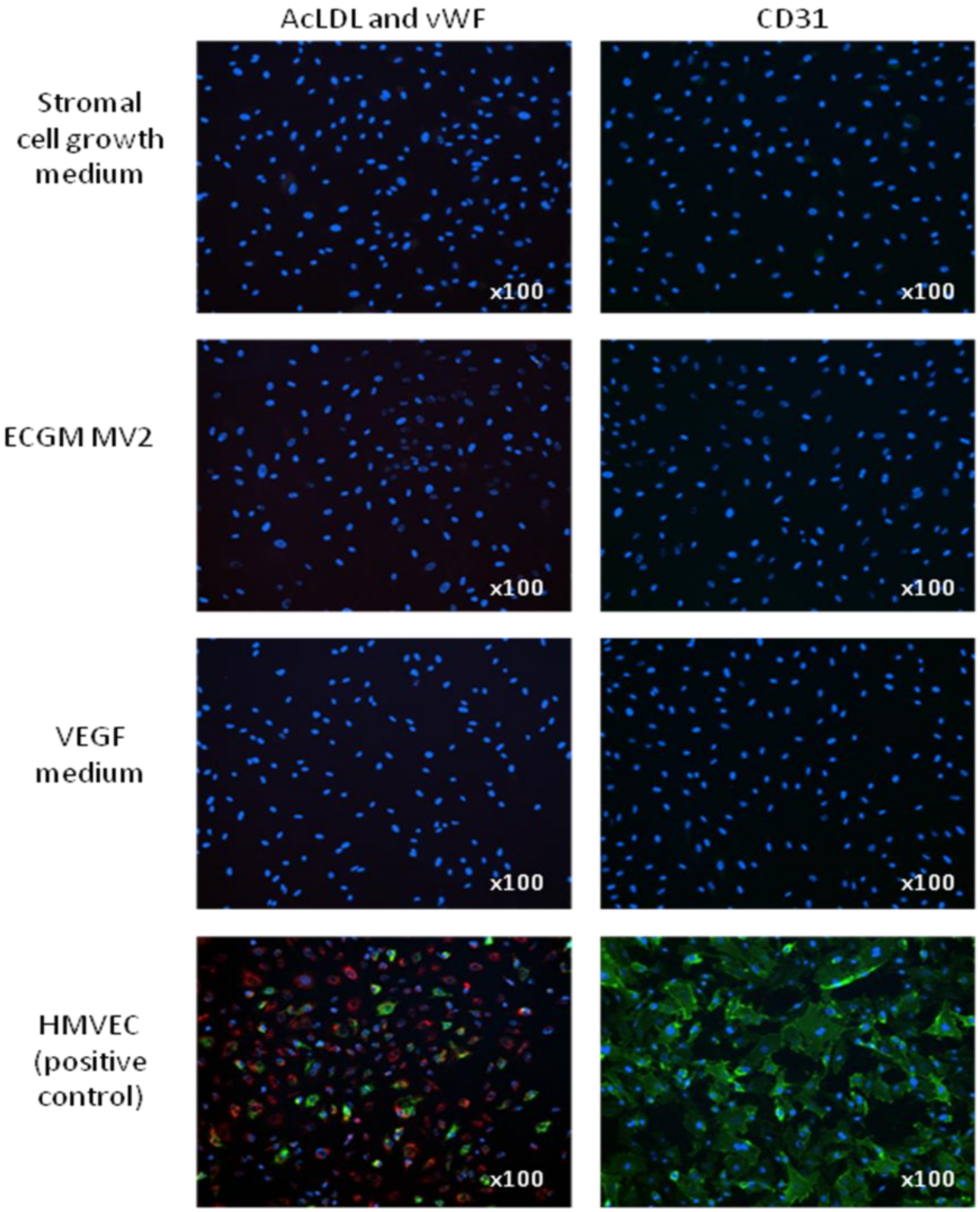

Figure 5.6: Immunocytochemistry of stromal cells maintained in different media for 21 days. Left hand panels show cells exposed to DiIAcLDL (red) and stained for vWF (green); nuclei counterstained with DAPI (blue). Right hand panels show cells stained for CD31 (green) and nuclei counterstained with DAPI (blue).

5.4.4 Behaviour of stromal cells in tube formation assays

Stromal cells were used for a tubule formation assay following exposure to different media types for 21 days. Surprisingly, all stromal cells (grown in stromal growth medium, ECGM MV2 and VEGF medium) formed tubes on basement membrane extract. The mean tube length was the only parameter that differed significantly between the three groups (p=0.01, Figure 5.7). Post-hoc analysis revealed a significant difference between tube length of cells grown in standard DMEM compared to ECGM MV2 (p=0.002) and media with VEGF (p=0.02). The absolute difference observed was small; therefore the relevance of this finding is uncertain.

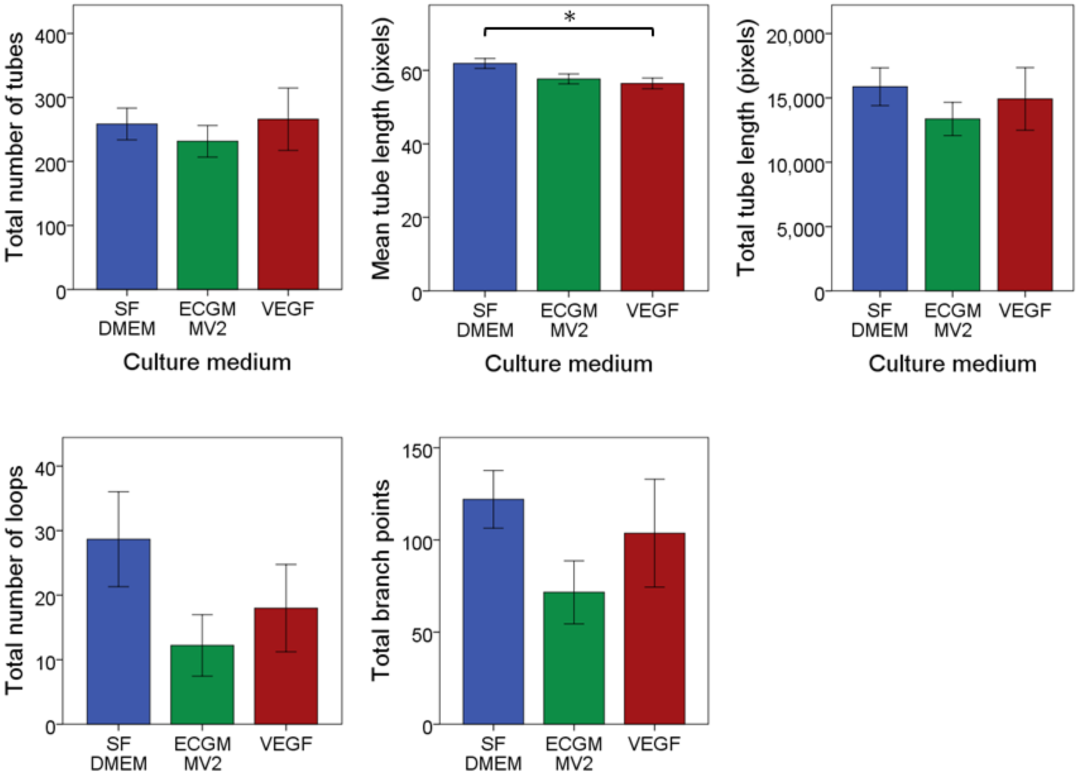

Figure 5.7: Tube formation parameters of stromal cells after culture in different media. SF DMEM = serum-free DMEM; ECGM MV2 = microvascular endothelial medium; VEGF = medium with 50ng/ml VEGF. * p=0.01.

These data suggest that stromal cells are not able to differentiate towards an endothelial-like phenotype by exposure to endothelial growth media or high VEGF levels. However, the ability of stromal cells to form tube-like structures on basement membrane extract indicates that these cells may in some way participate in vessel development.

5.4.5 Tube formation in stromal cells derived from women with and without endometriosis

The ability of cultured stromal cells to form tubes on basement membrane extract may reflect pro-angiogenic activity, despite their inability to differentiate towards an endothelial-like phenotype. Therefore, the tube forming ability of stromal cells derived from women with (n=10) and without endometriosis (n=10) was compared. Demographics for samples included are shown below (Table 5-B). No significant differences were noted in age, BMI or smoking status between the two groups. By design, five women in each group were in the follicular phase of the cycle, and five in the luteal phase. Findings in the control group included: a normal pelvis (n = 2); adhesions (n = 4); ovarian cysts (n = 2); pelvic inflammatory disease (n=1) and fibroids (n = 1).

Table 5-B: Demographics for endometriosis and control subjects.

Variable	**Controls (n=10)**	**Endometriosis (n=10)**	**P value**
Age in years, mean (± SD)	33.80 (±6.76)	36.40 (±4.27)	0.248
BMI, mean (± SD)	25.82(±5.57)	28.60(±4.84)	0.318
Number of smokers (%)	6 (60%)	4 (40%)	0.314
Follicular phase	n=5	n=5	1
Luteal phase	n=5	n=5	1
Stage of disease	n/a	n=7 stage 1-2 n=3 stage 3-4	n/a
Presenting symptoms			
Pain	3 (30%)	5 (50%)	0.650
Subfertility	6 (60%)	8 (80%)	0.628
Ovarian cyst	2 (20%)	2 (20%)	1

Comparison of tube formation revealed no significant differences between cells derived from women with and without endometriosis. Figure 5.8 shows representative images of tubes formed by stromal cells from one subject with and one without endometriosis. Figure 5.9 shows the comparison of tube forming ability by cells derived from women with and without endometriosis. Data are shown in Table 7-F (appendix).

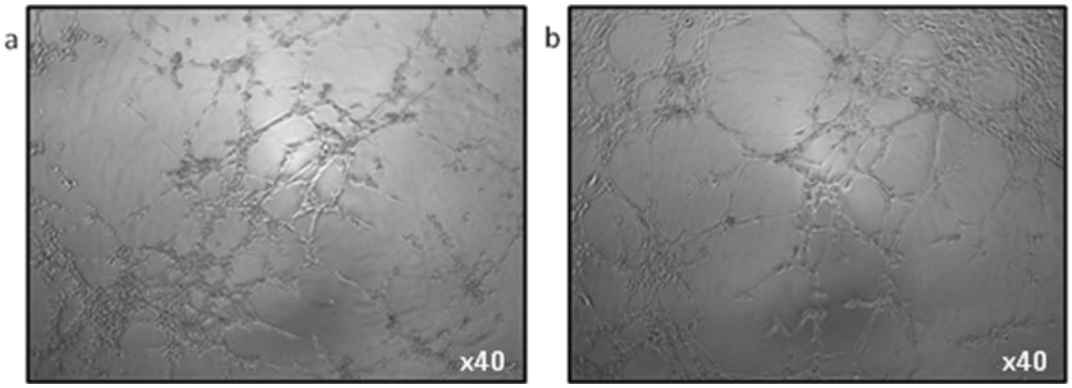

Figure 5.8: (a) stromal cells from a control subject (i.e. no endometriosis); (b) cells from a subject with stage 4 endometriosis.

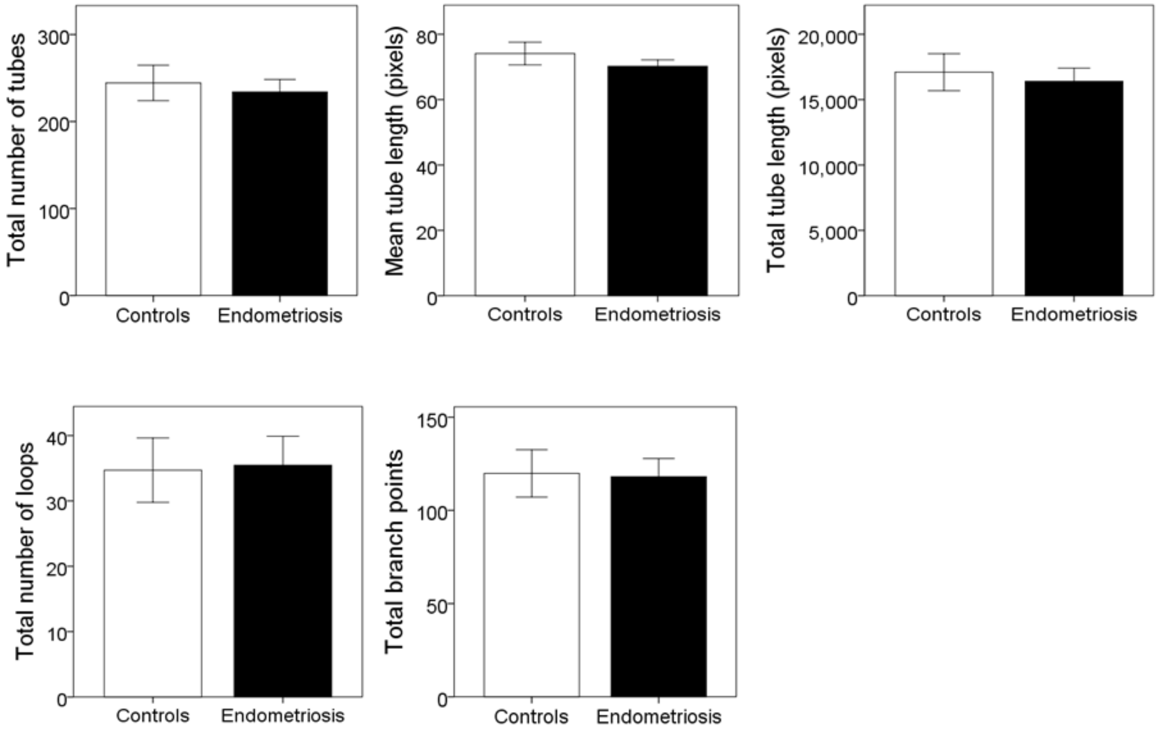

Figure 5.9: Tube formation parameters of stromal cells derived from women with (n=10) and without endometriosis (n=10).

Furthermore, no significant differences were found between stromal cells isolated during the follicular and luteal phases of the cycle (Figure 5.10, Table 7-G [appendix]).

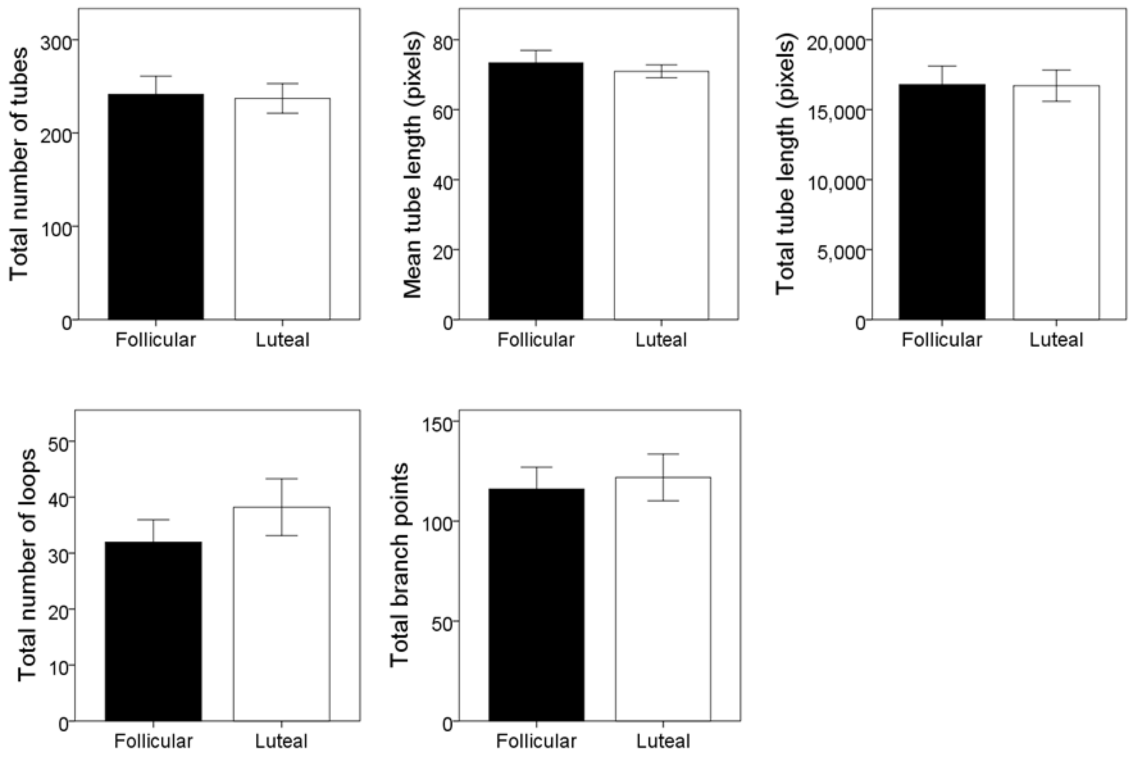

Figure 5.10: Tube formation parameters of stromal cells derived from follicular (n=10) and luteal (n=10) phases.

5.4.6 Tube formation in co-cultures of HMVEC and stromal cells

Stromal cells from women with and without endometriosis were plated with HMVEC to assess whether these cells could co-operate to form tubes. Comparisons were made across three groups (HMVEC alone, HMVEC with control stromal cells and HMVEC with endometriosis stromal cells).

Demographics for samples included are shown below (Table 5-C). No significant differences were present between the two groups. Findings in the control group included: a normal pelvis (n = 2); polycystic ovarian syndrome (n = 2); adhesions (n = 1) and dermoid cyst (n = 1).

Table 5-C: Demographics for endometriosis and control subjects for tube formation with HMVEC.

Variable	**Controls (n=6)**	**Endometriosis (n=6)**	**P value**
Age in years, mean (±SD)	31.50 (±5.17)	36.00(±3.29)	0.102
BMI, mean (±SD)	28.03(±4.04)	26.01(±3.29)	0.364
Stage of disease	n/a	All stage 3-4	n/a
Number of smokers (%)	2 (33.3%)	0 (0%)	0.227
Presenting symptoms			
Pain	1 (17%)	5 (83%)	0.08
Subfertility	5 (83%)	2 (33%)	0.242
Ovarian cyst	1 (17%)	2 (33%)	1

No significant differences were seen in any tube formation parameters between the three groups. The addition of stromal cells, therefore, did not appear to affect tube formation by HMVEC, regardless of the origin of the stromal cells (endometriosis subject or control subject)(Figure 5.11, Table 7-H[appendix]).

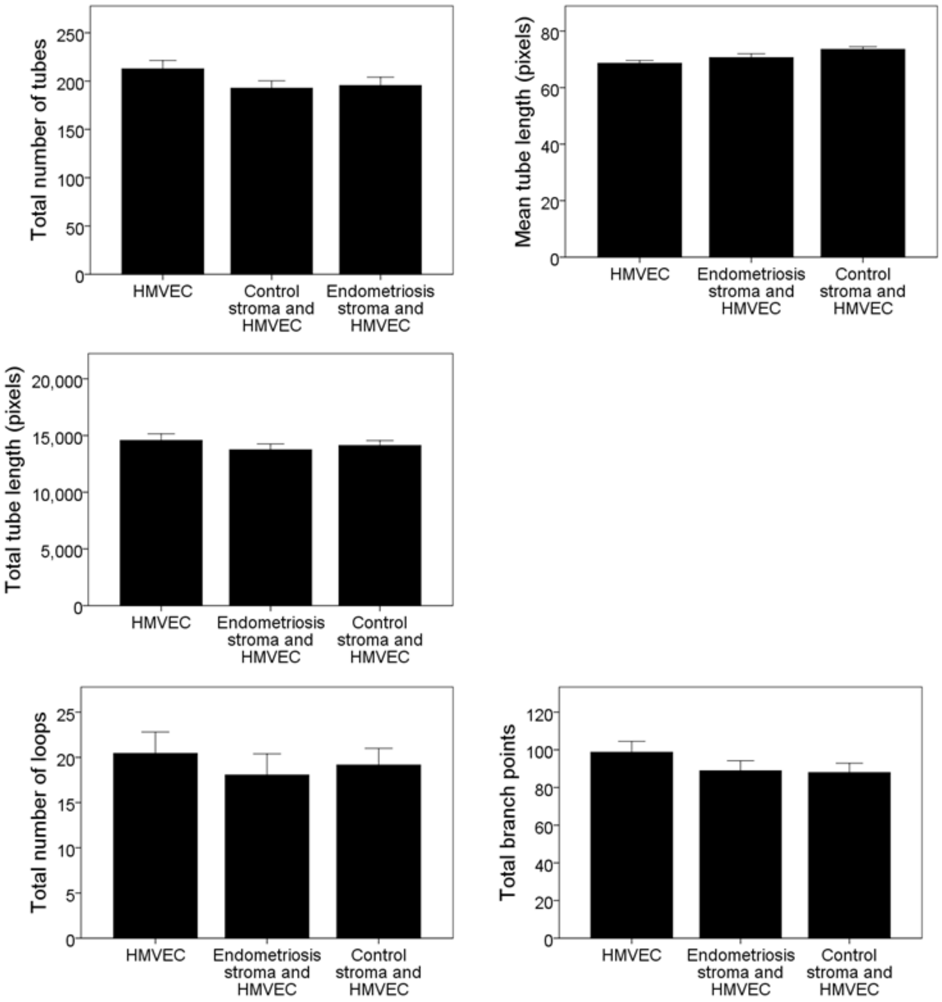

Figure 5.11: Tube formation by HMVEC alone (n=6), and HMVEC mixed with stromal cells derived from women with endometriosis (n=6) and controls (n=6).

5.4.7 Tube formation in conditioned media from stromal cells

Finally, conditioned medium obtained from cultures of control stromal cells and endometriosis stromal cells was used with HMVEC in a tubule formation assay. The samples used were identical to those described in 5.4.6. Results were compared to tube formation in serum free DMEM or ECGM MV2 (Figure 5.12, Table 7-I [appendix]).

The only significant difference between the cultures was in the total number of loops formed (p=0.003). Post-hoc analysis showed the difference was between HMVEC forming tubes in serum free DMEM and tube formation in ECGM MV2 (p=0.002), control conditioned medium (p=0.02) or endometriosis conditioned medium (p=0.05).

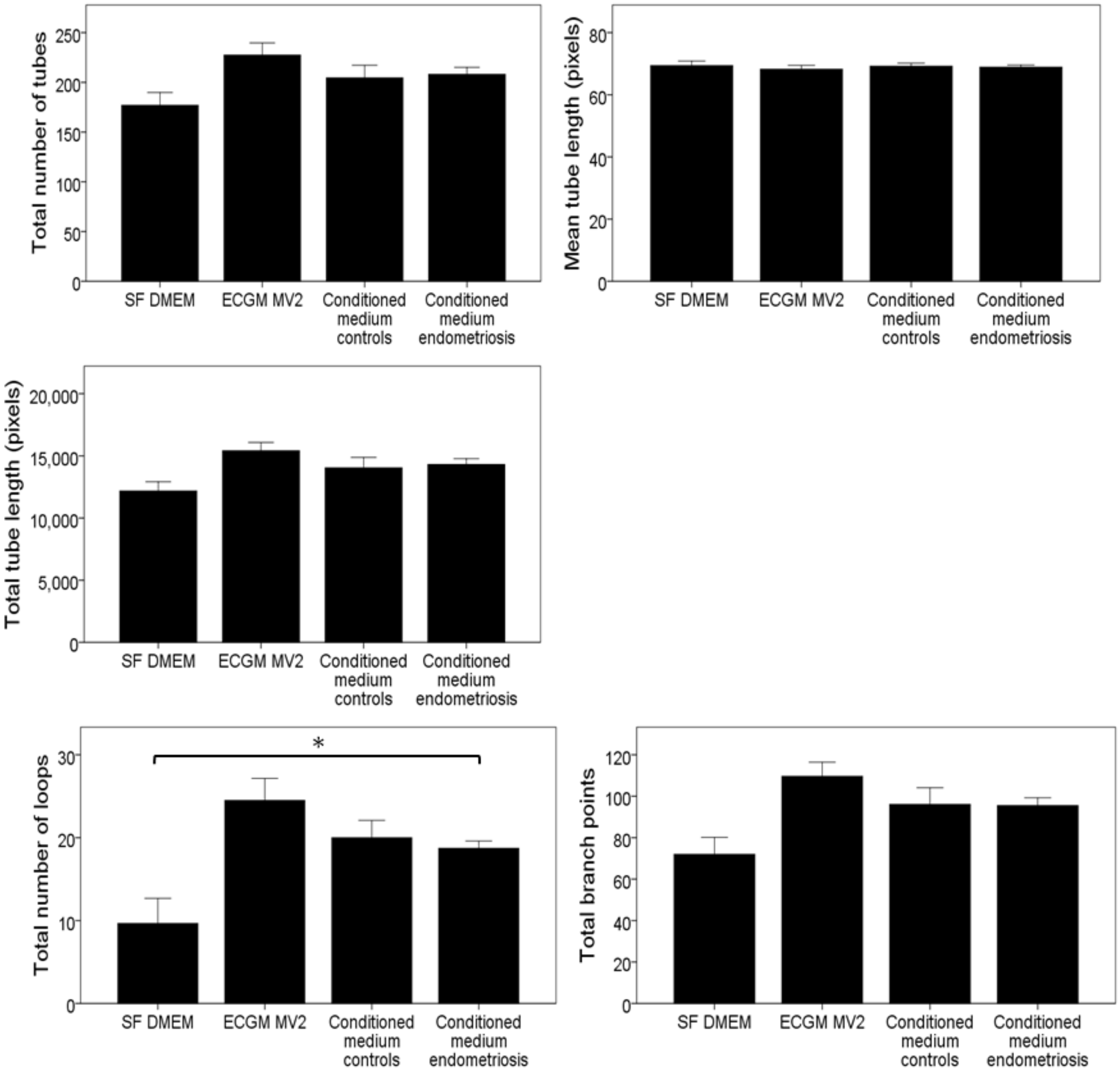

Figure 5.12: Tube formation parameters by HMVEC in serum free DMEM (SF DMEM, n=4), microvascular endothelial medium (n=4), and conditioned medium from stromal cells of women with endometriosis (n=6) and controls (n=6) *p<0.01.

5.5 Discussion

In this chapter, the plasticity of cultured endometrial stromal cells was assessed, with particular regard to their ability to differentiate into endothelial cells. Furthermore, the ability of stromal cells to participate in, and promote, tube formation was studied to investigate their pro-angiogenic ability.

5.5.1 Cultured endometrial stromal cells express surface markers of stromal stem cells

Evidence for the presence of a stem cell population within eutopic endometrium is increasing, and research continues to identify the functions of this cell population in a variety of endometrial disorders. Previous studies have reported CD146 and PDGFR-β as markers that enrich for colony forming cells within the endometrium – putative endometrial stromal stem cells (Schwab and Gargett, 2007; Schwab et al., 2008). Here, it is shown that a high proportion of endometrial stromal cells obtained through standard methods (enzymatic digestion of endometrial biopsies) are positive for these markers. There is also strong stromal cell expression of other markers commonly found on endometrial stem cells, including CD90 (99.10±0.79%) and CD105 (88.00±7.73%). This suggests that cultured endometrial stromal cells are likely to be enriched in endometrial stem cells, without the need for further purification. Previous data also support the presence of these markers at high levels in early passage stromal cell cultures (Santamaria et al., 2011; Wolff et al., 2011). Maintenance of stromal cells in culture is likely to provide some selection pressure to enrich the culture for rapidly dividing cells. Cells which are senescent and unable to divide will be quickly lost from the culture during subsequent passages.

5.5.2 Cultured stromal cells cannot be differentiated into endothelial cells

Several markers found on endometrial stem cells, including CD105 and CD90, are also expressed by MSC (Haasters et al., 2009; Lee et al., 2009). This has led to the suggestion that endometrial stromal stem cells may constitute a population of MSC (Schwab and Gargett, 2007). MSC derived from tissues other than endometrium can be differentiated towards an endothelial-like phenotype, under the appropriate conditions (Oswald et al., 2004; Gang et al., 2006; Alviano et al., 2007). As pluripotency of endometrial stromal stem cells has also been shown, it is possible that they may be induced to express markers consistent with endothelial cells. Given that angiogenesis is critically important in both endometrial regeneration and endometriosis, stromal stem cells may directly participate in angiogenesis *in vivo*.

Two types of media were used to induce endothelial differentiation. Firstly, standard microvascular endothelial cell medium was used. Secondly, medium with low serum (2%) and high levels of VEGF (50ng/ml) was used. This medium has been reported to induce differentiation of bone marrow derived mesenchymal stem cells towards an endothelial phenotype (Oswald et al., 2004).

However, the experiments described above were unable to demonstrate significant changes in either morphology, cell surface marker expression or tube formation activity of stromal cells cultured in endothelial differentiation media. This indicates that stromal stem cells cannot adopt an endothelial phenotype under these conditions.

Of note, a recent publication has suggested that sorted endometrial stem cells may be able to differentiate towards an endothelial phenotype (Masuda et al., 2012). These authors used stem cells identified with a novel marker (W5C5) and cultured them in microvascular cell medium for 4 weeks. Positive immunostaining for CD31 was noted in all three samples assessed. However, the paper did not investigate other markers of endothelial phenotype, either by immunocytochemistry or flow cytometry, and conducted no functional assays to confirm the endothelial behaviour of the differentiated cells. Furthermore, stem cells grown in control medium were also shown to express CD31, although at lower levels than the differentiated cells: 5.3% of cells in the initial population of W5C5$^+$ cells were shown to be CD31$^+$. Importantly, endometrial stem cells have never previously been shown to express CD31 (Chan et al., 2004; Gargett et al., 2009), raising the possibility that W5C5$^+$ cells represent a heterogeneous cell population, potentially comprising a small number of endothelial progenitors, as well as stromal stem cells. All stromal cell cultures in the present study were negative for CD31 before exposure to differentiation medium, which may mean that cells with the potential to differentiate to endothelial cells were not present.

Masuda and colleagues also maintained stromal stem cells in microvascular medium for 4 weeks before CD31 staining (Masuda et al., 2012), compared to only 3 weeks in the present study. It is possible that culture for a further week could have stimulated endothelial differentiation, although this seems unlikely. Firstly, expression of endothelial antigens CD31 and CD144 was analysed at four time points, and there was no trend to increasing expression over the time course of the experiment, even to a small degree. Secondly, previous authors have induced endothelial markers in mesenchymal stem cells with only 7 days of differentiation medium, indicating that this would be sufficient to induce the phenotype (Oswald et al., 2004).

Not all studies have successfully induced endothelial differentiation from MSC. One study reported enhanced tube formation after differentiation, but inconsistent uptake of acetylated LDL, and no expression of vWF or CD144 (König et al., 2012). Similarly, an *in vivo* study of MSC in angiogenesis revealed that these cells participate in vessel formation as pericytes, rather than differentiating directly into endothelial cells (Au et al., 2008).

A potential criticism of the results described in this chapter is that a pure population of endometrial stem cells was not used. Consequently, any effect of endothelial differentiation media on the stem cell population may have been masked by the lack of effect on fully differentiated stromal cells. However, as described above, the majority of endometrial stromal cells used did express markers consistent with a stem cell phenotype. Furthermore, although CD146 and PDGFR-β are expressed by stromal stem cells, these markers are not specific for stem cells. Therefore, even isolating double positive cells (for example by FACS) would not generate a pure stem cell culture. Culturing cells in a clonogenic assay may better enrich for a uniform stem cell population, although this process risks the differentiation of cells with increasing time in culture.

5.5.3 Stromal cells directly participate in tube formation assays, but this is not affected by endometriosis

However, the experiments did result in the novel finding that endometrial stromal cells can form tubes in a basement membrane extract assay, in a manner analogous to endothelial cells. This observation has not previously been reported, and may indicate a novel role for stromal cells in angiogenesis without the need for differentiation. MSC have previously been shown to participate in tube formation, and have other pro-angiogenic effects, including in animal models of myocardial ischaemia (Nagaya et al., 2004; Tang et al., 2006) and wound healing (Wu et al., 2007). However, whilst stromal cells participated in tube formation, they were not shown to enhance tube formation by microvascular endothelial cells – either when cultured together, or when stromal cell conditioned medium was added to endothelial cell cultures. This is in keeping with one previous paper which used a two-chamber co-culture system to assess the effect of endometrial stroma on tube formation by endothelial cells (Albrecht et al., 2003). These authors found that the addition of stromal cells to the lower chamber did not promote microvascular endothelial tube formation in the upper chamber. Therefore, currently, the significance of tube formation in endometrial stromal cells is unclear. Potentially their co-operation with endothelial cells would be revealed by other stimuli, such as hypoxia or pro-angiogenic cytokines.

Crucially, no differences were identified between cells derived from women with and without endometriosis, for what may be several reasons. Firstly, stromal cells from women with and without endometriosis may be equivalent with respect to angiogenesis. Whilst differences in other physiological processes have been identified - such as decidualisation and cell migration (Aghajanova et al., 2009; Gentilini et al., 2010) - there are no reports of different pro-angiogenic properties of stromal cells. It may be that stromal cells from women with endometriosis are not especially pro-angiogenic, but simply respond to signals from the local environment during the formation of endometriotic lesions. The peritoneal environment of women with endometriosis is known to differ considerably from that in healthy women (Oral et al., 1996; Gazvani and Templeton, 2002). It may also be that factors within the peritoneum are able to promote different angiogenic behaviour in stromal cells, rather than intrinsic properties of the cells themselves.

Culture conditions *in vitro* do not truly mimic the environment *in vivo*, and this may also mask differences between the two cell types. Culturing stromal cells in relative hypoxia may be more representative of the intra-peritoneal environment to which sloughed stromal cells are exposed. Other authors have suggested that conditioned medium from MSC cultured in hypoxic conditions may be better able to promote tube formation than that derived from cells cultured in normoxia (Hung et al., 2007; Zhang et al., 2012). Shear stress is also known to play an important role in vessel development and has been found to enhance endothelial differentiation of amniotic-fluid derived stem cells (Zhang et al., 2009). Exposing endometrial stromal cells to shear forces may potentially increase their ability to differentiate. Similarly, exposing the stromal cells to peritoneal fluid from women with or without endometriosis may reveal differences in their behaviour in tube assays.

5.5.4 Summary

In conclusion, this chapter demonstrates that the majority of cultured stromal cells express cellular markers consistent with a stromal stem cell phenotype, but are unable to differentiate into endothelial cells. Nonetheless, stromal cells do exhibit angiogenic activity in a well-recognised *in vitro* model of angiogenesis. However, this capacity is unaffected by the presence of endometriosis.

Chapter 6: Discussion

The data presented here indicate that angiogenesis is equivalent in women with and without endometriosis; at least as far as CAC levels and stromal cell behaviour are concerned. This is contrary to the original hypothesis - that differences in angiogenesis may underlie the development of endometriosis.

6.1 Pro-angiogenic phenotypes

It was hypothesised that women with endometriosis have a pro-angiogenic phenotype, which facilitates vessel formation. Consequently, refluxed endometrial tissue would be better vascularised in these women, facilitating the development of endometriotic lesions.

Studies of angiogenesis in mice with a variety of genetic backgrounds have revealed striking differences between strains, indicating that aspects of angiogenesis are genetically determined (Rohan et al., 2000; Rogers et al., 2003). Those mice with the highest response to a pro-angiogenic stimulus have subsequently been shown to have the greatest number of CACs (Shaked et al., 2005). Genetic differences in angiogenic ability are also present in humans. For example, retinopathy of prematurity is a disorder of angiogenesis affecting premature babies where exposure to high oxygen concentrations results in extensive retinal neovascularisation and long-term visual impairment. A twin study has indicated that genetic factors predispose to the development of this condition, (Bizzarro et al., 2006) and CAC levels have been recently identified as increased in affected neonates (Safranow et al., 2012).

Specific genetic variants of angiogenic factors have also been shown to be associated with disease. Single nucleotide polymorphisms of VEGF and VEGF receptors have been associated with the development of tumours, and may predict response to anti-angiogenic drugs (reviewed in Schneider et al., 2009). Genetic susceptibility to endometriosis has also been postulated (Uno et al., 2010; Painter et al., 2011). One recent study has identified a significant association between endometriosis and certain alleles of the fibroblast growth factor genes (FGF-1 and 2), which are known to be important in vessel development (Kang et al., 2010).

However, the unchanged CAC levels reported in this thesis suggest that endometriosis is not associated with a widespread pre-disposition to angiogenesis, i.e. women who develop the disease do not have a pro-angiogenic phenotype.

This would be in keeping with epidemiological data on the association between endometriosis and other diseases. If women with endometriosis had a general enhancement of angiogenesis they might also be predisposed to tumour development, as tumours would have a growth advantage by being better vascularised. Although certain specific tumours are associated with endometriosis (notably subtypes of ovarian cancer), no association has been found between the occurrence of endometriosis and overall cancer incidence (Somigliana et al., 2006).

Enhanced angiogenesis would also be expected to show some benefits, such as better wound repair or a lower incidence of cardiovascular disease. No longitudinal studies assessing the incidence of cardiovascular disease in women with endometriosis have been conducted. However, recent data shows that endometriosis patients may actually have higher cardiovascular risk than healthy controls, in terms of endothelial dysfunction and pro-atherogenic lipid profile (Melo et al., 2010; Santoro et al., 2012). Similarly disorders such as pre-eclampsia, with associated inadequate placental vascularisation, may be expected to be reduced in women with endometriosis. Whilst a small case-control study suggested this may be the case, a more recent population based study showed no protective effect of endometriosis on the incidence of pre-eclampsia (Brosens et al., 2007; Hadfield et al., 2009).

Women with endometriosis, therefore, do not appear to have generally enhanced angiogenesis, although subtle genetic changes may contribute to better vessel development in specific circumstances.

6.2 Angiogenic activity in the eutopic endometrium

Studies of the eutopic endometrium have previously identified significant changes in vessel formation in women with endometriosis, for example increased endometrial microvessel density (Khan et al., 2003; Burlev et al., 2005; Bourlev et al., 2006b). Endothelial cell proliferation was also increased in women with endometriosis in one study (Wingfield et al., 1995), but not in another (Bourlev et al., 2006b). These changes could reflect increased angiogenic activity locally, within the endometrium itself. If so, reflux of pro-angiogenic endometrium into the peritoneal cavity would stimulate vessel formation, which would in turn facilitate lesion development.

However, data from this thesis show that the angiogenic activity of stromal cells is equivalent in women with and without the disease. The eutopic stromal cells of women with endometriosis do not form better tubes, nor do they stimulate endothelial cell tube formation better than cells derived from healthy women. This suggests that alterations in eutopic stromal cells do not contribute to the vessel development seen in endometriosis.

It is possible that other cell types may contribute to vascular changes in the endometrium, for example epithelial or endothelial cells. Endometrial epithelial cells are known to secrete VEGF, and hypoxia has been shown to greatly stimulate this process (Sharkey et al., 2000). Glandular cells may therefore play a greater role than stromal cells in promoting angiogenesis, which may be further enhanced by the relative hypoxia induced on transport into the peritoneal cavity. Similarly, endothelial cells derived from women with endometriosis have been shown to be altered when compared to cells from healthy women, with respect to gene expression and response to sex steroids (Luk et al., 2005; Sha et al., 2007; Luk et al., 2010). Unfortunately, I was unable to extend these investigations in this thesis.

Further study of the eutopic endometrium is warranted, particularly with regard to endothelial and epithelial cell behaviour. Although these cells are challenging to culture there may be crucial differences in angiogenesis that contribute to the development of endometriosis. Eutopic endometrial stromal cells were used in this thesis. However, it would also be of interest to assess ectopic stromal cells, derived from endometriotic lesions. The retrograde menstruation hypothesis suggests that these cells originate from the same location but transplantation of cells to the peritoneal cavity may cause phenotypic and functional changes, which promote angiogenesis. Therefore, stromal cells from the lesions themselves may be more active in promoting angiogenesis than those derived from eutopic endometrium.

6.3 Equivalence of angiogenic features

It remains the case that angiogenesis is a key feature of endometriosis. Evaluation of endometriotic lesions at both macroscopic and microscopic levels reveals intense vascularity (Taylor et al., 2009). Interruption of vessel development in murine models also prevents the development of the disease (Nap et al., 2004; Becker et al., 2005). Consequently the apparent lack of altered angiogenesis in women with endometriosis is surprising; however, several possibilities may account for this, as discussed below.

6.3.1 Angiogenesis during lesion growth

A small amount of vessel growth is constantly required in endometriotic lesions as they are exposed to the same hormones as eutopic endometrium and undergo similar cyclical vessel growth (Nieminen, 1962). However, significant angiogenesis may occur only when the lesions are actively growing. Our current understanding of lesion growth and the natural history of endometriosis is very limited. Whilst stages of endometriosis are recognised, the progression of disease from one stage to the next is not well described. “Second-look” laparoscopy was previously used to assess the outcome of treatment in women with endometriosis, and revealed that some lesions spontaneously regress, whilst others remain unchanged or worsen with time (Thomas and Cooke, 1987; Mahmood and Templeton, 1990).

The equivalence of pro-angiogenic features in women with and without endometriosis may reflect the need for vessel development only when lesions are growing. Therefore, markers of angiogenesis may be normal in a woman with severe endometriosis, but elevated in women with less extensive - but more active - disease. It is assumed that small lesions eventually develop into extensive disease in women with more severe endometriosis, but the speed of lesion growth is not known, probably due to the need for surgery to assess the disease. It may be that more sensitive imaging techniques (such as MRI) will be able to detect small changes in disease burden, and help to assess whether angiogenic markers correlate specifically with lesion growth.

Endothelial cell proliferation is likely to give some idea of angiogenic activity. Growing vessels should exhibit a large number of proliferating endothelial cells. However, this has not been shown to be the case in endometriosis. Matsuzaki and colleagues reported that only 5 out of 52 peritoneal lesions contained any proliferating endothelial cells (Matsuzaki et al., 2001). Furthermore, proliferating cells (where present) comprised less than 1% of the total number of endothelial cells in the lesions. This indicates that active vessel growth is not occurring in the majority of peritoneal lesions; hence markers of angiogenesis may not be affected by the disease.

6.3.2 Angiogenesis in specific types of endometriotic lesion

Another indicator of angiogenesis is the presence of mature blood vessels. Initial vessel formation depends on the formation of an endothelial cell tube, but this becomes enclosed by surrounding pericytes when mature (reviewed in Conway et al., 2001). The lack of pericytes can, therefore, be used to demonstrate that vessels are in the process of developing or remodelling.

Matsuzaki and colleagues identified approximately 30% of vessels in red endometriotic lesions as mature (28.9% in proliferative phase, and 34.2% in secretory phase) as compared to approximately 70% mature vessels in black lesions (76.2% proliferative phase, 68.5% secretory phase) (Matsuzaki et al., 2001). This suggests that angiogenesis is likely to be more active in red than black lesions. One study assessed ovarian lesions and found pericytes associated with only 16% of vessels, indicating that angiogenesis is occurring in these lesions (Hull et al., 2003). It is unclear whether these lesions were superficial or from endometriomas; nevertheless, vessel maturity and consequently angiogenesis may be related to the type of endometriotic lesion.

Black, red and white peritoneal lesions have also been shown to contain different vessel densities and sizes of vessels (Nisolle et al., 1993). Similarly, VEGF levels are increased in red, compared to black lesions (Donnez et al., 1998) and rectovaginal endometriosis was found to contain increased numbers of vessels, compared to ovarian or bladder disease (Machado et al., 2008).

Vessel growth is, therefore, likely to vary depending on the location and histology of the lesions. Consequently, markers of angiogenesis may relate more to the specific lesion type rather than the stage of disease.

6.3.3 A balance of pro- and anti-angiogenic features

It is possible that women with endometriosis show both pro- and anti-angiogenic features, and that these counteract each other. Whilst one paper reported no significant differences in biochemical markers of cardiovascular disease between women with endometriosis and those with other gynaecological pathology (Pretta et al., 2007), two more recent studies have indicated that cardiovascular risk may be increased in affected women (Kinugasa et al., 2011; Santoro et al., 2012).

Importantly, the women with endometriosis who are studied are almost exclusively pre-menopausal, and their background risk for cardiovascular disease is likely to be low. Use of traditional markers of cardiovascular health (such as blood pressure and cholesterol levels) may, therefore, be inappropriate in these women. Instead, newer techniques may detect endothelial dysfunction, which precedes other changes. The recent studies specifically assessed endothelial function in women with endometriosis by measuring flow mediated dilatation (FMD) - the reactive dilation of the brachial artery following a period of ischaemia (Kinugasa et al., 2011; Santoro et al., 2012). Both of these papers identified a significant reduction in FMD in women with endometriosis, as compared with women without the disease. The authors suggest that this demonstrates an underlying endothelial dysfunction that predates the overt appearances of atherosclerosis. Other metabolic markers of endothelial function and inflammation were also shown to be adversely affected by endometriosis, including ICAM-1 and VCAM-1 (Santoro et al., 2012).

It is possible that two elements of the pathogenesis of endometriosis are counterbalancing each other. Firstly, the disease is pro-angiogenic - relying on the development of new vessels to supply lesions with the oxygen and nutrients they require to grow. Secondly, the pro-inflammatory nature of the disease may be anti-angiogenic and promote endothelial dysfunction and damage, predisposing to later cardiovascular problems. The opposing actions of these two processes may account for the apparently unchanged angiogenic markers.

6.4 The future of anti-angiogenic agents in endometriosis

The possibility of using anti-angiogenic therapies in endometriosis has been an attractive alternative to hormonal treatments (Ferrero et al., 2006; Becker and D'Amato, 2007) especially as murine data suggest that these treatments may prevent disease progression (Hull et al., 2003; Becker et al., 2005; Becker et al., 2008).

However, data from this thesis indicate that the angiogenic factors studied are unchanged in endometriosis. Consequently the use of these agents to treat the disease may not be justified. Angiogenesis may only be a useful target in treating endometriosis at times when lesions are actively growing. Potentially this could mean that anti-angiogenic agents could be used as an adjunct to standard treatment, but they are unlikely to replace it.

Secondly, if women with endometriosis are at elevated risk of cardiovascular disease then the use of anti-angiogenic agents in this group may further increase this risk. The long-term effects of such treatments are not yet known, but it would seem prudent to avoid their use in subjects with an increased background risk.

Together, these data indicate that anti-angiogenic therapies may not be an appropriate treatment for women with endometriosis. These agents have the potential to disrupt physiological angiogenesis in the reproductive tract and they are associated with a considerable risk of teratogenicity in the developing embryo. Even if these risks are acceptable to an individual woman, the treatment may only be successful in those with actively growing lesions or specific lesion types.

6.5 Further work

Future work should focus on the role of other cell types in promoting angiogenesis. Fluorescence activated cell sorting (FACS) of endometrial endothelial cells should isolate a pure population of cells for further study - to assess their behaviour in angiogenesis assays and monitor their response to recognised endometriosis treatments. Epithelial cells may also be involved in promoting a pro-angiogenic environment in endometriosis and their role warrants further investigation.

CAC levels are not a useful biomarker of endometriosis and other markers of angiogenesis may also be unchanged in the periphery. The higher level of physiological angiogenesis in young women may mask small changes that occur due to pathological processes. Therefore, this aspect of the disease may not yield promising results in the search for disease biomarkers.

The pro-angiogenic nature of stromal cells was demonstrated by their participation in tube formation assays. This finding may also have relevance for other disorders of the endometrium, including heavy menstrual bleeding and endometrial carcinoma. Further investigation into the role of these cells in angiogenesis is therefore required.

References

Aghajanova L, Hamilton A, Kwintkiewicz J, Vo KC, Giudice LC. Steroidogenic enzyme and key decidualization marker dysregulation in endometrial stromal cells from women with versus without endometriosis. *Biology of Reproduction* 2009; **80**: 105-114.

Albrecht ED, Babischkin JS, Lidor Y, Anderson LD, Udoff LC, Pepe GJ. Effect of estrogen on angiogenesis in co-cultures of human endometrial cells and microvascular endothelial cells. *Human Reproduction* 2003; **18**: 2039-2047.

Alev C, Ii M, Asahara T. Endothelial progenitor cells: a novel tool for the therapy of ischemic diseases. *Antioxididants and Redox Signaling* 2011; **15**: 949-965.

Allen C, Hopewell S, Prentice A, Gregory D. Nonsteroidal anti-inflammatory drugs for pain in women with endometriosis. *Cochrane Database of Systematic Reviews* 2010.

Alviano F, Fossati V, Marchionni C, Arpinati M, Bonsi L, Franchina M, Lanzoni G, Cantoni S, Cavallini C, Bianchi F, Tazzari PL, Pasquinelli G, Foroni L, Ventura C, Grossi A, Bagnara GP. Term amniotic membrane is a high throughput source for multipotent mesenchymal stem cells with the ability to differentiate into endothelial cells in vitro. *BMC Developmental Biology* 2007; **7**: 11.

American Society for Reproductive Medicine. Revised American Society for Reproductive Medicine classification of endometriosis: 1996. *Fertility and Sterility* 1997; **67**: 817-821.

Andreesen R, Picht J, Löhr GW. Primary cultures of human blood-borne macrophages grown on hydrophobic teflon membranes. *Journal of Immunological Methods* 1983; **56**: 295-304.

Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 1997; **275**: 964-966.

Au P, Tam J, Fukumura D, Jain RK. Bone marrow–derived mesenchymal stem cells facilitate engineering of long-lasting functional vasculature. *Blood* 2008; **111**: 4551-4558.

Barcelos LS, Duplaa C, Kränkel N, Graiani G, Invernici G, Katare R, Siragusa M, Meloni M, Campesi I, Monica M, Simm A, Campagnolo P, Mangialardi G, Stevanato L, Alessandri G, Emanueli C, Madeddu P. Human CD133+ Progenitor Cells Promote the Healing of Diabetic Ischemic Ulcers by Paracrine Stimulation of Angiogenesis and Activation of Wnt Signaling. *Circulation Research* 2009; **104**: 1095-1102.

Bazot M, Jarboui L, Ballester M, Touboul C, Thomassin-Naggara I, Daraï E. The value of MRI in assessing parametrial involvement in endometriosis. *Human Reproduction* 2012; **27**: 2352-2358.

Becker CM, Beaudry P, Funakoshi T, Benny O, Zaslavsky A, Zurakowski D, Folkman J, D'Amato RJ, Ryeom S. Circulating endothelial progenitor cells are up-regulated in a mouse model of endometriosis. *The American Journal of Pathology* 2011; **178**: 1782-1791.

Becker CM and D’Amato RJ. Angiogenesis and antiangiogenic therapy in endometriosis. *Microvascular Research* 2007; **74**: 121-130.

Becker CM, Rohwer N, Funakoshi T, Cramer T, Bernhardt W, Birsner A, Folkman J, D'Amato RJ. 2-Methoxyestradiol Inhibits Hypoxia-Inducible Factor-1α and Suppresses Growth of Lesions in a Mouse Model of Endometriosis. *The American Journal of Pathology* 2008; **172**: 534-544.

Becker CM, Sampson DA, Rupnick MA, Rohan RM, Efstathiou JA, Short SM, Taylor GA, Folkman J, D’Amato RJ. Endostatin inhibits the growth of endometriotic lesions but does not affect fertility. *Fertility and Sterility* 2005; **84**: 1144-1155.

Beekhuizen H and van Furth R. Growth characteristics of cultured human macrovascular venous and arterial and microvascular endothelial cells. *Journal of Vascular Research* 1994; **31**: 230-239.

Benschop L, Farquhar C, van der Poel N, Heineman MJ. Interventions for women with endometrioma prior to assisted reproductive technology. *Cochrane Database of Systematic Reviews* 2010.

Berkkanoglu M and Arici A. Immunology and endometriosis. *American Journal of Reproductive Immunology* 2003; **50**: 48-59.

Bhaskar A, Gupta R, Kumar L, Sharma A, Sharma MC, Kalaivani M, Thakur SC. Circulating endothelial progenitor cells as potential prognostic biomarker in multiple myeloma. *Leukemia & Lymphoma* 2012; **53**: 635-640.

Bizzarro MJ, Hussain N, Jonsson B, Feng R, Ment LR, Gruen JR, Zhang H, Bhandari V. Genetic susceptibility to retinopathy of prematurity. *Pediatrics* 2006; **118**: 1858-1863.

Bland M. An Introduction to Medical Statistics, Third Edition. (2000): Oxford University Press.

Bourlev V, Iljasova N, Adamyan L, Larsson A, Olovsson M. Signs of reduced angiogenic activity after surgical removal of deeply infiltrating endometriosis. *Fertility and Sterility* 2010; **94**: 52-57.

Bourlev V, Larsson A, Olovsson M. Elevated levels of fibroblast growth factor-2 in serum from women with endometriosis. *American Journal of Obstetrics and Gynecology* 2006a; **194**: 755-759.

Bourlev V, Volkov N, Pavlovitch S, Lets N, Larsson A, Olovsson M. The relationship between microvessel density, proliferative activity and expression of vascular endothelial growth factor-A and its receptors in eutopic endometrium and endometriotic lesions. *Reproduction* 2006b; **132**: 501-509.

Bredhult C, Bäcklin B-M, Olovsson M. Effects of some endocrine disruptors on the proliferation and viability of human endometrial endothelial cells in vitro. *Reproductive Toxicology* 2007; **23**: 550-559.

Brosens IA, De Sutter P, Hamerlynck T, Imeraj L, Yao Z, Cloke B, Brosens JJ, Dhont M. Endometriosis is associated with a decreased risk of pre-eclampsia. *Human Reproduction* 2007; **22**: 1725-1729.

Buck Louis GM, Hediger ML, Peterson CM, Croughan M, Sundaram R, Stanford J, Chen Z, Fujimoto VY, Varner MW, Trumble A, Giudice LC. Incidence of endometriosis by study population and diagnostic method: the ENDO study. *Fertility and Sterility* 2011; **96**: 360-365.

Bulut D, Albrecht N, Imöhl M, Günesdogan B, Bulut-Streich N, Börgel J, Hanfeld C, Krieg M, Mügge A. Hormonal status modulates circulating endothelial progenitor cells. *Clinical Research in Cardiology* 2007; **96**: 258-263.

Burlev VA, Il'yasova NA, Dubinskaya ED. Proliferative activity of microvessels and angiogenesis in eutopic endometrium in patients with peritoneal endometriosis. *Bulletin of Experimental Biology and Medicine* 2005; **139**: 727-731.

Capobianco A, Monno A, Cottone L, Venneri MA, Biziato D, Di Puppo F, Ferrari S, De Palma M, Manfredi AA, Rovere-Querini P. Proangiogenic Tie2+ macrophages infiltrate human and murine endometriotic lesions and dictate their growth in a mouse model of the disease. *The American Journal of Pathology* 2011; **179**: 2651-2659.

Chan RWS, Schwab KE, Gargett CE. Clonogenicity of human endometrial epithelial and stromal cells. *Biology of Reproduction* 2004; **70**: 1738-1750.

Chapron C, Querleu D, Bruhat M, Madelenat P, Fernandez H, Pierre F, Dubuisson J. Surgical complications of diagnostic and operative gynaecological laparoscopy: a series of 29,966 cases. *Human Reproduction* 1998; **13**: 867-872.

Chen M-C, Yip H-K, Chen C-J, Yang C-H, Wu C-J, Cheng C-I, Chen Y-H, Chai H-T, Lee C-P, Chang H-W. No age-related change in circulating endothelial progenitor cells in healthy subjects. *International Heart Journal* 2006; **47**: 95-105.

Cho S, Oh Y, Nam A, Kim H, Park J, Kim J, Park K, Cho D, Lee B. Evaluation of serum and urinary angiogenic factors in patients with endometriosis. *American Journal of Reproductive Immunology* 2007; **58**: 497-504.

Chu KK, Chen FP, Chang SD. Prevalence of endometriosis among women undergoing laparoscopic procedures. *Diagnostic and Therapeutic Endoscopy* 1995; **2**: 35-57.

Clark EA and Ledbetter JA. Leukocyte cell surface enzymology: CD45 (LCA, T200) is a protein tyrosine phosphatase. *Immunology Today* 1989; **10**: 225-228.

Conway EM, Collen D, Carmeliet P. Molecular mechanisms of blood vessel growth. *Cardiovascular Research* 2001; **49**: 507-521.

Cosin R, Gilabert-Estelles J, Ramon LA, Espana F, Gilabert J, Romeu A, Estelles A. Vascular endothelial growth factor polymorphisms (-460C/T, +405G/C, and 936C/T) and endometriosis: their influence on vascular endothelial growth factor expression. *Fertility and Sterility* 2009; **92**: 1214-1220.

Cosín R, Gilabert-Estellés J, Ramón LA, Gómez-Lechón MJ, Gilabert J, Chirivella M, Braza-Boïls A, España F, Estellés A. Influence of peritoneal fluid on the expression of angiogenic and proteolytic factors in cultures of endometrial cells from women with endometriosis. *Human Reproduction* 2010; **25**: 398-405.

D'Hooghe TM, Mihalyi AM, Simsa P, Kyama CK, Peeraer K, De Loecker P, Meeuwis L, Segal L, Meuleman C. Why we need a noninvasive diagnostic test for minimal to mild endometriosis with a high sensitivity. *Gynecologic and Obstetric Investigation* 2006; **62**: 136-138.

Darai E, Detchev R, Hugol D, Quang NT. Serum and cyst fluid levels of interleukin (IL) -6, IL-8 and tumour necrosis factor-alpha in women with endometriomas and benign and malignant cystic ovarian tumours. *Human Reproduction* 2003; **18**: 1681-1685.

de Ziegler D, Borghese B, Chapron C. Endometriosis and infertility: pathophysiology and management. *The Lancet* 2010; **376**: 730-738.

Desai A, Glaser A, Liu D, Raghavachari N, Blum A, Zalos G, Lippincott M, McCoy JP, Munson PJ, Solomon MA, Danner RL, Cannon RO. Microarray-Based Characterization of a Colony Assay Used to Investigate Endothelial Progenitor Cells and Relevance to Endothelial Function in Humans. *Arteriosclerosis, Thrombosis, and Vascular Biology* 2009; **29**: 121-127.

Dessapt-Baradez C, Reza M, Sivakumar G, Hernandez-Fuentes M, Markakis K, Gnudi L, Karalliedde J. Circulating vascular progenitor cells and central arterial stiffness in polycystic ovary syndrome. *PLoS ONE* 2011; **6**: e20317.

Di Carlo C, Bonifacio M, Tommaselli GA, Bifulco G, Guerra G, Nappi C. Metalloproteinases, vascular endothelial growth factor, and angiopoietin 1 and 2 in eutopic and ectopic endometrium. *Fertility and Sterility* 2009; **91**: 2315-2323.

Dimitrov R, Timeva T, Kyurkchiev D, Stamenova M, Shterev A, Kostova P, Zlatkov V, Kehayov I, Kyurkchiev S. Characterization of clonogenic stromal cells isolated from human endometrium. *Reproduction* 2008; **135**: 551-558.

Dmowski WP and Braun DP. Immunology of endometriosis. *Best Practice and Research Clinical Obstetrics and Gynaecology* 2004; **18**: 245-263.

Dome B, Timar J, Ladanyi A, Paku S, Renyi-Vamos F, Klepetko W, Lang G, Dome P, Bogos K, Tovari J. Circulating endothelial cells, bone marrow-derived endothelial progenitor cells and proangiogenic hematopoietic cells in cancer: From biology to therapy. *Critical Reviews in Oncology/Hematology* 2009; **69**: 108-124.

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006; **8**: 315-317.

Donnez J, Smoes P, Gillerot S, Casanas-Roux F, Nisolle M. Vascular endothelial growth factor (VEGF) in endometriosis. *Human Reproduction* 1998; **13**: 1686-1690.

Dormon BH, Varma VA, Siegfried JM, Melin SA, Adamec TA, Norton CR, Kaufman DG. Morphology and growth potential of stromal cell cultures derived from human endometrium. *In Vitro* 1982; **18**: 919-928.

Duda D, Cohen K, Scadden D, Jain R. A protocol for phenotypic detection and enumeration of circulating endothelial cells and circulating progenitor cells in human blood. *Nature Protocols* 2007; **2**: 805-810.

Elsheikh E, Sylvén C, Ericzon B, Palmblad J, Mints M. Cyclic variability of stromal cell-derived factor-1 and endothelial progenitor cells during the menstrual cycle. *International Journal of Molecular Medicine* 2011; **27**: 221-226.

Estes M, Mund J, Ingram D, Case J. Identification of endothelial cells and progenitor cell subsets in human peripheral blood. *Current Protocols in Cytometry* 2010a; **9**: 1-11.

Estes ML, Mund JA, Mead LE, Prater DN, Cai S, Wang H, Pollok KE, Murphy MP, An CST, Srour EF, Ingram DA, Case J. Application of polychromatic flow cytometry to identify novel subsets of circulating cells with angiogenic potential. *Cytometry Part A* 2010b; **77A**: 831-839.

Fadini GP, de Kreutzenberg S, Albiero M, Coracina A, Pagnin E, I B, Cignarella A, Bolego C, Plebani M, Nardelli GB, Sartore S, Agostini C, Avogaro A. Gender differences in endothelial progenitor cells and cardiovascular risk profile: the role of female estrogens. *Arteriosclerosis, Thrombosis, and Vascular Biology* 2008; **28**: 997-1004.

Fadini GP, de Kreutzenberg SV, Coracina A, Baesso I, Agostini C, Tiengo A, Avogaro A. Circulating CD34+ cells, metabolic syndrome, and cardiovascular risk. *European Heart Journal* 2006; **27**: 2247-2255.

Fadini GP, Losordo D, Dimmeler S. Critical reevaluation of endothelial progenitor cell phenotypes for therapeutic and diagnostic use. *Circulation Research* 2012; **110**: 624-637.

Farha S, Asosingh K, Laskowski D, Licina L, Sekiguchi H, Losordo D, Dweik R, Wiedemann H, Erzurum S. Pulmonary gas transfer related to markers of angiogenesis during the menstrual cycle. *Journal of Applied Physiology* 2007; **103**: 1789-1795.

Fauconnier A, Chapron C, Dubuisson J-B, Vieira M, Dousset B, Bréart G. Relation between pain symptoms and the anatomic location of deep infiltrating endometriosis. *Fertility and Sterility* 2002; **78**: 719-726.

Ferguson B, Bennington J, Haber S. Histochemistry of mucosubstances and histology of mixed müllerian pelvic lymph node glandular inclusions. Evidence for histogenesis by müllerian metaplasia of coelomic epithelium. *Obstetrics and Gynecology* 1969; **33**: 617-625.

Ferrero S, Arena E, Morando A, Remorgida V. Prevalence of newly diagnosed endometriosis in women attending the general practitioner. *International Journal of Gynecology and Obstetrics* 2010; **110**: 203-207.

Ferrero S, Ragni N, Remorgida V. Antiangiogenic therapies in endometriosis. *British Journal of Pharmacology* 2006; **149**: 133-135.

Fleming H and Gurpide E. Growth characteristics of primary cultures of stromal cells from human endometrium. *Journal of Steroid Biochemistry* 1982; **16**: 717-720.

Folkman J. What is the evidence that tumors are angiogenesis dependent? *Journal of the National Cancer Institute* 1990; **82**: 4-7.

Foresta C, De Toni L, Di Mambro A, Ferlin A, Perilli L, Bertuzzi I, Galan A, Zuccarello D. Role of estrogen receptors in menstrual cycle–related neoangiogenesis and their influence on endothelial progenitor cell physiology. *Fertility and Sterility* 2010; **93**: 220-228.

Fràter-Schröder M, Risau W, Hallmann R, Gautschi P, Böhlen P. Tumor necrosis factor type alpha, a potent inhibitor of endothelial cell growth in vitro, is angiogenic in vivo. *Proceedings of the National Academy of Sciences* 1987; **84**: 5277-5281.

Fukumura D, Xavier R, Sugiura T, Chen Y, Park E-C, Lu N, Selig M, Nielsen G, Taksir T, Jain RK, Seed B. Tumor induction of VEGF promoter activity in stromal cells. *Cell* 1998; **94**: 715-725.

Gagne D, Page M, Robitaille G, Hugo P, Gosselin D. Levels of vascular endothelial growth factor (VEGF) in serum of patients with endometriosis. *Human Reproduction* 2003; **18**: 1674-1680.

Gang E, Jeong J, Han S, Yan Q, Jeon C-J, Kim H. In vitro endothelial potential of human UC blood-derived mesenchymal stem cells. *Cytotherapy* 2006; **8**: 215-227.

Gargett C and Rogers P. Human endometrial angiogenesis. *Reproduction* 2001; **121**: 181-186.

Gargett CE, Schwab KE, Zillwood RM, Nguyen HPT, Wu D. Isolation and culture of epithelial progenitors and mesenchymal stem cells from human endometrium. *Biology of Reproduction* 2009; **80**: 1136-1145.

Gazvani R and Templeton A. Peritoneal environment, cytokines and angiogenesis in the pathophysiology of endometriosis. *Reproduction* 2002; **123**: 217-226.

Gentilini D, Vigano P, Somigliana E, Vicentini LM, Vignali M, Busacca M, Di Blasio AM. Endometrial stromal cells from women with endometriosis reveal peculiar migratory behavior in response to ovarian steroids. *Fertility and Sterility* 2010; **93**: 706-715.

George J, Shmilovich H, Deutsch V, Miller H, Keren G, Roth A. Comparative analysis of methods for assessment of circulating endothelial progenitor cells. *Tissue Engineering* 2006; **12**: 331-335.

Gilabert-Estelles J, Ramon LA, Espana F, Gilabert J, Vila V, Reganon E, Castello R, Chirivella M, Estelles A. Expression of angiogenic factors in endometriosis: relationship to fibrinolytic and metalloproteinase systems. *Human Reproduction* 2007; **22**: 2120-2127.

Giudice LC. Endometriosis. *New England Journal of Medicine* 2010; **362**: 2389-2398.

Goodger AM and Rogers PAW. Endometrial endothelial cell proliferation during the menstrual cycle. *Human Reproduction* 1994; **9**: 399-405.

Grümmer R. Animal models in endometriosis research. *Human Reproduction Update* 2006; **12**: 641-649.

Grümmer R, Schwarzer F, Bainczyk K, Hess-Stumpp H, Regidor P-A, Schindler AE, Winterhager E. Peritoneal endometriosis: validation of an in-vivo model. *Human Reproduction* 2001; **16**: 1736-1743.

Gruppo Italiano per lo Studio dell Endometriosi. Ablation of lesions or no treatment in minimal–mild endometriosis in infertile women: a randomized trial. *Human Reproduction* 1999; **14**: 1332-1334.

Gruppo Italiano per lo Studio dell Endometriosi. Relationship between stage, site and morphological characteristics of pelvic endometriosis and pain. *Human Reproduction* 2001; **16**: 2668-2671.

Haasters F, Prall WC, Anz D, Bourquin C, Pautke C, Endres S, Mutschler W, Docheva D, Schieker M. Morphological and immunocytochemical characteristics indicate the yield of early progenitors and represent a quality control for human mesenchymal stem cell culturing. *Journal of Anatomy* 2009; **214**: 759-767.

Hadfield RM, Lain SJ, Raynes-Greenow CH, Morris JM, Roberts CL. Is there an association between endometriosis and the risk of pre-eclampsia? A population based study. *Human Reproduction* 2009; **24**: 2348-2352.

Halme J, Hammond M, Hulka J, Raj S, Talbert L. Retrograde menstruation in healthy women and in patients with endometriosis. *Obstetrics and Gynecology* 1984; **64**: 151-154.

Härkki-Sirén P and Kurki T. A nationwide analysis of laparoscopic complications. *Obstetrics and Gynecology* 1997; **89**: 108-112.

Hii LL and Rogers PA. Endometrial vascular and glandular expression of integrin alpha(v)beta3 in women with and without endometriosis. *Human Reproduction* 1998; **13**: 1030-1035.

Hill J, Zalos G, Halcox J, Schenke W, Waclawiw M, Quyyumi A, Finkel T. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *New England Journal of Medicine* 2003; **348**: 593-600.

Hirschi KK, Ingram DA, Yoder MC. Assessing identity, phenotype, and fate of endothelial progenitor cells. *Arteriosclerosis, Thrombosis, and Vascular Biology* 2008; **28**: 1584-1595.

Hoffman RA, Kung PC, Hansen WP, Goldstein G. Simple and rapid measurement of human T lymphocytes and their subclasses in peripheral blood. *Proceedings of the National Academy of Sciences* 1980; **77**: 4914-4917.

Holmes K, Roberts OLI, Thomas AM, Cross MJ. Vascular endothelial growth factor receptor-2: structure, function, intracellular signalling and therapeutic inhibition. *Cellular Signalling* 2007; **19**: 2003-2012.

Holyoake TL and Alcorn MJ. CD34+ positive haematopoietic cells: biology and clinical applications. *Blood Reviews* 1994; **8**: 113-124.

Hull ML, Charnock-Jones DS, Chan CLK, Bruner-Tran KL, Osteen KG, Tom BDM, Fan T-PD, Smith SK. Antiangiogenic agents are effective inhibitors of endometriosis. *Journal of Clinical Endocrinology & Metabolism* 2003; **88**: 2889-2899.

Hung S-C, Pochampally RR, Chen S-C, Hsu S-C, Prockop DJ. Angiogenic effects of human multipotent stromal cell conditioned medium activate the PI3K-Akt pathway in hypoxic endothelial cells to inhibit apoptosis, increase survival, and stimulate angiogenesis. *Stem Cells* 2007; **25**: 2363-2370.

Hur J, Yang H-M, Yoon C-H, Lee C-S, Park K-W, Kim J-H, Kim T-Y, Kim J-Y, Kang H-J, Chae I-H, Oh B-H, Park Y-B, Kim H-S. Identification of a Novel Role of T Cells in Postnatal Vasculogenesis: Characterization of Endothelial Progenitor Cell Colonies. *Circulation* 2007; **116**: 1671-1682.

Ingram DA, Mead LE, Tanaka H, Meade V, Fenoglio A, Mortell K, Pollok K, Ferkowicz MJ, Gilley D, Yoder MC. Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. *Blood* 2004; **104**: 2752-2760.

Iruela-Arispe ML, Rodriguez-Manzaneque JC, Abu-Jawdeh G. Endometrial endothelial cells express estrogen and progesterone receptors and exhibit a tissue specific response to angiogenic growth factors. *Microcirculation* 1999; **6**: 127-140.

Jacobson TZ, Duffy JMN, Barlow D, Koninckx PR, Garry R. Laparoscopic surgery for pelvic pain associated with endometriosis. *Cochrane Database of Systematic Reviews* 2009.

Jacobson TZ, Duffy JMN, Barlow DH, Farquhar C, Koninckx PR, Olive D. Laparoscopic surgery for subfertility associated with endometriosis. *Cochrane Database of Systematic Reviews* 2010.

Jain S, Ward MM, O'Loughlin J, Boeck M, Wiener N, Chuang E, Cigler T, Moore A, Donovan D, Lam C, Cobhma MV, Schneider S, Christos P, Baergen RN, Swistel S, Lane ME, Mittal V, Rafii S, Vahdat LT. Incremental increase in VEGFR1+ hematopoietic progenitor cells and VEGFR2+ endothelial progenitor cells predicts relapse and lack of tumor response in breast cancer patients. *Breast Cancer Research and Treatment* 2012; **132**: 235-242.

Jansen FW, Kapiteyn K, Trimbos-Kemper T, Hermans J, Trimbos JB. Complications of laparoscopy: a prospective multicentre observational study. *British Journal of Obstetrics and Gynaecology* 1997; **104**: 595-600.

Kalu E, Sumar N, Giannopoulos T, Patel P, Croucher C, Sherriff E, Bansal A. Cytokine profiles in serum and peritoneal fluid from infertile women with and without endometriosis. *Journal of Obstetrics and Gynaecology Research* 2007; **33**: 490-495.

Kang S, Li S-Z, Wang N, Zhou R-M, Wang T, Wang D-J, Li X-F, Bui J, Li Y. Association between genetic polymorphisms in fibroblast growth factor (FGF)1 and FGF2 and risk of endometriosis and adenomyosis in Chinese women. *Human Reproduction* 2010; **25**: 1806-1811.

Kayisli UA, Luk J, Guzeloglu-Kayisli O, Seval Y, Demir R, Arici A. Regulation of angiogenic activity of human endometrial endothelial cells in culture by ovarian steroids. *Journal of Clinical Endocrinology & Metabolism* 2004; **89**: 5794-5802.

Kennedy S, Bergqvist A, Chapron C, D'Hooghe T, Dunselman G, Greb R, Hummelshoj L, Prentice A, Saridogan E. ESHRE guideline for the diagnosis and treatment of endometriosis. *Human Reproduction* 2005; **20**: 2698-2704.

Khan KN, Masuzaki H, Fujishita A, Kitajima M, Hiraki K, Miura S, Sekine I, Ishimaru T. Peritoneal fluid and serum levels of hepatocyte growth factor may predict the activity of endometriosis. *Acta Obstetricia et Gynecologica Scandinavica* 2006; **85**: 458-466.

Khan KN, Masuzaki H, Fujishita A, Kitajima M, Sekine I, Ishimaru T. Immunoexpression of hepatocyte growth factor and c-Met receptor in the eutopic endometrium predicts the activity of ectopic endometrium. *Fertility and Sterility* 2003; **79**: 173-181.

Kim S-W, Kim H, Cho H-J, Lee J-U, Levit R, Yoon Y-S. Human peripheral blood-derived CD31+ cells have robust angiogenic and vasculogenic properties and are effective for treating ischemic vascular disease. *Journal of the American College of Cardiology* 2010; **56**: 593-607.

Kim S-W, Zhang H-Z, Kim CE, An HS, Kim J-M, Kim MH. Amniotic mesenchymal stem cells have robust angiogenic properties and are effective in treating hindlimb ischaemia. *Cardiovascular Research* 2012; **93**: 525-534.

Kinugasa S, Shinohara K, Wakatsuki A. Increased asymmetric dimethylarginine and enhanced inflammation are associated with impaired vascular reactivity in women with endometriosis. *Atherosclerosis* 2011; **219**: 784-788.

König J, Huppertz B, Desoye G, Parolini O, Fröhlich JD, Weiss G, Dohr G, Sedlmayr P, Lang I. Amnion-derived mesenchymal stromal cells show angiogenic properties but resist differentiation into mature endothelial cells. *Stem Cells and Development* 2012; **21**: 1309-1320.

Koolwijk P, Kapiteijn K, Molenaar B, van Spronsen E, van der Vecht B, Helmerhorst FM, van Hinsbergh VWM. Enhanced angiogenic capacity and urokinase-type plasminogen activator expression by endothelial cells isolated from human endometrium. *Journal of Clinical Endocrinology & Metabolism* 2001; **86**: 3359-3367.

Krause D, Fackler M, Civin C, May W. CD34: structure, biology, and clinical utility. *Blood* 1996; **87**: 1-13.

Kyama CM, Overbergh L, Debrock S, Valckx D, Vander Perre S, Meuleman C, Mihalyi A, Mwenda JM, Mathieu C, D'Hooghe TM. Increased peritoneal and endometrial gene expression of biologically relevant cytokines and growth factors during the menstrual phase in women with endometriosis. *Fertility and Sterility* 2006; **85**: 1667-1675.

Lamont T, Watts F, Panesar S, MacFie J, Matthew D. Early detection of complications after laparoscopic surgery: summary of a safety report from the National Patient Safety Agency. *British Medical Journal* 2011; **342**.

Laschke M, Giebels C, Nickels R, Scheuer C, Menger M. Endothelial progenitor cells contribute to the vascularisation of endometriotic lesions. *American Journal of Pathology* 2011; **178**: 442-450.

Lee HJ, Choi BH, Min B-H, Park SR. Changes in surface markers of human mesenchymal stem cells during the chondrogenic differentiation and dedifferentiation processes in vitro. *Arthritis & Rheumatism* 2009; **60**: 2325-2332.

Lemieux C, Cloutier I, Tanguay J. Menstrual cycle influences endothelial progenitor cell regulation: a link to gender differences in vascular protection? *International Journal of Cardiology* 2009; **136**: 200-210.

Li A, Dubey S, Varney ML, Dave BJ, Singh RK. IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis. *The Journal of Immunology* 2003; **170**: 3369-3376.

Li T, Saravelos H, Richmond M, Cooke I. Complications of laparoscopic pelvic surgery: recognition, management and prevention. *Human Reproduction Update* 1997; **3**: 505-515.

Lipton B, Bensch K, Karasek M. Microvessel endothelial cell transdifferentiation: phenotypic characterization. *Differentiation* 1991; **46**: 117-133.

Liu JW, Dunoyer-Geindre S, Serre-Beinier V, Mai G, Lambert J-F, Fish RJ, Pernod G, Buehler L, Bounameaux H, Kruithof EKO. Characterization of endothelial-like cells derived from human mesenchymal stem cells. *Journal of Thrombosis and Haemostasis* 2007; **5**: 826-834.

Liu X, Li Y, Liu Y, Luo Y, Wang D, Annex BH, Goldschmidt-Clermont PJ. Endothelial progenitor cells (EPCs) mobilized and activated by neurotrophic factors may contribute to pathologic neovascularization in diabetic retinopathy. *The American Journal of Pathology* 2010; **176**: 504-515.

Liu Y, Lu L, Zhu G. Angiogenesis of eutopic and ectopic endometria in endometriosis. *Journal of Huazhong University of Science and Technology [Medical Sciences]* 2003; **23**: 190-191.

Ludwig A, Jochmann N, Kertesz A, Kuhn C, Mueller S, Gericke C, Baumann G, Stangl K, Stangl V. Smoking decreases the level of circulating CD34+ progenitor cells in young healthy women - a pilot study. *BMC Women's Health* 2010; **10**: 20.

Luk J, Seval Y, Kayisli U, Ulukus M, Ulukus C, Arici A. Regulation of interleukin-8 expression in human endometrial endothelial cells: a potential mechanism for the pathogenesis of endometriosis. *Journal of Clinical Endocrinology & Metabolism* 2005; **90**: 1805-1811.

Luk J, Seval Y, Ulukus M, Ulukus C, Arici A, Kayisli U. Regulation of monocyte chemotactic protein-1 expression in human endometrial endothelial cells by sex steroids: a potential mechanism for leukocyte recruitment in endometriosis. *Reproductive Sciences* 2010; **17**: 278-287.

MacEneaney OJ, Kushner EJ, Van Guilder GP, Greiner JJ, Stauffer BL, DeSouza CA. Endothelial progenitor cell number and colony-forming capacity in overweight and obese adults. *International Journal of Obesity* 2008; **33**: 219-225.

Machado DE, Abrao MS, Berardo PT, Takiya CM, Nasciutti LE. Vascular density and distribution of vascular endothelial growth factor (VEGF) and its receptor VEGFR-2 (Flk-1) are significantly higher in patients with deeply infiltrating endometriosis affecting the rectum. *Fertility and Sterility* 2008; **90**: 148-155.

Mahmood T and Templeton A. Prevalence and genesis of endometriosis. *Human Reproduction* 1991; **6**: 544-549.

Mahmood TA and Templeton A. The impact of treatment on the natural history of endometriosis. *Human Reproduction* 1990; **5**: 965-970.

Marcoux S, Maheux R, Bérubé S. Laparoscopic surgery in infertile women with minimal or mild endometriosis. *New England Journal of Medicine* 1997; **337**: 217-222.

Markham R and Fraser I. The measurement of tumour necrosis factor alpha in patients with endometriosis. *Australian Journal of Medical Science* 1997a; **18**: 56-59.

Markham R and Fraser I. Blood and peritoneal fluid concentrations of TNFalpha and RANTES in patients with and without endometriosis. *Australian Journal of Medical Science* 1997b; **18**: 116-118.

Martin JJ and Hauck A. Endometriosis in the male. *The American Journal of Surgery* 1985; **51**: 426-430.

Masouleh BK, Baraniskin A, Schmiegel W, Schroers R. Quantification of circulating endothelial progenitor cells in human peripheral blood: establishing a reliable flow cytometry protocol. *Journal of Immunological Methods* 2010; **357**: 38-42.

Masuda H, Anwar SS, Bühring HJ, Rao JR, Gargett CE. A novel marker of human endometrial mesenchymal stem-like cells. *Cell Transplantation* 2012.

Matalliotakis I, Neonaki M, Zolindaki A, Hassan E, Georgoulias V, Koumantakis E. Changes in immunologic variables (TNF-a, sCD8 and sCD4) during danazol treatment in patients with endometriosis. *International Journal of Fertility and Women's Medicine* 1997; **42**: 211-214.

Matalliotakis IM, Goumenou AG, Koumantakis GE, Athanassakis I, Dionyssopoulou E, Neonaki MA, Vassiliadis S. Expression of serum human leukocyte antigen and growth factor levels in a Greek family with familial endometriosis. *Journal of the Society of Gynecologic Investigation* 2003; **10**: 118-121.

Matsubara K, Abe E, Matsubara Y, Kameda K, Ito M. Circulating endothelial progenitor cells during normal pregnancy and pre-eclampsia. *American Journal of Reproductive Immunology* 2006; **56**: 79-85.

Matsuzaki S, Canis M, Murakami T, Dechelotte P, Bruhat MA, Okamura K. Immunohistochemical analysis of the role of angiogenic status in the vasculature of peritoneal endometriosis. *Fertility and Sterility* 2001; **76**: 712-716.

May KE, Conduit-Hulbert SA, Villar J, Kirtley S, Kennedy SH, Becker CM. Peripheral biomarkers of endometriosis: a systematic review. *Human Reproduction Update* 2010; **16**: 651-674.

May KE, Villar J, Kirtley S, Kennedy SH, Becker CM. Endometrial alterations in endometriosis: a systematic review of putative biomarkers. *Human Reproduction Update* 2011; **17**: 637-653.

McLaren J, Prentice A, Charnock-Jones DS, Smith SK. Vascular endothelial growth factor (VEGF) concentrations are elevated in peritoneal fluid of women with endometriosis. *Human Reproduction* 1996; **11**: 220-223.

Melo AS, Rosa-e-Silva JC, Rosa-e-Silva AC, Poli-Neto OB, Ferriani RA, Vieira CS. Unfavorable lipid profile in women with endometriosis. *Fertility and Sterility* 2010; **93**: 2433-2436.

Meuleman C, Vandenabeele B, Fieuws S, Spiessens C, Timmerman D, D'Hooghe T. High prevalence of endometriosis in infertile women with normal ovulation and normospermic partners. *Fertility and Sterility* 2009; **92**: 68-74.

Minguell JJ, Erices A, Conget P. Mesenchymal stem cells. *Experimental Biology and Medicine* 2001; **226**: 507-520.

Mirshahi P, Rafii A, Vincent L, Berthaut A, Varin R, Kalantar G, Marzac C, Calandini OA, Marie j-P, Soria C, Soria J, Mirshahi M. Vasculogenic mimicry of acute leukemic bone marrow stromal cells. *Leukemia* 2009; **23**: 1039-1048.

Moen MH and Muss KM. Endometriosis in pregnant and non-pregnant women at tubal sterilization. *Human Reproduction* 1991; **6**: 699-702.

Mohamed M, El Behery M, Mansour S. Comparative study between VEGF-A and CA-125 in diagnosis and follow-up of advanced endometriosis after conservative laparoscopic surgery. *Archives of Gynecology and Obstetrics* 2013; **287**: 77-82.

Mol BWJ, Bayram N, Lijmer JG, Wiegerinck MAHM, Bongers MY, van der Veen F, Bossuyt PMM. The performance of CA-125 measurement in the detection of endometriosis: a meta-analysis. *Fertility and Sterility* 1998; **70**: 1101-1108.

Moore J, Copley S, Morris J, Lindsell D, Golding S, Kennedy S. A systematic review of the accuracy of ultrasound in the diagnosis of endometriosis. *Ultrasound in Obstetrics and Gynecology* 2002; **20**: 630-634.

Morrison SJ, Shah NM, Anderson DJ. Regulatory mechanisms in stem cell biology. *Cell* 1997; **88**: 287-298.

Müller-Ehmsen J, Braun D, Schneider T, Pfister R, Worm N, Wielckens K, Scheid C, Frommolt P, Flesch M. Decreased number of circulating progenitor cells in obesity: beneficial effects of weight reduction. *European Heart Journal* 2008; **29**: 1560-1568.

Na Y-J, Yang S-H, Baek D-W, Lee D-H, Kim K-H, Choi Y-M, Oh S-T, Hong Y-S, Kwak J-Y, Lee K-S. Effects of peritoneal fluid from endometriosis patients on the release of vascular endothelial growth factor by neutrophils and monocytes. *Human Reproduction* 2006; **21**: 1846-1855.

Nagaya N, Fujii T, Iwase T, Ohgushi H, Itoh T, Uematsu M, Yamagishi M, Mori H, Kangawa K, Kitamura S. Intravenous administration of mesenchymal stem cells improves cardiac function in rats with acute myocardial infarction through angiogenesis and myogenesis. *American Journal of Physiology - Heart and Circulatory Physiology* 2004; **287**: H2670-H2676.

Naito H, Kidoya H, Sakimoto S, Wakabayashi T, Takakura N. Identification and characterization of a resident vascular stem/progenitor cell population in preexisting blood vessels. *The European Molecular Biology Organization Journal* 2012; **31**: 842-855.

Nakatsu MN, Sainson RCA, Aoto JN, Taylor KL, Aitkenhead M, Pérez-del-Pulgar S, Carpenter PM, Hughes CCW. Angiogenic sprouting and capillary lumen formation modeled by human umbilical vein endothelial cells (HUVEC) in fibrin gels: the role of fibroblasts and angiopoietin-1. *Microvascular Research* 2003; **66**: 102-112.

Nap AW, Griffioen AW, Dunselman GAJ, Bouma-Ter Steege JCA, Thijssen VLJL, Evers JLH, Groothuis PG. Antiangiogenesis Therapy for Endometriosis. *Journal of Clinical Endocrinology & Metabolism* 2004; **89**: 1089-1095.

Nap AW, Groothuis PG, Demir AY, Maas JWM, Dunselman GAJ, de Goeij AFPM, Evers JLH. Tissue integrity is essential for ectopic implantation of human endometrium in the chicken chorioallantoic membrane. *Human Reproduction* 2003; **18**: 30-34.

Neufeld G, Cohen T, Gengrinovitch S, Poltoral Z. Vascular endothelial growth factor (VEGF) and its receptors. *The Journal of the Federation of American Societies for Experimental Biology* 1999; **13**: 9-22.

Nieminen U. Studies on the vascular pattern of ectopic endometrium with special reference to cyclic changes. *Acta Obstetricia et Gynecologica Scandinavica* 1962; **41**: 1-81.

Nikitenko LL, MacKenzie IZ, Rees MCP, Bicknell R. Adrenomedullin is an autocrine regulator of endothelial growth in human endometrium. *Molecular Human Reproduction* 2000; **6**: 811-819.

Nisolle M, Casanas-Roux F, Anaf V, Mine JM, Donnez J. Morphometric study of the stromal vascularization in peritoneal endometriosis. *Fertility and Sterility* 1993; **59**: 681-684.

Nisolle M and Donnez J. Peritoneal endometriosis, ovarian endometriosis, and adenomyotic nodules of the rectovaginal septum are three different entities. *Fertility and Sterility* 1997; **68**: 585-596.

Novella-Maestre E, Carda C, Ruiz-Sauri A, Garcia-Velasco JA, Simon C, Pellicer A. Identification and quantification of dopamine receptor 2 in human eutopic and ectopic endometrium: a novel molecular target for endometriosis therapy. *Biology of Reproduction* 2010; **83**: 866-873.

Olive D and Henderson D. Endometriosis and Müllerian anomalies. *Obstetrics and Gynecology* 1987; **69**: 412-415.

Oosterlynck DJ, Meuleman C, Sobis H, Vandeputte M, Koninckx PR. Angiogenic activity of peritoneal fluid from women with endometriosis. *Fertility and Sterility* 1993; **59**: 778-782.

Oral E, Olive D, Arici A. The peritoneal environment in endometriosis. *Human Reproduction Update* 1996; **2**: 385-398.

Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, Carey VJ, Richardson AL, Weinberg RA. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell* 2005; **121**: 335-348.

Oswald J, Boxberger S, Jørgensen B, Feldmann S, Ehninger G, Bornhäuser M, Werner C. Mesenchymal stem cells can be differentiated into endothelial cells in vitro. *Stem Cells* 2004; **22**: 377-384.

Othman EE-D, Hornung D, Salem HT, Khalifa EA, El-Metwally TH, Al-Hendy A. Serum cytokines as biomarkers for nonsurgical prediction of endometriosis. *European Journal of Obstetrics, Gynecology, & Reproductive Biology* 2008; **137**: 240-246.

Overton WR. Modified histogram subtraction technique for analysis of flow cytometry data. *Cytometry* 1988; **9**: 619-626.

Painter JN, Anderson CA, Nyholt DR, Macgregor S, Lin J, Lee SH, Lambert A, Zhao ZZ, Roseman F, Guo Q, Gordon SD, Wallace L, Henders AK, Visscher PM, Kraft P, Martin NG, Morris AP, Treloar SA, Kennedy SH, Missmer SA, Montgomery GW, Zondervan KT. Genome-wide association study identifies a locus at 7p15.2 associated with endometriosis. *Nature Genetics* 2011; **43**: 51-54.

Paranya G, Vineberg S, Dvorin E, Kaushal S, Roth SJ, Rabkin E, Schoen FJ, Bischoff J. Aortic valve endothelial cells undergo transforming growth factor-β-mediated and non-transforming growth factor-β-mediated transdifferentiation in vitro. *The American Journal of Pathology* 2001; **159**: 1335-1343.

Pellicer A, Albert C, Mercader A, Bonilla-Musoles F, Remohl J, Simon C. The follicular and endocrine environment in women with endometriosis: local and systemic cytokine production. *Fertility and Sterility* 1998; **70**: 425-431.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. *Science* 1999; **284**: 143-147.

Pizzo A, Salmeri F, Ardita F, Sofo V, Tripepi M, Marsico S. Behaviour of cytokine levels in serum and peritoneal fluid of women with endometriosis. *Gynecologic and Obstetric Investigation* 2002; **54**: 2002.

Povsic TJ, Zavodni KL, Vainorius E, Kherani JF, Goldschmidt-Clermont PJ, Peterson ED. Common endothelial progenitor cell assays identify discrete endothelial progenitor cell populations. *American Heart Journal* 2009; **157**: 335-344.

Pradhan KR, Mund JA, Johnson C, Vik TA, Ingram DA, Case J. Polychromatic flow cytometry identifies novel subsets of circulating cells with angiogenic potential in pediatric solid tumors. *Cytometry Part B: Clinical Cytometry* 2011; **80B**: 335-338.

Pretta S, Remorgida V, Abbamonte LH, Anserini P, Ragni N, Del Sette M, Gandolfo C, Ferrero S. Atherosclerosis in women with endometriosis. *European Journal of Obstetrics & Gynecology and Reproductive Biology* 2007; **132**: 226-231.

Print C, Valtola R, Evans A, Lessan K, Malik S, Smith S. Soluble factors from human endometrium promote angiogenesis and regulate the endothelial cell transcriptome. *Human Reproduction* 2004; **19**: 2356-2366.

Pupo-Nogueira A, de Oliveira RM, Petta CA, Podgaec S, Dias JA, Jr., Abrao MS. Vascular endothelial growth factor concentrations in the serum and peritoneal fluid of women with endometriosis. *International Journal of Gynecology and Obstetrics* 2007; **99**: 33-37.

Rehman J, Li J, Orschell CM, March KL. Peripheral blood “endothelial progenitor cells” are derived from monocyte/macrophages and secrete angiogenic growth factors. *Circulation* 2003; **107**: 1164-1169.

Ridley J and Edwards I. Experimental endometriosis in the human. *American Journal of Obstetrics and Gynecology* 1958; **76**: 783-789.

Risau W. Mechanisms of angiogenesis. *Nature* 1997; **386**: 671-674.

Robb AO, Mills NL, Smith IB, Short A, Tura-Ceide O, Barclay GR, Blomberg A, Critchley HO, Newby DE, Denison FC. Influence of menstrual cycle on circulating endothelial progenitor cells. *Human Reproduction* 2009; **24**: 619-625.

Roederer M. Spectral compensation for flow cytometry: Visualization artifacts, limitations, and caveats. *Cytometry* 2001; **45**: 194-205.

Roederer M. How many events is enough? Are you positive? *Cytometry Part A* 2008; **73A**: 384-385.

Rogers MS, Rohan RM, Birsner AE, D'Amato RJ. Genetic loci that control vascular endothelial growth factor-induced angiogenesis. *The Journal of the Federation of American Societies for Experimental Biology* 2003; **17**: 2112-2114.

Rogers P. Structure and function of endometrial blood vessels. *Human Reproduction Update* 1996; **2**: 57-62.

Rogers P, Lederman F, Taylor N. Endometrial microvascular growth in normal and dysfunctional states. *Human Reproduction Update* 1998; **4**: 503-508.

Rogers PAW, D'Hooghe TM, Fazleabas A, Gargett CE, Giudice LC, Montgomery GW, Rombauts L, Salamonsen LA, Zondervan KT. Priorities for Endometriosis Research: Recommendations From an International Consensus Workshop. *Reproductive Sciences* 2009; **16**: 335-346.

Rogers PAW and Gargett CE. Endometrial angiogenesis. *Angiogenesis* 1998; **2**: 287-294.

Rohan RM, Fernandez A, Udagawa T, Yuan J, D’Amato RJ. Genetic heterogeneity of angiogenesis in mice. *The Journal of the Federation of American Societies for Experimental Biology* 2000; **14**: 871-876.

Rousseau A, Ayoubi F, Deveaux C, Charbit B, Delmau C, Christin-Maitre S, Jaillon P, Uzan G, Simon T. Impact of age and gender interaction on circulating endothelial progenitor subsets in healthy subjects. *Fertility and Sterility* 2010; **93**: 843-846.

Ryan I, Tseng J, Schriock E, Khorram O, Landers D, Taylor R. Interleukin-8 concentrations are elevated in peritoneal fluid of womon with endometriosis. *Fertility and Sterility* 1995; **63**: 929-932.

Safranow K, Kotowski M, Lewandowska J, Machalińska A, Dziedziejko V, Czajka R, Celewicz Z, Rudniki J, Machaliński B. Circulating endothelial progenitor cells in premature infants: is there an association with premature birth complications? *Journal of Perinatal Medicine* 2012; **40**: 455-462.

Salahuddin SZ, Markham PD, Gallo RC. Establishment of long-term monocyte suspension cultures from normal human peripheral blood. *Journal of Experimental Medicine* 1982; **155**: 1842-1857.

Salcedo R, Ponce ML, Young HA, Wasserman K, Ward JM, Kleinman HK, Oppenheim JJ, Murphy WJ. Human endothelial cells express CCR2 and respond to MCP-1:

direct role of MCP-1 in angiogenesis and tumor progression. *Blood* 2000; **96**: 34-40.

Salzman GC, Crowell JM, Martin JC, Trujillo TT, Romero A, Mullaney PF, LaBauve PM. Cell classification by laser light scattering: identification and separation of unstained leukocytes. *Acta Cytologica* 1975; **19**: 374-377.

Sampson J. Peritoneal endometriosis due to the menstrual dissemination of endometrial tissue into the peritoneal cavity. *American Journal of Obstetrics and Gynecology* 1927a; **14**: 422-469.

Sampson J. Metastatic or embolic endometriosis due to menstrual dissemination of endometrial tissue into the venous circulation. *American Journal of Pathology* 1927b; **3**: 93-109.

Sanchez-Guijo F, Oterino E, Barbado M, Carrancio S, Lopez-Holgado N, Muntion S, Hernandez-Campo P, Sanchez-Abarca L, Perez-Simon J, San Miguel J, Briñon J, del Cañizo M. Both CD133(+) cells and monocytes provide significant improvement for hindlimb ischemia, although they do not transdifferentiate into endothelial cells. *Cell Transplantation* 2010; **19**: 103-112.

Santamaria X, Massasa EE, Feng Y, Wolff E, Taylor HS. Derivation of insulin producing cells from human endometrial stromal stem cells and use in the treatment of murine diabetes. *Molecular Therapy* 2011; **19**: 2065-2071.

Santoro L, D'Onofrio F, Campo S, Ferraro PM, Tondi P, Campo V, Flex A, Gasbarrini A, Santoliquido A. Endothelial dysfunction but not increased carotid intima-media thickness in young European women with endometriosis. *Human Reproduction* 2012; **27**: 1320-1326.

Sasson IE and Taylor HS. Stem Cells and the Pathogenesis of Endometriosis. *Annals of the New York Academy of Sciences* 2008; **1127**: 106-115.

Schatz F, Soderland C, Hendricks-Muñoz K, Gerrets R, Lockwood C. Human endometrial endothelial cells: isolation, characterization, and inflammatory-mediated expression of tissue factor and type 1 plasminogen activator inhibitor. *Biology of Reproduction* 2000; **62**: 691-697.

Schmidt-Lucke C, Fichtlscherer S, Aicher A, Tschöpe C, Schultheiss H-P, Zeiher AM, Dimmeler S. Quantification of circulating endothelial progenitor cells using the modified ISHAGE protocol. *PLoS ONE* 2010; **5**: e13790.

Schmidt-Lucke C, Rössig L, Fichtlscherer S, Vasa M, Britten M, Kämper U, Dimmeler S, Zeiher AM. Reduced number of circulating endothelial progenitor cells predicts future cardiovascular events. *Circulation* 2005; **111**: 2981-2987.

Schneider BP, Radovich M, Miller KD. The role of vascular endothelial growth factor genetic variability in cancer. *Clinical Cancer Research* 2009; **15**: 5297-5302.

Schwab KE and Gargett CE. Co-expression of two perivascular cell markers isolates mesenchymal stem-like cells from human endometrium. *Human Reproduction* 2007; **22**: 2903-2911.

Schwab KE, Hutchinson P, Gargett CE. Identification of surface markers for prospective isolation of human endometrial stromal colony-forming cells. *Human Reproduction* 2008; **23**: 934-943.

Seeber B, Sammel MD, Fan X, Gerton GL, Shaunik A, Chittams J, Barnhart KT. Panel of markers can accurately predict endometriosis in a subset of patients. *Fertility and Sterility* 2008; **89**: 1073-1081.

Sha G, Wu D, Zhang L, Chen X, Lei M, Sun H, Lin S, Lang J. Differentially expressed genes in human endometrial endothelial cells derived from eutopic endometrium of patients with endometriosis compared with those from patients without endometriosis. *Human Reproduction* 2007; **22**: 3159-3169.

Sha G, Zhang Y, Zhang C, Wan Y, Zhao Z, Li C, Lang J. Elevated levels of gremlin-1 in eutopic endometrium and peripheral serum in patients with endometriosis. *Fertility and Sterility* 2009; **91**: 350-358.

Shaked Y, Bertolini F, Man S, Rogers MS, Cervi D, Foutz T, Rawn K, Voskas D, Dumont DJ, Ben-David Y, Lawler J, Henkin J, Huber J, Hicklin DJ, D'Amato RJ, Kerbel RS. Genetic heterogeneity of the vasculogenic phenotype parallels angiogenesis: implications for cellular surrogate marker analysis of antiangiogenesis. *Cancer Cell* 2005; **7**: 101-111.

Sharkey AM, Day K, McPherson A, Malik S, Licence D, Smith SK, Charnock-Jones DS. Vascular endothelial growth factor expression in human endometrium is regulated by hypoxia. *Journal of Clinical Endocrinology & Metabolism* 2000; **85**: 402-409.

Shmelkov SV, St. Clair R, Lyden D, Rafii S. AC133/CD133/Prominin-1. *International Journal of Biochemistry and Cell Biology* 2005; **37**: 715-719.

Smith NR, Baker D, James NH, Ratcliffe K, Jenkins M, Ashton SE, Sproat G, Swann R, Gray N, Ryan A, Jürgensmeier JM, Womack C. Vascular endothelial growth factor receptors VEGFR-2 and VEGFR-3 are localized primarily to the vasculature in human primary solid cancers. *Clinical Cancer Research* 2010; **16**: 3548-3561.

Smythe J, Fox A, Fisher N, Frith E, Harris AL, Watt SM. Measuring angiogenic cytokines, circulating endothelial cells, and endothelial progenitor cells in peripheral blood and cord blood: VEGF and CXCL12 correlate with the number of circulating endothelial progenitor cells in peripheral blood *Tissue Engineering Part C: Methods* 2008; **14**: 59-67.

Somigliana E, Vigano P, Parazzini F, Stoppelli S, Giambattista E, Vercellini P. Association between endometriosis and cancer: A comprehensive review and a critical analysis of clinical and epidemiological evidence. *Gynecologic Oncology* 2006; **101**: 331-341.

Somigliana E, Viganò P, Rossi G, Carinelli S, Vignali M, Panina-Bordignon P. Endometrial ability to implant in ectopic sites can be prevented by interleukin-12 in a murine model of endometriosis. *Human Reproduction* 1999; **14**: 2944-2950.

Sommer M, Gerth J, Stein G, Wolf G. Transdifferentiation of endothelial and renal tubular epithelial cells into myofibroblast-like cells under in vitro conditions: a morphological analysis. *Cells Tissues Organs* 2005; **180**: 204-214.

Stanford EJ, Koziol J, Feng A. The prevalence of interstitial cystitis, endometriosis, adhesions, and vulvar pain in women with chronic pelvic pain. *Journal of Minimally Invasive Gynecology* 2005; **12**: 43-49.

Steff AM, Gagne D, Page M, Rioux A, Hugo P, Gosselin D. Serum concentrations of insulin-like growth factor-1, soluble tumor necrosis factor receptor-1 and angiogenin in endometriosis patients. *American Journal of Reproductive Immunology* 2004; **51**: 166-173.

Takehara M, Ueda M, Yamashita Y, Terai Y, Hung YC, Ueki M. Vascular endothelial growth factor A and C gene expression in endometriosis. *Human Pathology* 2004; **35**: 1369-1375.

Talbi S, Hamilton AE, Vo KC, Tulac S, Overgaard MT, Dosiou C, Le Shay N, Nezhat CN, Kempson R, Lessey BA, Nayak NR, Giudice LC. Molecular phenotyping of human endometrium distinguishes menstrual cycle phases and underlying biological processes in normo-ovulatory women. *Endocrinology* 2006; **147**: 1097-1121.

Tan XJ, Lang JH, Liu DY, Shen K, Leng JH, Zhu L. Expression of vascular endothelial growth factor and thrombospondin-1 mRNA in patients with endometriosis. *Fertility and Sterility* 2002; **78**: 148-153.

Tang J, Xie Q, Pan G, Wang J, Wang M. Mesenchymal stem cells participate in angiogenesis and improve heart function in rat model of myocardial ischemia with reperfusion. *European Journal of Cardio-Thoracic Surgery* 2006; **30**: 353-361.

Taylor RN, Jie Yu, Torres PB, Schickedanz AC, Park JK, Mueller MD, Sidell N. Mechanistic and therapeutic implications of angiogenesis in endometriosis. *Reproductive Sciences* 2009; **16**: 140-146.

Taylor RN, Lebovic DI, Hornung D, Mueller MD. Endocrine and paracrine regulation of endometrial angiogenesis. *Annals of the New York Academy of Sciences* 2001; **943**: 109-121.

Thomas E and Cooke I. Impact of gestrinone on the course of asymptomatic endometriosis. *British Medical Journal (Clinical Research edition)* 1987; **294**: 272-274.

Timmermans F, Plum J, Yöder MC, Ingram DA, Vandekerckhove B, Case J. Endothelial progenitor cells: identity defined? *Journal of Cellular and Molecular Medicine* 2009; **13**: 87-102.

Timmermans F, Van Hauwermeiren F, De Smedt M, Raedt R, Plasschaert F, De Buyzere ML, Gillebert TC, Plum J, Vandekerckhove B. Endothelial Outgrowth Cells Are Not Derived From CD133+ Cells or CD45+ Hematopoietic Precursors. *Arteriosclerosis, Thrombosis, and Vascular Biology* 2007; **27**: 1572-1579.

Tögel F, Weiss K, Yang Y, Hu Z, Zhang P, Westenfelder C. Vasculotropic, paracrine actions of infused mesenchymal stem cells are important to the recovery from acute kidney injury. *American Journal of Physiology - Renal Physiology* 2007; **292**: F1626-F1635.

Tsoumpou I, Kyrgiou M, Gelbaya TA, Nardo LG. The effect of surgical treatment for endometrioma on in vitro fertilization outcomes: a systematic review and meta-analysis. *Fertility and Sterility* 2009; **92**: 75-87.

Tuder RM, Karasek MA, Bensch KG. Cyclic adenosine monophosphate levels and the function of skin microvascular endothelial cells. *Journal of Cellular Physiology* 1990; **142**: 272-283.

Uno S, Zembutsu H, Hirasawa A, Takahashi A, Kubo M, Akahane T, Aoki D, Kamatani N, Hirata K, Nakamura Y. A genome-wide association study identifies genetic variants in the CDKN2BAS locus associated with endometriosis in Japanese. *Nature Genetics* 2010; **42**: 707-710.

Venneri MA, Palma MD, Ponzoni M, Pucci F, Scielzo C, Zonari E, Mazzieri R, Doglioni C, Naldini L. Identification of proangiogenic TIE2-expressing monocytes (TEMs) in human peripheral blood and cancer. *Blood* 2007; **109**: 5276-5285.

Vercellini P, De Benedetti F, Rossi E, Colombo A, Trespidi L, Crosignani PG. Tumor necrosis factor in plasma and peritoneal fluid of women with and without endometriosis. *Gynecologic and Obstetric Investigation* 1993; **36**: 39-41.

Vercellini P, Fedele L, Aimi G, Pietropaolo G, Consonni D, Crosignani PG. Association between endometriosis stage, lesion type, patient characteristics and severity of pelvic pain symptoms: a multivariate analysis of over 1000 patients. *Human Reproduction* 2007; **22**: 266-271.

Williams ARW, Critchley HOD, Osei J, Ingamells S, Cameron IT, Han C, Chwalisz K. The effects of the selective progesterone receptor modulator asoprisnil on the morphology of uterine tissues after 3 months treatment in patients with symptomatic uterine leiomyomata. *Human Reproduction* 2007; **22**: 1696-1704.

Wingfield M, Macpherson A, Healy DL, Rogers PA. Cell proliferation is increased in the endometrium of women with endometriosis. *Fertility and Sterility* 1995; **64**: 340-346.

Wolff EF, Gao X-B, Yao KV, Andrews ZB, Du H, Elsworth JD, Taylor HS. Endometrial stem cell transplantation restores dopamine production in a Parkinson's disease model. *Journal of Cellular and Molecular Medicine* 2011; **15**: 747-755.

Wu M and Ho H. The role of cytokines in endometriosis. *American Journal of Reproductive Immunology* 2003; **49**: 285-296.

Wu Y, Chen L, Scott PG, Tredget EE. Mesenchymal stem cells enhance wound healing through differentiation and angiogenesis. *Stem Cells* 2007; **25**: 2648-2659.

Xavier P, Belo L, Beires J, Rebelo I, Martinez-de-Oliveira J, Lunet N, Barros H. Serum levels of VEGF and TNF-a and their association with C-reactive protein in patients with endometriosis. *Archives of Gynecology and Obstetrics* 2006; **273**: Jan.

Yoder MC. Is endothelium the origin of endothelial progenitor cells? *Arteriosclerosis, Thrombosis, and Vascular Biology* 2010; **30**: 1094-1103.

Yoder MC, Mead LE, Prater D, Krier TR, Mroueh KN, Li F, Krasich R, Temm CJ, Prchal JT, Ingram DA. Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals. *Blood* 2007; **109**: 1801-1809.

Zhang B, Yang S, Zhang Y, Sun Z, Xu W, Ye S. Co-culture of mesenchymal stem cells with umbilical vein endothelial cells under hypoxic condition. *Journal of Huazhong University of Science and Technology [Medical Sciences]* 2012; **32**: 173-180.

Zhang P, Baxter J, Vinod K, Tulenko TN, Di Muzio PJ. Endothelial differentiation of amniotic-fluid derived stem cells: synergism of biochemical and shear force stimuli. *Stem Cells and Development* 2009; **18**: 1299-1308.

Zhu W-H, Iurlaro M, MacIntyre A, Fogel E, Nicosia RF. The mouse aorta model: influence of genetic background and aging on bFGF- and VEGF-induced angiogenic sprouting. *Angiogenesis* 2003; **6**: 193-199.

Zong L, Li Y, Ha X. Determination of HGF concentration in serum and peritoneal fluid in women with endometriosis. *Di Yi Jun Yi Da Xue Xue Bao* 2003; **23**: 757-760.

Chapter 7: Appendices

7.1 Appendix 1

7.1.1 Antibodies

Antibodies used in Chapter 2 are detailed in Table 7-A, below.

Antibody	**Fluorophore**	**Manufacturer**	**Catalogue number**	**Monoclonal (M)/ Polyclonal (P)**	**Final concentration**
CD34	PE-Cy7	BD	560710	m	1.25μg/ml
Mouse IgG1κ Isotype Control	PE-Cy7	BD	557872	m	1.25μg/ml
VEGFR-2	AF647	BD	560495	m	2.5μg/ml
Mouse IgG1κ Isotype Control	AF-647	BD	557714	m	2.5μg/ml
VEGFR-2	APC	R&D	FAB357A	m	2.5μg/ml
Mouse IgG1 Isotype Control	APC	R&D	IC002A	m	2.5μg/ml
VEGFR-2	AF-647	New England Biolabs	3628S	m	1μg/ml
Rabbit mAb Isotype control	AF-647	New England Biolabs	2985S	m	1μg/ml
CD133	PE	Miltenyi Biotec	130-080-801	m	2.5μg/ml
Mouse IgG1 Isotype control	PE	Miltenyi Biotec	130-092-212	m	2.5μg/ml
CD45	PerCP-Cy5.5	eBioscience	45-9459-42	m	1.25μg/ml
Mouse IgG1 Isotype Control	PerCP-Cy5.5	eBioscience	45-4714-80	m	1.25μg/ml
CD144	FITC	BD	560411	m	2.5μg/ml
Mouse IgG1κ Isotype Control	FITC	BD	555748	m	2.5μg/ml
CD31	APC-Cy7	BioLegend	303120	m	2.5μg/ml
Mouse IgG1 κ Isotype Control	APC-Cy7	BioLegend	400128	m	2.5μg/ml
YO-PRO 1 (nucleic acid stain)	FITC	Invitrogen	Y3603	n/a	10nM

Table 7-A: Antibodies used in Chapter 2.

7.1.2 Optimisation of staining – antibody titration

Antibody concentrations were optimised by titration on populations of cells known to express the antigen of interest. PBMCs were used to titrate CD45; HUVECs were used to titrate CD34, CD144, CD31 and VEGFR-2, and apoptotic PBMCS were used to titrate the viability dye (YO-PRO-1). Serial dilutions of each antibody of interest were incubated with appropriate cells for 30 minutes on ice. Equivalent concentrations of isotype control antibodies were used to identify background staining for comparison. After incubation, cells were washed three times with 2% FCS before analysis on the flow cytometer. After excluding dead cells and debris based on forward and side scatter, a gate was set from the isotype control tube, at an arbitrary cut-off of 1% positive events. This gate was then compared to the results in the corresponding sample (containing the antibody of interest, at equivalent concentration). The percentage of positive cells in the samples was recorded. In addition, a signal:noise ratio was calculated for each sample by the following equation:

$$\frac{\text{MFI stained - MFI unstained}}{\text{standard deviation of MFI unstained}}$$

Where:

MFI stained = mean fluorescence intensity of cells in positive gate from sample tube

MFI unstained = mean fluorescence intensity of cells in positive gate from isotype control

A representative example, demonstrating titration of CD31-APC-Cy7 on HUVEC is shown in Figure 7.1. The antibody concentration that demonstrated the greatest percentage of positive events with high signal: noise ratio (indicating clear distinction between stained and unstained cells) was chosen for future use.

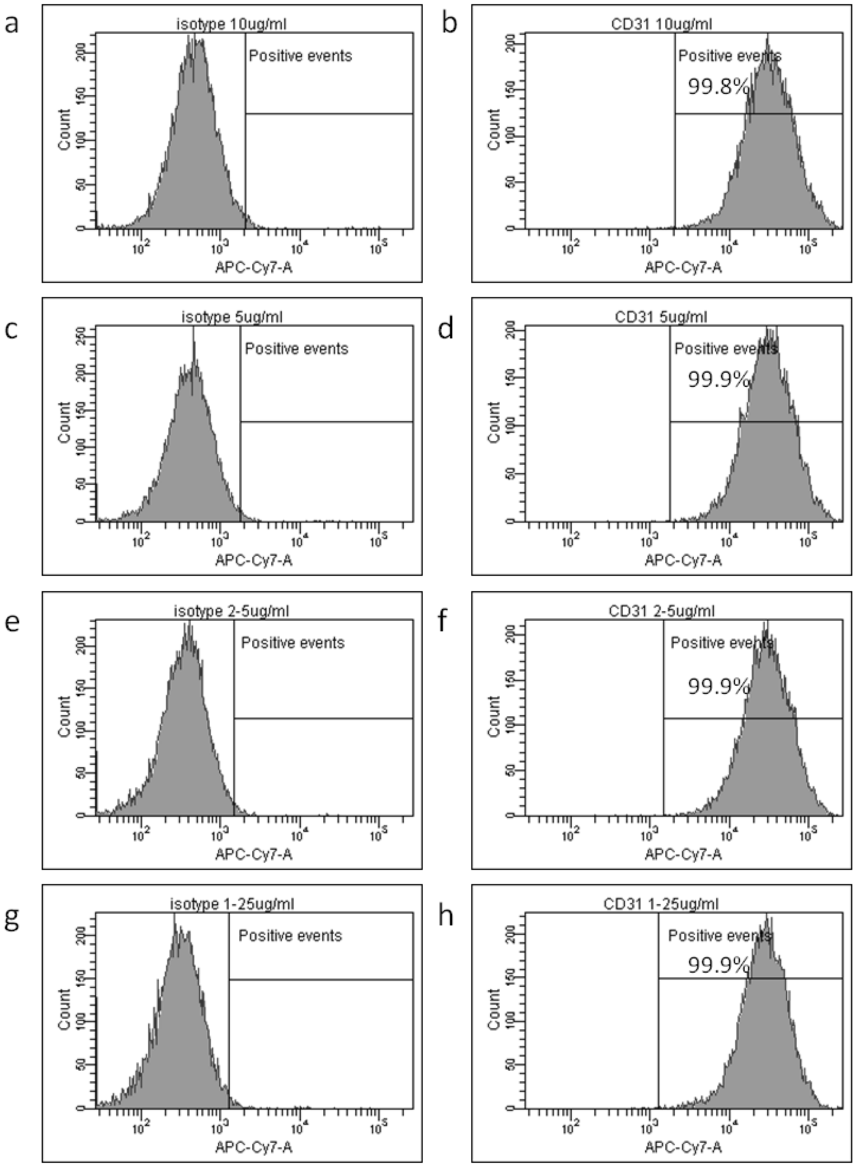

Figure 7.1 Titration of CD31. HUVEC were stained with different concentrations of CD31 antibody and equivalent concentrations of isotype control to determine the optimum concentration for staining. Left hand images show isotype controls. (a) and (b) 99.8% positive events with signal: noise ratio 1.47 at 10μg/ml; (c) and (d) 99.9% positive events with signal: noise ratio 4.48 at 5μg/ml; (e) and (f) 99.9% positive events with signal: noise ratio 7.66 at 2.5μg/ml; (g) and (h) 99.9% positive events with signal: noise ratio 2.75 at 1.25μg/ml. Adequate positive staining was obtained with the highest signal: noise ratio at 2.5μg/ml, therefore this concentration was used for all experiments.

7.1.3 Alternative protocol to identify CACs

Preliminary experiments used a different protocol to identify CACs as $CD34^{+}CD133^{+}CD144^{+}VEGFR\text{-}2^{+}CD45^{-/dim}$ cells. Positive staining was based on FMO+isotype control tubes – including all antibodies bar one. The missing antibody was substituted by the corresponding isotype control for that fluorophore. Antibody combinations are shown in table 7-B.

Tube	**CD45**	**CD34**	**CD133**	**CD144**	**VEGFR-2**	**Isotype control**	**Buffer (2% FCS)**
Blank	-	-	-	-	-	-	55µl
CACs	✓	✓	✓	✓	✓	-	-
CD45 FMO + isotype	-	✓	✓	✓	✓	Mouse IgG1- PerCP-Cy5.5 Isotype Control	4.375µl
CD34 FMO + isotype	✓	-	✓	✓	✓	Mouse IgG1- PE-Cy7 Isotype Control	2.5µl
CD133 FMO + isotype	✓	✓	-	✓	✓	Mouse IgG1- PE Isotype Control	3.75µl
CD144 FMO + isotype	✓	✓	✓	-	✓	Mouse IgG1- FITC Isotype Control	15µl
VEGFR-2 FMO + isotype	✓	✓	✓	✓	-	Mouse IgG1- AF-647 Isotype Control	17.5µl

Table 7-B: Combinations of antibodies and buffers for preliminary CAC experiments.

7.1.3.1 Results from alternative CAC panel

CACs were identified from this panel as $CD34^{+}CD133^{+}CD144^{+}VEGFR\text{-}2^{+}CD45^{-/dim}$ within a PBMC gate (n = 11). However, very sparse staining with either CD144 or VEGFR-2 antibodies was seen with this method. The CAC numbers identified were in the range of 0-10 cells per sample. When compared to the number of non-specifically stained cells in isotype control tubes, the numbers of CACs were found to be the same or even lower than “CACs” identified in control tubes. This indicated a potential problem with the antibody panel.

7.1.4 Titration of VEGFR-2

Whilst VEGFR-2 is commonly used to identify CACs, some authors have described problems with commercially available antibodies (Estes et al., 2010a; Smith et al., 2010). In view of this, and considering the problems with the preliminary antibody panel, different VEGFR-2 antibodies were tested to determine the reliability of staining.

Three antibodies against VEGFR-2 were titrated using HUVECs (kindly donated by Dr. Dionne Tanetta, Nuffield Department of Obstetrics and Gynaecology, Oxford), which were thawed and washed free of DMSO before plating in T75 tissue culture flasks. Flasks were pre-coated with 1% gelatin before plating and cells were grown in Endothelial Cell Growth Medium-2MV (Promocell, Heidelberg, Germany). Flasks were coated by adding 1.5ml of 1% bovine gelatin solution (diluted in PBS, Sigma-Aldrich, Poole, UK) to a flask and incubating at 37°C for at least 30 minutes. Residual gelatin was then aspirated, and the flasks were washed once with sterile PBS before being used to culture endothelial cells. At confluence, cells were harvested with trypsin, centrifuged into pellets and resuspended in 2% FCS prior to staining. Each antibody was used at four concentrations (0.5, 1, 2 and 4 times that recommended by the manufacturer). Equivalent concentrations of the appropriate isotype control antibodies were used for each sample.

7.1.4.1 Staining of VEGFR-2 antibodies

In total, three commercially available antibodies were tested by titration on HUVECs. Figure 7.2 shows the percentage of cells that stained positive with individual antibodies at each concentration. The percentage positivity was seen to be strongly dependent on concentration for both the BD Bioscience and R&D antibodies, which are both raised against the same VEGFR-2 epitope. This suggests a lack of antibody specificity. Very little staining was seen with the third antibody (New England Biolabs), even at the highest concentration. This antibody has subsequently been withdrawn by the manufacturer due to insufficient difference in staining of positive and negative cells (personal communication, Adam Peltan, Technical Support, New England Biolabs).

In view of these findings, the available antibodies were considered unreliable for flow cytometry and the antibody panel was revised.

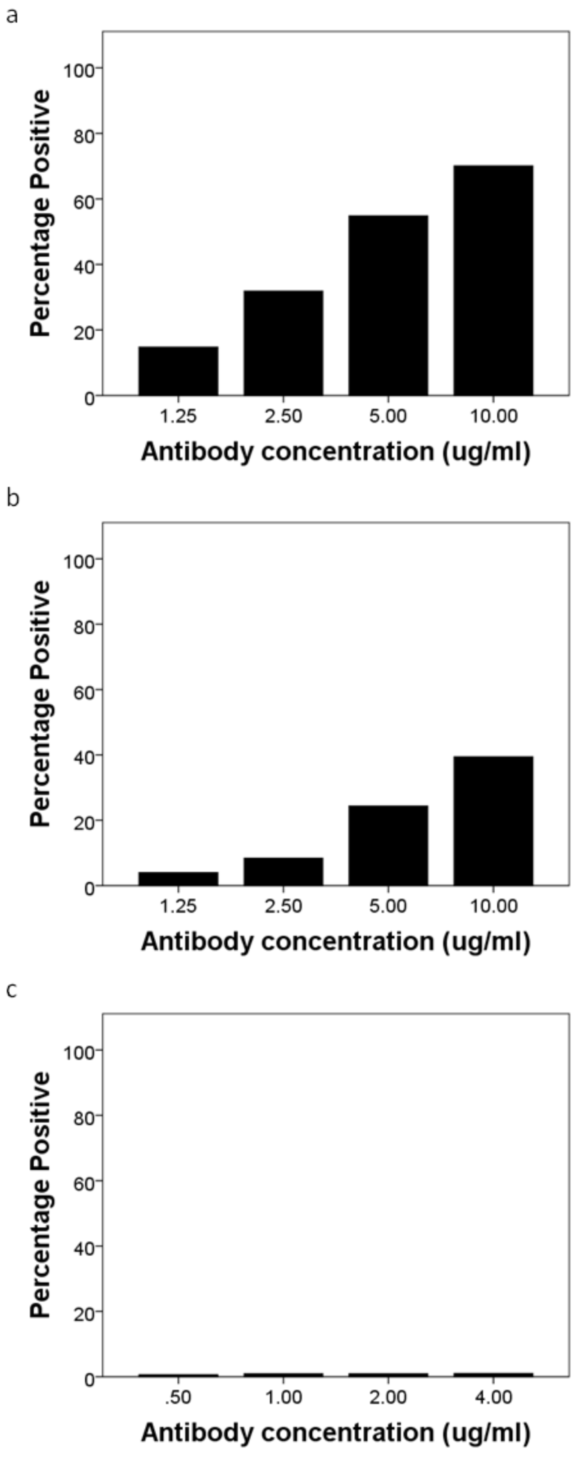

Figure 7.2 Dose dependent staining is seen with VEGFR-2 antibodies. Percentage of cells identified as VEGFR-2 positive with (a) BD Biosciences VEGFR-2 antibody; (b) RnD VEGFR-2 antibody; (c) New England Biolabs VEGFR-2 antibody.

7.2 Appendix 2

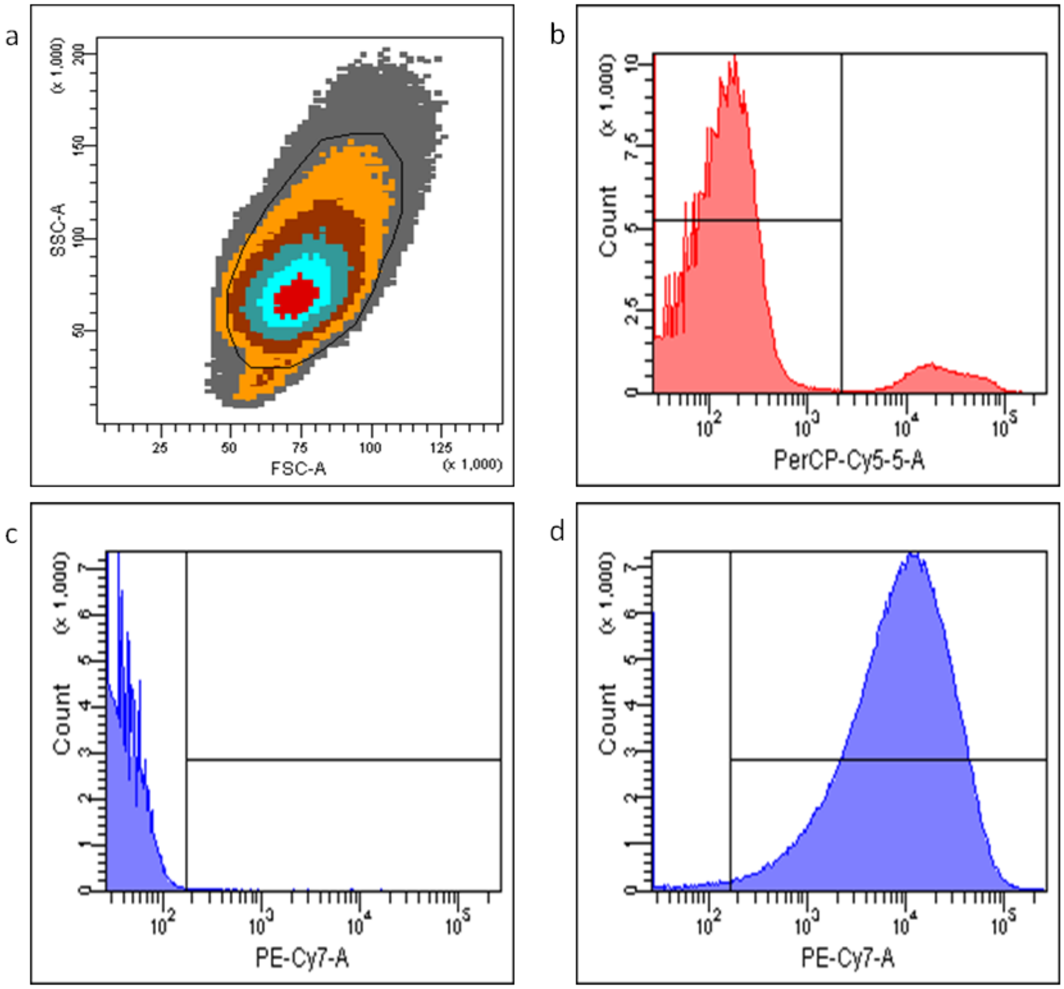

Figure 7.3: CD34 is expressed by freshly isolated endometrial stromal cells. (a) Forward/side scatter plot identifying moderate FSC/SSC population; (b)CD45-PerCPCy5.5 histogram, showing identification of CD45 negative cells from moderate FSC/SSC population; (c) FMO tube without CD34 antibody; (d) sample tube with CD34 antibody – note almost entire population of CD45 negative "stromal" cells are CD34 positive.

7.3 Appendix 3

Antibodies used in chapter 4 are shown in Table 7-C below

Antibody	**Fluorophore**	**Manufacturer**	**Catalogue number**	**Monoclonal (M)/ Polyclonal (P)**	**Final concentration**
CD34	PE-Cy7	BD	560710	m	1.25μg/ml
Mouse IgG1κ Isotype Control	PE-Cy7	BD	557872	m	1.25μg/ml
CD45	PerCP-Cy5.5	eBioscience	45-9459-42	m	1.25μg/ml
Mouse IgG1 Isotype Control	PerCP-Cy5.5	eBioscience	45-4714-80	m	1.25μg/ml
CD90	FITC	BioLegend	328108	m	5μg/ml
Mouse IgG1κ Isotype Control	FITC	BioLegend	400109	m	5μg/ml
CD144	FITC	BD	560411	m	2.5μg/ml
Mouse IgG1κ Isotype Control	FITC	BD	555748	m	2.5μg/ml
CD31	APC-Cy7	BioLegend	303120	m	2.5μg/ml
Mouse IgG1 κ Isotype Control	APC-Cy7	BioLegend	400128	m	2.5μg/ml
CD105	FITC	BioLegend	323203	m	20μg/ml
CD10	FITC	BioLegend	312207	m	20μg/ml
CD14	AF-647	BioLegend	325611	m	5μg/ml

Table 7-C: Antibodies used in Chapter 4.

7.4 Appendix 4

7.4.1 Antibodies used in Chapter 5

Antibodies used in Chapter 5 are shown in Table 7-D, below.

Antibody	**Fluorophore**	**Manufacturer**	**Catalogue number**	**Monoclonal (M)/ Polyclonal (P)**	**Final concentration**
CD34	PE-Cy7	BD	560710	m	1.25μg/ml
Mouse IgG1κ Isotype Control	PE-Cy7	BD	557872	m	1.25μg/ml
CD45	PerCP-Cy5.5	eBioscience	45-9459-42	m	1.25μg/ml
Mouse IgG1 Isotype Control	PerCP-Cy5.5	eBioscience	45-4714-80	m	1.25μg/ml
CD90	FITC	BioLegend	328108	m	5μg/ml
CD105	FITC	BioLegend	323203	m	20μg/ml
CD10	FITC	BioLegend	312207	m	20μg/ml
Mouse IgG1κ Isotype Control	FITC	BioLegend	400109	m	5μg/ml
CD144	FITC	BD	560411	m	2.5μg/ml
Mouse IgG1κ Isotype Control	FITC	BD	555748	m	2.5μg/ml
CD31	APC-Cy7	BioLegend	303120	m	2.5μg/ml
Mouse IgG1 κ Isotype Control	APC-Cy7	BioLegend	400128	m	2.5μg/ml
CD146	PE	BioLegend	342003	m	10μg/ml
Mouse IgG2a κ Isotype Control	PE	BioLegend	400211	m	10μg/ml
PDGFR-β	PE	BioLegend	323605	m	20μg/ml
Mouse IgG1 κ Isotype Control	PE	BioLegend	400113	m	20μg/ml

Table 7-D: Antibodies used in Chapter 5.

7.4.2 Components of ECGM MV2 medium

Fetal Calf Serum	0.05 ml / ml (5%)
Epidermal Growth Factor (recombinant human)	5 ng / ml
Basic Fibroblast Growth Factor (recombinant human)	10 ng / ml
Insulin-like Growth Factor (Long R3 IGF)	20 ng / ml
Vascular Endothelial Growth Factor 165 (recombinant human)	0.5 ng / ml
Ascorbic Acid	1 µg / ml
Hydrocortisone	0.2 µg / ml

7.4.3 Data for tube formation assays

Table 7-E: Expression of cell surface markers in cultures exposed to Dynabeads and those not exposed.

Antigen	Cultures exposed to Dynabeads Percentage positive (mean ± SD)	Cultures not exposed to Dynabeads Percentage positive (mean ± SD)
CD90	98.80 ± 1.11	99.39 ± 0.25
CD105	90.43 ± 6.85	85.57 ± 9.19
CD10	92.34 ± 6.20	78.42 ± 12.94
CD146	79.56 ± 14.49	82.59 ± 12.08
PDGFR-β	52.95 ± 21.53	75.60 ± 11.40
CD34	15.90 ± 10.19	5.92 ± 5.95
CD45	1.16 ± 0.71	8.03 ± 1.89
CD31	0.76 ± 0.83	0.39 ± 0.20

Table 7-F: Comparison of tube formation parameters between stromal cells derived from women with and without endometriosis.

Variable Mean ± SD	Controls (n=10)	Endometriosis (n=10)	P value
Total tube number	244.40 ± 64.39	234.05 ± 45.43	0.683
Mean tube length (pixels)	74.10 ± 10.95	70.20 ± 6.12	0.339
Total tube length (pixels)	17096.60 ± 4472.05	16404.50 ± 3166.37	0.694
Total branching points	119.85 ± 40.20	118.05 ± 31.00	0.912
Total loops	34.7 ± 15.59	35.45 ± 14.04	0.911

Table 7-G: Comparison of tube formation parameters between stromal cells derived from follicular and luteal phases.

Variable Mean ± SD	Follicular phase (n=10)	Luteal phase (n=10)	P value
Total tube number	241.40 ± 61.22	237.05 ± 50.11	0.864
Mean tube length (pixels)	73.35 ± 11.35	70.95 ± 5.82	0.559
Total tube length (pixels)	16788.25 ± 4216.11	16712.85 ± 3537.30	0.966
Total branching points	116.05 ± 34.55	121.85 ± 36.97	0.721
Total loops	31.95 ± 12.67	38.20 ± 16.08	0.347

Table 7-H: Comparison of tube formation by HMVEC alone, HMVEC with stroma from control subjects and HMVEC with stroma from endometriosis subjects.

Variable Mean ± SD	HMVEC alone (n=6)	HMVEC + control stroma (n=6)	HMVEC + endometriosis stroma (n=6)	P value
Total tube number	212.78 ± 21.08	192.67 ± 18.75	195.39 ± 21.34	0.214
Mean tube length (pixels)	68.61 ± 2.45	73.56 ± 2.21	70.61 ± 3.32	0.020
Total tube length (pixels)	14576.83 ± 1403.18	14113.94 ± 1075.27	13743.05 ± 1252.02	0.527
Total branching points	98.72 ± 14.15	87.94 ± 12.09	88.89 ± 13.06	0.317
Total loops	20.44 ± 5.79	19.17 ± 4.50	18.06 ± 5.71	0.747

Table 7-I: Tube formation parameters in serum-free DMEM, microvascular endothelial medium, and conditioned medium (CM) from stromal cells of women with and without endometriosis.

Variable Mean ± SD	Serum-free DMEM (n=4)	ECGM MV2 (n=4)	CM control stroma (n=6)	CM endometriosis stroma (n=6)	P value
Total tube number	176.92 ± 25.64	227.5 ± 24.22	204.56 ± 30.99	208.00 ± 17.29	0.075
Mean tube length (pixels)	69.42 ± 2.83	68.25 ± 2.47	69.22 ± 2.31	68.89 ± 1.68	0.887
Total tube length (pixels)	12178.33 ± 1469.76	15418.42 ± 1330.87	14040.22 ± 2048.28	14314.94 ± 1144.24	0.063
Total branching points	72.00 ± 16.33	109.58 ± 13.71	96.06 ± 19.77	95.56 ± 9.18	0.023
Total loops	9.67 ± 6.04	24.5 ± 5.31	20.00 ± 5.10	18.72 ± 2.15	0.003

7.5 Appendix 5

7.5.1 Research output resulting from this thesis

Levels of Circulating Angiogenic Cells are not altered in women with endometriosis. Webster KE, Kennedy, SH, Becker CM. Human Reproduction. 2013 (EPub Jan 14)

Endometrial alterations in endometriosis: a systematic review of putative biomarkers. May KE, Villar J, Kirtley S, Kennedy SH, Becker CM. Human Reproduction Update. 2011 Sep-Oct;17(5):637-53.

Peripheral biomarkers of endometriosis: a systematic review. May KE, Conduit-Hulbert SA, Villar J, Kirtley S, Kennedy SH, Becker CM. Human Reproduction Update. 2010 Nov-Dec;16(6):651-74.

- Poster presentation at the Annual Academic Meeting of the RCOG December 2011. "Endothelial progenitor cells in endometriosis" May KE, Kennedy, SH, Becker CM.

- Poster presentation at the World Congress of Endometriosis (WCE) September 2011. “Endothelial progenitor cells in endometriosis” May KE, Kennedy, SH, Becker CM.
- Recipient of Rodolph Maheaux Travel Award to enable attendance at the WCE.