**Endometrial vascular development in heavy menstrual bleeding: altered spatio-temporal expression of endothelial cell markers and extracellular matrix components**

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**Abstract**

**STUDY QUESTION:** Are there any phenotypic and structural/architectural changes in the vessels of endometrium and superficial myometrium during the normal menstrual cycle in healthy women and those with heavy menstrual bleeding (HMB)?

**SUMMARY ANSWER:** Spatial and temporal differences in protein levels of endothelial cell (EC) markers and components of the extracellular matrix (ECM) were detected across the menstrual cycle in healthy women and these are altered in HMB.

**WHAT IS KNOWN ALREADY:** HMB affects 30% of women of reproductive age with approximately 50% of cases being idiopathic. We have previously shown that the differentiation status of endometrial vascular smooth muscle cells (VSMCs) is altered in women with HMB, suggesting altered vessel maturation compared to controls. Endometrial arteriogenesis requires the co-ordinated maturation not only of the VSMCs but also the underlying ECs and surrounding ECM. We hypothesized that there are spatial and temporal patterns of protein expression of EC markers and vascular ECM components in the endometrium across the menstrual cycle, which are altered in women with HMB.

**STUDY DESIGN, SIZE, DURATION:** Biopsies containing endometrium and superficial myometrium were taken from hysterectomy specimens from both healthy control women without endometrial pathology and women with subjective HMB in the proliferative (PP), early secretory (ESP), mid secretory (MSP) and late secretory (LSP) phases (N=5 for each cycle phase and subject group). Samples were fixed in formalin and embedded in paraffin wax.

**PARTICIPANTS/MATERIALS, SETTING, METHODS:** Serial sections (3μm thick) were immunostained for EC markers (factor VIII related antigen (F8RA), CD34, CD31 and ulex europaeus-agglutinin I (UEA-1) lectin), structural ECM markers (osteopontin, laminin, fibronectin, and collagen IV) and for Ki67 to assess proliferation. Immunoreactivity of vessels in superficial myometrium, endometrial stratum basalis, stratum functionalis and luminal region was scored using either a modified Quickscore or by counting the number of positive vessels.

**MAIN RESULTS AND THE ROLE OF CHANCE:** In control samples, all four EC markers showed a dynamic expression pattern according to menstrual cycle phase, in both endometrial and myometrial vessels. EC protein marker expression was altered in women with HMB compared with controls, especially in the secretory phase in the endometrial luminal region and stratum functionalis. For example, in the LSP expression of UEA-1 and CD31 in the luminal region decreased in HMB (mean quickscore: 1 and 5, respectively) compared with controls (3.2 and 7.4, respectively) (both *P*=0.008), while expression of F8RA and CD34 increased in HMB (1.4 and 8, respectively) compared with controls (0 and 5.8, respectively) (both *P*=0.008). There was also a distinct pattern of expression of the vascular structural ECM protein components osteopontin, laminin, fibronectin and collagen IV in the superficial myometrium, stratum functionalis and stratum basalis during the menstrual cycle, which was altered in HMB. In particular, compared with controls, osteopontin expression in HMB was higher in stratum functionalis in the LSP (7.2 and 11.2, respectively *P*=0.008), while collagen IV expression was reduced in stratum basalis in the MSP (4.6 and 2.8, respectively *P*=0.002) and in stratum functionalis in the ESP (7 and 3.2, respectively *P*=0.008).

**LIMITATIONS, REASONS FOR CAUTION:** The protein expression of vascular EC markers and ECM components was assessed using a semi-quantitative approach in both straight and spiral arterioles. In our hospital, HMB is determined by subjective criteria and levels of blood loss were not assessed.

**WIDER IMPLICATIONS OF THE FINDINGS:** Variation in the protein expression pattern between the four EC markers highlights the importance of choice of EC marker for investigation of endometrial vessels. Differences in expression of the different EC markers may reflect developmental stage dependent expression of EC markers in endometrial vessels, and their altered expression in HMB may reflect dysregulated vascular development. This hypothesis is supported by altered expression of ECM proteins within endometrial vessel walls, as well as our previous data showing a dysregulation in VSMC contractile protein expression in the endometrium of women with HMB. Taken together these data support the suggestion that HMB symptoms are associated with weaker vascular structures, particularly in the LSP of the menstrual cycle, which may lead to increased and extended blood flow during menstruation.

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**Key words:** endometrium; heavy menstrual bleeding; spiral arteries; endothelial cell markers; extracellular matrix

**Introduction**

Heavy menstrual bleeding (HMB) affects approximately 10 million women annually in the USA, including 30% of women of reproductive age ([Garside *et al*., 2004](#_ENREF_21); [Mansfield *et al*., 2004](#_ENREF_32); [Group *et al*., 2007](#_ENREF_24); [NICE, 2007](#_ENREF_35)). By 60 years of age, one in five women in the UK and one in three in the USA have undergone hysterectomy, HMB being the underlying cause in at least 50-70% of cases ([Duckitt and McCully, 2004](#_ENREF_12); [El-Hemaidi *et al*., 2007](#_ENREF_13)). While HMB may result from clinical conditions such as uterine fibroids, approximately 50% of the cases remain unexplained ([Rees, 1987](#_ENREF_44)) and current treatment options often compromise fertility.

Understanding the mechanisms of HMB requires insight into the mechanisms of normal menstrual bleeding. Although knowledge of the structure and function of endometrial blood vessels has improved ([Bartelmez 1933](#_ENREF_6); [Markee, 1940](#_ENREF_33); [Chennazhi and Nayak, 2009](#_ENREF_10); [Fraser and Duncan, 2009](#_ENREF_17); [Girling and Rogers, 2009](#_ENREF_22); [Demir *et al*., 2010](#_ENREF_11); [Plaisier, 2011](#_ENREF_40)), much remains to be elucidated. During the menstrual cycle, angiogenesis is spatially and temporally regulated: with vascular repair in the stratum basalis in the menstrual phase; angiogenesis in the stratum functionalis of the proliferative phase supporting endometrial growth; and growth and coiling of the spiral arterioles in the secretory phase ([Gargett and Rogers, 2001](#_ENREF_20)). HMB may be due to increased or earlier, out-of-phase breakdown of endometrial tissue and vessels at the beginning of menstruation, failure in endometrial repair at the end of menstruation, and/or an increased rate of blood flow through structurally or functionally impaired blood vessels ([Abberton *et al*., 1999](#_ENREF_1)a; [Hurskainen *et al*., 1999](#_ENREF_26)). Uterine blood vessels are comprised of outer layers of vascular smooth muscle cells (VSMCs) and extracellular matrix (ECM), with an inner endothelial cell (EC) layer. Several previous studies have highlighted the potential importance of endometrial vessel number and morphology, as well as ECs and VSMCs and their state of differentiation, in relation to the menstrual cycle and to some extent in HMB (Kooy *et al*., 1996; [Abberton *et al*., 1999](#_ENREF_1)a; [Abberton *et al*., 1999](#_ENREF_2)b; [Rogers and Abberton, 2003](#_ENREF_46); [Kawai-Kowase and Owens, 2007](#_ENREF_29); [Biswas Shivhare *et al*., 2014](#_ENREF_9)).

ECs express various molecules that were shown to be differentially expressed in luteal phase endometrium from women suffering recurrent reproductive failure ([Quenby *et al*., 2009](#_ENREF_43)). These include factor VIII related antigen (F8RA), CD34 and CD31 as well as glycoproteins which bind the lectin, ulex europaeus-agglutinin I (UEA-1). While there have been several studies on expression of different EC markers in endometrium and myometrium during the menstrual cycle ( [Rees *et al*., 1993](#_ENREF_45); [Rogers *et al*., 1993](#_ENREF_47); [Tawia *et al*., 1993](#_ENREF_53); [Nikitenko *et al*., 2000](#_ENREF_36); [Zhang *et al*., 2002](#_ENREF_59)), a detailed phenotypic analysis of endothelial cells in the different layers of the endometrium (luminal region, stratum functionalis, stratum basalis) and superficial myometrium throughout the menstrual cycle and a comparison with HMB has not been reported.

As a major component of blood vasculature, ECM is integral to angiogenesis, tissue stability as well as regulation of cell growth and differentiation, and also provides mechanical properties maintaining vascular tone. The main constituents of the human endometrial vascular basement membrane include collagen, fibronectin, and laminin ([Faber *et al*., 1986](#_ENREF_14); [Aplin *et al*., 1988](#_ENREF_3)) and have been shown to be expressed during the menstrual phase ([Kelly *et al*., 1995](#_ENREF_30)). Since the pattern of angiogenesis differs between the endometrial layers and menstrual cycle phases, the vascular ECM may also show spatio-temporal alterations. Although expression of endometrial vascular ECM components has been studied in the menstrual cycle ([Aplin *et al*., 1988](#_ENREF_3); [Kelly *et al*., 1995](#_ENREF_30); [Oefner *et al*., 2015](#_ENREF_38)), osteopontin, laminin, collagen IV and fibronectin expression in blood vessels have not been studied in detail in the context of HMB.

We hypothesised that the endometrial vascular EC phenotype and ECM component expression is altered in HMB. To test this hypothesis we compared the protein expression pattern of various EC markers and vascular ECM components in the endometrium and superficial myometrium across the menstrual cycle in controls and women with HMB.

**Materials and Methods**

## Samples

Endometrial biopsies were obtained from women undergoing hysterectomy at the Royal Victoria Infirmary, Newcastle upon Tyne Hospitals NHS Foundation Trust, Newcastle upon Tyne, UK, or at the Liverpool Women’s Hospital, Liverpool, UK. The study was approved by Newcastle and North Tyneside Research Ethics Committee (Ref: 10/H0906/71) and Liverpool Adult Ethics committee (Ref: 09/H1005/55) and all subjects gave written informed consent. The experimental group consisted of women with a history of HMB, defined as excessive menstrual blood loss which interferes with the woman’s physical, emotional, social and material quality of life, and which can occur alone or in combination with other symptoms ([NICE, 2007](#_ENREF_35)). Diagnosis of HMB was based on subjective criteria and blood loss volume was not recorded. The control group were women with no uterine pathology undergoing hysterectomy for prolapse, cystocele, rectocele, or urinary or stress incontinence. Women with any conditions potentially associated with an endometrial abnormality (e.g. endometriosis, adenomyosis, leiomyomata, heavy irregular bleeding) were excluded from the study, as were those who had received hormone treatment within 3 months of the operation. The menstrual cycle phase was staged according to standard morphological criteria ([Noyes *et al*., 1975](#_ENREF_37)) by a specialist gynaecologist histopathologist (JNB). Five samples were obtained in each of proliferative (PP), early secretory (ESP), mid secretory (MSP) and late secretory phases (LSP) for both study groups. Biopsies were fixed in 10% neutral buffered formalin for 24hrs, routinely processed and embedded in paraffin wax. Patient details are shown in Table I.

## Immunohistochemistry

Serial 3µm sections were dewaxed, rehydrated, incubated in 1% H2O2 in water for 10 min to block endogenous peroxidase activity and washed in 0.15M Tris-buffered 0.05M saline, pH 7.6 (TBS) for 5 min. Sections were immunostained using an avidin-biotin-peroxidase technique (Vectastain Elite ABC kit; Vector Laboratories, Peterborough, UK), which has been described in detail previously ([Schiessl *et al*., 2009](#_ENREF_48)). Details of source, dilution, and pre-treatment for all primary antibodies are provided in Table II. All primary antibodies were incubated for 60 min at room temperature and washes and dilutions were performed in TBS. The reaction was developed for 1-2 min with 3,3**′**-diaminobenzidine (DAB; Sigma Chemical Co. Dorset, UK) containing 0.01% H2O2 to give a brown reaction product. Sections were lightly counterstained with Mayer’s haematoxylin for 30 sec, dehydrated, cleared in xylene and mounted with DPX synthetic resin (Raymond A. Lamb Ltd., London, UK). Positive controls were included in each staining run and negative (replacement of the primary antibody by appropriate non-immune serum) controls were performed for all samples. Ideally negative controls would include non-immune isotype Ig controls, however given that the range of antibodies used had differential staining patterns we are confident of their specificity.

## Lectin histochemistry

Sections were dewaxed, rehydrated, incubated in 1% H2O2 in water as above and then incubated in trypsin buffer (pH 7.8) for 15 min at 37°C. After blocking non-specific binding by 10 min incubation with non-immune rabbit serum (Vector Laboratories), sections were washed and incubated for 60 min with 1:20 biotinylated UEA-1 lectin (Sigma-Aldrich Co., Dorset, UK), washed for 5 min in TBS and then incubated for 30 min with 1:100 streptavidin/horse-radish peroxidase (HRP; Streptavidin P0397, Dako Cytomation, Cambridgeshire, UK). After two further 5 min washes in TBS, the reaction was developed with DAB. Sections were lightly counterstained with Mayer’s haematoxylin, dehydrated, cleared and mounted with DPX synthetic resin. Negative controls were performed by omission of UEA-1 lectin.

## Quantitative image analysis

Sections were examined using a Nikon Eclipse 80i microscope. Vessels identified by their surrounding layer(s) of smooth muscle cells were assessed in the entire tissue by SBS, who was blinded to the origin of the sample. Only vessels with a visible lumen were included in the analysis. Entire full thickness immunostained tissue sections were analysed semi-quantitatively using a modified ‘Quickscore’ method ([Schiessl *et al*., 2009](#_ENREF_48)) taking into account both the percentage of vessels stained (1 = 0-25%, 2 > 25-50%, 3 > 50-75%, 4 > 75-100%) and the staining intensity for each percentage (0=negative, 1=weak, 2=moderate, 3=strong). The percentage and intensity scores were then multiplied and summed to give a possible score range of 0-12. Superficial myometrium, stratum basalis (within one 200x field of myometrium), stratum functionalis and, for ECs, luminal region (within one 200x field from surface epithelium) were scored separately. To try and understand which portions of the endometrial blood vessels were the youngest, or least mature, and which were the oldest, or more mature vessels, proliferation was assessed using the open-source ImageJ software package (Version 1.46, NIH, MD, USA) in combination with the ‘cell counter’ plugin to determine the percentage of Ki67+ vessels compared with the total number of UEA-1, F8RA, CD34 or CD31-positive vessels in three randomly selected 200x fields within the luminal region and stratum functionalis in each sample. As this was a minor part of the study, only three samples from control women at each phase of the menstrual cycle were randomly selected from the N=5 available in each group. All captured images were assembled using NIS Elements Viewer (NIKON Instruments Inc., Surrey, UK).

## Statistical analysis

Statistical analyses were performed using Statistical Package for Social Sciences version 15.0 (SPSS Inc., Chicago, IL, USA). Results are presented as mean ± SEM. Kruskal-Wallis with a post-hoc test was used to determine differences between menstrual cycle phases, myometrial/endometrial layers, or ECM components. Mann-Whitney U test was used to determine differences between control and HMB groups. Differences were considered statistically significant at *P*≤0.05.

**Results**

## Expression of uterine EC markers during the menstrual cycle in control women and those with HMB

Representative immunostaining patterns for the different EC markers in endometrial stratum functionalis are illustrated in Figure 1. In control women the expression patterns of the different EC markers were similar, with expression tending to increase from the luminal region to the superficial myometrium during the menstrual cycle (Figure 2).

*UEA-1:* In control women, UEA-1 reactivity was present across most of the menstrual cycle but was lower in the luminal region of MSP endometrium when compared with LSP (*P*=0.006) and in the stratum functionalis when compared with ESP (*P*=0.002) (Figure 2A). Myometrial UEA-1 reactivity was persistently higher than the luminal region of the endometrium (PP *P*<0.001, ESP *P*=0.001, LSP *P*=0.001) (Figure 2A).

Compared with controls, ECs in the luminal region and stratum functionalis showed reduced UEA-1 reactivity in HMB in both PP (lumen *P*=0.008; stratum functionalis *P*=0.008) and LSP (lumen *P*=0.008; stratum functionalis *P*=0.008) (Figure 2A).

*F8RA:* In controls, F8RA protein levels did not vary between PP, ESP and MSP but was reduced in LSP compared with ESP in the luminal region (*P*=0.006), stratum functionalis (*P*=0.003), stratum basalis (*P*=0.003) and myometrium (*P*=0.002) (Figure 2B). F8RA protein levels were also lower in the luminal region compared with myometrium in PP (*P*=0.002), ESP (*P*=0.002) and LSP (*P*=0.004) (Figure 2B).

In women with HMB, protein levels of F8RA were increased in the luminal region during the LSP (*P*=0.008), but were decreased in the myometrium during the ESP (*P*=0.008) (Figure 2B). No alterations in protein levels were observed in the stratum functionalis or stratum basalis.

*CD34:* In control women, CD34 protein levels were stable across the different phases of the menstrual cycle in endometrium and superficial myometrium (Figure 2C). Protein levels of CD34 were lower in the luminal area compared with stratum functionalis in PP (*P*=0.002) and with myometrium in LSP (*P*=0.001) (Figure 2C).

In women with HMB, CD34 protein levels were increased in the luminal region in LSP (*P*=0.008), in the stratum functionalis in MSP (*P*=0.008), in the stratum basalis in ESP (*P*=0.008) and LSP (*P*=0.008), and in the myometrium at all stages of the menstrual cycle (all *P*=0.008) compared with controls (Figure 2C).

*CD31:* CD31 protein levels were increased in LSP compared with PP in the luminal region (*P*=0.007) and stratum functionalis (*P*=0.002) (Figure 2D). CD31 protein levels in the myometrial vessels were reduced in LSP compared with PP (*P*=0.003) (Figure 2D).

In women with HMB, CD31 protein levels were reduced in the luminal and stratum functionalis regions in both MSP (both *P*=0.008) and LSP (both *P*=0.008) compared with controls (Figure 2D). In the stratum basalis, CD31 protein levels were also reduced in LSP (*P*=0.008) compared with controls (Figure 2D). In contrast, CD31 protein levels in myometrial vessels were increased in LSP (*P*=0.008) compared with controls (Figure 2D).

## Proliferation rates differ in vessels expressing different EC markers

To determine whether immunoreactivity of the different EC markers reflected vessel maturation status, Ki67 immunostaining was performed on three control samples from each menstrual cycle phase. It is assumed that the newer and less mature a vessel is the higher the rate of EC proliferation. Vessels in the luminal region and the stratum functionalis were assessed separately. At all stages of the menstrual cycle in both the luminal region and stratum functionalis, proliferating EC were most numerous in vessels that were UEA-1 reactive (Figure 3A-C). In the luminal region proliferation in UEA-1 positive ECs was significantly higher than in F8RA positive ECs at all stages of the menstrual cycle (PP *P*=0.04; ESP *P*=0.01; MSP *P*=0.02; LSP *P*=0.001), as well as both CD31 and CD34 positive ECs in ESP (CD31 *P*=0.0009, CD34 *P*=0.003) and LSP (CD31 *P*=0.01, CD34 *P*=0.005) endometrium (Figure 3B). A similar pattern was also observed in the stratum functionalis; proliferation in UEA-1 positive ECs was significantly higher than in F8RA positive ECs at all stages of the menstrual cycle (PP *P*=0.05; ESP *P*=0.0004; LSP *P*=0.002), apart from the MSP, as well as both CD31 and CD34 positive ECs in ESP (CD31 *P*=0.0006, CD34 *P*=0.0009) and LSP (CD31 *P*=0.01, CD34 *P*=0.01) endometrium (Figure 3C).

## Expression of uterine vascular ECM proteins during the menstrual cycle in control women and those with HMB

Vascular expression of the different ECM components differed between stratum functionalis, stratum basalis and myometrium, and across the menstrual cycle (Figure 4, Figure 5).

*Osteopontin:* In control women in PP, vascular osteopontin protein levels were highest in stratum functionalis compared with myometrium (*P*=0.002; Figure 5A), although this was reversed in the MSP (*P*=0.02; Figure 5A). In the stratum functionalis, osteopontin protein levels were highest in PP, decreasing in MSP (*P*=0.003; Figure 5A). In women with HMB, vascular osteopontin protein levels increased in the LSP (*P=*0.008) in stratum functionalis but decreased in the ESP in stratum basalis (*P=*0.008) compared with controls (Figures 4A, 4B).

*Laminin:* In general laminin immunoreactivity was weak. In control samples, laminin protein levels were increased in ESP stratum functionalis compared with stratum basalis (*P*=0.005; Figures 4C, 4D, 5B). There were no cyclic changes in laminin protein levels within the stratum functionalis, stratum basalis or myometrium. In addition, endometrial vascular laminin protein levels were not altered in women with HMB compared with controls.

*Fibronectin:* Fibronectin protein levels in PP endometrium of healthy controls did not differ between the three layers (Figure 5C), while protein levels were higher in ESP and LSP stratum functionalis compared with stratum basalis (ESP *P*=0.003; LSP *P*=0.002; Figures 4E, 4F, 5C). In MSP controls, fibronectin was highly expressed in the stratum functionalis but decreased in the myometrium (*P*=0.004; Figure 5C). In control women there were no cyclic changes in fibronectin protein levels within the stratum functionalis or myometrium (Figure 5C). In the stratum basalis of controls, fibronectin protein levels were low, and increased in MSP compared with LSP (*P*=0.005; Figure 5C). Compared with controls, vascular fibronectin protein levels were reduced in MSP stratum basalis (*P=*0.006) in HMB.

*Collagen IV:* In control samples, collagen IV was absent from the stratum basalis in the PP, with higher protein levels in myometrium (*P*=0.01; Figure 5D). In ESP, collagen IV expression was highest in stratum functionalis and lowest in myometrium (*P*=0.01; Figure 5D). In MSP and LSP, there was no variation in expression between the three layers. There were no cyclic changes in collagen IV protein levels within the stratum functionalis, stratum basalis or myometrium (Figure 5D). In women with HMB, collagen IV protein levels were decreased in the stratum functionalis in ESP ((Figures 4G, 4H); *P=*0.008) and in the stratum basalis in ESP (*P=*0.007) and MSP (*P=*0.002) compared with healthy controls.

**Discussion**

This study compared endometrial vascular protein levels of different EC markers and ECM components in stratum functionalis, stratum basalis and myometrium in controls and women with HMB. Vascular EC markers and ECM components differed across the menstrual cycle and between different endometrial/myometrial layers, and distinct differences were observed in HMB compared to controls. A limitation of the study is that diagnosis of HMB was based on subjective criteria and blood loss volume was not recorded. Therefore, while conclusions can be drawn for women with subjectively diagnosed idiopathic HMB we cannot definitely state that the endometrial vascular alterations observed are associated with increased blood loss during menstruation. The uterine vasculature plays an integral role in maintaining normal endometrial function and structural abnormalities in endometrial blood vessels have been implicated in HMB ([Abberton *et al*., 1999](#_ENREF_1)a; [Abberton *et al*., 1999](#_ENREF_2)b; [Hurskainen *et al*., 1999](#_ENREF_26); [Rogers and Abberton, 2003](#_ENREF_46); [Kawai-Kowase and Owens, 2007](#_ENREF_29); [Biswas Shivhare *et al*., 2014](#_ENREF_9)). Comparison of these cyclically developing vessels in HMB and controls may provide clues to the mechanisms that underlie HMB. For example, we have recently shown that, although the muscle content of vessels in the stratum functionalis was unchanged in HMB, expression of vascular calponin was significantly decreased, suggesting altered VSMC differentiation status ([Biswas Shivhare *et al*., 2014](#_ENREF_9)).

Of the EC markers studied, CD31 and CD34 were those predominantly expressed within the endometrial vasculature. CD31 has previously been reported to be expressed by both large and small endometrial vessels ([Rees *et al*., 1993](#_ENREF_45); [Zhang *et al*., 2002](#_ENREF_59)). In contrast, CD34 was primarily detected in small and medium vessels and capillaries, and UEA-1 was reactive with all types of vessels ([Zhang *et al.*, 2002](#_ENREF_59)). F8RA was expressed only by larger vessels ([Rees *et al*., 1993](#_ENREF_45)) with weak expression across the menstrual cycle ([Zhang *et al*., 2002](#_ENREF_59)). In the present study, F8RA expression was weak and decreased during the secretory phase, especially in the luminal region and stratum functionalis in the LSP. These observations agree with previous reports by Zhu and Zhao (1988) and Zhang *et al*. (2002) but differ from Rees *et al*. (1993), who found no difference between menstrual cycle phases. CD31 expression increased from the PP to the LSP in the luminal region and the stratum functionalis, again in agreement with Zhang *et al*. (2002). In contrast, [Tawia *et al*. (1993](#_ENREF_71)) did not detect altered CD31 expression across the menstrual cycle, having quantified staining intensity in the entire endometrium. In the current study, expression of each EC marker differed in a spatio-temporal manner in healthy controls; therefore care must be exercised in selecting EC markers for studies of endometrial vessels, especially in the superficial functional parts of the endometrium.

UEA-1 reactivity differed from F8RA, CD34 and CD31 protein levels, especially in the luminal region and stratum functionalis. To investigate whether these differences reflected vessel development, we used Ki67 immunostaining to assess the proportion of proliferating vessels in the stratum functionalis and luminal region at each stage of the menstrual cycle. UEA-1 was the most prominent EC marker expressed by the proliferating vessels, being significantly higher than in F8RA positive vessels at all stages of the menstrual cycle as well as in CD31 and CD34 positive vessels in both ESP and LSP. Based on these data it can be suggested that UEA-1 is the first EC marker expressed by developing endometrial vessels during the menstrual cycle. However, the order in which the other markers are expressed is not clear and further analysis with double labelling immunohistochemistry and investigation of vessel size is required.

Since endometrial blood vessels have been implicated to play a role in the pathogenesis of HMB, an alteration of this protein expression pattern may also contribute to HMB. Vascular expression of CD34, a transmembrane glycoprotein, was increased in both endometrium and myometrium in HMB samples compared with controls. Interestingly, a previous study using double staining for CD34 and proliferating cell nuclear antigen reported increased endometrial EC proliferation in HMB ([Kooy *et al*., 1996](#_ENREF_31)), highlighting possible dysregulation of CD34+ endometrial vessels in the context of vascular development in HMB. However, we did not investigate proliferation of EC specifically in the current study and these studies should be performed in the future. In contrast, UEA-1+ and CD31+ vessels were generally reduced in HMB in the secretory phase. CD31 has been implicated in the binding of leukocytes to ECs as well as maintaining cell-cell contact ([Tabibzadeh and Poubouridis, 1990](#_ENREF_51)). Since both UEA-1 and CD31 may stain small and large vessels, altered CD31+ expression may reflect dysregulation from an early stage of angiogenesis, possibly giving rise to leaky vessels owing to weaker lumen formation. F8RA was reduced in ESP myometrium but was increased in the LSP in the luminal region in HMB. F8RA enables platelet adhesion to injured vessel walls ([Sixma and de Groot, 1991](#_ENREF_49)); altered expression may be indicative of adverse endothelial changes in these superficial endometrial vessels in HMB. From these results it can be speculated that there is dysregulation of endometrial vascular endothelium in HMB. This may alter EC-VSMC signalling, which in turn may affect recruitment and/or differentiation of VSMCs, ultimately resulting in dysfunctional vascular tone and consequently altered blood flow.

Angiogenesis is regulated both spatially and temporally in cycling endometrium; hence vascular ECM must also make corresponding alterations to sustain these vascular changes, although little is known about the mechanisms underlying ECM regulation in endometrium. In agreement with Kelly *et al*. (1995), in our study vascular laminin and fibronectin protein levels were low throughout the menstrual cycle, with few differences observed between either phases or endometrium/myometrium. Our results for fibronectin and laminin protein levels in stratum functionalis are similar to those of Bilalis *et al*. (1996), the only difference being complete absence of both 10 days after the LH surge in their study. Since we examined ESP, MSP and LSP endometrium that was staged using histologic criteria (Noyes *et al*., 1975) rather than by timing from the LH surge, this difference can be explained by sample selection. Osteopontin was expressed at high levels in the stratum functionalis with little variation in the secretory phase. In contrast, collagen IV protein levels varied in both stratum functionalis and basalis, with increased levels in the secretory phase. While these results are similar to those of Bilalis *et al*. (1996), they differ from Kelly *et al*. (1995), who concluded that the number of collagen IV+ vessels remained similar across the menstrual cycle. A more recent study reported increased collagen IV expression in endometrial vessels from proliferative to secretory phase in whole endometrium ([Oefner *et al*., 2015](#_ENREF_38)), while we demonstrated increased collagen IV protein levels in the ESP and LSP in stratum functionalis. The failure to distinguish stratum functionalis and basalis and many other methodological differences between these studies, including antibody specificity, endometrial staging and image quantification, is likely to account for the differences in the results.

The variation in ECM component protein levels suggests that, in common with ECs and VSMCs, vascular ECM is strictly regulated depending on the cyclic needs of the endometrial vasculature. The ECM is known to interact with cellular receptors that determine cell shape, migration, proliferation and even apoptosis ([Timpl, 1996](#_ENREF_54)). Regulation of angiogenesis for the maintenance of normal endometrial function may result in distinctly separate ECM signatures in the stratum functionalis, stratum basalis and myometrium.

A major function of vascular ECM is to provide vascular stability; if ECM component expression is tightly regulated in the endometrial vasculature, any variation in this ECM signature could lead to altered vessel stability, which could lead to abnormal bleeding in HMB. Laminin and fibronectin protein levels in stratum functionalis and myometrium were comparable in controls and HMB. Both laminin and fibronectin protein levels in LSP stratum basalis were higher in HMB, although fibronectin was decreased in MSP. Laminin and fibronectin are both adhesion glycoproteins and possess similar biological properties such as epithelial cell attachment to basement membrane and stromal cell adhesion to interstitial matrix ([Beliard *et al*., 1997](#_ENREF_7)). Altered expression of these two molecules may reflect altered structural properties of endometrial vessels in HMB. Laminin has also been implicated in endometrial epithelial remodelling ([Tanaka *et al*., 2009](#_ENREF_52)), inhibition of endometrial stromal differentiation ([Mizuno *et al*., 1999](#_ENREF_34)) and regulation of trophoblast proliferation and differentiation in angiogenesis during pregnancy ([Bischof *et al*., 1994](#_ENREF_8); [Qin *et al*., 2003](#_ENREF_42); [Kaloglu and Onarlioglu, 2010](#_ENREF_27)). Fibronectin is known to promote matrix metalloproteinase-2 (MMP-2) protein expression resulting in ECM breakdown during menses ([Hoffmann *et al*., 2006](#_ENREF_25)). However, when fibronectin is targeted by MMP-3 and broken into proteolytic fragments, it has been shown to induce apoptosis ([Fukai *et al*., 1995](#_ENREF_18)). Therefore, increased fibronectin expression in LSP stratum basalis in HMB may reflect a response to increased local ECM breakdown and/or enhanced cellular apoptosis. It can be speculated that compared with control women, vessels arising within the stratum basalis at the end of menses have altered ECM composition, and therefore function, in HMB. Finally, this cyclic variation of laminin and fibronectin protein levels is also an indication that their production is under the direct or indirect regulation of ovarian steroid hormones.

Collagen IV protein levels were generally decreased in both stratum functionalis and stratum basalis in HMB. Collagen IV networks are a major architectural feature of the vascular ECM and maintain mechanical stability. Therefore, reduced collagen IV would suggest a weaker endometrial vascular structure in HMB, while increased myometrial collagen IV expression may represent a compensatory mechanism to stabilise the vascular bed.

Vascular osteopontin was higher in ESP than in LSP in controls but this pattern was reversed in HMB, with osteopontin reduced in ESP stratum basalis and increased in LSP stratum functionalis compared with controls. Osteopontin is induced in VSMCs in cardiovascular diseases ([Waller *et al*., 2010](#_ENREF_56)) and increased osteopontin has been implicated in renal diseases and linked with the activity of several oncogenes. Moreover, activated lymphocytes and macrophages synthesize osteopontin, accounting for its elevated expression in traumatised tissue ([Sodek *et al*., 2000](#_ENREF_50)). Thus, increased osteopontin expression in the LSP could reflect increased breakdown of endometrial vascular ECM. A study using murine aortic VSMCs has shown that osteopontin down-regulates calponin, a VSMC differentiation/contractile marker, through an extracellular signalling pathway ([Gao *et al*., 2012](#_ENREF_19)). We recently reported reduced vascular calponin expression in LSP stratum functionalis in HMB ([Biswas Shivhare *et al*., 2014](#_ENREF_9)). It may be speculated that osteopontin may down-regulate calponin expression in LSP endometrium in HMB, but *in vitro* mechanistic studies are required to confirm this suggestion.

In conclusion, the levels of a number of key proteins in endometrial vascular EC and ECM varied during the menstrual cycle and differed between control and HMB endometrium. While laminin and fibronectin did not show major changes, osteopontin was generally increased and collagen IV was decreased in uterine vessels in HMB. Altered vascular EC and ECM component expression likely reflects a dysregulated development and structural instability of endometrial vessels, and consequently altered function, which could contribute to abnormal bleeding in HMB. Investigation of the uterine vasculature in HMB may provide important clues in the development of alternative treatments for a condition that affects quality of life for thousands of women worldwide.

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**Authors’ roles**

S.B.S was involved in study design, execution, data collection, and analysis and wrote the manuscript. J.N.B. played a role in primary histopathological assessment of all sections, study design and contributed towards critical discussion of the manuscript. B.A.I. was involved in sample collection and preparation. D.K.H. contributed towards the critical discussion of the manuscript. G.E.L. played a role in study design and contributed towards critical discussion of the manuscript.

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**Conflict of interest**

There are no competing interests to declare.

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**Figure Legends**

**Figure 1**: **Immunostaining for endothelial cell markers differed across the menstrual cycle, and between controls and women with subjective heavy menstrual bleeding.**

Representative photomicrographs of immunostaining for the different endothelial cell (EC) markers in endometrial stratum functionalis. **(A,B)** ulex europaeus-agglutinin I (UEA-1) immunoreactivity in late secretory phase (LS) **(A)** control and **(B)** heavy menstrual bleeding (HMB) endometrium; **(C,D)** factor VIII related antigen (F8RA) immunoreactivity in early secretory phase (ES) **(C)** and late secretory **(D)** phase (LS) control endometrium; **(E,F)** CD34 immunoreactivity in mid secretory phase (MS) **(E)** control and **(F)** HMB endometrium; **(G,H)** CD31 immunoreactivity in LSP **(G)** control and **(H)** HMB endometrium. Original magnification 200x; inserts on each image show higher power (400x) of one vessel cross-section. NPA = no primary antibody negative control.

**Figure 2**: **Semi-quantitative** **analysis of** **immunostaining for EC markers revealed differences across the menstrual cycle, and between controls and women with subjective HMB.**

Graphical representation (mean±SEM) of Quickscore assessment of staining for EC markers (N=5, each phase) in control and HMB endometrium (proliferative (Prolif), ES, MS, LS) in the uterine tissue layers (luminal, stratum functionalis, stratum basalis, myometrium). Bars labelled with the same letter are significantly different from each other. **A)** UEA-1, a,c,d,f *P*=0.008, b *P*=0.006, e *P*=0.002, A *P*<0.001, B,C *P*=0.001; **B)** F8RA, a *P*=0.006, b,f *P*=0.008, c,d *P*=0.003, e *P*=0.002, A,B *P*=0.002, C *P*=0.004; **C)** CD34, a-h *P*=0.008, A *P*=0.002, B *P*=0.001; **D)** CD31, a *P*=0.007, b,c,e,f,g,i *P*=0.008, d *P*=0.002, h *P*=0.003. Kruskal-Wallis with a post-hoc test was used to determine differences between menstrual cycle phases, or myometrial/endometrial layers. Mann-Whitney U test was used to determine differences between control and HMB groups. Differences were considered statistically significant at *P*≤0.05.

**Figure 3**: **Blood vessels expressing EC markers have different rates of proliferation.**

Representative photomicrographs of a blood vessel from LS stratum functionalis of a healthy control immunostained (darker brown) for **A)** F8RA, **B)** Ki67, **C)** UEA-1 (original magnification 200x). Graphical representation (mean±SEM) of percentage assessment of immunohistochemical staining for a proliferation marker (Ki67, N=3, each phase) in vessels expressing UEA-1, F8RA, CD34 and CD31 in the menstrual cycle of healthy control women in **D)** luminal region (a *P*=0.04, b,h *P*=0.01, c *P*=0.003, d *P*=0.0009, e *P*=0.02, f *P*=0.001, g *P*=0.005) and **E)** stratum functionalis (a *P*=0.05, b *P*=0.0004, c *P*=0.0009, d *P*=0.0006, e *P*=0.002, f,g *P*=0.01). Kruskal-Wallis with a post-hoc test was used to determine differences between menstrual cycle phases. Differences were considered statistically significant at *P*≤0.05.

**Figure 4**: **Immunostaining for extracellular matrix proteins differed across the menstrual cycle, and between controls and women with subjective HMB.**

Representative photomicrographs of immunostaining for the different extracellular matrix (ECM) markers. **(A,B)** Osteopontin immunoreactivity in LS **(A)** control and **(B)** HMB stratum functionalis; **(C,D)** Laminin immunoreactivity in ES control **(C)** stratum functionalis and **(B)** stratum basalis; **(E,F)** Fibronectin immunoreactivity in ES control **(E)** stratum functionalis and **(F)** stratum basalis; **(G,H)** Collagen IV immunoreactivity in ES **(G)** control and **(H)** HMB stratum functionalis. Original magnification 400x. **I)** Negative control with no primary antibody.

**Figure 5**: **Semi-quantitative** **analysis of** **immunostaining for ECM proteins revealed differences across the menstrual cycle, and between controls and women with subjective HMB.**

Graphical representation (mean±SEM) of Quickscore assessment of staining for ECM markers (N=5, each phase) in control and HMB endometrium in the uterine tissue layers (stratum functionalis, stratum basalis, myometrium). Bars labelled with the same letter are significantly different from each other. **A)** Osteopontin, a *P*=0.002, b *P*=0.003, c *P*=0.02, d,e *P*=0.008; **B)** Laminin, a *P*=0.005; **C)** Fibronectin, a *P*=0.003, b *P*=0.004, c *P*=0.002, d *P*=0.005, e *P*=0.006; **D)** Collagen IV, a,c *P*=0.01, b *P*=0.008, d *P*=0.007, e *P*=0.002. Kruskal-Wallis with a post-hoc test was used to determine differences between menstrual cycle phases, or myometrial/endometrial layers. Mann-Whitney U test was used to determine differences between control and HMB groups. Differences were considered statistically significant at *P*≤0.05.