Altered vascular smooth muscle cell differentiation in the endometrial vasculature in menorrhagia

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Study Design, Size, Duration: Endometrial biopsies were taken from hysterectomy specimens or by pipelle biopsy prior to hysterectomy in controls without endometrial pathology and in women with menorrhagia (n = 7 for each of proliferative, early-secretory, mid-secretory and late-secretory phases for both groups). Biopsies were formalin fixed and embedded in paraffin wax.

Participants/Materials, Setting, Methods: Paraffin-embedded sections were immunostained for α smooth muscle actin (αSMA), myosin heavy chain (MyHC), H-caldesmon, desmin, smoothelin and calponin (h1 or basic). VSMC content was measured in 25 αSMA+ vascular cross sections per sample and expressed as a ratio of the muscular area: gross vascular cross-sectional area. VSMC differentiation was analysed by the presence/absence of differentiation markers compared with αSMA expression. Smoothelin and calponin expression was also analysed in relation to total number of blood vessels by double immunostaining for endothelial cell markers.

Main Results and the Role of Chance: Study of VSMC differentiation markers revealed decreased expression of calponin both in αSMA+ vessels (P = 0.008) and in relation to total number of vessels (P = 0.001) in late secretory phase endometrium in menorrhagia compared with controls. Smoothelin expression in αSMA+ vessels was increased (P = 0.03) in menorrhagia, although this was not significant in relation to the total number of vessels. In normal endometrium, the proportion of blood vessels expressing αSMA increased from 63% in proliferative endometrium to 81% in the late secretory phase (P = 0.002). The overall arterial muscle content did not differ between control and menorrhagia at any phase of the menstrual cycle, occupying 78–81% of gross vascular cross-sectional area during the different menstrual cycle phases.

Limitations, Reasons for Caution: This study included both straight and spiral arterioles and analysed only stratum functionalis. The VSMC differentiation with respect to αSMA expression is an observational study and the data are presented as presence or absence of the differentiation markers in each field of view, corresponding with the vascular cross sections included in the study of vascular muscle content.

Wider Implications of the Findings: Smoothelin and calponin have been widely implicated as important regulators of vascular tone, vascular contractility and rate of blood flow. Our results have uncovered a disparate pattern of calponin expression, potentially indicating a dysfunctional contraction mechanism in the endometrial blood vessels in menorrhagia, thus implicating calponin as a potential therapeutic target.
Introduction

The human menstrual cycle can be divided into three phases: menstrual (Days 1–4), proliferative (or follicular, Days 5–14) and secretory (or luteal, Days 14–28) phases. This cycle encompasses the structural and functional remodelling of the endometrium under the dynamic influence of the ovarian steroid hormones, estrogen and progesterone. The endometrium, which is one of the fastest growing tissues in the human, forms the inner lining of the body of the uterus. This complex mucosal lining consists of two major layers: a germinal layer or stratum basalis, which persists from one menstrual cycle to the next, and the transient superficial layer, the stratum functionalis (Heller, 1994). The endometrial vasculature includes the basal arteries supplying the stratum basalis and the spiral arteries supplying the stratum functionals. The blood vessels comprise several layers: the innermost endothelial cell layer, the basement membrane and vascular smooth muscle cells (VSMCs). The coiled shape of the spiral arteries and arterioles helps to reduce blood pressure and rate of flow along its length, maintaining a steady blood flow to the stratum functionals (Aplin et al., 2008).

Menorrhagia, defined as excessively heavy menstrual bleeding (menstrual blood loss $>80$ ml per cycle), which interferes with a woman’s physical, social, emotional and/or material quality of life, affects $~10$ million women annually in the USA including $30\%$ of women of reproductive age (Garside et al., 2004; Mansfield et al., 2004; Collins and Crosignani, 2007; NICE, 2007). By the age of 60 years, one in five women in the UK and one in three in the USA have had a hysterectomy, menorrhagia being the underlying cause in at least $50–70\%$ (Duckitt and McCully, 2004; El-Hemaidi et al., 2007). While menorrhagia may result from clinical conditions such as uterine fibroids, $~50\%$ of the cases remain unexplained (Rees, 1987). Abnormal menstrual bleeding accounts for $3.5$ million working days lost annually in the UK (Collins and Crosignani, 2007) and the annual treatment costs are $>65$ (US$109$) million, resulting in an enormous economic burden for the UK National Health Service. Current surgical or medical treatment options for menorrhagia are unsuitable for women who wish to retain fertility. Therefore study of the underlying mechanisms of menorrhagia remains central not only to the understanding of this common condition but also to the possible development of new fertility sparing, less invasive, and more effective treatments for thousands of women suffering from unexplained menorrhagia.

Women with menorrhagia bleed longer but, more critically, exhibit an increased (3- to 4-fold increase) rate of blood loss than women with normal menstrual cycles (Abberton et al., 1999a). Consistent with this, Hurskainen et al. (1999) showed that women with menorrhagia had a lower uterine artery pulsatility index (PI) compared with controls. Correlation between menstrual blood loss (MBL) and PI suggests that abnormal structure and/or function of the endometrial spiral arteries, including inefficient vasodilation or vasoconstriction may contribute to menorrhagia. VSMCs are a major constituent of the endometrial blood vessels and their stage of differentiation contributes to normal blood flow and pressure. Failure of these vessels to control the rate of blood loss could be attributed to a failure in normal vasoconstriction, which in turn may be explained by abnormal arteriogenesis together with impaired VSMC maturation and function. However, while vessel number and morphology has been studied previously (Abberton et al., 1999a,b; Rogers and Abberton, 2003), VSMC differentiation in endometrial blood vessels has not been extensively investigated across the normal menstrual cycle or in menorrhagia.

Alpha smooth muscle actin ($\alpha$SMA), a contractile cytoskeletal protein, is the earliest marker of VSMC differentiation and is located in both small and large blood vessels. During their differentiation VSMCs express a series of contractile proteins including $\alpha$SMA, calponin, H-caldesmon (H-cal), myosin heavy chain (MyHC), desmin and smoothelin, amongst others. Changes in the state of VSMC differentiation have been implicated in vascular diseases, while the co-operative signal transduction pathways underlying VSMC differentiation and proliferation have been widely studied (Owens, 1995; Owens et al., 2004; Kawai-Kowase and Owens, 2007). Mature VSMCs have the capacity to de-differentiate to fulfi immediate needs for growth and remodelling (Owens, 1995). Mature VSMCs may therefore alternate between a contractile state expressing a high level of contractile proteins and a secretory state, which involves proliferation and secretion of basement membrane proteins (Owens, 1995; Rogers and Abberton, 2003).

Following recruitment and differentiation, VSMC proliferation is the key to growth of an arteriole. Studies using proliferating cell nuclear antigen (PCNA) have shown that VSMC proliferation in spiral arterioles remained consistently low during the early menstrual cycle and increased during the mid-late secretory phase, explaining vessel growth and coiling; however, VSMC proliferation remained constant for straight arterioles (Abberton et al., 1999b). In women with menorrhagia VSMC proliferation was significantly reduced in the mid and late secretory phase in both the spiral and straight arterioles (Abberton et al., 1999b). This suggests that the spiral arterioles have an altered growth pattern in menorrhagia, since it is in the secretory phase that spiral arterioles grow and gain their distinctive coiled structure. Interestingly, the total number of VSMCs in spiral arterioles was similar between control and menorrhagia tissue and consequently absolute wall thickness and $\alpha$SMA actin expression pattern was similar. In addition, expression of MyHC, a late marker of VSMC differentiation, was lower in the early secretory phase in women with menorrhagia, suggesting that VSMCs mature at different rates in the spiral arterioles of menorrhagia compared with normal endometrium (Abberton et al., 1999b).

We hypothesized that blood vessel structure is altered in the endometrium of women with menorrhagia. To test this hypothesis we examined the thickness of the blood vessel wall and the differentiation status of the VSMCs in endometrium across the menstrual cycle in healthy, fertile controls and women with menorrhagia.
Materials and Methods

Samples

Endometrial biopsies were obtained with informed consent from women undergoing hysterectomy at the Royal Victoria Infirmary, Newcastle upon Tyne or at the Liverpool Women’s Hospital, Liverpool. The experimental group consisted of women with a history of menorrhagia, 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). The control group were fertile women without any uterine pathology potentially associated with endometrial abnormality (e.g. endometriosis, adenomyosis, leiomyomata, heavy irregular bleeding or menorrhagia) undergoing hysterectomy due to prolapse, cystocele, rectocele, urinary or stress incontinence. Any women who had received hormone treatment within 3 months prior to the operation were excluded from the study. The menstrual cycle phase was staged according to standard morphological criteria (Noyes et al., 1975) by a specialist gynaecologist histopathologist (JNB). Patient details are shown in Table I. Endometrial biopsies (n = 7, in each of proliferative, early, mid and late secretory phases) were obtained from both control and menorrhagic women. The biopsies were fixed in 10% neutral buffered formalin for 24–48 h, routinely processed and embedded in paraffin wax, and 3 μm serial sections were cut for immunohistochemistry. The study was approved by Newcastle and North Tyneside Research Ethics Committee (Ref:10/H0906/71) and were cut for immunohistochemistry. The study was approved by Newcastle and North Tyneside Research Ethics Committee (Ref:10/H0906/71) and were cut for immunohistochemistry. The study was approved by Newcastle and North Tyneside Research Ethics Committee (Ref:10/H0906/71) and were cut for immunohistochemistry.

Immunohistochemistry

Paraffin sections (3 μm) were dewaxed in xylene, rehydrated through descending concentrations of alcohol (99, 90, 70%) and incubated in 1% H2O2 in water for 10 min to block endogenous peroxidase activity. All washes and incubations were performed in TBS at room temperature. Non-specific binding was blocked by treating the sections with rabbit serum (Vectastain Elite ABC kit; Vector Laboratories) for 10 min. Following this sections were washed twice for 5 min in TBS and incubated with biotinylated Ulex Europaeus-Agglutinin I (UEA-I lectin); diluted in TBS for 60 min (Supplementary data, Table SI). The sections were then further washed and incubated for 30 min with streptavidin/horse radish peroxidase (HRP) diluted 1:100 in TBS, pH7.6 (Streptavidin P0397, Dako Cytomation, Cambridgeshire, UK). The sections were then washed in TBS, pH7.6 and the reaction was developed for 1–2 min with 3,3’-diaminobenzidine (DAB; Sigma Chemical Co.) containing 0.01% H2O2 to give a brown reaction product. Negative controls showed no immunostaining with any of the protocols used.

Lectin histochemistry

Paraffin sections (3 μm) were dewaxed in xylene, rehydrated through descending concentrations of alcohol (99, 90, 70%) and incubated in 1% H2O2 in water for 10 min to block endogenous peroxidase activity. All washes and incubations were performed in TBS at room temperature. Non-specific binding was blocked by treating the sections with rabbit serum (Vectastain Elite ABC kit; Vector Laboratories) for 10 min. Following this sections were washed twice for 5 min in TBS and incubated with biotinylated Ulex Europaeus-Agglutinin I (UEA-I lectin); diluted in TBS for 60 min (Supplementary data, Table SI). The sections were then further washed and incubated for 30 min with streptavidin/horse radish peroxidase (HRP) diluted 1:100 in TBS, pH7.6 (Streptavidin P0397, Dako Cytomation, Cambridgeshire, UK). The sections were then washed in TBS, pH7.6 and the reaction was developed for 1–2 min with 3,3’-diaminobenzidine (Sigma Chemical Co.) containing 0.01% H2O2 to give a brown reaction product. The sections were then lightly counterstained with Mayer’s haematoxylin for 30 s, dehydrated, cleared in xylene and mounted with DPX synthetic resin (Raymond A. Lamb Ltd). Negative controls were performed by omission of Ulex lectin.

Double immunohistochemical labelling

Double immunohistochemical labelling was used to identify the proportion of vessels identified using endothelial cell markers (CD31 or biotinylated UEA-I lectin) that were immunopositive for the smooth muscle markers (αSMA, smoothelin or calponin). The antibody combinations for double labelling were selected based on pretreatment compatibility as follows: CD31/αSMA, calponin/ulex and smoothelin/CD31. All antibody details are provided in Supplementary data, Table SI. The first immunostain was visualized using DAB containing 0.01% H2O2 to give a brown reaction product and the second with Vector Silver substrate kit for peroxidase (Cat: SK-4700, Vector Laboratories, Inc.). Appropriate positive and negative controls were performed for each antibody run, including double immunohistochemistry labelling with each antibody used. Negative controls showed no immunostaining for any of the protocols used.

Quantitative image analysis

Sections were examined using a Nikon Eclipse 80i microscope with a ×20 objective and ×10 eyepiece. Since tissue sections varied in size, in order to standardize the analysis keeping a consistent sample size between control and menorrhagia, for each tissue section images were captured of different fields of view that contained 25 αSMA positively immunostained vessel cross sections. Images were then captured of the same fields of view in the corresponding serial sections immunostained with the different VSMC differentiation markers. All captured images were assembled using NIS Elements Viewer and NIS Elements Ar Viewer (NIKON Instruments, Inc., Surrey, UK). The thickness of the vessel wall muscle was assessed in sections immunostained for αSMA. While other methods have previously been documented (Abberton et al., 1999a; Stephanie et al., 2007), we have utilized a different method of assessment. Measurements were taken of the stained muscular area and the inner luminal cross-sectional area using NIS Elements Ar (NIKON Instruments, Inc.). Vessel thickness was expressed as the ratio of the outer stained muscular area to the total area (stained area + inner luminal area) of the vascular cross section. For each individual cross section, measurements were obtained in triplicate and the mean was used

Table I Patient demographic information for control and menorrhagia groups.

<table>
<thead>
<tr>
<th>Demographic criteria</th>
<th>Normal control (n = 45*)</th>
<th>Menorrhagia (n = 44***)</th>
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<tbody>
<tr>
<td>Ethnicity</td>
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<td>European</td>
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<td>Smoking history</td>
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<td>Non-smoker</td>
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<tr>
<td></td>
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<td>31</td>
</tr>
<tr>
<td>Age (mean range)</td>
<td>40.9 (27–51)</td>
<td>41.3 (29–51)</td>
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<tr>
<td>BMI (mean range)</td>
<td>28.4 (19.7–45.5)</td>
<td>29.3 (17.5–45)</td>
</tr>
<tr>
<td>Parity (mean range)</td>
<td>2.3 (1–5)</td>
<td>2.2 (0–6)</td>
</tr>
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*Total patient numbers, since patient groups differed for the VSMC thickness study and VSMC differentiation study.
**Information available for 44/45 samples.
***Information available for 43/44 samples.
for the final calculations. The number of vessels expressing αSMA was also quantified as a proportion of the total number of vessels as determined by double immunostaining with CD31 in the stratum functionalis of the endometrium.

VSMC differentiation markers were analysed by their absence or presence in vascular cross sections in each field of view of a particular tissue section, corresponding with the αSMA$^+$ vascular cross sections. Vessels expressing smoothelin and calponin were also measured as a proportion of total number of vessels as determined by double immunostaining with CD31 and Ulex europaeus, respectively, in the stratum functionalis.

**Statistical analysis**

Data are presented as means with standard errors. Statistical calculations were performed using the Prism software package (GraphPad Software, San Diego, CA, USA). Fisher’s test followed by unpaired Student’s t-test or Mann–Whitney U-test was used to determine statistical significance, as appropriate. Differences were considered statistically significant at $P < 0.05$.

**Results**

**Vascular smooth muscle content remained unchanged during the normal menstrual cycle and in menorrhagia**

Endometrial samples ($n = 4$ each menstrual cycle phase) were double immunostained for αSMA and an endothelial cell marker (CD31) (Fig. 1A–H). During the normal menstrual cycle 63% of the CD31-positive vessels in the stratum functionalis expressed αSMA in the proliferative phase, increasing to 81% in the late secretory phase ($P = 0.002$) (Fig. 1I). During the menstrual cycle of women with menorrhagia 63% of vessels in the stratum functionalis expressed smooth muscle marker αSMA in proliferative endometrium, increasing to 79% in the late secretory phase ($P = 0.02$) (Fig. 1I). There was no difference in this percentage between the two groups at any stage of the menstrual cycle.

Endometrial vascular smooth muscle thickness was analysed in serial sections from endometrial biopsies, collected from both normal and menorrhagia groups ($n = 7$ for each phase of menstrual cycle) (Fig. 2A–J). The overall vascular smooth muscle cell content (positive αSMA staining) in both control and menorrhagia groups remained relatively stable, accounting for 78–81% of the total vascular cross-sectional area at all cycle phases (Fig. 2K). There was no difference in the muscle content of endometrial vessels between control and menorrhagia groups during the proliferative, early, mid or late secretory phases of the menstrual cycle (Fig. 2K).

**Differentiation status of vascular smooth muscle cells in endometrial vasculature is altered in menorrhagia compared with controls**

Serial tissue sections were immunostained for the different VSMC differentiation markers: H-cal, calponin, MyHC, desmin and smoothelin (Fig. 3). Tissue sections, corresponding with those used for the measurement of thickness (αSMA$^+$), were imaged in ≥5 fields of views per section. Differentiation markers were analysed by means of ‘presence’ or ‘absence’ in the corresponding areas of the sections. Desmin immunostaining was absent from all tissues (Fig. 3). Amongst the VSMC differentiation markers, MyHC was the most commonly present (≥84% of all vascular sections) in both control and menorrhagia tissue (Fig. 4A). H-cal was present in >26% of all vascular sections in both groups (Fig. 4B). The expression of MyHC was relatively consistent (85–96%) across the four menstrual cycle phases, while that of H-cal varied considerably (26–50% in control; 35–61% in menorrhagia), during proliferative and early secretory phases. (Fig. 4A and B). None of these variations were significantly different between control and menorrhagia groups.

In contrast to MyHC and H-cal, expression of smoothelin and calponin differed markedly between the control and menorrhagia tissues and also varied between the different menstrual cycle phases. In the control group calponin was expressed in 11–18% of vessels in the proliferative and early secretory phases, before increasing to be expressed in 37% vessels in the late secretory phase. In comparison, in the menorrhagia group, calponin was expressed in 12% vessels in the proliferative phase, increasing to 32 and 39% in the early and mid secretory phases, respectively, but was seen in only 11% of αSMA$^+$ vessels in the late secretory phase (Fig. 4C). In the controls, while smoothelin was expressed in 36% of the αSMA$^+$ vessels in proliferative endometrium, this decreased to 17% in the early secretory phase and decreased further to expression in only 5% αSMA$^+$ vessels in the late secretory phase. In contrast, in menorrhagia smoothelin remained stable in proliferative (18%), early secretory (18%), mid secretory (23%) phases, before increasing to be expressed in 47% αSMA$^+$ vessels in the late secretory phase (Fig. 4D). There was an apparent inverse relationship between expression of smoothelin and calponin in the αSMA$^+$ vessels in the late secretory phase in both control and menorrhagia groups (Fig. 4C and D). While smoothelin was increased in αSMA$^+$ vessels ($P = 0.03$), calponin was decreased ($P = 0.008$), in menorrhagia compared with controls. The most marked difference between control and menorrhagia groups in calponin and smoothelin expression was in the late secretory phase.

A double immunohistochemical labelling study ($n = 5$ samples per subject group) was undertaken to investigate smoothelin and calponin expression in conjunction with the endothelial cell markers CD31 or Ulex europaeus, respectively, in the stratum functionalis in late secretory phase endometrium (Fig. 4E–I). The proportion of vessels expressing calponin and smoothelin was expressed as a percentage of the total number of vessels in the stratum functionalis. This demonstrated that the proportion of vessels expressing calponin was significantly decreased ($P = 0.001$) in cases of menorrhagia compared with controls (Fig. 4E). No significant differences in the proportion of vessels identified using the endothelial markers were observed for smoothelin.

**Discussion**

The current study showed that the muscle content of vessels in the stratum functionalis measured as a proportion of vascular cross-sectional area increased from the proliferative to the late secretory phase. However, this pattern remained unchanged between control and menorrhagia groups. Moreover, vascular calponin expression in the late secretory phase, in relation to both the total number of vessels and to αSMA$^+$ vessels was significantly decreased in women with menorrhagia, suggesting an alteration in vascular smooth muscle cell differentiation status. Vascular smooth muscle cells form an integral part of the structure and normal functioning of the endometrial spiral arteries and arterioles and therefore may play an important role in abnormal bleeding disorders.
such as menorrhagia. Understanding the healthy functioning of the uterus, especially the endometrial vasculature may thus provide significant clues to advance the development of effective and less invasive treatments, to improve the quality of life for thousands of women suffering from menorrhagia. This study investigated two aspects of endometrial blood vessel development in the stratum functionalis in menorrhagia: VSMC content and VSMC differentiation status.

Previous studies have suggested that the structure and/or function of endometrial spiral arteries and arterioles may play a role in menorrhagia, but little is known about the underlying mechanisms. Abberton et al. (1999b) and Hurskainen et al. (1999) reported that menorrhagic women had increased volume and rate of blood flow through the endometrial vessels. This increased blood flow rate may be explained by either increased vasodilation or poor vasoconstriction. If vasodilation is caused by relaxation of the vascular smooth muscle cells of the vascular wall, it is possible that differences may lie in the vascular muscle content and thickness in menorrhagia.

The current study examined whether the thickness of VSMCs differed between controls and women with menorrhagia during the different menstrual cycle phases. Previous studies have shown no difference in the endometrial microvascular density in women with menorrhagia compared with controls (Abberton et al., 1999b). This study has extended these findings to demonstrate that, while the percentage of muscularised blood vessels increased across the menstrual cycle, there was no

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**Figure 1 (A–H)** Representative double immunostaining CD31/αSMA (alpha smooth muscle actin) (brown/silver grey) of blood vessels in control (A–D) and menorrhagia (G–J). The red and black arrows indicate CD31⁺/αSMA⁺ (brown/silver) vessels and CD31⁺/αSMA⁻ only vessels, respectively. Images were captured at × 200 magnification. (I) The percentage of αSMA⁺ vessels in each phase of the menstrual cycle for control and menorrhagia groups (mean ± SEM). The proportion of vessels expressing αSMA in the normal menstrual cycle increased from 63% in the proliferative to 81% in the late secretory phase, while in menorrhagia αSMA⁺ vessels increased from 63% in the proliferative to 79% in the late secretory phase. Fisher’s test followed by unpaired Student’s t-test was used to determine statistical significance, as appropriate. Differences were considered statistically significant at $P < 0.05$ (denoted by ‡).
difference between controls and women with menorrhagia. In addition, in αSMA+ vessels, the proportion of vascular cross-sectional area accounted for by VSMCs did not differ between controls and women with menorrhagia. This result is partially consistent with that of Abberton et al. (1999a) who reported no difference in the muscle thickness of spiral arterioles between control and menorrhagic tissue during the menstrual cycle, although there was a decrease in muscle thickness in straight arterioles in the late secretory phase in menorrhagia. These differences may be explained by the fact that our study included both straight and spiral arteries/arterioles, while Abberton et al. (1999a) analysed these separately. Stratum functionalis primarily consists of spiral arteries/arterioles, and straight arteries do not undergo vascular remodelling. Therefore any changes shown in our data may be expected to correspond to the spiral arteries/arterioles. Furthermore, assessment of vessel muscle content by Abberton et al. (1999a) was based on the number of VSMC layers surrounding the vessel rather than as a ratio of the total vessel cross-sectional area, as reported in the current study. Overall, these data suggest that the number and muscle content of endometrial blood vessels is not substantially altered in menorrhagia and that any underlying vascular abnormalities are more subtle.

VSMCs play a major role in contraction and control of blood flow by monitoring vascular tonicity and diameter and consequently in maintenance of blood pressure. They express a range of proteins as differentiation markers. αSMA is one of the earliest markers of VSMC differentiation, while calponin and H-caldesmon are expressed later (Glukhova et al., 1986, 1990; Frid et al., 1992; Owens, 1995). Myosin heavy chain 1 (MyHC1) is expressed prior to MyHC2 (Kocher et al., 1991; Aikawa et al., 1993; Miano et al., 1994; Owens, 1995; Owens et al., 2004), whereas smoothelin is expressed in 10- to 11-week placental tissue (van der Loop et al., 1997). Combining these a chronological

Figure 2 (A–J) Vessels (arrowed) immunopositive for alpha smooth muscle actin (αSMA) in control and menorrhagia endometrium in the proliferative, early secretory, mid secretory and late secretory phases of the menstrual cycle. Images were captured at × 200 magnification. The total vascular smooth muscle content did not differ between control and menorrhagia endometrium samples and was 78–81% of a gross vascular cross-sectional area (K). 'N' represents the number of samples. In (K), the bar chart with a standard error of mean (SEM) represents the mean muscle content in each phase of the menstrual cycle for both control and menorrhagia groups. Fisher’s test followed by unpaired multiple Student’s t-test, with Holm-Sidak correction method to account for multiple comparisons, indicated no statistical significance (P > 0.05).
pattern of expression of VSMC differentiation markers could be deduced as αSMA, H-caldesmon and calponin, MyHC1, smoothelin and finally MyHC2. However, it is not known whether the order of this sequential differentiation is similar in endometrial vessels to that in other vessels nor is it known whether a change in the differentiation status can potentially lead to altered vascular function.

Desmin is the major intermediate filament in smooth muscle and is thought to contribute to maintaining mechanical strength and contractility (reviewed by Paulin and Li et al. (2004)). However, desmin was not expressed by endometrial blood vessels at any stage of the menstrual cycle, perhaps suggesting an alternative mechanism of maintaining mechanical integrity and contractile function in these vessels. MyHC (both SM1 and SM2 were detected by the antibody used) was the most abundantly detected VSMC marker throughout the menstrual cycle in both subject groups, while the expression patterns of smoothelin and calponin were the most highly variable, both with menstrual cycle stage and between control and menorrhagia groups. This difference suggests a change of differentiation status of VSMCs in menorrhagia that may reflect altered function, particularly in the late secretory phase of the menstrual cycle just prior to menstruation. In contrast to our findings, Abberton et al. (1999a) reported decreased MyHC expression in spiral arterioles in the early secretory phase in menorrhagia

Figure 3  Vascular smooth muscle cell (VSMC) differentiation markers during the menstrual cycle in menorrhagia showing vessels (arrowed) immuno-positive for H-caldesmon (A–D), calponin (E–H), myosin heavy chain (MyHC) (I–L), desmin (M–P), smoothelin (Q–T), in menorrhagia endometrium during the proliferative, early secretory, mid secretory and late secretory phases of the menstrual cycle. Images were captured at ×200 original magnification.
Vascular muscle differentiation in menorrhagia

A

Percentage MyHC* compared to αSMA* vessels

B

Percentage H-Ca* compared to αSMA* vessels

C

Percentage Calponin* compared to αSMA* vessels

D

Percentage Smoothelin* compared to αSMA* vessels

E

Percentage of total number of vessels

*P = 0.008

*P = 0.03

*P = 0.001

Calponin

Control | Menorrhagia

Smoothelin

Control | Menorrhagia

F

G

H

I

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et al. (1999a). However, as stated, our study included spiral and straight arterioles and recorded MyHC expression only in vessels that expressed αSMA, while Abberton et al. (1999a) measured MyHC expression independent of αSMA using a quick scoring method (integrated optical density) \( \times \) (proportional area). While both methods have merits, by analysing expression of VSMC differentiation markers in relation to all αSMA+ vessels, we hoped to also detect differences in the chronological pattern of differentiation in the VSMCs between control and menorrhagia samples.

There was an inverse relationship between smoothelin and calponin expression in the late secretory phase; the proportion of αSMA+/smoothelin− vessels increased in the late secretory phase in menorrhagia compared with controls, whereas that of αSMA+/calponin+ vessels decreased. Double immunohistochemical labelling also showed a decrease in the proportion of calponin expressing vessels in menorrhagia, compared with the total number of vessels detected using endothelial markers. The fact that some of these differentiation markers manifest a more restricted distribution than others in endometrial vessels raises an important question; do the differences in distribution indicate their stage of differentiation or do they reflect functional differences? Further future functional studies examining these possibilities therefore are warranted, which could in turn indicate potential therapeutic targets.

Smoothelin is a major contributor to the contractile properties of VSMCs and has two isoforms: smoothelin A, expressed in viscosa, and smoothelin B, which is specifically present in VSMCs (Rensen et al., 2002; van Eys et al., 2007). Rensen et al. (2008) reported that smoothelin B deficient mice had significantly reduced arterial contractility and increased basal mean arterial pressure (MAP), which may be attributed to a simultaneous reduction in vasodilation. The rate of muscle contraction is determined by actomyosin cross-bridge cycling and the rate of muscle shortening (shortening velocity); the co-operation between these actomyosin cross-bridges in turn depends on proteins such as H-caldesmon and calponin (Butler and Siegman, 1998; Fitzsimons et al., 2001; Morgan and Gangopadhyay, 2001). Interestingly, smoothelin B consists of a ‘calponin homology domain’, which is capable of binding to αSMA in VSMCs (van Eys et al., 2007). In support of this, Schildmeyer et al. (2000) showed that αSMA null mice had abnormal vasoconstriction as well as impaired vasodilation. Therefore, deficiency or aberrant expression of smoothelin B may result in impaired vasodilation (Li and Hui, 2009). Increased smoothelin may facilitate contractility and vasodilation leading to increased blood flow. In this study we have shown that an increase in smoothelin in the late secretory phase, perhaps indicating increased vasoconstriction and vasodilation as an underlying mechanism for menorrhagia.

Calponin h1 (or basic calponin) is a smooth muscle specific protein, which binds to actin and plays a significant role in regulation of the contractile apparatus in VSMCs (Walsh et al., 1993; Lu et al., 1995; Stafford et al., 1995). In aortic smooth muscle, shortening velocity is a direct measure of rate of vessel contraction and consequently blood flow; increased shortening velocity increases the rate of vessel contraction, thereby increasing the rate of blood flow per unit time. Takahashi et al. (2000) reported that calponin plays a role in regulating cross-bridge cycling and shortening velocity and consolidated previous findings that calponin slowed shortening velocity in both tonic and phasic smooth muscle (Jaworowski et al., 1995; El-Mezgueldi and Marston, 1996; Malmqvist et al., 1997; Takahashi et al., 2000). The same group also reported that decreased calponin leads to an increase in cross-cycle duration, i.e. increased shortening velocity and therefore increased rate of contraction and blood flow (Fujishige et al., 2002). In the present study there was a significant decrease in calponin in the late secretory phase of the menstrual cycle in menorrhagia. It is possible that reduced calponin leads to increased shortening velocity leading to an increased rate of contraction and blood flow in menorrhagia. This hypothesis is also in line with our findings on smoothelin; increased smoothelin expression may facilitate contractility and vasodilation leading to increased blood flow. It should be noted, however, that in contrast with the pump vessels investigated in the studies described above, the endometrial vessels are thought to be resistance vessels and the mechanism of blood flow in these vessels may therefore differ. An alternative explanation for the present results could be that the changes in the pattern of expression of the differentiation markers may have caused a functional change in the endometrial vasculature, such that these vessels operate in a pumping manner similar to those in the cardiovascular system.

Vascular smooth muscle cell differentiation results from the coordinated transcriptional activation of an array of contractile proteins including calponin, MyHC, αSMA. Most of these genes carry the transcription binding code, CArG box (Minty and Kedes, 1986; Sun et al., 2006). This binds to the serum response factor (SRF) transcription factor with differing affinity, thus regulating its activation (Miano et al., 2007). However SRF expression is not specific to VSMCs; cell-specific activation is achieved through the expression of a SMC-specific co-factor, myocardin (MYCOD) (Wang et al., 2001; Chen et al., 2002). In VSMCs, the CArG-SRF-MYCOD acts as the primary transcriptional switch which maintains a physiologically normal functioning SMC phenotype (Long et al., 2008). The fact that these contractile genes share a co-ordinated regulation may lead to the assumption that their expression pattern (presence or absence) may also be co-ordinated. However, our results indicate a decrease of calponin without any significant alteration

**Figure 4** Differential expression of vascular smooth muscle cell (VSMC) differentiation markers during the normal menstrual cycle and in menorrhagia (A–D). The bar charts represent the mean percentage of vessels immunoreactive for the different VSMC differentiation markers related to alpha smooth muscle actin (αSMA), in each phase of the menstrual cycle for control and menorrhagia groups, respectively. There was an inverse relationship between the presence of calponin and smoothelin in the late secretory phase (C and D); Double immunolabeling studies (smoothelin/CD31; calponin/ulex) revealed that in the late secretory phase the proportion of vessels expressing calponin was decreased \( (P = 0.001) \) in menorrhagia compared with normal controls (E). In (E), the bar chart with a standard error mean (SEM) represents the mean percentage of smoothelin+ or calponin+ vessels, respectively, in the late secretory phase of the menstrual cycle for both control and menorrhagia groups. Mann–Whitney U-test and Fisher’s test followed by unpaired Student’s t-test were used to determine statistical significance, as appropriate. Differences were considered statistically significant at \( P < 0.05 \) (denoted by ‘\(*\)’). Double immunolabeling of endometrial blood vessels in control (F and H) and menorrhagia samples (G and I). Calponin (F and G) and smoothelin (H and I) are silver/grey and endothelial cells, ulex (F and G) and CD31 (H and I) in brown. The black arrows indicate calponin+/ulex+ and smoothelin+/CD31+ vessels. Images were captured at \( \times 200 \) original magnification.
in expression of the other contractile proteins between the control and menorrhagia groups. One explanation for this could be differences in the CArg positioning between calponin and the other contractile protein encoding genes (Miano et al., 2000). Moreover, calponin has another layer of complexity such that the positioning of CArg elements must also follow a strict positional relationship with other genomic elements (Long et al., 2011). Therefore the specific dysregulation of calponin expression shown in this study indicates the possibility of a novel calponin regulatory pathway.

In conclusion, endometrial vascular muscle content was unchanged during the menstrual cycle in menorrhagia compared with controls. However, there were characteristic differences in late secretory phase endometrium in the differentiation stage of VSMCs between menorrhagia and control endometrium, with smoothelin being increased and calponin expression decreased in muscularised vessels in menorrhagia and calponin expression also being reduced in relation to the total vessel number. This altered VSMC differentiation may lead to altered function, which could contribute to the abnormal bleeding. These results may therefore indicate calponin as a potential therapeutic target; however, detailed functional studies are required to establish this. Moreover, further studies of these vessels may lead to treatment advances, which will provide for a better quality of life for thousands of women worldwide.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors’ roles

S.B.S was involved in study design, execution, data collection, 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

None declared.

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