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Citation for published version:
Maybin, J, Boswell, L, Young, VJ, Duncan, W & Critchley, H 2017, 'Reduced Transforming Growth Factor Beta Activity in the Endometrium of Women with Heavy Menstrual Bleeding', J Clin Endocrinol Metab, vol. 102, no. 4, pp. 1299-1308. https://doi.org/10.1210/jc.2016-3437

Digital Object Identifier (DOI):
10.1210/jc.2016-3437

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Peer reviewed version

Published In:
J Clin Endocrinol Metab

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The Journal of Clinical Endocrinology & Metabolism
Endocrine Society

Submitted: October 12, 2016
Accepted: December 28, 2016
First Online: January 03, 2017

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Reduced Transforming Growth Factor Beta Activity in the Endometrium of Women with Heavy Menstrual Bleeding

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Received 12 October 2016. Accepted 28 December 2016.

Endometrial TGFβ1 and Heavy Menstrual Bleeding

Context: Heavy menstrual bleeding (HMB) is common and incapacitating. Aberrant menstrual endometrial repair may result in HMB. The TGFβ superfamily contributes to tissue repair, but its role in HMB is unknown. Objective: We hypothesised that TGFβ1 is important for endometrial repair and women with HMB have aberrant TGFβ1 activity at menses. Participants/Setting: Endometrial biopsies were collected from women and menstrual blood loss objectively measured (HMB >80ml/cycle, normal menstrual bleeding, NMB <80ml). Design: Immunohistochemistry and RT-PCR examined endometrial TGFβ1 ligand, receptors and downstream SMADs in women with NMB and HMB. The function and regulation of TGFβ1 was examined using primary stromal cell culture. Results: TGFB1 mRNA was maximal immediately prior to menses, but no differences were observed between women with NMB and HMB at any cycle stage. Histoscopy of TGFB1 revealed reduced staining in the stromal compartment during menses in women with HMB (p<0.05). There were no significant differences in TGFBR1/2 or TGFBR1/2 immunostaining. Cortisol increased activation of TGFβ1 in the supernatant of human endometrial stromal cells (HES, P<0.05) via thrombospondin-1. Endometrial SMAD2 and SMAD3 were lower in women with HMB during menstruation (P<0.05) and decreased phosphorylated SMAD2/3 immunostaining was seen in glandular epithelial cells during the late secretory phase (p<0.05). Wound scratch assays revealed increased repair in HES cells treated with TGFβ1 versus control (P>0.05). Conclusions: Women with HMB had decreased TGFβ1 and SMADs peri-menstrually. Cortisol activated latent TGFβ1 to enhance endometrial stromal cell repair. Decreased TGFβ1 activity may hinder repair of the denuded menstrual endometrium, resulting in HMB.

PRECIS: TGFβ1 and downstream SMADs were decreased in perimenstrual endometrium from women with heavy menstruation versus controls. TGFβ1 was activated by cortisol in endometrial cells and enhanced repair.

Introduction

The human endometrium is a complex and dynamic tissue. Throughout the reproductive years of a woman’s life it responds to steroid hormones to prepare for implantation, shed its luminal portion in the absence of pregnancy and efficiently regenerate for the subsequent menstrual cycle. Menstruation occurs as a result of the sharp decline in progesterone as the corpus luteum regresses. This progesterone withdrawal stimulates an influx of inflammatory cells and release of matrix metalloproteinases, resulting in tissue destruction and menstrual bleeding (1,2).

The regulation of endometrial repair after shedding remains undefined. Scanning electron microscopy and hysteroscopy analysis revealed that luminal epithelial cell migration precedes stromal expansion, but that breakdown and repair occur simultaneously in adjacent sections of the human endometrium during active bleeding (3). Initiation of endometrial repair therefore occurs during the menstrual phase, when ovarian hormone levels remain low. Indeed, in the mouse model of simulated menstruation repair occurred without delay when both exogenous and endogenous estrogens were removed (4).
The transforming growth factor beta (TGFβ) superfamily includes TGFβs, activins, nodal and bone morphogenic proteins (BMPs). This superfamily have been implicated in cell motility, proliferation, apoptosis, immune response and differentiation [reviewed in (5)]. Therefore, they are attractive candidates for the co-ordination of endometrial repair at menses.

TGFβ is synthesised as a dimeric pre-proprotein and is released in a latent form. It is activated in a tissue specific fashion by a variety of mechanisms, including extremes of pH or via plasmin or thrombospondin-1 (6,7). Once activated, it binds to type II transmembrane serine/threonine kinase receptors, which then form a heterotetrameric complex with dimers of type I receptors. This leads to phosphorylation and activation of intracellular regulatory SMADs (SMAD-2 and -3), which in turn interact with the co-mediator SMAD-4 and translocate to the nucleus to regulate transcription of target genes. TGFβ ligands and receptors are present in the human endometrium with maximal levels found during menstruation (8). TGFβ ligand expression was found to be suppressed by progesterone (8), meaning endometrial induction following progesterone withdrawal is expected. Despite the low levels of circulating progesterone and estradiol at menses, local generation of steroids in the endometrium may play a vital role in menstrual physiology. Endometrial expression of the enzyme 11βHSD1, necessary for local generation of cortisol, and the expression of the cortisol receptor GR have both been reported to be up-regulated at the time of menses (9). The role of cortisol in the regulation of TGFβ remains undetermined.

Heavy menstrual bleeding of endometrial origin (HMB-E) is a common condition with a significant impact on the quality of life of otherwise healthy women (10). The financial costs to women, their families and employers are marked (11). HMB-E can be contributed, at least in part, to delayed or ineffective endometrial repair at menses. Identification of the mechanisms involved in endometrial repair and aberrations in women with HMB-E will lead to new, effective medical therapies for the many women suffering from this debilitating condition.

Herein we hypothesise that TGFβ1, its receptors and downstream SMADs are important for endometrial repair at menses and that women with HMB-E have aberrant expression of this superfamily prior to and during the menstrual phase. To investigate this we used well-categorised endometrial whole tissue biopsies from women with objectively measured normal (<80ml) and heavy (>80ml) menstrual blood loss alongside in vitro endometrial cell culture and functional assays.

Materials and Methods

Tissue collection

Endometrial biopsies were collected with an endometrial suction curette (Pipelle, Laboratorie CCD, Paris, France) from 91 healthy women of reproductive age, who were predominantly White/Caucasian. Written informed consent was obtained and ethical approval granted from Lothian Research Ethics Committee (LREC/07/S1103/29). Participants were aged 22 to 50 years (median 41; mean 41). All reported regular menstrual cycles (21 to 35 days) and had not taken any exogenous hormones or used an intrauterine device for 3 months prior to tissue collection. Women with large fibroids (>3cm) and endometriosis were excluded.

Immediately after collection tissue was divided when possible and placed in (i) RNA later stabilisation solution (Ambion (Europe) Ltd., Warrington, UK) and stored at -70°C for RNA extraction, (ii) neutral buffered formalin (NBF) prior to paraffin wax embedding and (iii) phosphate buffered saline (PBS) for stromal cell extraction. If limited tissue was obtained (which often occurred with menstrual phase collection), NBF fixation was prioritised.

Menstrual stage was carefully categorised according to (i) histological appearance based on the criteria of Noyes et al. (12), assessed by a consultant pathologist (ii) the participant’s
reported last menstrual period (LMP) and (iii) serum progesterone and estradiol levels at the time of biopsy (see supplementary methods). Consistency for all three parameters was necessary before inclusion. Six endometrial tissue samples were excluded due to inconsistent dating and one sample due to detection of hyperplasia. Biopsies were classified as proliferative (P), early-mid secretory (E-MS), late secretory (LS) or menstrual (M) for analysis (Supplementary Table 1).

**Objective menstrual blood loss measurement**

A subset of women (n=78) also had objective measurement of their menstrual blood loss (MBL) using the modified alkaline haematin method, as previously published (13,14). In brief, women were given the same brand of tampon and/or pad (Tampax tampons and Always towels, Proctor and Gamble, UK), with verbal and written instruction on collection. Used sanitary products were added to a measured volume of 5% sodium hydroxide. The contents were left for 24h to allow conversion of haemoglobin to haematin. During the same time period, a 1 in 200 dilution of the patient’s venous blood in 5% sodium hydroxide was made and stored separately. The optical density (OD) of the samples were then measured using spectrophotometry at 546nm \((A_{546})\). MBL was calculated using the following equation (13):

\[
MBL = \frac{\text{OD of menstrual blood solution} \times \text{total volume of added NaOH}}{\text{OD of venous blood} \times 200}
\]

Greater than 80ml was classified as heavy menstrual bleeding (HMB) and less than 80ml as normal (NMB).

**Immunohistochemistry**

5µm tissue sections were deparaffinised in xylene and rehydrated. Slides for TGFβRI and II were loaded into a Celerus Riptide de-cloaking chamber (Celerus Diagnostics Carpinteria, CA, United States) epitope retrieval was performed using Novocastra Epitope Retrieval solution Ph6 (Leica Microsystems GmbH, Ernst-Leitz-Straße, Wetzlar, Germany). Slides were loaded onto Leica Bond-Max automated immunostainer (Leica Microsystems GmbH, Ernst-Leitz-Straße, Wetzlar, Germany). Primary antibodies were applied for 2h at 37°C (see supplementary Table 2) and negative control tissues incubated with isotype-matched IgG at the same concentration as the primary antibody. The presence of antigen was visualised with Bond Polymer refine detection kit (Leica Microsystems GmbH, Ernst-Leitz-Straße, Wetzlar, Germany). TGFβ1 detection was performed on the laboratory bench after pH9 antigen retrieval. The ImmPRESS polymerised reporter system (Vector Laboratories) was utilised before liquid diaminobenzidine (DAB) kit (Zymed Laboratories, San Francisco, CA, USA) detection. Sections were counterstained with haematoxylin, dehydrated and mounted with Pertex (Cellpath plc, Hemel Hempstead, UK).

**Semi-quantitive histoscore**

Localisation and intensity of immunostaining was evaluated by two independent, masked observers (15). The intensity of staining was graded with a three-point scale (0 = no staining, 1 = mild staining, 2 = strong staining). This was applied to the glands and stromal cells, as well as the surface epithelium and endothelial cells where visualised (N.B. the latter two cellular components were often absent in menstrual phase tissue, accounting for the lower n-numbers in these groups). The percentage of tissue in each intensity scale was recorded (15). A value was derived for each of the cellular compartments by using the sum of these percentages after multiplication by the intensity of staining.
Cell culture
Primary human endometrial stromal (HES) cells were isolated from secretory endometrial tissue (n=6) by enzymatic digestion as previously described (16). These women met the criteria detailed above in “tissue collection” but did not undergo objective measurement of their menstrual blood loss. Cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% FCS, 1% 200mM L-Glutamine and 500mg/ml Gentamycin.

Secretory phase HES cells from three patients with a subjective complaint of HMB and not using oral or inhaled corticosteroids were plated at 3x10^5 cells per well in 6 well plates in 10% RPMI 1640. The next day, cells were washed in PBS and incubated in serum free media overnight. Cells were then treated for 24h in duplicate with (i) vehicle (1:1000 ETOH), (ii) 1μM cortisol (17) or (iii) 1μM cortisol plus 5μM LSKL, a TSP-1 inhibitor (following a 2h pre-treatment with 5uM LSKL alone). The cell supernatant was collected for ELISA and RNA extracted from cells.

Q RT PCR
Total RNA from cells and endometrial biopsies was extracted using the RNeasey Mini Kit (Qiagen Ltd, Sussex, UK) with on column DNasel digestion according to manufacturer’s instructions. RNA samples were reverse transcribed using the Superscript VILO cDNA synthesis kit (Invitrogen, Paisley, UK) according to manufacturer’s instruction with appropriate controls. Primers for each gene of interest were designed using the Universal Probe Library Assay Design Center (Roche Applied Science, Burgess Hill, UK) (see supplementary Table 3) and purchased from Eurofins (MGW Operon, Ebergsberg, Germany). PCR was carried out using ABI Prism 7900 (Applied Biosystems). Samples and controls were analysed in triplicate using Sequence Detector version 2.3 (PE Biosystems), using the comparative threshold method. Messenger RNA transcripts were normalised relative to the geomean of two appropriate housekeeping genes 18S and ATP5B as determined by geNorm assay (Primerdesign Ltd., Southampton, UK) and quantified relative to a positive human liver cDNA sample.

ELISA
A TGF-β1 ELISA was performed using a Human TGF-β1 Quantikine Kit (DB100B; R&D Systems) according to the manufacturer's instructions. Samples were analysed without activation and with latent TGF-β1 activated to the immunoreactive form using 1m HCl and neutralized with 1.2m NaOH/0.5m HEPES buffer. Samples were assayed in duplicate, and after development assays were measured on a Lab Systems Multiscan EX Microplate reader at 450nm with wavelength correction at 540nm. Values were determined by standard curve analysis. Intra-assay CV was 2.5%, and the between-batch CV was 8.3% for cell culture supernatants.

Wound scratch assay
Secretory phase HES from three participants (passage <5) were seeded at 2 × 10^5 per well in 12 wells plates in appropriate supplemented media (see above) and 16h before scratch, medium was changed to serum-free. Each well of cells was scratched with a sterile 200μl pipette tip, washed with PBS and then incubated in serum free media with vehicle, 1ng human recombinant TGFβ1 (Peprotech, London, UK) or 10μg/ml of the TGFβ type I activin receptor-like kinase (ALK) receptors inhibitor SB 431542 hydrate (Sigma Aldridge, Dorset, UK) (n = 3 participants, triplicate wells for each). For each well, 4-5 images were captured along the length of each wound at 0 and 24h using an Axiovert 200M inverted microscope (Carl Zeiss, Jena, Germany). Images were analyzed using AxioVision release 4.72, and calculations of average distance closed for each sample were based on three measurements at identical positions along each wound image at 0 and 24h.
Statistical Analysis
Analysis was carried out using GraphPad Prism Software, Inc., San Diego, CA. For comparison of multiple data sets with two grouping variables (i.e. HMB vs NMB and stage of menstrual cycle, mRNA and immunohistochemistry data) a two way ANOVA was utilised, with Bonferroni’s multiple comparisons test. A paired one way ANOVA with Tukey’s multiple comparisons test was used to compare cell culture treatments. Tissue and cell endometrial mRNA results were expressed as the quantity relative to a comparator sample of RNA from human liver. A value of P<0.05 was considered significant.

Results
There is increased transcription of TGFB1 in the late secretory phase
TGFB1 mRNA was examined by qRT PCR in whole endometrial biopsies from women sampled at various stages of the menstrual cycle who had objectively determined menstrual blood loss. Overall the stage of the menstrual cycle had a significant impact on TGFB1 expression (P=0.0025, F=5.339), with the late secretory phase resulting in significantly higher levels of TGFB1 than endometrium from the proliferative (p<0.001) or early-mid secretory (p<0.01) phases (Figure 1). There was no significant difference between the late secretory and menstrual phase. The increased transcription of TGFB1 in the late secretory phase did not continue into the menstrual phase.

Women with HMB do not have altered endometrial TGFB1 transcription or TGFB1 reception
We compared the expression of TGFB1 in women with NMB and HMB (Figure 1) and found no significant difference in TGFB1 expression between the two groups at any cycle stage. In addition the two major TGFB1 receptors, type I and type II, were examined in the late secretory and menstrual endometrial samples. Neither TGFB1I nor TGFB1II expression was significantly different in endometrium from women with HMB versus NMB (Figure 2A,B). Immunohistochemical staining revealed maximal staining of TGFB1R1 in surface and glandular epithelial cells, with lower intensity staining in the stromal compartment (Figure 2C). TGFB1R2 showed a similar pattern, with highest immunostaining in epithelial and endothelial cells (Figure 2D). Semi-quantitative histoscoriing by two masked independent observers confirmed no differences in either receptor when comparing women with HMB and NMB throughout the perimenstrual phase (Figure 2E,F).

Women with HMB have reduced perimenstrual endometrial stromal TGFB1
As the numerous cell types in the endometrium differentially expressed TGFB1 receptors, we examined the localisation of TGFB1 by immunohistochemistry. TGFB1 could be immunolocalized to the cytoplasm of the surface epithelium, glandular epithelium, stromal cells and endothelial cells throughout the perimenstrual phase of the cycle in women with NMB (<80ml) and HMB (>80ml) (Figure 3A). Semi quantitative histoscoring revealed that protein in the menstrual phase was similar to late secretory phase. There was significantly reduced TGFB1 staining in the stromal cell compartment of endometrium from women with HMB versus those with NMB (Figure 3B). This suggests some post transcriptional regulation of TGFB1 in stromal cells.

Cortisol increases stromal TGFB1 activity via thrombospondin-1
To further investigate the post-transcriptional regulation of TGFB1 we collected primary HES cells from three women in the secretory phase of the menstrual cycle for in vitro analysis. Peri-menstrual serum progesterone and estradiol levels were not significantly different between women with HMB or NMB (Supplementary Table 1). However, we have previously shown that cortisol is involved both in endometrial repair and the regulation of endometrial thrombospondin-1 (TSP-1) (14), a known regulator of TGFB1 activity (6). Cortisol or cortisol plus LSKL (a TSP-1 inhibitor) produced a significant decrease in TGFB1 expression
in HES cells (p<0.05, Figure 4A) but there was no difference in the amount of latent TGFB1 secreted, detected by pH activation of culture supernatants prior to detection of activated TGFB1 by ELISA (Figure 4B). However analysis of unactivated cell culture supernatants revealed an increase in activation of TGFB1 protein on treatment with cortisol, which was prevented with co-treatment of cells with the TSP-1 inhibitor LSKL (p>0.05, Figure 4C). These data reveal cortisol does not increase the transcription or latent protein levels of stromal cell TGFB-β but has a role in the activation of latent TGFB-β1 in human endometrial stromal cells, via TSP-1.

**Women with HMB have reduced perimenstrual endometrial SMAD 2/3**

TGFB1 activity increases the expression and phosphorylation of the regulatory SMADs (SMAD2 and SMAD3). These activated pSMADs then interact with the co-mediator SMAD-4 and translocate to the nucleus to regulate transcription of target genes (5). Examination of SMAD2 and SMAD3 expression revealed significant decreases in women with HMB versus NMB during the menstrual phase of the cycle (P<0.05, Figures 5A,B). SMAD2 was significantly increased in women with HMB versus NMB during the late secretory phase. (Figure 5A). Immunohistochemical staining for phosphorylated SMAD 2/3 again revealed localisation to the glandular epithelium, surface epithelial cells, stromal compartment and endothelial cells (Figure 5C,D). Histoscore revealed a significant reduction in activated SMAD 2/3 protein levels in the endometrial glandular epithelial cells in women with HMB versus NMB during the late secretory phase of the menstrual cycle (Figure 5D).

**TGFB1 accelerates wound healing in primary endometrial cells**

To examine the functional effects of increased TGFB1 activity, primary HES were subjected to a wound scratch assay. As these cells are sources of TGFB1 they were studied in the presence of vehicle, SB-431542 (to block endogenously stimulated phosphorylation of SMAD proteins ) or TGFB1. HES cells showed significantly increased wound closure with TGFB1 treatment versus SB-431542 treated cells (p<0.05, Figure 6A,B).

**Discussion**

Herein we detail significant differences in TGFB1 downstream of local steroid action in the endometrium of women with HMB during menstruation. Endometrium from women with objectively measured HMB had decreased TGFB1 protein levels, unaltered TGFB receptor presence and a significant reduction in both SMAD2 and -3 mRNA expression and SMAD 2/3 protein phosphorylation before/during the menstrual phase when compared to women with NMB. We provide mechanistic data supporting TGFB1 protein activation by cortisol in endometrial cells, via thrombospondin-1. In addition, our functional studies reveal that a suboptimal TGFB response in the local endometrial environment may decrease post-menstrual repair of the stromal compartment and lead to heavy, prolonged menstrual bleeding (Figure 7).

Previous studies have detailed that TGFB1 levels in endometrial tissue explants are suppressed by progesterone (8). These authors found secretory explants cultured for 24h in the absence of progesterone and estrogen, a milieu analogous to the menstrual phase, significantly increased TGFB1 mRNA. Our results support these findings, with significantly greater TGFB1 mRNA prior to and during menstruation when compared to the proliferative and early-mid secretory phases, consistent with up-regulation following progesterone withdrawal. We did not observe any significant difference in endometrial TGFB1 mRNA between women with HMB and normal blood loss during menstruation, although we acknowledge our n-numbers are small. However, we did observe significantly decreased TGFB protein in the stromal compartment of women with HMB versus NMB during
menstruation. We acknowledge that menstrual biopsy n-numbers are low but these tissues are meticulously classified and have objective measurement of participant menstrual blood loss to aid precision of data. Our results suggest differences in TGFβ1 protein in women with HMB and NMB are not due to transcriptional regulation, but that post-transcriptional regulation may be aberrant.

Interestingly, there were no significant differences in serum progesterone or estradiol levels between women with HMB and NMB. In addition, no significant differences in endometrial estradiol receptor or progesterone receptor expression were previously detected in women with measured menstrual blood loss (18). Therefore, we hypothesised that local cortisol action may influence TGFβ1 activity during menses.

TGFβ is synthesised as a dimeric pre-proprotein and is released in a latent form. Thrombospondin-1 (TSP-1) is known to activate TGFβ1 and is thought to do so by inducing a conformational change in the latent protein (6). Our laboratory has previously published that women with HMB have significantly reduced endometrial TSP-1 mRNA levels when compared to women with normal bleeding (14). Previous studies from our laboratory have also found that cortisol increases TSP-1 mRNA expression in primary human endometrial stromal cells (14). Direct measurement of cortisol levels in the endometrium of women with HMB and NMB has not yet been carried out, but an enhanced local inactivation of cortisol by 11βHSD2 may be present in the endometrium of women with heavy menses (14). 11βHSD2 mRNA was increased 2.5 fold in women with HMB versus NMB, predicting substantially lower local cortisol concentrations. Therefore, we examined if cortisol was a local regulator of TGFβ1 activity via TSP-1. On examination of cell culture supernatants from HES cells treated with physiological levels of cortisol (17), activated TGFβ1 was significantly increased. This increase was abrogated by the addition of a TSP-1 inhibitor to culture. Interestingly, acid activation of latent TGFβ1 in the culture supernatant prior to ELISA resulted in no differences in TGFβ1 levels with any of the treatments used. This is consistent with cortisol stimulated TSP-1 production acting on latent TGFβ1 protein to increase its activity, rather than increasing the transcription or translation of TGFβ1. Indeed, cortisol and cortisol plus TSP-1 inhibitor treatment both significantly decreased TGFβ1 mRNA was not significantly different in the endometrium of women with NMB versus HMB during the peri-menstrual phase, but there was a trend towards increased TGFβ1 mRNA concentrations in women with HMB at this time, consistent with lower endometrial cortisol levels (14).

Next we examined the functional significance of TGFβ1 protein levels on endometrial cells. After shedding, endometrial cells migrate to cover the exposed surface of the endometrium and the stromal compartment regenerates (19). The wound scratch assay mimics this process in vitro, providing a means of quantifying stromal cell migration across a wounded surface. We found that TGFβ1 increased wound healing of primary stromal cell cultures. As we detected reduced phosphorylation of SMAD 2/3 in the endometrium of women with HMB versus NMB, we blocked TGF-β-mediated activation of SMAD proteins with SB 431542 and showed a decrease in stromal cell wound migration, which was significantly less that that seen with the addition of TGFβ1. We propose that women with HMB may have defective or delayed repair of the stromal cell compartment following shedding of their functional endometrium at menses.

In addition to its functional role in proliferation, it is clear that the TGFβ superfamily play an important role in endothelial cell function and blood loss. Greater than 50% of TGFβ1 knockout mice die during embryogenesis due to yolk sac defects affecting vasculogenesis and resulting in vessel fragility (20). In humans, mutation of the TGFβ receptor I ALK1 or of the endothelial accessory receptor endoglin causes hereditary haemorrhagic telangiectasia (HHT), an autosomal dominant vascular disease (21). The resulting aberrant TGFβ
superfamily signalling results in epistaxis, telangiectasia and arteriovenous malformations. Interestingly, previous histochemical and microscopic examination of endometrial blood vessels from women with normal and heavy menstrual bleeding revealed increased endothelial gaps in women with heavy loss (22). The role of the TGFβ superfamily in this pathology remains to be determined, but the observational data contained herein suggest that low late secretory/menstrual TGFβ1 protein levels and decreased pSMAD 2/3 may be involved. Previous results from our centre support a role for TGFβ1 in the generation of vasoactive factors in in women with endometriosis (23,24) and it may have a similar, if more regulated, role in the endometrium to ensure physiological menstruation.

We have previously shown that cortisol is angiostatic, preventing endothelial tube-like structure formation in vitro (14). Furthermore, SiRNA silencing of TSP-1 in uterine endothelial cells reversed the anti-angiogenic effect. In combination with data contained herein, we propose that cortisol may activate endometrial TGFβ1 via TSP-1 during menses to prevent an excessive angiogenic response and increase vascular integrity. Further experiments are required to definitively test this hypothesis.

In conclusion, we show that women with objectively measured HMB have decreased endometrial TGFβ1 protein and downstream SMADs during the late secretory/menstrual phase when compared to women with NMB. This may partially explain the increased menstrual blood loss experienced by many women. In addition, we show that cortisol has a mechanistic role in the activation of endometrial TGFβ1 at this time (Figure 7). Our in vitro results are consistent with TGFβ1 having a functional role in repair of the denuded endometrial surface at menstruation and we propose that women with HMB may benefit from therapies that increase TGFβ during menses.

Acknowledgements
We thank Ronnie Grant for assistance with figure preparation, Sheila Milne for manuscript formatting, Catherine Murray and Sharon McPherson for help with patient recruitment and Reena Murgai, Alison Murray and Moira Nicol for excellent technical support. University of Edinburgh undergraduate student Irene Sucquart performed some of these studies.

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Grants: This study was funded by The Society of Endocrinology and the Barbour Watson Fund with additional support from the Wellcome Trust (100646/Z/12/Z) and Medical Research Council (G1002033, MR/N022556/1).

Disclosure Statement: JAM, LB, VJY have nothing to disclose. HODC has clinical research support for laboratory consumables and staff from Bayer Pharma Ag and provides consultancy advice (but with no personal remuneration) for Bayer Pharma Ag, PregLem SA, Gideon Richter, Vifor Pharma UK Ltd, AbbVie Inc. WCD has received research funding from GSK for an unrelated project.

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Figure 1. **TGFβ1 in the human endometrium.** TGFβ1 mRNA expression in endometrium from across the menstrual cycle in women with HMB (blood loss >80ml) and NMB (blood loss <80ml). P proliferative, E/MS early-mid secretory, LS late secretory, M menstrual. ***P<0.001, **P<0.01.

Figure 2. **TGFβRI and TGFβRII in the human endometrium before and during menstruation.** (A) TGFβRI mRNA expression in endometrium from women with normal (NMB; <80ml) and heavy (HMB; >80ml) menstrual bleeding during the late secretory (LS) and menstrual (M) phases (B) TGFβRII mRNA expression (C) Immunohistochemical staining of TGFβRI in endometrium from the late secretory phase. SE: surface epithelium, GE: glandular epithelium, St: stromal cell compartment, arrow: endothelial cells (D) Immunohistochemical staining of TGFβRII in endometrium from the late secretory phase, inset: negative control (E) Immunohistochemical histoscore of TGFβRI in human endometrium from women with heavy and normal bleeding during the late secretory and menstrual phases (F) Immunohistochemical histoscore of TGFβRII in human endometrium from women with heavy and normal bleeding during the late secretory and menstrual phases. (N.B. lower n-numbers appear in SE and endothelial cell scoring due to the inability to identify these cells in some tissues).

Figure 3. **Immunohistochemistry for TGFβ1 in human endometrium from the perimenstrual phase.** (A) Staining of late secretory and menstrual phase endometrium from women with HMB (>80ml) and NMB (<80ml). GE glandular epithelium, SE surface epithelium, St stromal compartment, arrows indicate endothelial cells. Inset: negative control (B) Semi quantitative histoscore of TGFβ1 immunohistochemistry staining.

Figure 4. **The regulation of TGFβ1 by cortisol in primary human endometrial stromal cells.** (A) TGFβ1 mRNA after 24h treatment with vehicle, cortisol (1μM) or cortisol (1μM) plus a thrombospondin-1 inhibitor (LSKL 5μM) (B) Active TGFβ1 protein levels in experimental culture supernatants following pre-ELISA acid activation of latent TGFβ1 (C). Active TGFβ1 protein levels in the same culture supernatants without pre-ELISA acid activation. (*P<0.05).

Figure 5. **SMAD2/3 in the human endometrium before and during menstruation.** (A) SMAD2 mRNA expression in endometrium from women with normal (NMB; <80ml) and heavy (HMB; >80ml) menstrual bleeding during the late secretory (LS) and menstrual (M)
phases (B) SMAD3 mRNA expression in endometrium from women with NMB and HMB in the LS and M phases (C) Phosphorylated SMAD2/3 immunohistochemical staining in late secretory endometrium from a woman with NMB. Inset: negative control. SE: surface epithelium, GE: glandular epithelium, St: stromal cell compartment, arrow: endothelial cells (D) Phosphorylated SMAD2/3 immunohistochemical staining in late secretory endometrium from a woman with HMB (E) Histoscoring of immunostaining for phosphorylated SMAD2/3. *P<0.05.

**Figure 6. The effect of TGFβ1 on human endometrial cell wound repair.** (A) Average wound scratch closure distance (scratch distance at 0h minus scratch distance at 24h) in human primary stromal endometrial cells after treatment with vehicle, the Alk receptor inhibitor SB431542 or 1ng TGFβ1. (B) Images of wound scratch in HES cells treated with 10μg/ml SB431542 for (i) 0h (ii) 24h and treated with 1ng TGFβ1 (iii) 0h and (iv) 24h.

**Figure 7. Proposed role of TGFβ1 in the human endometrium at menstruation.** Red stars represent findings in women with HMB and potential impact on endometrial function.
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**Activated Supernatant**

- Vehicle
- Cortisol
- Cortisol + LSKL

**Unactivated Supernatant**

- Vehicle
- Cortisol
- Cortisol + LSKL

*Significant difference
