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ORIGINAL ARTICLE

# Identification of genes differentially expressed in menstrual breakdown and repair

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**STUDY QUESTION:** Does the changing molecular profile of the endometrium during menstruation correlate with the histological profile of menstruation.

**SUMMARY ANSWER:** We identified several genes not previously associated with menstruation; on Day 2 of menstruation (early-menstruation), processes related to inflammation are predominantly up-regulated and on Day 4 (late-menstruation), the endometrium is predominantly repairing and regenerating.

**WHAT IS KNOWN ALREADY:** Menstruation is induced by progesterone withdrawal at the end of the menstrual cycle and involves endometrial tissue breakdown, regeneration and repair. Perturbations in the regulation of menstruation may result in menstrual disorders including abnormal uterine bleeding.

**STUDY DESIGN, SIZE DURATION:** Endometrial samples were collected by Pipelle biopsy on Days 2 ($n = 9$), 3 ($n = 9$) or 4 ($n = 6$) of menstruation.

**PARTICIPANTS/MATERIALS, SETTING, METHODS:** RNA was extracted from endometrial biopsies and analysed by genome wide expression Illumina Sentrix Human HT12 arrays. Data were analysed using 'Remove Unwanted Variation-inverse (RUV-inv)'. Ingenuity pathway analysis (IPA) and the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 were used to identify canonical pathways, upstream regulators and functional gene clusters enriched between Days 2, 3 and 4 of menstruation. Selected individual genes were validated by quantitative PCR.

**MAIN RESULTS AND THE ROLE OF CHANCE:** Overall, 1753 genes were differentially expressed in one or more comparisons. Significant canonical pathways, gene clusters and upstream regulators enriched during menstrual bleeding included those associated with immune cell trafficking, inflammation, cell cycle regulation, extracellular remodelling and the complement and coagulation cascade. We provide the first evidence for a role for glutathione-mediated detoxification (glutathione-S-transferase mu 1 and 2; GSTM1 and GSTM2) during menstruation. The largest number of differentially expressed genes was between Days 2 and 4 of menstruation ($n = 1176$). We identified several genes not previously associated with menstruation including lipopolysaccharide binding protein, serpin peptidase inhibitor, clade B (ovalbumin), member 3 (SERPINB3) and -4 (SERPINB4), interleukin-17C (IL17C), V-set domain containing T-cell activation inhibitor 1 (VTCN1), proliferating cell nuclear antigen factor (KIAA0101/PAF), trefoil factor 3 (TFF3), laminin alpha 2 (LAMA2) and serine peptidase inhibitor, Kazal type 1 (SPINK1). Genes related to inflammatory processes were up-regulated on Day 2 (early-menstruation), and those associated with endometrial repair and regeneration were up-regulated on Day 4 (late-menstruation).

**LIMITATIONS, REASONS FOR CAUTION:** Participants presented with a variety of endometrial pathologies related to bleeding status and other menstrual characteristics. These variations may also have influenced the menstrual process.

†The authors consider that the first two authors should be regarded as joint first authors.

**WIDER IMPLICATIONS OF THE FINDINGS:** The temporal molecular profile of menstruation presented in this study identifies a number of genes not previously associated with the menstrual process. Our findings provide valuable insight into the menstrual process and may present novel targets for therapeutic intervention in cases of endometrial dysfunction.

**LARGE SCALE DATA:** All microarray data have been deposited in the public data repository Gene Expression Omnibus (GSE86003).

**STUDY FUNDING AND COMPETING INTEREST(S):** Funding for this work was provided by a National Health and Medical Research Council of Australia (NHMRC) Project Grant APP1008553 to M.H., P.R. and J.G. M.H. is supported by an NHMRC Practitioner Fellowship. P.P. is supported by a NHMRC Early Career Fellowship. The authors have no conflict of interest to declare.

**Key words:** endometrium / menstruation / gene array / RUV-inv / inflammation / repair

# Introduction

Menstruation is induced by progesterone withdrawal at the end of the menstrual cycle and involves endometrial tissue breakdown, regeneration and repair. It is a highly regulated process comprising a series of interdependent molecular and cellular events of an inflammatory nature (Evans and Salamonsen, 2012). In countries with ready access to contraception, women may experience over 400 episodes of menstruation during their reproductive life. In contrast, in areas where women are less able to regulate their own fertility, most women will be amenorrheic for a large proportion of their lives due to later puberty, multiple pregnancies and ensuing periods of lactational amenorrhoea (Jabbour *et al.*, 2006).

A clearer understanding of the regulation of normal endometrial function, including normal menstruation, is important to better understand the mechanisms responsible for endometrial dysfunction including abnormal uterine bleeding, dysmenorrhoea as well as early pregnancy loss and infertility. Several studies have investigated the molecular mechanisms of normal menstruation, particularly the initiation of menstruation. These studies have used progesterone antagonists and/or physiological progesterone withdrawal *in vivo* (Catalano *et al.*, 2007) and *in vitro* (Critchley *et al.*, 2003; Evans and Salamonsen, 2014) and in mouse models of decidualization and menstruation (Cheng *et al.*, 2007; Wang *et al.*, 2013; Xu *et al.*, 2013). Molecular pathways identified include those associated with NF-kappa(k) B, prostaglandins (PGs), chemokines, Wnt signalling, matrix metalloproteases (MMPs, Critchley *et al.*, 2003; Catalano *et al.*, 2007; Cheng *et al.*, 2007; Wang *et al.*, 2013; Xu *et al.*, 2013; Evans and Salamonsen, 2014) and haemostasis (the balance between coagulation and fibrinolysis) (Livingstone and Fraser, 2002). This knowledge has been exploited clinically. For example, PG synthesis inhibitors (mefenamic acid; Cameron *et al.* 1990) and anti-fibrinolytic therapy with tranexamic acid (Gleeson *et al.* 1993) have shown efficacy in the treatment of heavy menstrual bleeding (HMB). However, the molecular pathways underlying such pathologies are still to be fully elucidated and new therapeutic targets are necessary.

There is limited information on the molecular mechanisms at play during the course of menstruation due to the difficulty of collecting timed endometrial samples during menstrual bleeding. Gaide Chevronnay *et al.* (2009) compared global gene expression profiles in glands and stroma microdissected from menstrual endometrium. However, this study only included three samples of menstrual endometrium. In this study, we collected 36 carefully characterized endometrial samples during menstruation. We used gene array analysis to investigate temporal changes in gene expression in human endometrium from Days 2, 3 and 4 of menstruation (where Day 1 is the first day of bleeding). We hypothesized that the changing molecular profile of the endometrium during menstruation would correlate with the histological profile of menstruation previously described (Garry *et al.*, 2009); Day 2 endometrium would be predominantly associated with genes related to breakdown, Day 4 endometrium, predominantly with genes related to repair and Day 3 endometrium with genes common to both. By investigating the changing molecular profile of the endometrium over Days 2, 3 and 4 of menstruation, we aimed to identify genes and pathways that would provide new insights into the menstrual process.

The temporal molecular profile of menstruation identified in this study supports the overall view that on Day 2 of menstruation, processes related to inflammation are up-regulated, whereas on Day 4 of menstruation, endometrial repair and regeneration are actively occurring.

# Methods

## Patient recruitment

Premenopausal women were recruited from outpatient clinics of The Royal Women's Hospital, a public tertiary women's hospital, and from local advertising for a study investigating endometrial gene expression in women with/without self-reported HMB (Girling *et al.*, 2016). Participant details are summarized in Table I.

Retrospective menstrual histories (frequency, duration, regularity and volume; Munro *et al.*, 2012) were collected and all subjects completed a 3-month prospective menstrual diary. For this study, subjects were included irrespective of comorbidities (fibroids, adenomyosis and/or ovarian cysts; Table I), bleeding status (the presence/absence of HMB) and menstrual characteristics (frequency, duration, regularity and volume). Participants were excluded if they had known endocrine or haematological abnormalities that may cause HMB or were using sex steroids or any medication likely to reduce menstrual blood loss or interfere with vascular stability or coagulation.

Endometrial biopsies were obtained using a pipelle suction curette (Pipelle, Cornier, France) on Days 2, 3 and 4 of menstruation (where Day 1 is the first day of menstrual bleeding). Bleeding but not spotting days, as denoted in the menstrual diary, was used to determine day of cycle (Table I). Endometrial biopsies were stored in RNAlater (Ambion, Life Technologies, Carlsbad, CA, USA) overnight before being frozen and stored at −80°C.

A total of 36 samples were collected and arrayed. Samples were excluded from analysis if the day of menstruation could not be ascertained from the menstrual diary. Of the 24 samples remaining ($n = 9$, Day 2; $n = 9$, Day 3; $n = 6$, Day 4), 22 were from women with regular menstrual

**Table I Summary of patient characteristics used in array analysis and PCR.**

| Group | n | Median age [range] | Bleeding type | Comorbidities |
|---|---|---|---|---|
| Day 2 | 9 | 38 [30–44] | n = 4 HMB | n = 1 adenomyosis |
| | | | | n = 1 ovarian cyst |
| Extras for PCR | 1 | 26 | n = 1 HMB | |
| Day 3 | 9 | 40 [23–45] | n = 6 HMB | n = 2 fibroids |
| | | | | n = 1 polycystic ovary |
| Extras for PCR | 2 | 29,37 | n = 1 HMB | |
| Day 4 | 6 | 41 [36–45] | n = 1 bleeding type unknown, n = 4 HMB | n = 2 fibroid, n = 1 polyp |
| Extras for PCR | 7 | 38 [24–42] | n = 4 HMB | n = 1 adenomyosis |
| | | | | n = 1 fibroid |

HMB, heavy menstrual bleeding (self-reported).

cycles (FIGO definition, cycle to cycle variation of between 2 and 20 days (Munro *et al.*, 2012; Table I), whereas 2 samples were from women with irregular menstrual cycles.

A separate cohort for validation could not be obtained due to logistical difficulties in obtaining samples during menstrual bleeding. An additional 10 samples were included for PCR validation. These included one Day 2, two Day 3 and seven Day 4 samples (Table I).

## Ethical approval

Ethical approval was obtained from The Royal Women's Hospital Human Research Ethics Committee (Project 11/13) and informed consent was obtained from all subjects.

## Gene array analysis

Total RNA was extracted using Trizol (Invitrogen, Life Technologies, Carlsbad, CA, USA) and the Ambion PureLink mini kit (Ambion, Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions. RNA was quantified using the Nanodrop ND1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and stored at -80°C.

Three micrograms of RNA were further analysed for quality and hybridized to Illumina Human HT12 v4 arrays at the Australian Genome Research Facility (AGRF, Victoria, Australia). Data were then analysed using the 'Remove Unwanted Variation-inverse (RUV-inv)' method (Gagnon-Bartsch and Speed, 2012; Gagnon-Bartsch *et al.*, 2013; Girling *et al.*, 2016). The re-annotation functions of the Illumina Human v4.db Bioconductor R package (Barbosa-Morais *et al.*, 2010) were used to remap the probes according to the current knowledge of the human genome (hg19). Genes were defined as differentially expressed if the Benjamini–Hochberg corrected *P*-value was <0.05 and the absolute value of $\log_{[base2]}$ of fold change (logFC) was >1 (fold change >2).

## Ingenuity pathway analysis

Gene lists derived from arrays were analysed using Qiagen's Ingenuity Pathway Analysis (IPA®, Qiagen Redwood City, www.qiagen.com/ingenuity). Canonical pathways were defined as significant if the Benjamini–Hochberg corrected *P*-value from right-tailed Fisher exact tests was <0.01. IPA Upstream Regulator Analysis identified potential upstream transcriptional regulators. Overlap *P*-values ($P < 0.05$ was considered significant) represented the significance of the overlap between genes in our gene lists and those genes previously identified as regulated by a particular factor.

## Gene cluster analysis

Gene lists were also examined using the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resources v6.7 (http://david.abcc.ncifcrf.gov/home.jsp) (Huang da *et al.*, 2009). As DAVID analysis does not differentiate between up- vs down-regulated genes, these gene lists were submitted separately. We used the functional annotation cluster (FAC) tool, which combines functionally similar annotations derived from various databases into a single cluster. We selected annotations related to Gene Ontology Biological Process Terms, to provide insight into the biological processes associated with our gene lists. For each cluster, DAVID provides an Enrichment Score (geometric mean expressed as $-(\log_{[base10]})$ of the mean of the *P*-values from each individual annotations within a cluster) and individual *P*-values or EASE scores calculated using a modified Fisher exact test. An EASE score/*P*-value (with Benjamini–Hochberg correction) of 0.01 was considered significant; this equated to an enrichment score of 2. Classification stringency was set at medium.

## Quantitative PCR

cDNA was generated from 1 ug of RNA using the GoScript Reverse Transcription System (Promega, Alexandria, NSW, Australia) according to manufacturer's instructions. An RNA spike was also added to the reverse transcription mix (Fu *et al.*, 2008; Craythorn *et al.*, 2009; Winnall *et al.*, 2009) to control for variations in cDNA transcription efficiency.

Real-time PCR was performed using commercially validated TaqMan® Gene Expression Assays (Applied Biosystems, Life Technologies, Carlsbad, CA, USA, Supplementary Table I) and the 7500 ABI (Applied Biosystems) detection system. Samples were run in duplicate along with appropriate controls (i.e. no template, no RT). Cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min.

Data were analysed using the comparative Ct method ($2^{-\Delta\Delta CT}$). Several housekeeping genes were examined and compared during the menstrual period as described in Girling *et al.* (2016). Data were normalized to the housekeeping gene RPL13A, which exhibited the lowest standard deviation during the menstrual period, and represented as median fold change relative to the Day 2 group. Statistical analysis was performed using a Kruskal–Wallis test followed by a Dunn's *post hoc* test. $P < 0.05$ was

considered significant. Statistical analyses were conducted using SPSS for Windows v. 21.0 (2012, Armonk, NY, IBM Corp.)

# Results

## Differential gene expression

Overall, 1753 genes were differentially expressed in one or more comparisons ($P < 0.05$, $\log_{[base2]}FC > 1$) (Figure 1). The top 20 most significantly up- or down-regulated genes for each comparison are provided in Table II. The largest number of differentially expressed genes (1176) was observed between Days 2 and 4. Of these, 738 genes were up-regulated, whereas 438 genes were down-regulated in Day 2 compared to Day 4. A number of known genes in the context of menstruation including fibrinogen A (FGA), fibrinogen B (FGB), complement component 4 binding protein, alpha (C4BPA), nitric oxide synthase 2 (NOS2), chemokine CXC ligand 6 or granulocyte chemotactic protein-2 (CXCL6), colony stimulating factor 3 (CSF3) and solute carrier family 18 (vesicular monoamine transporter), member 2 (SLC18A2) were identified. Genes not previously associated in the context of menstruation were also identified (Table III). These included lipopolysaccharide binding protein (LBP) and serpin peptidase inhibitor, clade B (ovalbumin), member 3 (SERPINB3) and -4 (SERPINB4), which were among the top up-regulated genes on Day 2 of menstruation. Vav guanine nucleotide exchange factor 3 (VAV3), major histocompatibility complex, class II, DR beta 1 (HLA-DRB1) and olfactomedin 4 (OLFM4) were among the top up-regulated genes on Day 3 of menstruation. Whereas glutathione-S-transferase mu 1 and 2 (GSTM1 and GSTM2), V-set domain containing T-cell activation inhibitor 1 (VTCN1), proliferating cell nuclear antigen factor (KIAA0101/PAF), trefoil factor 3 (TFF3) and laminin alpha 2 (LAMA2) were among the top up-regulated genes on Day 4 of menstruation (Table III).

There were 738 genes differentially expressed on Day 2 vs Day 3 (380 up-regulated, 358 down-regulated) and 559 genes on Day 3 vs Day 4 (210 up-regulated, 349 down-regulated). There were 357 (309 + 48) genes with significantly different expression on Day 2 vs Days 3 and 4, 111 (63 + 48) genes on Day 3 vs Days 2 and 4 and 300 (252 + 48) genes on Day 4 vs Days 2 and 3. Forty-eight genes were significantly different in all three comparisons.

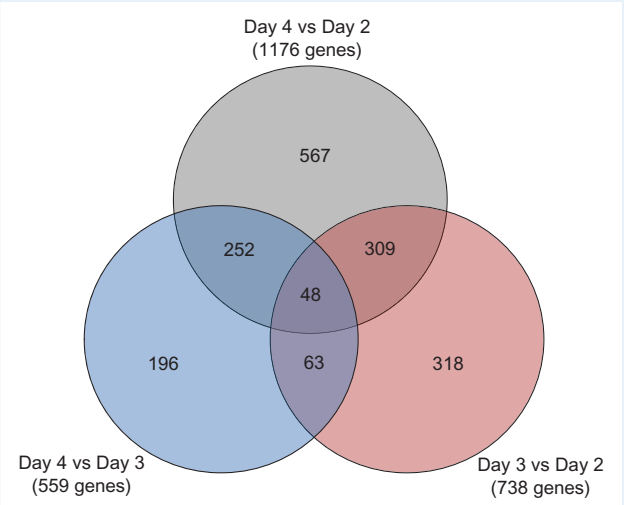


**Figure 1** Venn diagram illustrating the distribution of differentially expressed genes in menstrual-phase endometrial biopsies from women from Day 2 ($n = 9$), Day 3 ($n = 9$) and Day 4 ($n = 6$) of menstruation.

## Ingenuity pathway analysis

### *Day 2 vs Day 3*

Significant canonical pathways identified using IPA related to the immune and inflammatory response (retinoic and liver X receptor activation), immune cell trafficking (granulocyte and agranulocyte adhesion and diapedesis), extracellular remodelling (inhibition of MMPs) and the coagulation cascade (extrinsic prothrombin activation pathway) (Table IV, Supplementary Table II). The top five upstream regulators (Table V, Supplementary Table III) included cytokines (interleukin-1 beta; IL1B, IL6) and other regulators (v-rel avian reticuloendotheliosis viral oncogene homologue A; RELA, protein kinase D1; PRKD1) also associated with inflammation.

### *Day 2 vs Day 4*

IPA analysis identified pathways related to the immune and inflammatory response (pathogenesis of multiple sclerosis), immune cell trafficking (granulocyte and agranulocyte adhesion and diapedesis), the coagulation cascade and glutathione-mediated detoxification (Table IV, Supplementary Table II).

The top five upstream regulators (Table V, Supplementary Table III) included those associated with inflammation (interleukin-1 beta; IL1B) and growth and cell survival (erb-b2 receptor tyrosine kinase 2; ERBB2, RAB, member RAS oncogene family-like 6; RABL6, transforming growth factor beta; TGFB1).

### *Day 3 vs Day 4*

Significant canonical pathways identified using IPA included glutathione-mediated detoxification, the complement and coagulation cascade and immune cell trafficking (granulocyte adhesion and diapedesis) (Table IV, Supplementary Table II). The top five upstream regulators (Table V, Supplementary Table III) included those associated with inflammation (oncostatin M receptor; OSMR, triggering receptor expressed on myeloid cells 1; TREM1 and ILB).

## Functional cluster analysis

Of the genes up-regulated on Day 2 of menstruation, we identified four significant clusters, using DAVID analysis (Table VI, Supplementary Table IVA). These included genes related to the positive regulation of cell migration, immune and inflammatory response and the coagulation cascade. These clusters were specifically associated with genes up-regulated on Day 2 compared to Day 4.

Of the genes up-regulated on Day 3 of menstruation, we identified two significant clusters associated with the cell cycle and chemotaxis. These clusters were specifically associated with genes up-regulated on Day 3 compared to Day 2. We also identified one significant cluster related to the negative regulation of cell growth associated with genes up-regulated on Day 3 compared to Day 4 (Table VI, Supplementary Table IVB).

Of the genes up-regulated on Day 4 of menstruation, we identified six significant clusters (Table VI, Supplementary Table IVC). These

**Table II Top 20 genes with the highest fold change (up- and down-regulated) in comparisons between Days 2, 3 and 4 of menstruation.**

| Gene symbol | Gene name | Fold change | Adj. *P*-value |
|---|---|---|---|
| Up-regulated on Day 2 vs Days 3 and 4 | | | |
| Day 2 vs Day 3 | | | |
| GSTT1 | Glutathione-S-transferase theta 1 | 20.3 | 4.00E-08 |
| XYLT1 | Xylosyltransferase I | 11.2 | 6.10E-08 |
| IL17C | Interleukin-17 C | 5.5 | 3.90E-09 |
| SCGB2A2 | Secretoglobin, family 2A, member 2 | 4.6 | 3.30E-06 |
| LBP | Lipopolysaccharide binding protein | 4.3 | 1.50E-06 |
| CYP2C9 | Cytochrome P450 family 2 subfamily C member 9 | 4.2 | 2.30E-08 |
| SERPINB4 | Serpin peptidase inhibitor, clade B (ovalbumin), member 4 | 4.1 | 6.30E-10 |
| C4BPA | Complement component 4 binding protein, alpha | 4.0 | 1.10E-08 |
| C2CD2 | C2 calcium-dependent domain containing 2 | 3.8 | 8.10E-08 |
| CSF3 | Colony stimulating factor 3 | 3.7 | 4.40E-08 |
| AGTPBP1 | ATP/GTP binding protein 1 | 3.6 | 3.90E-08 |
| TMEM233 | Transmembrane protein 233 | 3.6 | 7.30E-07 |
| SLC18A2 | Solute carrier family 18 (vesicular monoamine transporter), member 2 | 3.6 | 7.80E-07 |
| DEFB4A | Defensin beta 4A | 3.5 | 4.50E-07 |
| FOLR3 | Folate receptor 3 | 3.5 | 3.90E-05 |
| VCAM1 | Vascular cell adhesion molecule 1 | 3.5 | 1.10E-08 |
| MROH5 (FLJ43860) | Maestro heat-like repeat family member 5 | 3.4 | 1.60E-08 |
| FGG | Fibrinogen G | 3.4 | 1.20E-07 |
| LIMS3 | LIM and senescent cell antigen-like domains 3 | 3.3 | 1.40E-07 |
| TCN1 | Transcobalamin 1 | 3.3 | 1.20E-07 |
| Day 2 vs Day 4 | | | |
| FGA | Fibrinogen A | 27.1 | 2.60E-14 |
| FGB | Fibrinogen B | 19.9 | 1.50E-10 |
| LBP | Lipopolysaccharide binding protein | 11.4 | 5.10E-09 |
| SERPINB4 | Serpin peptidase inhibitor, clade B (ovalbumin), member 4 | 10.2 | 9.90E-13 |
| PCSK1 | Proprotein convertase subtilisin/kexin type 1 | 8.3 | 6.50E-12 |
| TCN1 | Transcobalamin 1 | 7.9 | 1.40E-10 |
| PRKAR1A | Protein kinase, cAMP-dependent, regulatory subunit type I alpha | 7.6 | 2.50E-07 |
| HSD17B2 | Hydroxysteroid (17-beta) dehydrogenase 2 | 6.9 | 1.70E-10 |
| IL17C | Interleukin-17C | 5.5 | 8.60E-09 |
| SLC18A2 | Solute carrier family 18 (vesicular monoamine transporter), member 2 | 5.5 | 5.40E-08 |
| C4BPA | Complement component 4 binding protein, alpha | 5.5 | 1.00E-09 |
| CALB2 | Calbindin 2 | 5.1 | 9.10E-08 |
| EN2 | Engrailed homeobox 2 | 5.0 | 4.40E-10 |
| SERPINB3 | Serpin peptidase inhibitor, clade B (ovalbumin), member 3 | 4.7 | 3.50E-09 |
| NOS2 | Nitric oxide synthase 2 | 4.5 | 5.40E-08 |
| GPX2 | Glutathione peroxidase 2 | 4.3 | 6.70E-07 |
| CXCL6 | Chemokine (C-X-C motif) ligand 6 | 4.3 | 8.90E-08 |
| PIP | Prolactin-induced protein | 4.2 | 7.70E-08 |
| SPINK1 | Serine peptidase inhibitor, Kazal type 1 | 4.1 | 5.70E-05 |
| CSF3 | Colony stimulating factor 3 | 4.0 | 2.80E-08 |
| Up-regulated on Day 3 vs Days 2 and 4 | | | |
| Day 3 vs Day 2 | | | |
| VAV3 | Vav guanine nucleotide exchange factor 3 | 12.0 | 5.90E-06 |

*Continued*

**Table II** *Continued*

| Gene symbol | Gene name | Fold change | Adj. *P*-value |
|---|---|---|---|
| ODF2L | Outer dense fibre of sperm tails 2-like | 9.0 | 4.40E-10 |
| HLA-DRB1 | Major histocompatibility complex, class II, DR beta 1 | 8.0 | 6.00E-04 |
| SNHG8 | Small nucleolar RNA host gene 8 | 7.0 | 1.90E-08 |
| OLFM4 | Olfactomedin 4 | 5.3 | 3.80E-06 |
| HLA-DRB6 | Major histocompatibility complex, class II, DR beta 6 (pseudogene) | 5.1 | 6.90E-04 |
| RSAD2 | Radical S-adenosyl methionine domain containing 2 | 5.0 | 1.90E-07 |
| ZBED2 | Zinc finger, BED-type containing 2 | 5.0 | 2.20E-09 |
| BBPIFB1 (C20orf114) | BPI fold containing family B member 1 | 4.9 | 2.10E-03 |
| HLA-DRB1 | Major histocompatibility complex, class II, DR beta 1 | 4.7 | 8.30E-03 |
| TFF3 | Trefoil factor 3 | 4.6 | 8.80E-04 |
| GBP5 | Guanylate binding protein 5 | 4.4 | 1.30E-07 |
| NPR3 | Natriuretic peptide receptor 3 | 4.0 | 4.60E-08 |
| NMU | Neuromedin U | 4.0 | 1.30E-09 |
| TAP2 | Transporter 2, ATP-binding cassette, subfamily B (MDR/TAP) | 3.9 | 5.10E-09 |
| APOBEC3A | Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3A | 3.8 | 4.30E-08 |
| CLECL1 | C-type lectin like 1 | 3.7 | 2.90E-08 |
| MAP2 | Microtubule associated protein-2 | 3.7 | 2.30E-09 |
| LOC100132288 | | 3.6 | 5.00E-09 |
| GSTM1 | Glutathione-S-transferase mu 1 | 3.6 | 2.50E-03 |
| Day 3 vs Day 4 | | | |
| FGB | Fibrinogen B | 15.7 | 1.80E-10 |
| FGA | Fibrinogen A | 8.8 | 4.90E-12 |
| HLA-DRB1 | Major histocompatibility complex, class II, DR beta 1 | 7.7 | 6.70E-04 |
| VAV3 | Vav guanine nucleotide exchange factor 3 | 6.0 | 2.00E-04 |
| PRKAR1A | Protein kinase, cAMP-dependent, regulatory subunit type I alpha | 5.1 | 2.90E-03 |
| CALB2 | Calbindin 2 | 5.1 | 2.00E-06 |
| HIST1H4E | Histone cluster 1, H4e | 5.0 | 3.70E-08 |
| HLA-A | Major histocompatibility complex, class I, A | 4.6 | 3.40E-09 |
| SLN | Sarcolipin | 4.3 | 3.40E-03 |
| RSAD2 | Radical S-adenosyl methionine domain containing 2 | 4.2 | 5.60E-09 |
| OLFM4 | Olfactomedin 4 | 4.2 | 6.70E-07 |
| FOXD1 | Forkhead box D1 | 4.0 | 2.40E-05 |
| PCDHB2 | Protocadherin beta 2 | 3.8 | 8.90E-08 |
| PCSK1 | Proprotein convertase subtilisin/kexin type 1 | 3.8 | 2.30E-06 |
| HIST1H2BG | Histone cluster 1, H2b | 3.7 | 1.00E-08 |
| SERPINB3 | Serpin peptidase inhibitor, clade B (ovalbumin), member 3 | 3.7 | 1.40E-09 |
| IL1RL1 | Interleukin-1 receptor-like 1 | 3.6 | 1.10E-08 |
| IGFBP1 | Insulin like growth factor binding protein 1 | 3.4 | 3.20E-05 |
| AI458759 | | 3.4 | 1.80E-05 |
| SLC16A6 | Solute carrier family 16, member 6 | 3.3 | 6.00E-07 |
| Up-regulated on Day 4 vs Days 2 and 3 | | | |
| Day 4 vs Day 2 | | | |
| GSTM1 | Glutathione-S-transferase mu 1 | 12.1 | 9.60E-08 |
| ODF2L | Outer dense fibre of sperm tails 2-like | 10.3 | 3.30E-10 |
| GRAMD2 | GRAM domain containing 2 | 7.7 | 1.10E-08 |
| CFAP77 | Cilia and flagella associated protein 77 | 7.0 | 9.00E-10 |
| TUBA4B | Tubulin alpha 4b | 6.6 | 1.20E-08 |

*Continued*

**Table II** *Continued*

| Gene symbol | Gene name | Fold change | Adj. *P*-value |
|---|---|---|---|
| UBE2C | Ubiquitin conjugating enzyme E2C | 6.5 | 3.40E-09 |
| CR591103 | | 6.4 | 5.10E-08 |
| VTCN1 | V-set domain containing T-cell activation inhibitor 1 | 5.9 | 4.90E-09 |
| KIAA0101 (PAF) | Proliferating cell nuclear antigen-associated factor | 5.9 | 4.00E-12 |
| CFAP43 (WDR96) | Cilia and flagella associated protein 43 | 5.7 | 3.50E-08 |
| LAMA2 | Laminin subunit alpha 2 | 5.6 | 1.00E-09 |
| GSTM2 | Glutathione-S-transferase mu 2 | 5.5 | 1.30E-08 |
| ARMC3 | Armadillo repeat containing 3 | 5.4 | 2.10E-07 |
| ADGRG7 (GPR128) | Adhesion G Protein-Coupled Receptor G7 | 5.3 | 2.00E-04 |
| ATP1A2 | ATPase, Na+/K+ transporting, alpha 2 polypeptide | 5.2 | 2.20E-07 |
| CAPSL | Calcyphosine-like | 5.2 | 2.50E-08 |
| CDHR4 | Cadherin-related family member 4 | 5.1 | 9.70E-08 |
| MORN5 | MORN repeat containing 5 | 5.1 | 7.60E-09 |
| TFF3 | Trefoil factor 3 | 5.1 | 7.70E-04 |
| ALDH1L1 | Aldehyde dehydrogenase 1 family member L1 | 5.1 | 9.10E-09 |
| Day 4 vs Day 3 | | | |
| SCGB1D2 | Secretoglobin, family 1D, member 2 | 6.0 | 6.20E-08 |
| NAPSB | Napsin B aspartic peptidase, pseudogene | 5.5 | 1.20E-08 |
| GSTT1 | Glutathione-S-transferase theta 1 | 5.4 | 3.80E-05 |
| TUBA4B | Tubulin alpha 4b | 4.9 | 5.50E-08 |
| C1orf194 | Chromosome 1 open reading frame 194 | 4.8 | 8.10E-08 |
| GSTM1 | Glutathione-S-transferase mu 1 | 4.7 | 1.60E-05 |
| CHGA | Chromogranin A | 4.6 | 1.90E-05 |
| GPR84 | G protein-coupled receptor 84 | 4.5 | 9.50E-07 |
| PAGE4 | PAGE family member 4 | 4.4 | 9.70E-08 |
| LIMS3 | LIM and senescent cell antigen-like domains 3 | 4.4 | 4.90E-09 |
| HBD | Haemoglobin subunit delta | 4.3 | 3.50E-05 |
| CNTN1 | Contactin 1 | 4.2 | 1.50E-09 |
| HBM | Haemoglobin subunit mu | 4.2 | 1.80E-05 |
| DPY19L2 | dpy-19-like 2 (*Caenorhabditis elegans*) | 4.0 | 8.70E-09 |
| ATP1A2 | ATPase, Na+/K+ transporting, alpha 2 polypeptide | 4.0 | 1.00E-06 |
| FAM118A | Family with sequence similarity 118 member A | 4.0 | 1.00E-07 |
| LINC00969 (AK128346) | Long intergenic non-protein coding RNA 969 | 4.0 | 5.30E-09 |
| MSMB | Microseminoprotein, beta | 3.8 | 3.60E-05 |
| KIR2DS5 | Killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, 5 | 3.8 | 3.70E-06 |
| HBG2 | Haemoglobin subunit gamma 2 | 3.8 | 5.50E-04 |

included multiple clusters associated with cell division, mitosis and cell migration. These clusters were specifically associated with genes up-regulated on Day 4 compared to Day 2. We also identified two significant clusters related to chemotaxis and complement activation associated with genes up-regulated on Day 4 compared to Day 3 (Table VI, Supplementary Table IVC).

## Quantitative PCR

To validate the microarray data, the expression of eight candidate genes was examined by qPCR (Figure 2). These included genes that were both up- and down-regulated. These genes were in agreement with the microarray data direction of change. Of these, six were significantly altered (Figure 2). These included FGA ($P = 0.008$), FGB ($P = 0.04$), C4BPA ($P = 0.04$) and LBP ($P = 0.0001$), which were significantly up-regulated on Day 2 vs Day 4. Whereas VTCN1 ($P = 0.004$) and GSTM2 ($P = 0.036$) were significantly up-regulated on Day 4 vs Day 2.

SERPINB4 expression was higher on Day 2 vs Day 4, whereas TFF3 expression was higher on Day 4 vs Day 2. However, these differences were not statistically significant. The expression of GSTM1 was variable, being detectable in only 2/9 Day 2 samples, 7/11 Day 3 samples and 6/13 Day 4 samples (data not shown).

**Table III Genes with known and novel biological roles during menstruation.**

| Factor of interest | Biological role | Biological process associated with menstruation | References |
|---|---|---|---|
| *Genes up-regulated on Day 2* | | | |
| FGA | Coagulation | Regeneration and repair | Davies and Kadir (2012) |
| FGB | Coagulation | Regeneration and repair | Davies and Kadir (2012) |
| C4BPA | Complement system | Tissue breakdown | Blom *et al.* (2004) |
| NOS2 | Vasodilation and tissue breakdown via nitrous oxide production | Tissue breakdown | Tschugguel *et al.* (1999), Chwalisz and Garfield (2000) |
| CXCL6 (GCP-2) | Neutrophil recruitment | Inflammation and tissue breakdown | Wuyts *et al.* (1997) |
| CSF3 | Neutrophil recruitment | Inflammation and tissue breakdown | Chiang *et al.* (2013) |
| SLC18A2 | Vasodilation via the uptake and release of monoamines | Tissue breakdown and bleeding | Bottalico *et al.* (2003) |
| LBP* | Activates NFKB signalling | Inflammation and tissue breakdown | Fitzgerald *et al.* (2003) |
| SERPINB3/4* | Activates NFKB signalling | Inflammation and tissue breakdown | Catanzaro *et al.* (2014) |
| IL17C* | Pro-inflammatory | Inflammation and tissue breakdown | Johnston *et al.* (2013) |
| XYLT1* | Enzyme involved in glycosaminoglycan synthesis during cartilage repair | Regeneration and repair | Venkatesan *et al.* (2009) |
| *Genes up-regulated on Day 3* | | | |
| VAV3* | Wound healing | Regeneration and repair | Sindrilaru *et al.* (2009) |
| OLFM4* | Cell cycle control, apoptosis, cell adhesion, cell migration | Regeneration and repair | Zhang *et al.* (2004) |
| HLA-DRB1* | Wound healing | Regeneration and repair | Brown and Bayat (2009) |
| *Genes up-regulated on Day 4* | | | |
| GSTM1* | ROS deactivation and cell proliferation/ apoptosis | Regeneration and repair | Kalinina *et al.* (2014) |
| GSTM2* | ROS deactivation and cell proliferation/ apoptosis | Regeneration and repair | Kalinina *et al.* (2014) |
| VTCN1* | Regulation of the immune response, cell proliferation and migration | Regeneration and repair | Prasad *et al.* (2003), Choi *et al.* (2003), Gao *et al.* (2015) |
| KIAA0101 (PAF)* | DNA synthesis, cell cycle progression | Regeneration and repair | Yu *et al.* (2001), Cheng *et al.* (2013), Zhu *et al.* (2013), Fan *et al.* (2015) |
| TFF3* | Mucosal repair, cell migration, inhibition of proliferation and apoptosis | Regeneration and repair | Thim and May (2005), Hoffman (2005), Taupin and Podolsky (2003) |
| LAMA2* | Muscle regeneration and repair | Regeneration and repair | Kuang *et al.* (1999) |
| SPINK1* | Mucousal integrity and repair | Regeneration and repair | Konturek *et al.* (1998), Marchbank *et al.* (1998) |

*Genes with novel potential roles during menstruation.

# Discussion

In this study, we provide the first comparative global gene expression profile data of human endometrium on Days 2, 3 and 4 of menstruation. For the first time, we have demonstrated in detail the temporal, molecular response of the endometrium during menstrual bleeding *in vivo*. The largest number of differentially expressed genes ($n = 1176$) was between Days 2 and 4 of menstruation, demonstrating distinct molecular profiles on these days. We found changes in the expression of several genes not previously associated with menstruation. Other gene clusters and pathways identified were consistent with known mechanisms of menstruation including inflammation, immune cell trafficking, extracellular remodelling, cell cycle regulation and coagulation.

From the molecular profile of menstruation delineated in this study, our findings suggest that on Day 2 of menstruation (early-menstruation), processes associated with inflammation are prominent and by Day 4 of menstruation (late-menstruation) inflammation is reduced and endometrial repair processes take precedence.

Pathways and upstream regulators related to the immune and inflammatory response including immune cell trafficking were enriched in all day-by-day comparisons. This is consistent with the inflammatory nature of menstruation (Evans and Salamonsen, 2012; Maybin and Critchley, 2015). Progesterone withdrawal leads to an inflammatory cascade of events characterized by the onset of oxidative stress, NFkB activation, PG synthesis, leucocyte recruitment and the activation of MMPs (Maybin and Critchley, 2015).

**Table IV Significant canonical pathways identified using IPA.**

| Ingenuity canonical pathways | −log(*P*-value)* | Ratio** |
|---|---|---|
| Day 2 vs Days 3 | | |
| Granulocyte adhesion and diapedesis | 4.03 | 0.10 |
| Agranulocyte adhesion and diapedesis | 3.23 | 0.09 |
| Inhibition of MMPs | 2.89 | 0.16 |
| Extrinsic prothrombin activation pathway | 2.84 | 0.25 |
| LXR/RXR activation | 2.72 | 0.09 |
| Day 2 vs Day 4 | | |
| Pathogenesis of multiple sclerosis | 5.79 | 0.67 |
| Granulocyte adhesion and diapedesis | 4.19 | 0.13 |
| Coagulation system | 4.18 | 0.26 |
| Glutathione-mediated detoxification | 3.74 | 0.29 |
| Agranulocyte adhesion and diapedesis | 3.39 | 0.12 |
| Day 3 vs Day 4 | | |
| Glutathione-mediated detoxification | 5.83 | 0.29 |
| Coagulation system | 3.66 | 0.17 |
| Complement system | 3.59 | 0.17 |
| Granulocyte adhesion and diapedesis | 2.99 | 0.07 |
| PXR/RXR activation | 2.99 | 0.11 |

*−log(*P*-value) after right-tailed Fisher exact tests and Benjamini–Hochberg multiple correction. A *P*-value of 0.01 equates to a −log(*P*-value) of 2. **Ratio of genes within our gene list to the total number of genes in the canonical pathway.

**Table V Top five significant upstream regulators identified using IPA.**

| Gene symbol | Gene name | *P*-value* |
|---|---|---|
| Day 2 vs Day 3 | | |
| IL1B | Interleukin-1 beta | 1.50E-06 |
| RELA | v-rel avian reticuloendotheliosis viral oncogene homologue A | 2.80E-05 |
| E2F5 | E2F transcription factor 5, p130-binding | 1.10E-04 |
| PRKD1 | Protein kinase D1 | 1.20E-04 |
| IL6 | Interleukin-6 | 1.40E-04 |
| Day 2 vs Day 4 | | |
| ERBB2 | erb-b2 receptor tyrosine kinase 2 | 1.50E-10 |
| TGFB1 | Transforming growth factor beta 1 | 1.60E-10 |
| RABL6 | RAB, member RAS oncogene family-like 6 | 2.20E-09 |
| IL1B | Interleukin-1 beta | 6.10E-08 |
| RNASE1 | Ribonuclease, RNase A family, 1 | 4.20E-07 |
| Day 3 vs Day 4 | | |
| OSMR | Oncostatin M receptor | 5.10E-06 |
| TREM1 | Triggering receptor expressed on myeloid cells 1 | 1.70E-05 |
| IL1B | Interleukin-1 beta | 4.30E-05 |
| PAF1 | PAF1 homologue, Paf1/RNA polymerase II complex component | 1.20E-04 |
| FOXO1 | Forkhead box O1 | 2.70E-04 |

**P*-value of overlap: determines whether there is a significant overlap between genes in our gene lists and those known to be regulated by a particular factor.

The observation that genes associated with the immune and inflammatory response were up-regulated on Day 2 compared to Day 4 suggests that, on Day 2 (early-menstruation), inflammatory processes signal the onset of tissue breakdown. Consistent with this hypothesis, NOS2, which is induced by the inflammatory milieu post-progesterone withdrawal (Tschugguel *et al.*, 1999) and stimulates NO production which plays roles in tissue breakdown and vasodilation (Chwalisz and Garfield, 2000), is up-regulated on Day 2. Furthermore, we saw similar up-regulation in the chemokines CXCL6 and CSF3, which stimulate the migration of neutrophils (Wuyts *et al.*, 1997; Chiang *et al.*, 2013), key mediators of the inflammatory response and the predominant leucocyte population present at the onset of menstruation (Salamonsen and Lathbury, 2000). SLC18A2, the vesicular monoamine transporter, which regulates the uptake and release of monoamines that influence vasodilation during menstruation, is also up-regulated (Bottalico *et al.*, 2003).

We are the first to report that the inflammatory genes LBP, IL17C and SERPINB3/4 are associated with menstruation. These genes were among the most up-regulated on Day 2 vs Day 4 of menstruation. LBP (involved in the acute phase response) and SERPINB3/B4 (a member of the serpin family of endogenous serine/cysteine proteinase inhibitors) have been shown to activate NFkB pathways by augmenting lipopolysaccharide signalling through TLR4- (Fitzgerald *et al.*, 2003) and RAS-mediated signalling, respectively (Catanzaro *et al.*, 2014). Similarly, a pro-inflammatory pathogenic role for IL17C in association with psoriasiform skin inflammation has been reported (Johnston *et al.*, 2013). An excessive or prolonged inflammatory response is postulated to lead to excessive tissue damage and may result in HMB (Maybin and Critchley, 2015). In support of this notion, PG synthesis inhibitors (e.g. mefenamic acid; Cameron *et al.*, 1990) are a popular treatment for HMB. Our findings point to novel genes associated with inflammation during menstruation. Further investigation of these in the endometrium is warranted.

Pathways related to the coagulation and complement cascade featured in all three day-by-day comparisons. FGA and FGB were the top up-regulated genes on Day 2 compared to Days 3 and 4. Fibrinogen biosynthesis is stimulated by acute phase reactants that accompany both acute and chronic inflammatory states (Ross and Beck, 2014). Cessation of menstruation relies on an intact endometrial coagulation system culminating in the formation of fibrin from fibrinogen to achieve haemostasis. Fibrinolysis is key in the regulation of haemostasis (Davies and Kadir, 2012). Disorders that interfere with systemic haemostasis have an impact on menstruation. Von Willebrand disease is the most common of these disorders with a prevalence of 13% in women with HMB (Shankar *et al.*, 2004). There is evidence that an overactive fibrinolytic system interferes with haemostasis and contributes to abnormal uterine bleeding (Gleeson *et al.*, 1993) consistent with the efficacy of tranexamic acid, an anti-fibrinolytic, as a treatment for HMB (Gleeson *et al.*, 1994). The finding that FGA and FGB are

**Table VI Summary of FAC derived from the DAVID Bioinformatics Resources 6.7: for up- and down-regulated genes between Days 2, 3 and 4 of the menstrual cycle (for specific details of each annotation within a cluster, see Supplementary Tables IVA–C).**[a]

| Annotation cluster | GOBPTERM FAT | Enrichment score | No. | % |
|---|---|---|---|---|
| Up-regulated in Day 2 vs Days 3 and 4 | | | | |
| Day 2 vs Day 4 | | | | |
| Cell migration | Regulation of cell migration | 3.57 | 14 | 3.67 |
| | Regulation of locomotion | | | |
| | Regulation of cell motion | | | |
| | Positive regulation of cell migration | | | |
| | Positive regulation of cell motion | | | |
| | Positive regulation of locomotion | | | |
| Immune and inflammatory response | Response to wounding | 2.95 | 47 | 12.30 |
| | Defence response | | | |
| | Immune response | | | |
| | Inflammatory response | | | |
| Regulation of inflammatory response | Regulation of inflammatory response | 2.64 | 11 | 2.89 |
| | Regulation of response to external stimulus | | | |
| | Regulation of acute inflammatory response | | | |
| Coagulation | Coagulation | 2.26 | 11 | 2.89 |
| | Blood coagulation | | | |
| | Wound healing | | | |
| | Haemostasis | | | |
| Up-regulated in Day 3 vs Days 2 and 4 | | | | |
| Day 3 vs Day 2 | | | | |
| Cell cycle | Cell cycle phase | 2.62 | 23 | 6.91 |
| | Cell cycle process | | | |
| | M phase | | | |
| | Cell cycle | | | |
| Chemotaxis | Chemotaxis | 2.29 | 11 | 3.30 |
| | Taxis | | | |
| | Locomotory behaviour | | | |
| Day 3 vs Day 4 | | | | |
| Negative regulation of cell growth | Regulation of cell growth | 2.72 | 11 | 5.85 |
| | Negative regulation of cell growth | | | |
| | Regulation of growth | | | |
| | Negative regulation of cell size | | | |
| Up-regulated in Day 4 vs Days 2 and 3 | | | | |
| Day 4 vs Day 2 | | | | |
| Cell division | Cell cycle phase | 8.71 | 55 | 8.04 |
| | Mitosis | | | |
| | Nuclear division | | | |
| | M phase of mitotic cell cycle | | | |
| | Cell division | | | |
| Cell migration/cell division | Microtubule-based process | 5.32 | 39 | 5.70 |
| | Microtubule cytoskeleton organization | | | |
| | Spindle organization | | | |
| | Cytoskeleton organization | | | |

*Continued*

**Table VI** *Continued*

| Annotation cluster | GOBPTERM FAT | Enrichment score | No. | % |
|---|---|---|---|---|
| Regulation of mitosis | Regulation of mitotic cell cycle | 5.27 | 29 | 4.24 |
| | Regulation of cell cycle process | | | |
| | Regulation of mitosis | | | |
| | Regulation of nuclear division | | | |
| | Positive regulation of mitosis | | | |
| Regulation of mitosis | Regulation of mitosis | 4.15 | 19 | 2.78 |
| | Regulation of nuclear division | | | |
| | Spindle checkpoint | | | |
| | Regulation of mitotic metaphase/anaphase transition | | | |
| | Negative regulation of nuclear division | | | |
| | Negative regulation of mitosis | | | |
| Cell migration | Locomotory behaviour | 3.77 | 47 | 6.87 |
| | Chemotaxis | | | |
| Cell division | Chromosome segregation | 3.73 | 17 | 2.49 |
| | Mitotic sister chromatid segregation | | | |
| | Sister chromatid segregation | | | |
| Day 4 vs Day 3 | | | | |
| Chemotaxis | Chemotaxis | 2.26 | 15 | 4.73 |
| | Taxis | | | |
| | Locomotory behaviour | | | |
| Complement cascade and immune response | Complement activation, classical pathway | 2.18 | 11 | 3.47 |
| | Humoral immune response mediated by circulating immunoglobulin | | | |
| | Inflammatory response | | | |

[a]FAC, functional annotation cluster.

up-regulated on Day 2 vs Day 4 is consistent with the up-regulation of inflammatory processes on Day 2 of menstruation (early-menstruation) and the cessation of clotting processes by Day 4 (late-menstruation). A recent study showed that increased circulating fibrinogen levels were associated with delayed clot formation in women with HMB (Gebhart *et al.*, 2015). However, further studies to evaluate the role of fibrinogen isoforms in normal menstruation are warranted.

C4BPA, a regulatory protein in the complement system also featured among the top up-regulated genes on Day 2 compared to Day 4. C4BPA inhibits the classical pathway of complement activation by limiting DNA release post-cell death thus suppressing inflammation (Blom *et al.*, 2004; Trouw *et al.*, 2005). The up-regulation of C4BPA is again consistent with the up-regulation of inflammatory processes. Interestingly, XYLT1, an enzyme with roles in cartilage repair specifically via glycosaminoglycan synthesis (Venkatesan *et al.*, 2009), was also up-regulated on Day 2 of menstruation suggesting some degree of overlap in breakdown and repair processes in Day 2 menstrual endometrium.

The glutathione-S-transferases (GSTM1, GSTM2) were among the top up-regulated genes on Day 4 vs Day 2. GSTs are cytosolic or membrane bound enzymes involved in the detoxification of reactive oxygen species (ROS) via conjugation with glutathione (Kalinina *et al.*, 2014). GSTs, therefore, play an important role in cellular protection against oxidative stress. They are also involved in redox-dependent cell signalling and regulate cellular processes including proliferation and apoptosis through interactions with kinases that are activated by oxidative stress (Kalinina *et al.*, 2014). Steroid hormone withdrawal is known to decrease mechanisms that protect against ROS-induced damage in decidualized stromal cells (Sugino *et al.*, 2002) and to elevate inflammatory signals such as cyclooxygenase-2 and PG-F-2alpha (Sugino *et al.*, 2004). Based on these observations, we hypothesize that by Day 4 of menstruation (late-menstruation) endometrial regeneration is occurring and the GSTs play roles in cell proliferation and the restoration of the redox balance post-endometrial breakdown.

Genes coding for GSTM1 are polymorphic. The GSTM1 null variant is present in ~45% of healthy individuals (Seidegard *et al.*, 1990; Brockmoller *et al.*, 1996; Coles and Kadlubar, 2003). GSTM1 mRNA was undetectable in a large proportion of our endometrial tissue samples as was observed also in fibroid tissues from women who were poor responders to mifepristone treatment (Engman *et al.*, 2013). Although inconsistent, several studies have suggested a correlation between endometriosis and the GSTM1-deficient genotype (Baranov *et al.*, 1996; Baranova *et al.*, 1999; Hsieh *et al.*, 2004; Hur *et al.*, 2005). The GSTM1 null variant is also associated with an increased risk of

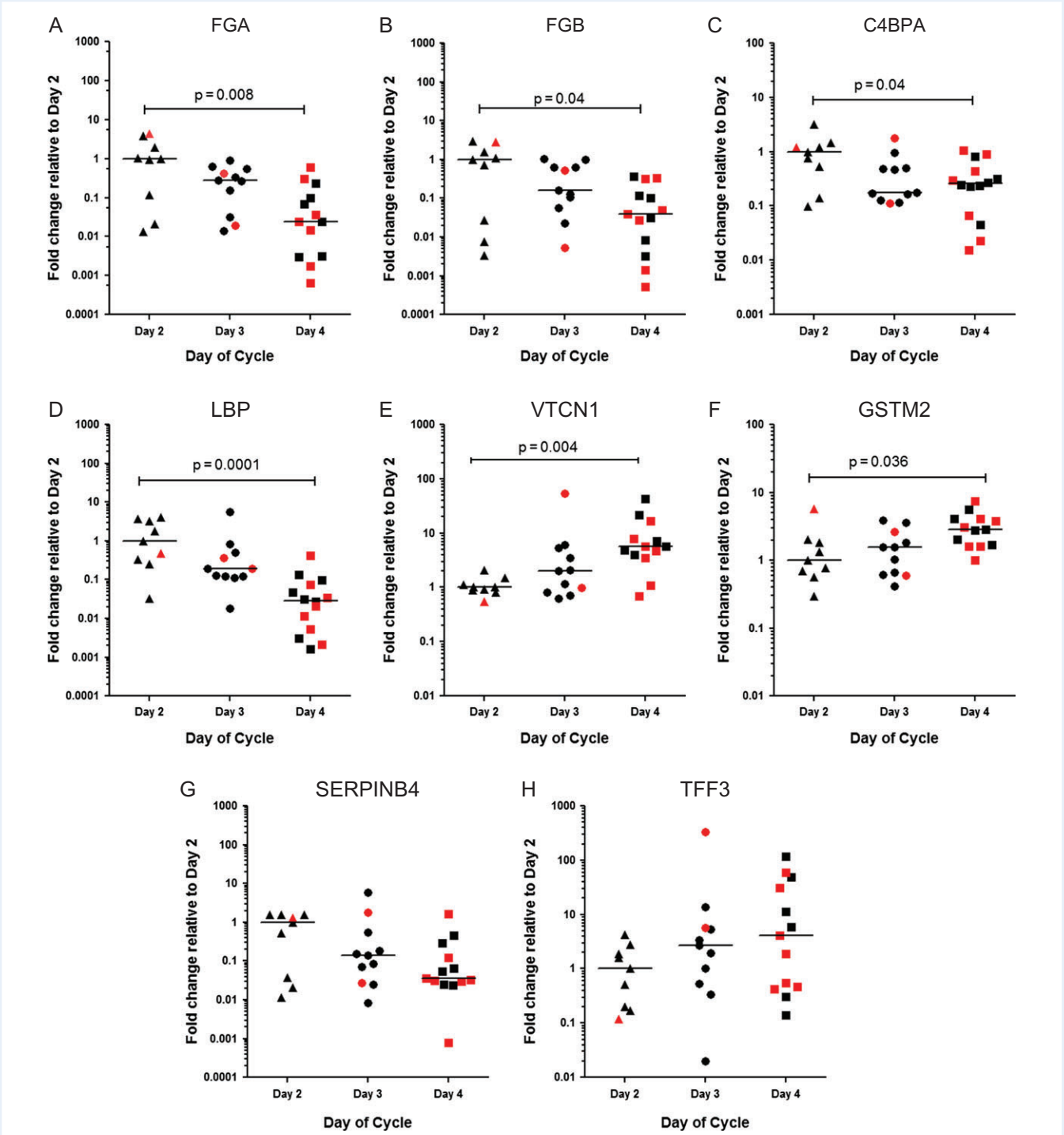


**Figure 2** Expression of FGA (**A**), FGB (**B**), C4BPA (**C**), LBP (**D**), VTCN1 (**E**), GSTM2 (**F**) SERPINB4 (**G**) and TFF3 (**H**) in menstrual-phase endometrial biopsies from women from Days 2, 3 and 4 of menstruation as determined by quantitative PCR. Median fold change was calculated using ΔΔCT with *RPL13A* as the housekeeping gene. Data points in red are additional samples that were not in the original gene array.

leiomyoma development (Huang *et al.*, 2010). Therefore, polymorphisms in these genes appear to be associated with various endometrial pathologies; however, the mechanisms involved remain to be elucidated. Future studies examining the specific roles of GSTs during menstruation are warranted.

Further evidence for endometrial regeneration by Day 4 of menstruation comes from DAVID analysis which indicates that several genes associated with cell division and mitosis are up-regulated at this time. We are the first to observe associations between these genes and menstruation. VTCN1 (also known as B7-H4, B7X or B7S1) is a

B7 family member that plays critical roles in regulating not only the immune response via T-cell activation and cytokine secretion (Prasad *et al.*, 2003; Choi *et al.*, 2003) but also cell proliferation and migration as demonstrated in tumour models *in vitro* (Gao *et al.*, 2015). KIAA0101 encodes a proliferating cell nuclear antigen-associated factor (Xie *et al.*, 2014), regulates DNA synthesis and cell cycle progression (Chang *et al.*, 2013) and has oncogenic roles in tumour progression (Yu *et al.*, 2001; Cheng *et al.*, 2013; Zhu *et al.*, 2013; Fan *et al.*, 2015). We also identified for the first time TFF3 and LAMA2 as potentially associated with endometrial repair during menstruation. TFF3 is a secreted protein, which functions in mucosal repair of the gastrointestinal tract (Taupin and Podolsky, 2003; Hoffmann, 2005; Thim and May, 2005) through the combined stimulation of cell migration and prevention of apoptosis and anoikis (Taupin and Podolsky, 2003). Dysregulated TFF3 expression has been implicated in numerous cancers (Regalo *et al.*, 2005; Perry *et al.*, 2008). Moreover, LAMA2, a subunit of the basement membrane protein laminin2/merosin, has been demonstrated to play a role in muscle regeneration and repair and defects in its expression are a major contributor to muscular dystrophy (Kuang *et al.*, 1999). Both TFF3 and LAMA2 are up-regulated on Day 4 vs Day 2, consistent with roles in endometrial regeneration and repair. In contrast, SPINK1, which has roles in gut mucosual repair and integrity, is down-regulated on Day 4 vs Day 2 (Konturek *et al.*, 1998; Marchbank *et al.*, 1998). These findings merit the further investigation of the roles of these factors in endometrial repair post-menstruation.

Identifying the processes occurring during menstruation is difficult for a number of reasons. The most significant of these is that shedding and repair can occur simultaneously in menstrual endometrium (Cheng *et al.*, 2007; Gaide Chevronnay *et al.*, 2009; Garry *et al.*, 2009). Therefore, menstrual endometrium is likely to contain areas of unshed, late secretory phase endometrium together with partially or completely sloughed and/or repaired endometrium (Garry *et al.*, 2009). Additionally, these processes proceed at different rates in different women, as reflected by variation in the length of the menstrual process. For example, repair in one woman may be complete by Day 3 of the menstrual cycle whereas repair may not be complete until Day 6 in another (McLennan and Rydell, 1965).

The women in this study also presented with a variety of endometrial pathologies related to bleeding status and other menstrual characteristics. These variations may also have influenced the menstrual process. It is also important to note that the current knowledge of canonical gene pathways cannot necessarily be related directly to a tissue as dynamic as the endometrium, which appears to have tissue-specialized processes (Ponnampalam *et al.* 2008) possibly also pertaining to menstruation. In addition, although endometrial sampling by pipelle biopsy provides a long ribbon of tissue, it is possible we may not have detected subtle changes in endometrial activity that occur very locally.

In spite of the practical difficulties of obtaining well-characterized clinical samples for this work, we have been able to provide new insights into the fundamental mechanisms underlying menstrual bleeding. Understanding the key regulators associated with menstruation is important for the development of new therapies that target aberrations in the expression of factors important for normal endometrial function.

# Supplementary data

Supplementary data are available at *Molecular Human Reproduction* online.

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# Authors' roles

P.P. was responsible for conducting all PCR experimentation, data analyses, interpreting the results and preparing the manuscript. M.L. undertook sample processing and PCR analyses, and contributed to data and cluster analysis. J.G. was responsible for study conception and design, data analysis and preparing the manuscript. M.O. undertook bioinformatics analyses and contributed to data interpretation. N.W. performed the pelvic ultrasounds and interpreted clinical results. J.M. assisted with development of the study database. M.H. was responsible for study conception and design, clinical aspects of the study including patient recruitment and biopsy collection, data interpretation and manuscript preparation. P.R. was responsible for study conception and design, study coordination, data interpretation and manuscript preparation. All authors made substantial contributions in critically revising the manuscript.

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# Conflict of interest

The authors declare no conflict of interest.

# References

Baranov VS, Ivaschenko T, Bakay B, Aseev M, Belotserkovskaya R, Baranova H, Malet P, Perriot J, Mouraire P, Baskakov VN *et al.* Proportion of the GSTM1 0/0 genotype in some Slavic populations and its correlation with cystic fibrosis and some multifactorial diseases. *Hum Genet* 1996;**97**:516–520.

Baranova H, Canis M, Ivaschenko T, Albuisson E, Bothorishvilli R, Baranov V, Malet P, Bruhat MA. Possible involvement of arylamine N-acetyltransferase 2, glutathione S-transferases M1 and T1 genes in the development of endometriosis. *Mol Hum Reprod* 1999;**5**:636–641.

Barbosa-Morais NL, Dunning MJ, Samarajiwa SA, Darot JF, Ritchie ME, Lynch AG, Tavare S. A re-annotation pipeline for Illumina BeadArrays: improving the interpretation of gene expression data. *Nucleic Acids Res* 2010;**38**:e17.

Blom AM, Villoutreix BO, Dahlback B. Functions of human complement inhibitor C4b-binding protein in relation to its structure. *Arch Immunol Ther Exp (Warsz)* 2004;**52**:83–95.

Bottalico B, Pilka R, Larsson I, Casslen B, Marsal K, Hansson SR. Plasma membrane and vesicular monoamine transporters in normal endometrium and early pregnancy decidua. *Mol Hum Reprod* 2003; **9**:389–394.

Brockmoller J, Kaiser R, Kerb R, Cascorbi I, Jaeger V, Roots I. Polymorphic enzymes of xenobiotic metabolism as modulators of acquired P53 mutations in bladder cancer. *Pharmacogenetics* 1996;**6**:535–545.

Brown JJ, Bayat A. Genetic susceptibility to raised dermal scarring. *Br J Dermatol* 2009;**161**:8–18.

Cameron IT, Haining R, Lumsden MA, Thomas VR, Smith SK. The effects of mefenamic acid and norethisterone on measured menstrual blood loss. *Obstet Gynecol* 1990;**76**:85–88.

Catalano RD, Critchley HO, Heikinheimo O, Baird DT, Hapangama D, Sherwin JR, Charnock-Jones DS, Smith SK, Sharkey AM. Mifepristone induced progesterone withdrawal reveals novel regulatory pathways in human endometrium. *Mol Hum Reprod* 2007;**13**:641–654.

Catanzaro JM, Sheshadri N, Zong WX. SerpinB3/B4: mediators of Ras-driven inflammation and oncogenesis. *Cell cycle* 2014;**13**:3155–3156.

Chang CN, Feng MJ, Chen YL, Yuan RH, Jeng YM. p15(PAF) is an Rb/E2F-regulated S-phase protein essential for DNA synthesis and cell cycle progression. *PloS one* 2013;**8**:e61196.

Cheng CW, Bielby H, Licence D, Smith SK, Print CG, Charnock-Jones DS. Quantitative cellular and molecular analysis of the effect of progesterone withdrawal in a murine model of decidualization. *Biol Reprod* 2007;**76**: 871–883.

Cheng Y, Li K, Diao D, Zhu K, Shi L, Zhang H, Yuan D, Guo Q, Wu X, Liu D *et al*. Expression of KIAA0101 protein is associated with poor survival of esophageal cancer patients and resistance to cisplatin treatment in vitro. *Lab Invest* 2013;**93**:1276–1287.

Chiang MF, Yang SY, Lin IY, Hong JB, Lin SJ, Ying HY, Chen CM, Wu SY, Liu FT, Lin KI. Inducible deletion of the Blimp-1 gene in adult epidermis causes granulocyte-dominated chronic skin inflammation in mice. *Proc Natl Acad Sci U S A* 2013;**110**:6476–6481.

Choi IH, Zhu G, Sica GL, Strome SE, Cheville JC, Lau JS, Zhu Y, Flies DB, Tamada K, Chen L. Genomic organization and expression analysis of B7-H4, an immune inhibitory molecule of the B7 family. *J Immunol* 2003; **171**:4650–4654.

Chwalisz K, Garfield RE. Role of nitric oxide in implantation and menstruation. *Human Reprod* 2000;**15**(Suppl 3):96–111.

Coles BF, Kadlubar FF. Detoxification of electrophilic compounds by glutathione S-transferase catalysis: determinants of individual response to chemical carcinogens and chemotherapeutic drugs? *BioFactors* 2003;**17**: 115–130.

Craythorn RG, Girling JE, Hedger MP, Rogers PA, Winnall WR., An RNA. spiking method demonstrates that 18S rRNA is regulated by progesterone in the mouse uterus. *Mol Hum Reprod* 2009;**15**:757–761.

Critchley HO, Kelly RW, Brenner RM, Baird DT. Antiprogestins as a model for progesterone withdrawal. *Steroids* 2003;**68**:1061–1068.

Davies J, Kadir RA. Endometrial haemostasis and menstruation. *Rev Endocr Metab Disord* 2012;**13**:289–299.

Engman M, Varghese S, Lagerstedt Robinson K, Malmgren H, Hammarsjo A, Bystrom B, Lalitkumar PG, Gemzell-Danielsson K. GSTM1 gene expression correlates to leiomyoma volume regression in response to mifepristone treatment. *PloS one* 2013;**8**:e80114.

Evans J, Salamonsen LA. Inflammation, leukocytes and menstruation. *Rev Endocr Metab Disord* 2012;**13**:277–288.

Evans J, Salamonsen LA. Decidualized human endometrial stromal cells are sensors of hormone withdrawal in the menstrual inflammatory cascade. *Biol Reprod* 2014;**90**:14.

Fan S, Li X, Tie L, Pan Y, Li X. KIAA0101 is associated with human renal cell carcinoma proliferation and migration induced by erythropoietin. *Oncotarget* 2015.

Fitzgerald KA, Rowe DC, Barnes BJ, Caffrey DR, Visintin A, Latz E, Monks B, Pitha PM, Golenbock DT. LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. *J Exp Med* 2003;**198**: 1043–1055.

Fu L, Girling JE, Rogers PA. Expression of Fox head protein 1 in human eutopic endometrium and endometriosis. *Reprod Sci* 2008;**15**:243–252.

Gagnon-Bartsch JA, Jacob L, Speed TP. Removing unwanted variation from high dimensional data with negative controls. *Statistics Technical Reports*. Department of Statistics, University of California, Berkley, 2013.

Gagnon-Bartsch JA, Speed TP. Using control genes to correct for unwanted variation in microarray data. *Biostatistics* 2012;**13**:539–552.

Gaide Chevronnay HP, Galant C, Lemoine P, Courtoy PJ, Marbaix E, Henriet P. Spatiotemporal coupling of focal extracellular matrix degradation and reconstruction in the menstrual human endometrium. *Endocrinology* 2009;**150**:5094–5105.

Gao A, Zhang L, Chen X, Chen Y, Xu Z, Liu Y, Zhu W. Effect of VTCN1 on progression and metastasis of ovarian carcinoma in vitro and vivo. *Biomed Pharmacother* 2015;**73**:129–134.

Garry R, Hart R, Karthigasu KA, Burke C. A re-appraisal of the morphological changes within the endometrium during menstruation: a hysteroscopic, histological and scanning electron microscopic study. *Human Reprod* 2009;**24**:1393–1401.

Gebhart J, Laczkovics C, Posch F, Ay C, Reitter-Pfoertner SE, Haslacher H, Muszbek L, Wolberg AS, Pabinger I. Plasma clot properties in patients with a mild-to-moderate bleeding tendency of unknown cause. *Ann Hematol* 2015;**94**:1301–1310.

Girling JE, Lockhart MG, Olshansky M, Paiva P, Woodrow N, Marino JL, Hickey M, Rogers PA. Differential gene expression in menstrual endometrium from women with self-reported heavy menstrual bleeding. *Reprod Sci* 2016.

Gleeson N, Devitt M, Sheppard BL, Bonnar J. Endometrial fibrinolytic enzymes in women with normal menstruation and dysfunctional uterine bleeding. *Br J Obstet Gynaecol* 1993;**100**:768–771.

Gleeson NC, Buggy F, Sheppard BL, Bonnar J. The effect of tranexamic acid on measured menstrual loss and endometrial fibrinolytic enzymes in dysfunctional uterine bleeding. *Acta Obstet Gynecol Scand* 1994;**73**:274–277.

Hoffmann W. Trefoil factors TFF (trefoil factor family) peptide-triggered signals promoting mucosal restitution. *Cell Mol Life Sci* 2005;**62**: 2932–2938.

Hsieh YY, Chang CC, Tsai FJ, Lin CC, Chen JM, Tsai CH. Glutathione S-transferase M1*null genotype but not myeloperoxidase promoter G-463A polymorphism is associated with higher susceptibility to endometriosis. *Mol Hum Reprod* 2004;**10**:713–717.

Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 2009;**4**:44–57.

Huang PC, Tsai EM, Li WF, Liao PC, Chung MC, Wang YH, Wang SL. Association between phthalate exposure and glutathione S-transferase M1 polymorphism in adenomyosis, leiomyoma and endometriosis. *Human Reprod* 2010;**25**:986–994.

Hur SE, Lee JY, Moon HS, Chung HW. Polymorphisms of the genes encoding the GSTM1, GSTT1 and GSTP1 in Korean women: no association with endometriosis. *Mol Hum Reprod* 2005;**11**:15–19.

Jabbour HN, Kelly RW, Fraser HM, Critchley HO. Endocrine regulation of menstruation. *Endocr Rev* 2006;**27**:17–46.

Johnston A, Fritz Y, Dawes SM, Diaconu D, Al-Attar PM, Guzman AM, Chen CS, Fu W, Gudjonsson JE, McCormick TS *et al*. Keratinocyte overexpression of IL-17C promotes psoriasiform skin inflammation. *J Immunol* 2013;**190**:2252–2262.

Kalinina EV, Chernov NN, Novichkova MD. Role of glutathione, glutathione transferase, and glutaredoxin in regulation of redox-dependent processes. *Biochemistry (Mosc)* 2014;**79**:1562–1583.

Konturek P, Brzozowski T, Konturek SJ, Taut A, Pierzchalski P, Elia G, Pajdo R, Yagi S, Hahn EG. Base variant of human pancreatic secretory trypsin inhibitor in healing of stress-induced gastric lesions in rats. *Regul Pept* 1998;**77**:95–103.

Kuang W, Xu H, Vilquin JT, Engvall E. Activation of the lama2 gene in muscle regeneration: abortive regeneration in laminin alpha2-deficiency. *Lab Invest* 1999;**79**:1601–1613.

Livingstone M, Fraser IS. Mechanisms of abnormal uterine bleeding. *Hum Reprod Update* 2002;**8**:60–67.

Marchbank T, Freeman TC, Playford RJ. Human pancreatic secretory trypsin inhibitor. Distribution, actions and possible role in mucosal integrity and repair. *Digestion* 1998;**59**:167–174.

Maybin JA, Critchley HO. Menstrual physiology: implications for endometrial pathology and beyond. *Hum Reprod Update* 2015;**21**:748–761.

McLennan CE, Rydell AH. Extent of endometrial shedding during normal menstruation. *Obstet Gynecol* 1965;**26**:605–621.

Munro MG, Critchley HO, Fraser IS. The FIGO systems for nomenclature and classification of causes of abnormal uterine bleeding in the reproductive years: who needs them? *Am J Obstet Gynecol* 2012;**207**:259–265.

Perry JK, Kannan N, Grandison PM, Mitchell MD, Lobie PE. Are trefoil factors oncogenic? *Trends Endocrinol Metab* 2008;**19**:74–81.

Ponnampalam AP, Gargett CE, Rogers PA. Identification and hormonal regulation of a novel form of NKp30 in human endometrial epithelium. *Eur J Immunol* 2008;**38**:216–226.

Prasad DV, Richards S, Mai XM, Dong C. B7S1, a novel B7 family member that negatively regulates T cell activation. *Immunity* 2003;**18**:863–873.

Regalo G, Wright NA, Machado JC. Trefoil factors: from ulceration to neoplasia. *Cell Mol Life Sci* 2005;**62**:2910–2915.

Ross RL, Beck AW. Fibrinogen and catheter-directed thrombolysis. *Semin Vasc Surg* 2014;**27**:182–195.

Salamonsen LA, Lathbury LJ. Endometrial leukocytes and menstruation. *Hum Reprod Update* 2000;**6**:16–27.

Seidegard J, Pero RW, Markowitz MM, Roush G, Miller DG, Beattie EJ. Isoenzyme(s) of glutathione transferase (class Mu) as a marker for the susceptibility to lung cancer: a follow up study. *Carcinogenesis* 1990;**11**:33–36.

Shankar M, Lee CA, Sabin CA, Economides DL, Kadir RA. von Willebrand disease in women with menorrhagia: a systematic review. *BJOG* 2004;**111**:734–740.

Sindrilaru A, Peters T, Schymeinsky J, Oreshkova T, Wang H, Gompf A, Mannella F, Wlaschek M, Sunderkotter C, Rudolph KL *et al*. Wound healing defect of Vav3-/- mice due to impaired {beta}2-integrin-dependent macrophage phagocytosis of apoptotic neutrophils. *Blood* 2009;**113**:5266–5276.

Sugino N, Karube-Harada A, Sakata A, Takiguchi S, Kato H. Different mechanisms for the induction of copper-zinc superoxide dismutase and manganese superoxide dismutase by progesterone in human endometrial stromal cells. *Human Reprod* 2002;**17**:1709–1714.

Sugino N, Karube-Harada A, Taketani T, Sakata A, Nakamura Y. Withdrawal of ovarian steroids stimulates prostaglandin F2alpha production through nuclear factor-kappaB activation via oxygen radicals in human endometrial stromal cells: potential relevance to menstruation. *J Reprod Dev* 2004;**50**:215–225.

Taupin D, Podolsky DK. Trefoil factors: initiators of mucosal healing. *Nat Rev Mol Cell Biol* 2003;**4**:721–732.

Thim L, May FE. Structure of mammalian trefoil factors and functional insights. *Cell Mol Life Sci* 2005;**62**:2956–2973.

Trouw LA, Nilsson SC, Goncalves I, Landberg G, Blom AM. C4b-binding protein binds to necrotic cells and DNA, limiting DNA release and inhibiting complement activation. *J Exp Med* 2005;**201**:1937–1948.

Tschugguel W, Schneeberger C, Unfried G, Brautigam G, Stonek F, Wieser F, Vytiska-Binstorfer E, Czerwenka K, Weninger W, Kaider A *et al*. Elevation of inducible nitric oxide synthase activity in human endometrium during menstruation. *Biol Reprod* 1999;**60**:297–304.

Venkatesan N, Barre L, Magdalou J, Mainard D, Netter P, Fournel-Gigleux S, Ouzzine M. Modulation of xylosyltransferase I expression provides a mechanism regulating glycosaminoglycan chain synthesis during cartilage destruction and repair. *FASEB J* 2009;**23**:813–822.

Wang Q, Xu X, He B, Li Y, Chen X, Wang J. A critical period of progesterone withdrawal precedes endometrial breakdown and shedding in mouse menstrual-like model. *Human Reprod* 2013;**28**:1670–1678.

Winnall WR, Muir JA, Liew S, Hirst JJ, Meachem SJ, Hedger MP. Effects of chronic celecoxib on testicular function in normal and lipopolysaccharide-treated rats. *Int J Androl* 2009;**32**:542–555.

Wuyts A, Van Osselaer N, Haelens A, Samson I, Herdewijn P, Ben-Baruch A, Oppenheim JJ, Proost P, Van Damme J. Characterization of synthetic human granulocyte chemotactic protein 2: usage of chemokine receptors CXCR1 and CXCR2 and in vivo inflammatory properties. *Biochemistry* 1997;**36**:2716–2723.

Xie C, Yao M, Dong Q. Proliferating cell unclear antigen-associated factor (PAF15): a novel oncogene. *Int J Biochem Cell Biol* 2014;**50**:127–131.

Xu X, Chen X, Li Y, Cao H, Shi C, Guan S, Zhang S, He B, Wang J. Cyclooxygenase-2 regulated by the nuclear factor-kappaB pathway plays an important role in endometrial breakdown in a female mouse menstrual-like model. *Endocrinology* 2013;**154**:2900–2911.

Yu P, Huang B, Shen M, Lau C, Chan E, Michel J, Xiong Y, Payan DG, Luo Y. p15(PAF), a novel PCNA associated factor with increased expression in tumor tissues. *Oncogene* 2001;**20**:484–489.

Zhang X, Huang Q, Yang Z, Li Y, Li CY. GW112, a novel antiapoptotic protein that promotes tumor growth. *Cancer Res* 2004;**64**:2474–2481.

Zhu K, Diao D, Dang C, Shi L, Wang J, Yan R, Yuan D, Li K. Elevated KIAA0101 expression is a marker of recurrence in human gastric cancer. *Cancer Sci* 2013;**104**:353–359.