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Date:
2017-01-01

Citation:
Girling, J. E., Lockhart, M. G., Olshansky, M., Paiva, P., Woodrow, N., Marino, J. L., Hickey, M. & Rogers, P. A. W. (2017). Differential Gene Expression in Menstrual Endometrium from Women with Self-Reported Heavy Menstrual Bleeding. Reproductive Sciences, 24 (1), pp.28-46. https://doi.org/10.1177/1933719116648217.

Persistent Link:
https://hdl.handle.net/11343/240677

Differential gene expression in menstrual endometrium from women with self-reported heavy menstrual bleeding

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Name of the institution where the work reported was done: Gynaecology Research Centre, The University of Melbourne Department of Obstetrics and Gynaecology and Royal Women's Hospital, Parkville, Victoria, Australia 3052

Funding

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 Hickey is supported by an NHMRC Practitioner Fellowship.

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ABSTRACT

Heavy menstrual bleeding (HMB) is a significant social and public health issue for menstruating women. Development of targeted treatments has been limited by poor understanding of local mechanisms underlying HMB. We aimed to determine how gene expression differs in menstrual phase endometrium from women with HMB. Menstrual phase endometrial biopsies were collected from women with (n=7) and without (n=10) HMB (regular menstrual cycles, no known pelvic pathology), as well as women with uterine fibroids (n=7, n=4 had HMB). Biopsies were analysed using Illumina Sentrix Human HT12 arrays and data 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 gene pathways, functional gene clusters and upstream regulators specific to the clinical groupings. Individual genes of interest were examined using quantitative PCR (qPCR). In total, 829 genes were differentially expressed in one or more comparisons. Significant canonical pathways and gene clusters enriched in Controls relative to both HMB and Fibroid groups suggest the mechanisms responsible for HMB include modifications of the endometrial inflammatory or infection response. In contrast, differentially expressed genes in women with fibroids suggest modifications of haemoglobin, antigen processing and the MHC complex (Class II, beta chain) activity. In conclusion, HMB associated with fibroids may be regulated by different endometrial mechanisms from HMB in women without fibroids and from normal menstrual bleeding. These novel data provide numerous testable hypotheses that will advance our understanding of the mechanisms responsible for HMB.

Keywords: Endometrium, heavy menstrual bleeding (HMB), menstruation, RUV-inv, uterine fibroids

INTRODUCTION

Heavy menstrual bleeding (HMB) is defined as 'excessive menstrual blood loss which interferes with a women's physical, social, emotional and/or material quality of life'.[1] This common condition affects 20-52% of women, with variations likely due to culture, clinical setting, and differences in how menstruation is perceived.[1,2] Complications of HMB may include iron deficiency anaemia, as well as adverse effects on personal, social and work life. In England and Wales, about 30,000 women undergo surgical treatment for HMB each year;[3] the economic burden of HMB is substantial.[2] For instance, conservative estimates from the USA (2005) suggest annual direct and indirect costs of US$1 billion and US$12 billion.[4] Surgical treatments for HMB, including hysterectomy, are effective but are unsuitable for women who wish to retain their fertility. Medical treatments may be effective, but many are also contraceptive. There is an urgent need for the development of fertility-preserving treatments for HMB.

A classification system for causes of abnormal uterine bleeding, which was recently developed by the International Federation of Gynecology and Obstetrics, highlights the diverse aetiology of various menstrual bleeding irregularities (PALM-COEIN: polyps, adenomyosis, leiomyoma, malignancy and hyperplasia – coagulopathy, ovulatory disorders, endometrial causes, iatrogenic, not classified).[1,5-7] HMB may be associated with irregular (usually anovulatory) menstrual cycles due to disturbances in ovarian function, or with regular HMB with no discernable pelvic pathology or associated with uterine fibroids. In this study, we address the hypothesis that different mechanisms are responsible for HMB associated with regular menstrual cycles, or with HMB associated with common pelvic pathology such as uterine fibroids.

Uterine fibroids are benign tumours of the myometrium that are commonly associated with HMB.[8] However, most medical treatments for HMB have either not been tested in women with uterine fibroids or are less likely to be effective in this population,[9] suggesting that fibroids may induce HMB by different mechanisms. Conservative surgery (uterine resection, endometrial ablation, myomectomy) may be effective but is not without risk. Hence, for many women, current

medical therapy for HMB is either inadequate or inappropriate, or is associated with unacceptable side effects.[1] Most previous studies have not differentiated bleeding associated with or without uterine fibroids. This is clinically important as understanding the different mechanisms responsible for HMB may help identify new targets for urgently needed therapies.

Understanding of the mechanisms of normal and abnormal uterine bleeding is incomplete.[10-13] Menstruation initiates after progesterone withdrawal from an estrogen-primed endometrium, with a subsequent cascade of molecular events culminating in endometrial breakdown, bleeding and repair. The process resembles an inflammatory response and includes leucocyte influx, cytokine production, extracellular matrix degradation and vascular and tissue remodelling. Menstruation involves multiple regulators, including prostaglandins; prostaglandin-mediated vasoconstriction of spiral arterioles minimises blood loss and causes hypoxia in the functionalis. Abnormal cyclooxygenase and prostaglandin production have been observed in women with HMB. Regulation of haemostasis is a key feature of menstruation. Of particular importance is the fibrinolytic pathway, which degrade fibrin deposits; key members of the fibrinolytic pathway (e.g. tissue plasminogen activator) are dysregulated in HMB, consistent with the efficacy of anti-fibrinolytics in reducing HMB.[10-13] In this study, we have examined menstrual phase biopsies from women with (1) regular menstrual cycles with self-reported HMB compared to those without HMB, and (2) women with uterine fibroids. To our knowledge, our study is the first to provide detailed analyses of endometrial gene expression from women with regular menstrual cycles and no known uterine comorbidities. In addition, no previous studies have examined variations in menstrual phase gene expression associated with the presence of uterine fibroids. We focused on carefully characterised samples collected on days 1-5 of the menstrual cycle (during menstruation). As endometrial gene and protein expression is highly dynamic with considerable heterogeneity among different phases of the menstrual cycle and between women, we have used innovative bioinformatics methodology called 'Remove Unwanted Variation-inverse' (RUV-inv) to examine gene array results.[14,15] RUV-inv is designed to overcome unwanted technical and biological variation otherwise inherent in gene array

analysis of highly variable tissues such as endometrium. The technique uses a group of control genes that are predicted to remain stable across different clinical groupings to identify and remove unwanted variation, even if the specific source of variation is not known.[14,15] RUV-inv is an extension of RUV4, which is itself an alternative to the basic RUV2 method. The RUV-inv method has allowed us to identify gene pathways, functional gene clusters and potential upstream regulators of the different bleeding patterns in these hard to obtain menstrual-phase endometrial biopsies. We hypothesised that the mechanisms responsible for HMB are specific to the different underlying pathology responsible for the abnormal bleeding.

METHODS

Patient Samples and Ethical Approval

Premenopausal women with and without self-reported HMB were recruited from The Royal Women's Hospital outpatient clinic and from local advertising over a four year period. Ethical approval for the study was obtained from the Royal Women's Hospital Human Research Ethics Committee (Project 11/13) and informed consent was obtained from all subjects. Participant details are summarised in Table 1.

Data about menstrual history were collected and subjects completed a menstrual diary. All subjects had a pelvic ultrasound performed by an experienced ultrasonologist (NW) within two weeks of recruitment using a Phillips iU22 using a C10-3v curved array transvaginal probe and a C9-4 or C5-2 curved array transabdominal probe (standardised ASUM protocol for gynaecological scanning[16]). The ultrasound reported the presence/absence, size and location of uterine fibroids and any apparent distortion of the endometrial cavity. The ovaries were examined for any cysts or masses and follicular dimensions and total number were recorded. Patients were classified as having uterine fibroids if any fibroids were detected, irrespective of number, size or location. Participants were excluded if they had other comorbidities (e.g. polyp, adenomyosis), unless explicitly stated in Table 1, were taking sex steroids, or had known endocrine or haematological

abnormalities that may cause HMB. We were not able to exclude endometriosis from this study as the women involved were not undergoing laparoscopic or other surgical procedures.

Endometrial biopsies were collected on days 1-5 of the menstrual cycle during a bleeding episode. Day-of-cycle was verified using the patient's menstrual diary (unless stated otherwise, Table 1). Endometrial biopsies were collected using a pipelle suction curette (Pipelle, Cornier, France) and stored in RNAlater (Ambion, Life Technologies, Carlsbad, CA, USA) overnight before being frozen and stored at -80°C.

A total of 36 samples were arrayed, of which 24 carefully characterised samples were used in the final array analysis. The remaining 11 samples were excluded due to irregular menstrual cycles (n=1), no menstrual diary provided (n=8), and/or detection of additional comorbidities (e.g. adenomyosis) (n=5). The 24 samples were separated into three groups (Table 1):

1. **Controls**: No heavy menstrual bleeding, regular menstrual cycles, no uterine comorbidities (n=7).
2. **HMB**: Heavy menstrual bleeding, regular menstrual cycles, no uterine comorbidities (n=10).
3. **Fibroids**: Mixed bleeding patterns and comorbidities (n=7).

Groups 1 and 2 were restricted to those with regular menstrual cycles, defined using standardised international criteria as cycle to cycle variation of between 2 and 20 days[6,7] and based on at least a 3 month menstrual diary (Table 1). Duration and frequency of menstruation were also noted, but not used as inclusion/exclusion criteria. Bleeding but not spotting days were used in calculations (Table 1).

Due to the logistical difficulties of obtaining these valuable samples, we were not able to obtain a completely separate cohort of samples for validation analyses. Instead, an additional 9 samples were included when individual genes of interest were examined using PCR. These included 2 Control, 6 HMB and 1 Fibroid samples. Details about these participants are included in Table 1.

RNA Extraction

RNA was isolated from tissues using Trizol (Invitrogen, Life technologies, Carlsbad, CA, USA) and the Ambion PureLink mini kit (Ambion, Life technologies, Carlsbad, CA, USA). Briefly, endometrial tissue was homogenised in Trizol. Chloroform (200µl) was added to homogenised tissue and vigorously mixed for 15 seconds, incubated at room temperature for 5 minutes and centrifuged at 12,000g for 15 minutes at 4˚C. The aqueous phase was then separated and the RNA was precipitated with isopropanol (500µl) and centrifugation at 12,000g (10 minutes, 4˚C). The supernatant was removed and the precipitated RNA was resuspended with 100µl of RNAse free water. Lysis buffer (100µl) and 70% ethanol (100µl) were added to the solution, mixed by gently pipetting and then placed onto an Ambion PureLink column (Life Technologies Carlsbad, CA, USA). RNA was then column purified according to the manufacturer's instructions and eluted into 50µl of RNAse free water. RNA quantity was determined using a Nanodrop (Thermo Scientific, Waltham, MA, USA) and stored at -80˚C.

Gene Array Analysis

RNA (3µg) was used for further analysis of RNA quality and hybridization to Illumina Human HT12 v4 arrays at the Australian Genome Research Facility (AGRF, Victoria, Australia) (minimum RNA integrity number (RIN) required by AGRF for gene array is 7, our samples: average RIN =8.75) . The array data were then analysed using the 'Remove Unwanted Variation-inverse (RUV-inv) method.[14,15,17] The housekeeping genes used with RUV-inv were a modified list of the genes noted in Eisenberg and Levanon.[18] The re-annotation functions of the Illumina Human v4.db Bioconductor R package[19] were used to remap the probes according to current knowledge of the human genome (hg19). Finally genes were declared Differentially Expressed (DE) if the Benjamimi-Hochberg adjusted p-value (FDR) was less than 0.05 and the absolute value of log2 of Fold Change (logFC) was greater than 1 (Fold Change greater than 2).

Pathway Analysis and Functional Cluster Analysis

Gene lists derived from the arrays were analysed using Ingenuity® Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity). Significant canonical pathways were derived after right-tailed Fisher exact tests and Benjamini-Hochberg multiple correction. IPA Upstream Regulator was also used to identify potential upstream transcription regulators. Overlap p-values were derived to determine whether there was a significant overlap between genes in our gene lists and those known to be regulated by a particular factor; a p-value smaller than or equal to 0.001 was considered significant. Where applicable, a z-score that predicts the activation status of the hypothesised regulator was also obtained.

Gene lists were also examined using The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (http://david.abcc.ncifcrf.gov/home.jsp).[20,21] We used the Functional Annotation Clustering Tool, which combines similar annotations derived from various different databases into a single cluster (e.g. Gene Ontology, KEGG pathways). For each cluster, an Enrichment Score (geometric mean expressed as –(log10) of the mean of p-values from individual annotations within a cluster) and individual p-values or EASE scores (modified Fisher exact test, provides an estimate of gene enrichment) were obtained. We used a minimum EASE score/p-value of 0.05 for all analyses; a geometric mean of individual annotations in a cluster equal to 0.05 would equate to an Enrichment Score of 1.30. Classification stringency was set at medium.

Quantitative PCR

Using RNA extracted as described above, cDNA was generated from 1 µg of total RNA from each sample using the GoSCript Reverse Transcription System (Promega, Alexandria, NSW, Australia) with 0.5 µg random primers and 3mM MgCl as per the manufacturer's instructions. An RNA spike was also added to the reverse transcription mix.[22-24] The spike was used as a control to measure fluctuations in cDNA transcription efficiency; it had a very low standard deviation across all samples demonstrating consistent efficiency of the reverse transcriptase.

Real time PCR was performed in duplicate in 10 µl reactions using Fast Start Universal Probe Master (ROX) (Roche, Castle Hill, NSW, Australia) and commercially validated Taqman® Gene Expression Assays from Applied Biosystems (Life Technologies, Carlsbad, CA, USA; Supplemental Table 1). The template cDNA was diluted 1 in 10 and 4 µl in each PCR reaction. Thermal cycling conditions were: initial hold (50°C, 2 min), activation (95°C, 10 min) and 40 cycles of denaturation (95°C, 15 sec; annealing and extension, 60°C, 1 min). Mean Ct values were calculated using the 7500 real-time PCR System (Applied Biosystems).

Several housekeeping controls were examined, including ribosomal protein 13 A (*RPL13A*), Beta-2-Microglobulin (*B2M*), 18S and the RNA spike. They were compared by NormFinder,[25] GeNorm,[26] BestKeeper[27] and delta CT[28] using an online reference gene finder (RefFinder, http://www.leonxie.com/referencegene.php). *RPL13A* was used in final analyses as it had a low standard deviation in raw microarray results, the lowest differences between bleeding groups, and the lowest ranking when compared with other housekeepers. Changes in gene expression were calculated using the ΔΔCT method with the Control group used as the calibrator; values are illustrated relative to median value in the Control group. Quantitative PCR data were examined using Mann Whitney U tests. A p-value <0.05 was considered significant.

RESULTS

Differential Gene Expression

A total of 829 genes were differentially expressed in one or more comparisons (False discovery rate (FDR) < 0.05, log of fold change ($|\log_{[base2]}FC|$) > 1) (Figure 1). There were 176 genes with significantly different expression in Control versus HMB groups (84 upregulated, 92 downregulated), 641 genes in Control versus Fibroid groups (235 upregulated, 406 downregulated), and 213 genes in HMB versus Fibroid groups (77 upregulated, 136 downregulated). There were 82 genes with significantly different expression in the Controls relative to both the HMB and Fibroid groups, 17 genes in the HMB relative to Controls and Fibroid groups, and 106 genes in the Fibroid

relative to Control and HMB groups (Figure 1). Four genes were significantly different in all comparisons (chemokine (C-C motif) ligand 4-like 2 [CCL4L2; HMB > Control > Fibroid], serum amyloid A2 [SAA2; Control > HMB > Fibroid], small Cajal body-specific RNA 27 [SCARNA27; Fibroid > HMB > Control], secretoglobin, family 3A, member 1 [SCGB3A1; Control > Fibroid > HMB]). The top 20 most significantly up or downregulated genes for each comparison are provided in Table 2.

Functional Clusters and Pathway Analysis

Of the differentially-expressed genes from the Control versus HMB groups, 163 genes (of 176 input) were annotated within IPA. Significant canonical pathways identified included acute phase response signalling and retinoid receptor activation (e.g. lipopolysaccharide binding protein [*LBP*], orosomucoid 1 [*ORM1*], serum amyloid A1 and A2 [*SAA1*, *SAA2*]) (Table 3). Several upstream regulators were also identified (Table 4) including the pleiotropic signal transcription regulator 'signal transducer and activator of transcription 5A' (STAT5A), several steroid hormones (e.g. androgen, dexamethasone, 17-alpha-ethinylestradiol), and several regulators related to immunity and response to pathogens (T-cell factors, interleukin 1 [IL1], poly rl:rc and lipopolysaccharide). Although there was a significant overlap for these regulators, the direction of change was not sufficiently consistent for a specific change in activation status to be calculated.

Of the differentially-expressed genes from the Control versus HMB groups, 164 genes (of 176 input) were annotated by the DAVID and 12 significant clusters were identified (Table 5, Supplemental Table 2). Key function-related clusters included genes involved in the inflammatory and immune response and, as with IPA analysis, included several acute phase proteins (*LBP*, *ORM1*, *SAA1*, *SAA2* – all downregulated in HMB relative to Controls). A further cluster included several cytokine-related genes (e.g. chemokine (C-C-motif) ligands: *CCL3L1*, *CCL3L3*, *CCL4L2*). Other clusters included genes related cell adhesion, the plasma membrane, cystine knots, lipoproteins, lipocalin, cell migration and proliferation and apoptosis.

The comparison with the largest number of differentially expressed genes was the Control versus Fibroid groups of which 568 genes (of 641 input) were annotated within IPA. Key canonical pathways included acute phase response signalling and pathways associated with B and T cell activity (e.g. B cell development, OX40 signalling, antigen presentation) (Table 3). These pathways reflected the presence of several human leukocyte antigen molecules (e.g. *HLA-A*, *HLA-C*, *HLA-DPB)*, which were also highlighted by analyses using DAVID (below). Several upstream regulators were also identified (Table 4), including STAT5A, interleukins, regulators suggesting the involvement of retinoic acid (tretinonin), and regulators associated with the immune response and pathogen recognition (resveratrol, surfactant protein 1 [SFTPA1]). For SFTPA1 and transforming growth factor beta 1 [TGFB1], analyses suggested that the activity of these regulators was inhibited and activated, respectively, in the Fibroid relative to Control groups.

Of the differentially-expressed genes from the Control versus Fibroid groups, 569 genes (of 641 input) were also annotated by DAVID; 26 significant clusters were identified (Table 5, Supplemental Table 3). Important functional clusters included genes associated with the plasma membrane (enrichment score 3.96), oxygen transport and haemoglobin including six haemoglobin subunits (Enrichment Score = 3.70; *HBD*, *HBG1, HBG2, HBM*, *HBQ1*, *HBZ)*; all six genes were upregulated in Fibroids relative to Controls. Other process-related clusters related to cell adhesion, the immune response and major histocompatibility (MH) complex, leucocyte activation, wound healing and coagulation, and von Willebrand factor. Of note was the large number of chemokines/cytokines/interleukins that were differentially expressed.

Of the differentially-expressed genes from the HMB versus Fibroid groups, 181 genes (of 213 input) were annotated within IPA, with significant canonical pathways including allograft rejection signalling, OX40 signalling, antigen presentation, graft-versus-host disease, CDC42 signalling and B cell development (reflecting the presence of several human leukocyte antigen molecules; Table 3). Other significant pathways included both agranulocyte (lymphocytes and monocytes) and granulocyte (neutrophils, basophils, eosinophils) adhesion and diapedesis, and communication

between innate and adaptive immune cells. As with comparison between Control and HMB, the upstream regulators identified included STAT5A and interleukins, regulators suggesting the involvement of retinoic acid, and regulators associated with the immune response and pathogen recognition; Table 4). For SFTPA1 and TGFB1, analyses suggested that the activity of these regulators was inhibited and activated, respectively, in the Fibroid relative to Control groups.

Of the differentially-expressed genes from the HMB versus Fibroid groups, 183 genes (of 213 input) were also annotated by DAVID. We identified 7 significant clusters (Table 5, Supplemental Table 4), including haemoglobin subunits and other genes related to oxygen transport. Other clusters included genes related to secreted/signalling peptides and other extracellular components, the immune response and MHC complex and a cluster of chemokines (e.g. *CCL2*, *CCL3L1*, *CCL3L3*, *CCL4*, *CCL4L2*, *CMTM1*, *CXCL5*).

Quantitative PCR

A set of 10 genes were chosen to be examined using quantitative PCR. With the exception of one sample from the Fibroid group with insufficient RNA, gene expression was examined in the same samples used in the array as well as the additional new samples (Table 1). Genes examined included five haemoglobin genes (*HBG1/2*, *HBD*, *HBM*, *HBQ1* and *HBZ*) and a gene associated with heme production (*ALAS2*). In the array analysis, these genes were all significantly upregulated in the Fibroid group relative to both HMB and Control groups. We also examined four secreted proteins (three secretoglobins: *SCGB3A1*, *SCGB1D2* and *SCGB1A2*; previously known as uteroglobin-related protein 2 or cytokine high in normal-1, lipophilin-B, and mammogloblin-A, respectively; and trefoil factor 3 (intestinal) [*TFF3*]), which were all upregulated in Control relative to HMB samples in array analysis; SCGB3A1 was also upregulated in Fibroids relative to the HMB group.

As expected, there was considerable variation among mRNA expression values determined by quantitative PCR, even within a group. However, all genes were expressed in reasonable quantities by endometrial tissues (Figures 2 and 3). For the haemoglobin related genes (Figure 2), there was a

an increase in mRNA expression that did not reach significance in the Fibroid relative to the Control group for HBG1/2, HBM, and HBQ1; there was also a non-significant increase in expression in the Fibroid relative to both Control and HMB groups for ALAS2 mRNA. For the secretoglobins (Figure 3), SCGB3A1 mRNA expression was significantly upregulated in the Control versus the HMB group. There was also a non-significant increase in the expression of TFF3 mRNA in the Control versus Fibroid groups.

DISCUSSION

Using gene arrays, we have demonstrated that endometrial genes, gene pathways, functional gene clusters, and potential upstream regulators are different among women with regular menstrual cycles with and without HMB and compared to women with uterine fibroids. Several of these groupings and regulators are consistent with known mechanisms associated with menstruation and HMB. However, by using a new bioinformatics technique (RUV-inv) that allows detection of more subtle changes, we have also identified novel genes/gene sets not previously associated with endometrial bleeding. This is the first application of these techniques to endometrial tissues, which are known for their patient-to-patient and cycle-stage variability. These techniques provide a valuable tool for future endometrial studies of endometrial function. Our findings have identified alternative gene groupings and tissue processes that may contribute to different patterns of uterine bleeding.

Our key comparison of interest was differences in gene expression between endometrium from women with regular menstrual cycles, no known uterine pathology and either with or without HMB. The analyses conducted using IPA and DAVID gave mostly consistent results. Of the process-based clusters identified by DAVID, the two most significant clusters grouped genes related to inflammation/immune response and cell adhesion (with several genes common to both lists). With IPA, the most significant canonical pathway identified was acute phase response signalling, which is the initial physiological response to infection or in inflammatory processes. Menstruation is often

likened to an inflammatory process and it has been hypothesised that HMB may reflect deviations in this response, including the activity of prostaglandins.[13,29-31] Whilst we did not identify genes specifically associated with prostaglandins, the various genes associated with the acute phase response were down regulated in HMB relative to Controls. This included orsomucoids 1 (*ORM1*) and serum amyloids A1 and A2 (*SAA1*, *SAA2*), which are major components of serum synthesised predominantly in the liver in response to inflammatory stimuli (e.g. interleukin 6 and tumor necrosis factor-α[32,33]). *ORM1* is produced by several non-hepatic cell types including microvascular endothelial cells and various leucocytes, whereas *SAAs* are also expressed by epithelia. These proteins have multiple functions during inflammation and immune modulation; *ORM1* and *ORM2* also form a component of the capillary barrier and have a role in vascular permeability.[32] It was also particularly interesting to note that the potential upstream regulators identified for Controls versus HMB included factors associated with infection and the innate/adaptive immune response, including T-cell factors, the pro-inflammatory cytokines IL1B and IL6 and components of pathogens recognised by the innate immune system (poly rel: rc, lipopolysaccharide). Although highly speculative at this stage, it is possible that HMB may reflect variations in the uterine microbiome and/or the uterine response to infection. This is not a new suggestion; Viniker hypothesised the presence of a common bacterial factor that may explain various gynaecological and obstetric pathologies.[34]. Although there is currently no data to support a role for the microbiome in HMB, recent studies have demonstrated a distinct microbiota in the human uterus, which is at least consistent with the possibility that unknown variations in the endometrial microbiome might contribute to common gynaecological pathologies.[35]

It is also important to note that cluster analysis is based on differentially expressed genes between the groups of interest and that our analyses included both up and downregulated genes (see Tables 3-5 for genes that were up or down regulated in Control versus HMB groups). In addition, the changes in gene expression between Control and HMB groups were not consistent enough to allow a z-score and therefore an activation status to be calculated for the various

upstream regulators identified. For this reason, further functional studies will be required to elucidate the regulatory mechanisms of inflammatory and immune response in HMB.

Also listed in the inflammation and adhesion clusters were the integrin *ITGAX* (CD11c) and the common leucocyte antigen *PTPRC* (CD45). *ITGAX* is a marker of dendritic cells and other leucocytes[36] and has been used to classify endometrial and decidual leucocyte populations,[37,38] whereas *PTPRC* is routinely used as a leucocyte marker in studies of endometrium. Variations in the expression of these genes may reflect differences in the relative density and phenotype of endometrial leucocyte populations between HMB and control endometrium; previous studies have already suggested that endometrial leucocyte (e.g. uterine natural killer cells) populations may be dysregulated in HMB.[39] Few of the genes listed in the inflammation and adhesion clusters (Table 3) have been examined in relation to menstruation or uterine bleeding. In addition, since the key functional differences of interest are likely reflected in patterns of protein production and post-translational protein modifications, it is important that these functional properties are considered in carefully characterised uterine tissues accounting for HMB of different aetiologies.

In addition to the upstream inflammatory regulators noted above, several steroid hormones were identified as potential regulators of the variations in bleeding volume associated with Controls versus HMB (androgen, dexamethasone, estrogen receptor 1, and 17-alpha-ethinylestradiol). Unfortunately, the direction of change of the different genes involved did not allow for the determination of a z-score for these hormones and therefore we do not know whether there has been a change in activation state. However, our observation is consistent with previous studies that have suggested that HMB may result from a deficiency in glucocorticoid within the endometrium that may limit vasoconstriction and angiogenesis.[13,40] Further, Rae et al. have demonstrated that the enzyme that inactivates cortisol (11βHSD2) is increased in the endometrium of women with HMB.[40] A clinical trial exploring the potential use of the glucocorticoid dexamethasone as a treatment for HMB is currently underway.[41]

Our analyses demonstrate that uterine fibroids are associated with differences in endometrial gene expression, suggesting different underlying mechanisms for HMB in this population. Canonical pathways identified in IPA as common to the Fibroid group included OX40 signalling (TNF receptor that functions as a T cell activator[42]), B cell development, the antigen signalling pathway and allograft rejection signalling. These pathways all reflect the presence of significant expression differences for a group of human leukocyte antigen (HLA) genes (discussed below) between the Fibroid and both Control and HMB groups. Further, functional clusters identified by DAVID as unique to the Fibroid group included genes related to haemoglobin and oxygen transport (see below), as well as antigen processing and the MHC complex. These common genes in HMB and Control groups relative to the Fibroid group are likely to be highly informative (as are the differences in gene expression) when considering the pathways responsible for fibroid-related symptoms and the mechanisms that may induce HMB.

The common MHC complex cluster differentially expressed in the Fibroid group included the HLA class II (beta chain) molecules *HLA-DPB1*, *HLA-DQA1*, *HLA-DQB1*, *HLA-DRB1*, *HLA-DRB4*. The class II HLA molecules are heterodimers containing an alpha and beta chain that are expressed on antigen presenting cells such as T cells and macrophages; expression may also be upregulated in other cells such as endothelial cells in response to interferon-γ during inflammation.[43] Whether the variations in HLA expression reflect functional abnormalities in antigen presentation or differences in the type and number of antigen-presenting cells present in the endometrium of women with uterine fibroids is unknown.

Unexpectedly, we found that a gene cluster related to oxygen transport and haemoglobin was upregulated in endometrial samples from women with uterine fibroids. This included the α-globin genes *HBM*, *HBQ1* and *HBZ*, the β-globin genes *HBG1/2* and *HBD*, and a gene associated with heme production (5-aminolevulinate synthase *2, ALAS2*).[44,45] However, as these particular β-globin genes are not currently annotated to specific canonical pathways in IPA, this finding was not detected by IPA analysis. Haemoglobins are abundant proteins whose production is traditionally thought to be

restricted to erythrocytes. They are made up of two alpha and two beta globin peptides and a heme group and have a high affinity for and the ability to reversibly bind with oxygen.[46] It is possible that the presence of a haemoglobin-related gene cluster reflects the large number of erythrocytes in menstrual-phase endometrial biopsies. However, studies have shown that mature human erythrocytes only contain low amounts of small-sized RNAs such as microRNAs, but lack the ribosomal and larger sized RNAs.[47] Further, the dogma that haemoglobin production is restricted to erythroid cells has been challenged. Haemoglobins are produced by several cell type (macrophages, various epithelia) and their long evolutionary history and presence early in embryonic development is hypothesised to support a broader role in protecting cells against oxygen,[48] in pathogen recognition, inflammation, and antimicrobial and antioxidative functions.[49] Haemoglobin expression has been demonstrated in normal human endometrium,[50] and in decidual tissues from pregnant women (5-8 weeks gestation)[51] with the Shiota et al. hypothesising a role for haemoglobins in oxygen supply prior to the development of foetal-placental circulation. The expression of haemoglobin-related genes in menstrual-phase endometrium and during pregnancy suggests these proteins have key roles in endometrial homeostasis. How and whether the presence of uterine fibroids impacts on their expression and function in endometrium warrants further attention.

These studies have provided the first data about global gene expression in menstrual phase endometrium from women with and without HMB. Our use of the sophisticated RUV-inv technique meant we could identify key genes and gene sets of interest for further analysis despite the variation inherent in our endometrial biopsies (day of menstruation when the biopsy was collected; duration of menstruation, patients definition of what constituted 'bleeding' or 'spotting', relative proportion of endometrial tissue that is breaking down, repairing or repaired in any one biopsy; mixed population of epithelial, stromal and other cells types).[17,30] However, it is not always possible to replicate the results obtained by RUV-inv when genes are individually examined using PCR. This is due, in part, to RUV-inv's ability to partially remove unwanted biological variability among women allowing more subtle gene expression changes to be detected; this variability among women cannot

be removed in analyses of individual genes using PCR. In the current studies, this meant the differences noted in the gene arrays were not statistically significant when individual genes of interest were examined using quantitative PCR; although, several of the genes examined showed patterns consistent with the RUV-inv observations from the gene array analysis. In addition, carefully characterised menstrual phase biopsies were logistically difficult to collect, particularly as considerable care was taken to ensure accurate details about the menstrual characteristics and presence/absence of comorbidities, which restricted the sample size and meant that we lacked a separate cohort of samples for validation. It should also be noted that women were classified as having HMB based on self-report rather than an objective measure of blood loss. While objective measures also have significant limitations, the perception of what constitutes heavy bleeding can vary among women and it is possible that some women reporting HMB were actually bleeding at levels at the upper limit or even within the normal range (≤80 ml). For these reasons, ongoing mechanistic studies will be required to determine how the identified genes, gene pathways, gene groupings and upstream regulators impact on menstrual bleeding patterns; of particular interest will be studies considering the role of infection or variations in the microbiome in HMB. Despite these limitations however, our data have provided numerous testable hypotheses that will advance our understanding of the mechanisms of HMB.

In summary, we have used gene array analysis and the bioinformatics technique RUV-inv to identify genes and functional gene clusters that vary in menstrual phase endometrial biopsies from women with regular menstrual cycles with and without HMB and in women with uterine fibroids. Comparisons between women with regular menstrual cycles and with/without HMB suggest that the excess bleeding may reflect deviations in the immune and inflammatory response. Our results also suggest that different mechanisms are responsible for the HMB seen in women with/without uterine fibroids with differentially expressed genes related to haemoglobin, antigen processing and the MHC complex identified in women with these tumours. Overall, our results provide alternative genes and gene groupings of interest for future research aimed at understanding the different molecular

mechanisms responsible for HMB and for identifying potential novel targeted therapies aimed at managing HMB of different aetiology.

ACKNOWLEDGMENTS

We would like to thank the women who generously donated tissue to this study. We would also like to acknowledge the role and expertise of the Research Nurses and Drs Catarina Ang, Wan Tinn Teh and Stefan Kane and other clinical staff at the Royal Women's Hospital who contributed to this study. We would like to acknowledge Marie Everest from AGRF for her assistance with the expression arrays and RNA quality determination.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

Figure Captions

Figure 1: Venn diagram illustrating the distribution of differentially expressed genes in menstrual-phase endometrial biopsies from women with regular menstrual cycles with (n=10 samples) and without (Controls, n=7 samples) heavy menstrual bleeding (HMB), and women with uterine fibroids (mixed bleeding patterns, n=7 samples).

Figure 2: Expression of *HBG1/2* (A), *HBD* (B), *HBM* (C), *HBQ1* (D), *HBZ* (E) and *ALAS2* (F) in menstrual-phase endometrial biopsies from women with and without heavy menstrual bleeding (HMB). A further group included endometrial biopsies from women with uterine fibroids. Fold change was calculated using ΔΔCT with *RPL13A* as the housekeeping gene. Numbers below group headings represent crossing threshold values (median [range]). Points coloured grey are extra samples that were not in the original gene array. In gene array analyses, all genes were upregulated in the Fibroid group relative to Control and HMB groups with no significant difference between the HMB and Fibroid groups.

Figure 3: Expression of *SCGB1D2* (A), *SCGB2A2* (B), *SCGB3A1* (C), and *TFF3* (D) in menstrual-phase endometrial biopsies from women with and without heavy menstrual bleeding (HMB). A further group included endometrial biopsies from women with uterine fibroids. Fold change was calculated using ΔΔCT with *RPL13A* as the housekeeping gene. Numbers below group headings represent crossing threshold values (median [range]) from quantitative PCR. Points coloured grey are extra samples that were not in the original gene array. In gene array analyses, all genes were upregulated in the Control group relative to HMB and Fibroid groups; *SCGB3A1* was also significantly upregulated in the Fibroid relative to the HMB group.

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Table 1: Summary of Patient Characteristics used in Array Analysis

Group	n	Age (Median [range])	Day of cycle (Median [range])	Uterine fibroids	Notes (including other comorbidities)
Control (regular menstrual cycles*)	7	38 [23-44]	3 [2-4]**	No	Ovarian cyst noted on one ovary of one patient
Extras for PCR	*2*	*29,36*	*3-4*	*No*	*No comorbidities detected*
HMB (regular menstrual cycles*)	10	38 [31-44]	3 [2-5]	No	No comorbidities detected
Extras for PCR	*6*	*32 [24-40]*	*4 [3-4]***	*No*	*No comorbidities detected*
Uterine fibroid	7	41 [34-47]	3 [1-4]***	Yes	• Five samples: Subjects with HMB of which four had regular menstrual cycles (including one with a uterine polyp) and one had non-defined cycle regularity. • One sample: Participant with a regular menstrual cycle and no HMB. • One sample: Participant whose bleeding pattern and regularity could not be defined.
Extras for PCR	*1*	*44*	*4*	*Yes*	*No comorbidities detected*

* Regular Menstrual cycles as per FIGO classification (Cycle-to-cycle variation of 2-20 days) (Munro, Critchley and Fraser, 2012).

** Note: The day of cycle could not be verified from a bleeding diary for one of these samples.

*** Note: The day of cycle could not be further verified from a bleeding diary for two of these samples.

HMB: Heavy Menstrual Bleeding (self-reported)

Table 2: Top 20 genes with the highest fold change (up and down regulated) in comparisons between Controls, heavy menstrual bleeding and Fibroids

Upregulated in HMB versus controls (top 20 of 92 genes)

Gene Symbol	Gene Name	Adj. p value	Fold Change
HLA-A*	Major histocompatibility complex, class I, A	2.0E-2	4.2
IL6ST	Interleukin 6 signal transducer	2.3E-4	3.3
APCDD1L	Adenomatosis polyposis coli down-regulated 1-like	4.6E-3	3.0
TBX3	T-box 3	1.0E-3	2.9
UBN1*	Ubinuclein 1	1.5E-4	2.8
DSP*	Desmoplakin	2.4E-4	2.7
SDCBP2	Syndecan binding protein (syntenin) 2	8.3E-4	2.7
EZH2	Enhancer of zeste 2 polycomb repressive complex 2 subunit	2.2E-4	2.7
UHMK1	U2AF homology motif (UHM) kinase 1	1.1E-3	2.6
NR2C2	Nuclear receptor subfamily 2, group C, member 2	4.5E-4	2.5
MIR1282*	MicroRNA 1282	8.2E-4	2.5
TMED6*	Transmembrane p24 trafficking protein 6	2.3E-4	2.5
VAV3	Vav 3 guanine nucleotide exchange factor	2.3E-2	2.5
CDC42BPA	CDC42 binding protein kinase alpha (DMPK-like)	4.5E-2	2.5
C17orf75	Chromosome 17 open reading frame 75	3.7E-4	2.4
MB21D1	Mab-21 domain containing 1	1.3E-3	2.4
PTPRC	Protein tyrosine phosphatase, receptor type, C	2.2E-4	2.4
WISP1	WNT1 inducible signaling pathway protein 1	3.8E-3	2.4
CDC27	Cell division cycle 27	1.4E-3	2.4

FSD1L	Fibronectin type III and SPRY domain containing 1-like	4.1E-4	2.4

Downregulated in HMB versus controls (top 20 of 84 genes)

Gene Symbol	Gene Name	Adj. p value	Fold Change
BPIFB1 (C20orf114)*	BPI fold containing family B, member 1	4.6E-3	9.3
SCGB3A1*	Secretoglobin, family 3A, member 1	2.7E-4	9.0
TFF3*	Trefoil factor 3 (intestinal)	7.0E-3	5.6
SCGB1D2*	Secretoglobin, family 1D, member 2	2.3E-4	5.6
SCGB2A2*	Secretoglobin, family 2A, member 2	2.7E-4	5.2
PRODH	Proline dehydrogenase (oxidase) 1	4.3E-4	4.2
MFF	Mitochondrial fission factor	2.3E-4	4.2
TSPAN8	Tetraspanin 8	9.6E-4	3.2
CXCL6*	Chemokine (C-X-C motif) ligand 6	2.0E-3	3.2
SPDYE2	Speedy/RINGO cell cycle regulator family member E2	7.6E-4	3.1
FCGBP	Fc fragment of IgG binding protein	6.4E-4	2.9
IRX6	Iroquois homeobox 6	4.4E-4	2.8
ADAM10	ADAM metallopeptidase domain 10	1.5E-4	2.8
MUC5AC*	Mucin 5AC, oligomeric mucus/gel-forming	1.2E-2	2.8
TRHDE-AS1 (LOC283392)	TRHDE antisense RNA 1	5.0E-4	2.8
CYP26A1	Cytochrome P450, family 26, subfamily A, polypeptide 1	8.6E-4	2.7
SAA2*	Serum amyloid A2	1.2E-3	2.7

MMEL1	Membrane metallo-endopeptidase-like 1	1.2E-4	2.6
GRIP2	Glutamate receptor interacting protein 2	1.2E-3	2.6
SAA1*	Serum amyloid A1	1.5E-3	2.6

Upregulated in Fibroid vs. Control (top 20 of 406 genes)

Gene Symbol	Gene Name	Adj. p value	Fold Change
HLA-A*	Major histocompatibility complex, class I, A	2.3E-4	14.2
SCARNA27*	Small Cajal body-specific RNA 27	3.5E-5	7.8
HLA-DQA1*	Major histocompatibility complex, class II, DQ alpha 1	1.8E-3	5.1
MIR1282*	MicroRNA 1282	1.7E-6	5.0
PRELID3B (SLMO2)	PRELI domain containing 3B	2.1E-5	4.4
SPRED* (LOC644701_)	Sprouty-related, EVH1 domain containing 1	8.3E-6	4.4
DSP*	Desmoplakin	1.9E-5	4.3
LUC7L	LUC7-like	8.1E-6	4.1
HBZ*	Hemoglobin, zeta	6.0E-5	4.0
ALAS2*	5'-aminolevulinate synthase 2	1.2E-3	4.0
PJA1	Praja ring finger 1, E3 ubiquitin protein ligase	8.6E-6	3.9
TMED6*	Transmembrane p24 trafficking protein 6	1.2E-3	3.8
HBG1*	Hemoglobin, gamma A	1.2E-3	3.8
HBD*	Hemoglobin, delta	4.3E-4	3.7
LOC400743	Uncharacterized LOC400743	6.4E-5	3.7
UBN1*	Ubinuclein 1	1.9E-5	3.5
HBM*	Hemoglobin, mu	6.3E-4	3.5

HMGA1	High mobility group AT-hook 1	9.6E-6	3.4
PITX1	Paired-like homeodomain 1	1.9E-5	3.3
FOXR1	Forkhead box R1	4.9E-5	3.3

Downregulated in Fibroid vs. Control (top 20 of 235 genes)

Gene Symbol	Gene Name	Adj. p value	Fold Change
HLA-DRB1*	Major histocompatibility complex, class II, DR beta 1	4.4E-4	24.4
BPIFB1 (C20orf114)*	BPI fold containing family B, member 1	2.2E-4	21.8
SCGB1D2*	Secretoglobin, family 1D, member 2	1.3E-5	9.7
TFF3*	Trefoil factor 3 (intestinal)	2.2E-3	6.3
CXCL6*	Chemokine (C-X-C motif) ligand 6	5.3E-5	5.5
SAA2*	Serum amyloid A2	1.6E-5	5.4
SCGB3A1*	Secretoglobin, family 3A, member 1	6.2E-4	5.1
MUC5AC*	Mucin 5AC, oligomeric mucus/gel-forming	4.0E-4	5.0
HP	Haptoglobin	9.4E-4	4.1
SCGB2A2*	Secretoglobin, family 2A, member 2	2.8E-4	4.0
MUC4	Mucin 4, cell surface associated	6.4E-4	3.9
MICA*	MHC class I polypeptide-related sequence A	2.3E-6	3.9
SCNN1G	Sodium channel, non voltage gated 1 gamma subunit	4.3E-6	3.7
SIGLEC14	Sialic acid binding Ig-like lectin 14	3.9E-3	3.6
LRRK2	Leucine-rich repeat kinase 2	4.6E-5	3.5
DEFB4A*	Defensin, beta 4A	1.1E-3	3.5
FOLR1	Folate receptor 1 (adult)	1.3E-4	3.4

SAA1*	Serum amyloid A1	9.6E-5	3.4
IL33	Interleukin 33	1.9E-5	3.4
GPR128	Adhesion G protein-coupled receptor G7	1.1E-2	3.3

Upregulated in HMB relative to Fibroids (top 20 of 77)

Gene Symbol	Gene Name	Adj. p value	Fold Change
HLA-DRB1*	Major histocompatibility complex, class II, DR beta 1	2.7E-2	5.8
CCL4L2	Chemokine (C-C motif) ligand 4-like 2	7.9E-5	5.2
TEKT4P2 (LOC100132288)	Tektin 4 pseudogene 2	2.7E-4	3.9
CCL3L1	Chemokine (C-C motif) ligand 3-like 1	2.3E-3	3.3
CFTR	Cystic fibrosis transmembrane conductance regulator (ATP-binding cassette sub-family C, member 7)	5.8E-5	3.0
GPR109B	Hydroxycarboxylic acid receptor 3	1.1E-3	2.9
CLEC12A	C-type lectin domain family 12, member A	3.0E-4	2.9
SERPINB7	Serpin peptidase inhibitor, clade B (ovalbumin), member 7	5.8E-3	2.8
VPS16	Vacuolar protein sorting 16 homolog (S. cerevisiae)	4.6E-5	2.8
XDH	Xanthine dehydrogenase	2.2E-4	2.8
GPR84	G protein-coupled receptor 84	7.6E-4	2.7
SCEL	Sciellin	3.1E-4	2.7
ODF2L	Outer dense fiber of sperm tails 2-like	3.7E-3	2.7
HECTD2	HECT domain containing E3 ubiquitin protein ligase 2	5.8E-5	2.7

DEFB4A*	Defensin, beta 4A	1.0E-2	2.5
RNASE1	Ribonuclease, RNase A family, 1 (pancreatic)	1.0E-3	2.5
MICA*	MHC class I polypeptide-related sequence A	7.9E-5	2.5
CTAGE6P	CTAGE family, member 6	3.1E-4	2.4
KRT6A	Keratin 6A, type II	1.5E-2	2.4
CLCA4	Chloride channel accessory 4	7.5E-4	2.4

Downregulated in HMB relative to Fibroids (top 20 of 136 genes)

Gene Symbol	Gene Name	Adj. p value	Fold Change
ALAS2*	5'-aminolevulinate synthase 2	2.9E-4	7.2
HLA-DQA1*	Major histocompatibility complex, class II, DQ alpha 1	1.4E-3	5.7
HLA-DRB4	Major histocompatibility complex, class II, DR beta 4	7.9E-3	4.8
HBM*	Hemoglobin, mu	5.6E-4	4.4
MIR1307	MicroRNA 1307	7.1E-4	4.0
HBG1*	Hemoglobin, gamma A	1.7E-3	3.8
DNASE1L1	Deoxyribonuclease I-like 1	2.9E-4	3.8
GSTT2B	Glutathione S-transferase theta 2B	1.9E-4	3.5
HBZ*	Hemoglobin, zeta	3.1E-4	3.4
SCARNA27*	Small Cajal body-specific RNA 27	1.8E-3	3.3
SPRED* (LOC644701_)	Sprouty-related, EVH1 domain containing 1	7.6E-5	4.4
TRAF4	TNF receptor-associated factor 4	2.2E-4	3.3
FOLR3	Folate receptor 3 (gamma)	1.8E-3	3.3
HBD*	Hemoglobin, delta	2.0E-3	3.2

HBG2	Hemoglobin, gamma G	3.3E-3	3.0
FOXE3	Forkhead box E3	4.6E-5	2.9
TOX2	TOX high mobility group box family member 2	3.9E-5	2.8
CILP	Cartilage intermediate layer protein, nucleotide pyrophosphohydrolase	6.3E-4	2.8
MMP26	Matrix metallopeptidase 26	3.6E-3	2.8
HLA-DQB1	Major histocompatibility complex, class II, DQ beta 1	1.0E-2	2.8

*Genes present in one or more top 20 up or down-regulated gene lists.

Table 3: Significant Canonical Pathways identified using Ingenuity Pathway Analysis

Ingenuity Canonical Pathways	-log(p-value)*	Ratio**	Molecules
CONTROL versus HMB			
Acute Phase Response Signalling *(rapid inflammatory response occurring soon after infection, trauma or other inflammatory processes)*	1.45	4.19E-02 (7/167)	C3, IL6ST, LBP, ORM1, OSM, SAA1, SAA2
LXR/RXR Activation *(Liver X and retinoid X receptors are involved in mediating the numerous retinoid actions including inflammation)*	1.45	4.96E-02 (6/121)	APOD, C3, LBP, ORM1, SAA1, SAA2
FXR/RXR Activation *(Farnesoid X and retinoid X receptors are involved with various metabolic pathways)*	1.45	4.8E-02 (6/125)	APOD, C3, CREBBP, ORM1, SAA1, SAA2
CONTROL versus FIBROID			
B Cell Development	3.11	3.04E-01 (7/23)	CD19, CD79A, HLA-DQA1, HLA-DQB1, HLA-DRB1, IL7R, PTPRC
Acute Phase Response Signalling	1.86	8.98E-02 (15/167)	CFB, FGA, FN1, HP, IL33, IL6ST, LBP, PDPK1, PLG, RBP7, SAA1, SAA2, SERPINA3, SOCS4, TNFRSF11B

OX40 Signalling Pathway	1.4	1.46E-01	CD3G, HLA-A, HLA-C, HLA-DPB1, HLA-DQA1, HLA-
(OX40 is involved in T cell activation)		(7/48)	DQB1,HLA-DRB1,
Antigen Presentation Pathway	1.4	1.67E-01	HLA-A, HLA-C, HLA-DPB1, HLA-DQA1, HLA-DRB1,
		(6/36)	TAP2
Allograft Rejection Signalling	1.39	1.54E-01	HLA-A, HLA-C, HLA-DPB1, HLA-DQA1, HLA-DQB1,
(Allorecognition of non-self antigens, involves a T cell response.)		(6/39)	HLA-DRB1
HMB versus FIBROID			
Allograft Rejection Signalling	2.56	1.28E-01	HLA-DPB1, HLA-DQA1, HLA-DQB1, HLA-DRB1, HLA-
		(5/39)	DRB4
Agranulocyte Adhesion and Diapedesis (lymphocytes and monocytes)	2.56	5.14E-02	CCL2, CCL3L1, CCL3L3, CCL4, CCL4L1/CCL4L2, CXCL5,
(Migration from vascular system to site of infection and inflammation)		(9/175)	IL1RN, MMP26, MYL3
Differential Regulation of Cytokine Production in Intestinal Epithelial Cells by	2.56	1.74E-01	CCL2,CCL4, DEFB103A/DEFB103B, DEFB4A/DEFB4B
IL-17A and IL-17F		(4/23)	
(IL-17A and IL-17F are interleukins linked to infection, inflammation and			
autoimmune disease)			
Communication between Innate and Adaptive Immune Cells	2.56	8.11E-02	CD8A, CCL3L3, CCL4, HLA-DRB4, HLA-DRB1, IL1RN
		(6/74)	

OX40 Signalling Pathway	2.54	1.04E-01 (5/48)	HLA-DPB1, HLA-DQA1, HLA-DQB1, HLA-DRB1, HLA-DRB4
Granulocyte Adhesion and Diapedesis (neutrophils, basophils and eosinophils)	2.38	4.85E-02 (8/165)	CCL2, CCL3L1, CCL3L3, CCL4, CCL4L1/CCL4L2CXCL5, IL1RN, MMP26
Antigen Presentation Pathway	2.05	1.11E-01 (4/36)	HLA-DPB1, HLA-DQA1, HLA-DRB1, HLA-DRB4
Graft-versus-Host Disease Signalling	1.98	1.03E-01 (4/39)	HLA-DQA1, HLA-DQB1, HLA-DRB1, IL1RN
CDC42 Signalling	1.71	4.88E-02 (6/123)	HLA-DPB1, HLA-DQA1, HLA-DQB1, HLA-DRB1, HLA-DRB4, MYL3
B Cell Development	1.63	1.3E-01 (3/23)	HLA-DQA1, HLA-DQB1, HLA-DRB1

*-log(p-value) after right-tailed Fisher Exact Tests and Benjamini-Hochberg Multiple Correction. A p-value of 0.05 equates to a –log(p-value) of 1.3; 0.01 equates to 2, and 0.001 to 3.

**Ratio of genes within our gene list to the total number of genes in the canonical pathway.

Table 4: Top 12 Significant Upstream Regulators identified using Ingenuity Pathway Analysis for each comparison

Upstream Regulator	Molecule Type	Predicted Activation State*	Activation z-score**	p-value of overlap***	Target molecules in dataset
HMB versus Control Groups					
STAT5A¥	Signal transducer and activator of transcription 5A (pleiotrophic transcription regulator)			8.80E-07	AGR2, CCL3L3, ERAP2, EZH2, FOSB, IL6ST, OSM, PIP, SLC34A2, TFF3, TNFRSF18, TSPAN8
TCF	Group of T-cell factor related transcription factors.			4.77E-06	APOD, CST4, HSD17B2, KRT5, SAA1, SCGB2A2, TSPAN8
Androgen	Steroid hormone		-1.206	2.07E-05	AGR2, APOD, DNMT3A, KAT5, PIP, PTPRA, PTPRC, VAV3
IL1B¥	Interleukin 1, beta: pro-inflammatory cytokine		-1.545	2.16E-05	C3, CCL3L1, CXCL6, DNMT3A, FOSB, HBEGF,HLA-A, IL19, LBP, MUC4, MUC5AC, NFAT5, ORM1, OSM, PIGR, PTGDS, SAA1, SAA2
ESR1	Estrogen receptor 1: ligand-dependent nuclear receptor		1.621	2.58E-05	BCLAF1, C3, CDC27, CDC42BPA, CREBBP, DST, FBXW7, FOLR1, FOSB, HBEGF, IGF2, IL6ST,

				ITGA2B, LBP, MUC5AC, NFAT5, NFATC1, NLGN4X, SLC39A8, SOSTDC1, TFF3, TNFRSF18, UHMK1, VAV3
MYD88	Myeloid differentiation primary response 88: involved in innate and adaptive immune response	-0.854	2.60E-05	C3, CCL3L3, CXCL6, DNMT3A, HLA-A, ITGAX, MUC5AC, SAA1, WISP1
Dexamethasone¥	Synthetic glucocorticoid	-1.898	4.36E-05	ADAM10, APOD, BCLAF1, C3, CCL3L3, CXCL6, DST, FOSB, HBEGF, IGF2, IL6ST, LBP, MT1H, MT1M, MUC5AC, NFATC1, ORM1, OSBPL1A, OSM, PTGDS, SAA1, SAA2, SLC5A1, TNFRSF18, TRPV1, UTS2
17-alpha-ethinylestradiol	Steroid hormone (estrogen)	-0.342	6.72E-05	C3, CCL3L3, CXCL6, PTGDS, SLC34A2, TFF3, UHMK1
IL6	Interleukin 6: pro-inflammatory cytokine	-0.319	8.48E-05	C3, CCL3L1, CCL3L3, CXCL6, EZH2, HLA-A, IGF2, IL6ST, LBP, ORM1, OSBPL1A, PTPRC, SAA1, SAA2, SCGB2A2
Poly rI:rC-RNA	Polyinosinic-cytidylic (poly(rI:rC)) dsRNA:	0.900	1.14E-04	ADAM10, C3, CCL3L1, CCL3L3, DNMT3A, HLA-A,

	synthetic mimic of double-stranded viral RNA			ITGAX, NLRP3, PIGR, ST8SIA4, TRPV1
Lipopolysaccharide[¥¥]	LPS: found in the outer membrane of gram-negative bacteria	-0.045	1.26E-04	AKAP2, C3, CCL3L3, CREBBP, CXCL6, FOSB, HBEGF, HLA-A, IL19, IL6ST, ITGAX, LBP, MUC5AC, NFAT5, NFATC1, NLRP3, ORM1, OSM, PIGR, PTGDS, SAA1, SAA2, SLC39A8, SLC5A1, WISP1
Phorbol myristate acetate[¥]	Phorbol myristate acetate: chemical drug with potent tumour promoter activity, activates protein kinase C and NF-kB.	-0.096	1.29E-04	C3, CCL3L1, CCL3L3, CREBBP, FOSB, HBEGF, HSD17B2, IGF2, ITGA2B, ITGAX, MUC4, MUC5AC, NFAT5, NFATC1, OSM, PTGDS, TNFRSF18, UTS2
Control versus Fibroid groups				
IL1B[¥]	Interleukin 1, beta: pro-inflammatory cytokine	0.356	7.61E-12	ANGPTL4, ARC, BAX, CCR5, CCR7, CFB, CFTR, CHI3L1, CXCL6, CYP1A1, DDIAS, DEFB4A/DEFB4B, FGFR3, FN1, FOSB, FOXO1, GATA3, GEM, GSTA1, HBEGF, HLA-A, HMGA1, HP, HSD11B1, HSPG2, IGFBP1, IL19, IL1RL1, IL23A, IL24, IL2RA, IL33, LBP, MMP14, MMP3, MUC4, MUC5AC, NR4A2, PIGR, PLA2G4A, PLK3, RORA, SAA1, SAA2, SCNN1G, SCX, SDC1, SERPINA3, SLC1A2, SLC1A3,

				SLCO1B1, TAP2, TGM1, TNFRSF11B, VCAM1, XYLT1
Phorbol myristate acetate[¥]	Phorbol myristate acetate: chemical drug with potent tumour promoter activity, activates protein kinase C and NF-kB.	1.644	5.43E-10	ANGPTL4, B3GNT5, BAX, BMP7, CA8, CCR7, CERK, CFTR, CPE, CTLA4, CYP1A1, CYP2A6, CYR61, DEFB4A/DEFB4B, FOSB, GATA3, GEM, GSTA1, HBEGF, HMGA1, HSD11B1, HTR7, IGF2, IGFBP1, IL17C, IL23A, IL24, IL2RA, IL7R, ITGA2B, KRT34, KRT83, LPL, MICA, MMP14, MMP3, MPZ, MUC4, MUC5AC, MYADML2, NCF1, NFATC1, NPR3, NR4A2, P2RY2, PABPC4L, PDPK1, POU2AF1, SERPINA3, SERPINB7, SHISA7, ST3GAL1, TGM1, TRAF4, ULBP2, UTS2, VCAM1
FLT3LG	Fms-related tyrosine kinase 3 ligand. Associated with haematopoiesis.	0.971	1.09E-08	CCR5, CCR7, CD19, CD3G, CD79A, HOXB4, IL1RL1, IL2RA, IL7R, PTPRC, ROCK1
TNF	Tumor necrosis factor: multifunctional pro-inflammatory cytokine	-0.523	1.88E-08	ALAD, ANGPTL4, APLN, ARC, ARSI, BAX, CCR5, CCR7, CD5, CDKN2C, CFB, CFTR, CHI3L1, CHST2, CTLA4, CXCL6, CYP1A1, CYR61, DDIAS,

				DEFB4A/DEFB4B, DLX1, FN1, FOSB, FUT3, GEM, GSTA1, HBEGF, HLA-A, HP, HSD11B1, HSPG2, IFNE, IGF2, IGFBP1, IL17C, IL19,IL 1RL1, IL23A, IL24, IL2RA, IL33, IL7R, KRT34, KRT6B, LBP, LPL, MECOM, MICA, MMP14, MMP3, MSLN, MST1, MTHFD2L, MUC4, MUC5AC, NCF1, NFATC1, NR4A2, PC, PIGR, PLA2G4A, PLK3, RABEP2, RRAD, SAA1, SCNN1G, SEMA3C, SERPINA3, SLC1A2, SLC1A3, TNFRSF11B, TNNC1, TRAF4, VCAM1, WISP1
IL1¥¥¥	Group of interleukin genes including IL1B.	-0.694	2.69E-08	BAX, CCR7, CD34,CFB, CHI3L1, CYP1A1, DEFB4A/DEFB4B, FN1, GGT1, HP, HSD11B1, IGFBP1, IL1RL1, IL2RA, IL33, KRT72, MMP3, PIGR, PLA2G4A, RORA, SAA1, SAA2, TNFRSF11B, UGT2B17, VCAM1, XDH
STAT5A¥	Signal transducer and activator of transcription 5A (pleiotrophic transcription	-0.808	4.75E-08	AGR2, BAX, CCR5, ERAP2, EZH2, FOSB, FOXA1, IL24, IL2RA, IL6ST, IL7R, MUC13, NR4A2, PROM2,

	regulator)			PRR15L, RNF39, RORA, SERPINA3, SLC34A2, SYTL2, TFF3, TNNC1, TRAF4, TRIM31, TSPAN8
Resveratrol	A polyphenol produced by several plans in response to injury or pathogens.	1.199	2.17E-07	BAX, CYP1A1, DEFB4A/DEFB4B, FN1, FOXO1, FOXO3, GSTA1, IGF2, IGFBP1, IL2RA, IL7R, ITGA2B, MICA, MMP3, MUC5AC, NFATC1, ROCK1, SCX, TGM1, TNFRSF11B, ULBP2, VCAM1, ZNF91
Tretinoin	A pharmaceutical form of retinoic acid.	-0.813	2.67E-07	ABCG1, ADAM10, ANGPTL4, ANXA8/ANXA8L1, APLN, CA8, CCL4L1/CCL4L2, CCR5, CD34, CDKN2C, CFTR, CGGBP1, CLC, CPE, CTLA4, CYP1A1, CYR61, DEFA1, DEFA4, DHRS9, DSC1, DSP, FAM153A/FAM153B, FN1, FOXA1, FOXO3, GGT1, GNAO1, HBEGF, HLA-A, HLA-C, HMGA1, HOXA2, HOXB4, HSD11B1, HSF4, IFIT2, IGF2, IL6ST, ITGA2B, KRT5, KRT72, LBP, LRRK2, MECOM, MMP14, MMP3, MSLN, MST1, MUC4, MUC5AC, NCF1, NFATC1, NR2C2, OLFM4,

					P2RY11, P2RY2, PDPK1, PLK3, PTPRC, RPL7, SHROOM1, SMAD5, SUMO1, TGM1, TNFRSF11B, VCAM1, VGLL3
SFTPA1	Surfactant protein A1: Lung surfactant protein that has a role in surfactant homeostasis and in defence against pathogens.	**Inhibited in Fibroid**	-2.887	4.33E-07	AGR3, ANGPTL4, CYR61, FOSB, HBEGF, HBG1, HBG2, IL1RL1, IL2RA, LGALS7/LGALS7B, SLC22A3, ST3GAL1
TGFB1	Transforming growth factor, beta 1: pleiotrophic cytokine	**Activated in Fibroid**	2.221	6.20E-07	ABCG1, ADAM10, ADGRB1, AGR2, ANGPTL4, ANKLE2, ANXA8/ANXA8L1, APLN, BAX, BMP7, CCR5, CCR7, CD34, CDKN2C, CHI3L1, CTLA4, CXADR, CYR61, DOK1, DSP, FN1, FOSB, FOXO1, FOXO3, GABBR1, GATA3, GEM, GNAO1, GRHL2, GRIN2B, HAS3, HBEGF, HLA-DQA1, HLA-DQB1, HMGA1, HOXA2, HSPG2, IGF2, IL1RL1, IL23A, IL2RA, IL33, KDELR3, LPL, LSR, MMP14, MMP3, MPZ, MSMB, MUC4, MUC5AC, MXI1, NCF1, NFATC1, NR4A2, OSR2, PLA2G4A, PORCN, PTPRC, RORA, RRAD, SDC1, SERPINA3, SLC16A9,

Dexamethasone[¥]	Synthetic glucocorticoid	0.277	7.13E-07	SLC1A2, SMTN, TBX3, TGFB1I1, TMOD3, TNFRSF11B, VCAM1, WISP1, XDH, XYLT1 ACKR4, ADAM10, ANGPTL4, APLN, B3GNT5, BAX, CA8, CFB, CGGBP1, CHI3L1, CPE, CXADR, CXCL6, CYP1A1, CYP2A6, CYR61, DEFB4A/DEFB4B, DOK1, DST, ECT2, FGA, FN1, FOSB, FOXO1, FOXO3, GATA3, GEM, GSTA1, HBEGF, HLA-DPB1, HLA-DQB1, HLA-DRB1, HMGA1, HP, HSD11B1, IGF2, IGFBP1, IL1RL1, IL2RA, IL33, IL6ST, IL7R, KIR2DS4, LBP, LPL, LRRK2, MMP14, MMP3, MSMB, MUC5AC, NFATC1, NPR3, NTN4, OSBPL1A, P2RY2, PC, PER3, RPL7, SAA1, SAA2, SCNN1G, SDS, SERPINA3, SLC1A2, SMAD5, TNFRSF11B, TNFSF13, TRIM31, UTS2,VCAM1,ZNF91
ATP	Adenosine triphosphate: essential for energy transfer in cells.	-0.682	7.21E-07	BAX, CCR5, CCR7, FOXO1, FOXO3, HBEGF, IL23A, IL2RA, IL33, MMP3, NFATC1, P2RY2, VCAM1

HMB versus Fibroid Groups					
Peptidoglycan	Bacterial cell wall constituent	**Activated in HMB**	2.399	7.35E-08	BCL2A1, CCL2, CCL3L3, CCL4, CXCL5, DEFB4A/DEFB4B, IL1RN, NLRP3, PRAME
Lipopolysaccharide[¥¥]	LPS: found in the outer membrane of gram-negative bacteria		1.990	8.34E-08	BCL2A1, CCL2, CCL3L3, CCL4, CD8A, CFTR, CXCL5, DEFB103A/DEFB103B, DEFB4A/DEFB4B, GPR84, HAMP, HCAR2, HCAR3, HLA-DQA1, HLA-DQB1, HP, IGFBP1, IL1RN, LRRK2, MICA, MYL3, NCF1, NLRP3, OGN, PRAME, SAA2, SCNN1G, SEPT1, SLC5A1, SPRR3, TCF7L2, TREM1, ULBP2, XDH
IL1[¥¥¥]	Group of interleukin genes including IL1B.	**Activated in HMB**	2.387	7.27E-07	CCL2, CCL4, CXCL5, DEFB103A/DEFB103B, DEFB4A/DEFB4B, HP, IGFBP1, IL1RN, PLA2G4A, S100A3, SAA2, UGT2B17, XDH
Fluticasone	Synthetic glucocorticoid.		1.245	2.87E-06	CCL2, CCL4L1/CCL4L2, CLEC12A, CXCL5, DEFB4A/DEFB4B, HLA-DPB1, HLA-DQA1, HLA-DRB4, IL1RN, RNASE1
CEBPB	CCAAT/enhancer binding protein (C/EBP), beta: important in regulation of genes		1.606	3.16E-06	BCL2A1, CCL2, CCL3L3, CCL4, CFTR, CXCL5, HAMP, HP, IGFBP1, IL1RN, PLA2G4A, SAA2, SST,

	involved in immune an dinflammatory responses.				XDH
STAT3	Signal transducer and activator of transcription 3 (acute-phase response factor): pleiotrophic transcription regulator		0.012	6.09E-06	ALAS2, CCL2, CCL3L3, CCL4, CYP26A1, DKK1, FGA, HAMP, HBG1, HLA-DQA1, HP, IGFBP1, IL1RN, MICA, PLA2G4A
SMC3	Structural maintenance of chromosome 3: important during mitosis			6.40E-06	HLA-DQA1, HLA-DQB1, HLA-DRB1
SELP	Selectin P (granule membrane protine 140kDa, antigen CD62): involved with platelet activation and degranulation, mediates interaction of endothelial cells with leucocytes.		1.342	7.85E-06	BCL2A1, CCL2, CCL3L3, CCL4, HCAR3
IL-17f dimer	Complex of IL-17f (interleukin 17F): expressed by activated T cells.	**Activated in HMB**	2.000	1.21E-05	CCL2, CCL4, DEFB103A/DEFB103B, DEFB4A/DEFB4B
STK40	Serine/threonine kinase 40: enzyme			1.88E-05	CCL2, CCL3L3, CCL4, CXCL5, HLA-DQA1
B2M	Beta-2-microglobulin: serum protein that associates with major histocompatibility		-1.067	2.41E-05	CCL3L3, CD8A, HLA-DQA1, HLA-DQB1

	complex class I heavy chain			
CD14	CD14 molecule: cell surface antigen that mediates innate immune response to bacterial LPS.	1.964	2.74E-05	BCL2A1, CCL2, CCL3L3, CCL4, DEFB4A/DEFB4B

*Predicted activation state

**Activation z-score: predicts the activation status of the hypothesised regulator (<-2: inhibited, ≥-2 and ≤2: no activation status inferred, >2: activated).

***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 (p-value ≤0.001 considered significant).

¥Upstream regulator identified for CONTROL versus HMB and FIBROID

¥¥Upstream regulator identified for HMB versus CONTROL and FIBROID

¥¥¥Upstream regulator identified for FIBROID versus CONTROL and HMB

Table 5: Summary of Functional Annotation Clusters derived from the DAVID Bioinformatics Resources 6.7 (for details of individual genes in each cluster, see Supplemental Tables 3-5)

Annotation Cluster*	Enrichment Score**	Number*** (and %¥) of genes in one or more annotations within a cluster
Control versus Heavy Menstrual Bleeding Group		
1. Functional and cellular component cluster including the extracellular space with secreted and signal peptides, disulfide bond and glycoprotein	8.13	75 (45%)
2. Inflammatory and Immune Response	2.49	28 (17%)
3. Inflammatory and Immune Response	2.39	17 (10%)
4. Cell Adhesion	2.35	14 (8.5%)
5. Cytokines	2.16	11 (6.7%)
6. Plasma Membrane	1.97	30 (18%)
7. Cystine Knot	1.77	3 (1.8%)
8. Lipoprotein	1.70	3 (1.8%)
9. Lipocalin	1.66	3 (1.8%)
10. Cell Proliferation and Migration	1.51	15 (9.1%)
11. Apoptosis	1.50	13 (7.9%)

12. Glycosaminoglycan Binding	1.48	5 (3.0%)
Control versus Fibroid Group		
1. Functional and cellular component cluster including the extracellular space with secreted and signal peptides, disulfide bond and glycoprotein	6.85	295 (52%)
2. Plasma Membrane	3.96	161 (28%)
3. Oxygen Transport and Haemoglobin	3.70	15 (2.6%)
4. Cell Adhesion	3.10	30 (5.3%)
5. Cystine knot and von Willebrand	2.81	8 (1.4%)
6. Cell migration and motility	2.75	23 (4.0%)
7. Immune Response, MHC Protein Complex	2.67	41 (7.2%)
8. Cell junctions	2.57	31 (5.4%)
9. Immunoglobulin	2.55	37 (6.5%)
10. MHC Protein Complex	2.36	12 (2.1%)
11. Protein-lipid Complex	2.16	6 (1.1%)
12. Cytokine Activity	2.02	20 (3.5%)
13. Leucocyte activation	1.98	21 (3.7%)
14. Cytoskeleton and Filament	1.97	8 (1.4%)

15. Wound Healing and Coagulation	1.90	19 (3.3%)
16. Morphogenesis	1.87	9 (1.6%)
17. von Willebrand factor	1.74	7 (1.2 %)
18. Development of Female Primary Sexual Characteristics	1.71	10 (1.8%)
19. Growth Factor Binding	1.64	9 (1.6%)
20. Inflammatory Response	1.63	17 (3.0%)
21. Chemotaxis	1.62	14 (2.5%)
22. Leucocyte Activation	1.59	18 (3.2%)
23. Glycosaminoglycan and Heparin Binding	1.56	17 (3.0%)
24. Regulation of Cell Growth	1.52	21 (3.7%)
25. Purinoreceptor	1.44	4 (0.7%)
26. Fork-head	1.36	5 (0.9%)
HMB versus Fibroid Group		
1. Oxygen Transport and Haemoglobin	5.23	14 (7.6%)
2. Functional and cellular component cluster including the extracellular space with secreted and signal peptides	4.44	68 (37%)
3. MHC Protein Complex	2.96	9 (4.9%)

4. Immune Response, MHC Protein Complex	2.71	24 (13%)
5. Chemokines, Chemotaxis and Viral Reproduction	2.37	17 (9.3%)
6. Response to External Stimuli	2.09	8 (4.4%)
7. Immunoglobulin	1.96	15 (8.2%)

*Annotation Cluster derived using a classification stringency of medium and an EASE threshold of 0.05 (modified Fisher Exact p-value)

**Enrichment Score: Negative log of the geometric mean of p-values (EASE values) for each annotation within a cluster. The EASE score determines whether the number of genes in an annotation is more than would be expected by random chance (relative to total genes in the annotation and the number of human genes overall), thereby providing an estimate of gene enrichment. We used a minimum EASE score/p-value of 0.05 for all analyses; a geometric mean of individual annotations in a cluster equal to 0.05 would equate to an Enrichment Score of 1.30.

***Count: Number of genes in the cluster (**Control versus HMB:** 176 genes input into DAVID of which 164 genes were annotated overall; **Control versus Fibroid:** 641 input of which 569 were annotated; **HMB versus Fibroid:** 213 input of which 183 were annotated).

¥Percent: Percent of genes annotated in this grouping relative to the number of genes annotated overall (**Control versus HMB:** 164 genes annotated, **Control versus Fibroid:** 569 genes annotated; **HMB versus Fibroid:** 183 genes annotated).

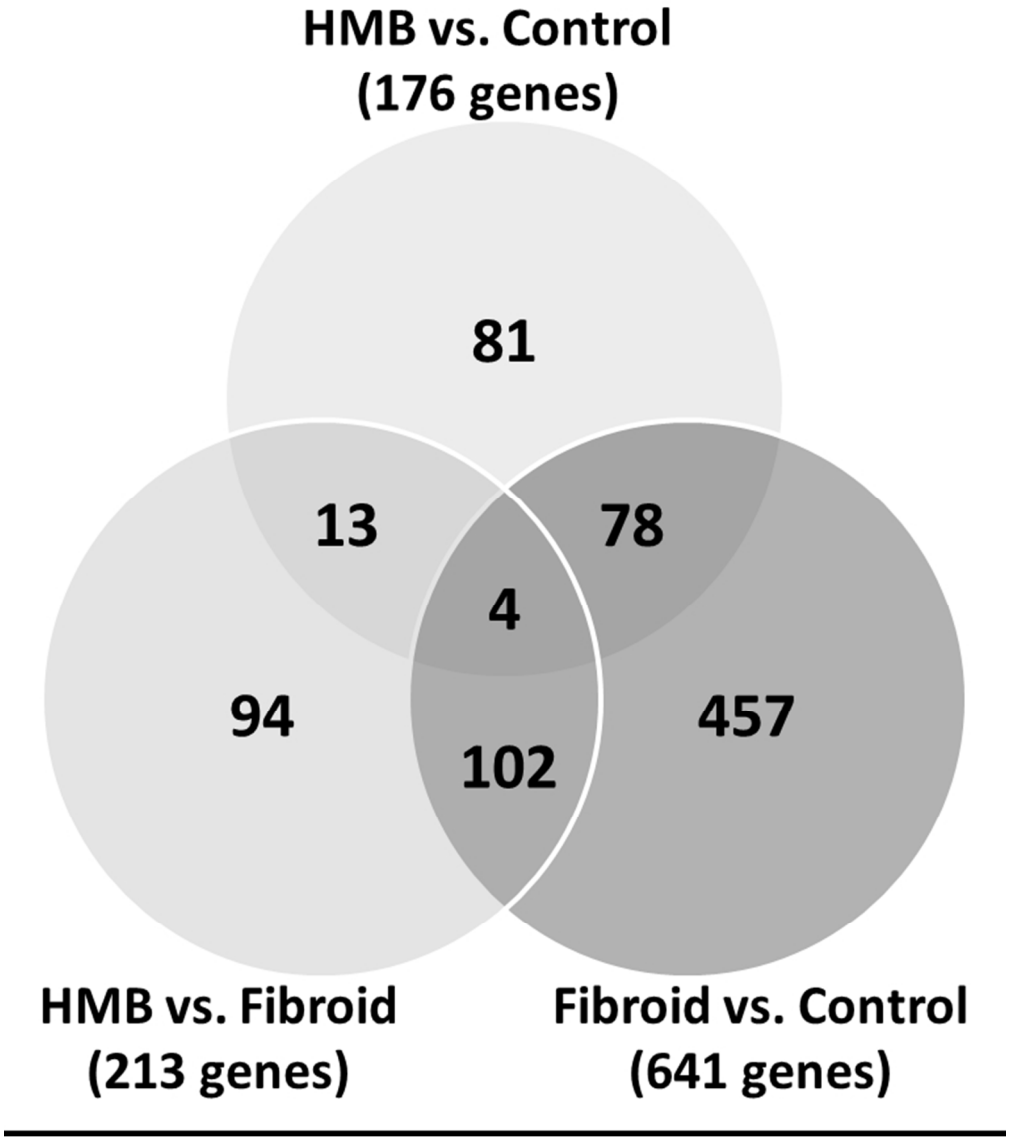

Figure 1: Venn diagram illustrating the distribution of differentially expressed genes in menstrual-phase endometrial biopsies from women with regular menstrual cycles with (n=10 samples) and without (Controls, n=7 samples) heavy menstrual bleeding (HMB), and women with uterine fibroids (mixed bleeding patterns, n=7 samples).
129x151mm (150 x 150 DPI)

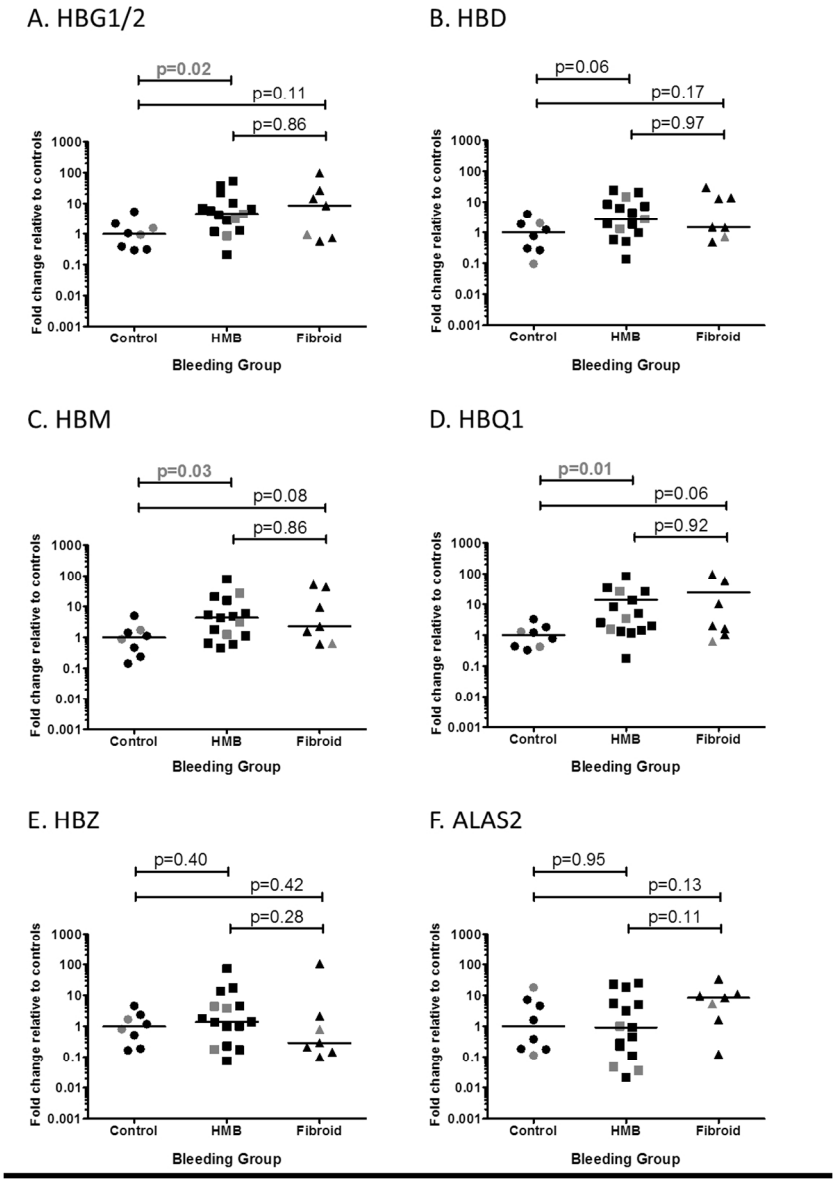

Figure 2: Expression of HBG1/2 (A), HBD (B), HBM (C), HBQ1 (D), HBZ (E) and ALAS2 (F) in menstrual-phase endometrial biopsies from women with and without heavy menstrual bleeding (HMB). A further group included endometrial biopsies from women with uterine fibroids. Fold change was calculated using ΔΔCT with RPL13A as the housekeeping gene. Numbers below group headings represent crossing threshold values (median [range]). Points coloured grey are extra samples that were not in the original gene array. In gene array analyses, all genes were upregulated in the Fibroid group relative to Control and HMB groups with no significant difference between the HMB and Fibroid groups.
191x277mm (150 x 150 DPI)

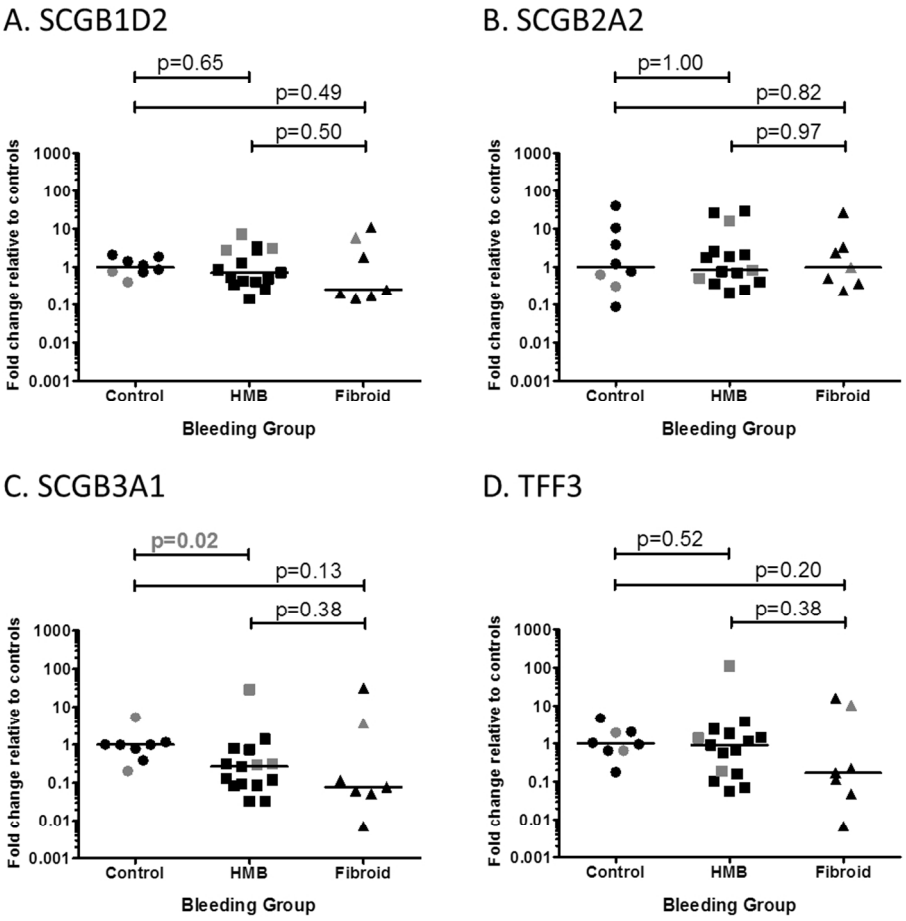

Figure 3: Expression of SCGB1D2 (A), SCGB2A2 (B), SCGB3A1 (C), and TFF3 (D) in menstrual-phase endometrial biopsies from women with and without heavy menstrual bleeding (HMB). A further group included endometrial biopsies from women with uterine fibroids. Fold change was calculated using ΔΔCT with RPL13A as the housekeeping gene. Numbers below group headings represent crossing threshold values (median [range]) from quantitative PCR. Points coloured grey are extra samples that were not in the original gene array. In gene array analyses, all genes were upregulated in the Control group relative to HMB and Fibroid groups; SCGB3A1 was also significantly upregulated in the Fibroid relative to the HMB group.
187x178mm (150 x 150 DPI)

Supplemental Table 1: Primer details for genes examined by quantitative PCR.

Gene	Gene name	Taqman Assay (Applied Biosystems, Life Technologies)
18S	18S Ribosomal RNA	Hs99999901_s1
B2M	Beta-2-microglobulin	Hs00984230_m1
RPL13A	Ribosomal protein 13 A	Hs01926559_g1
SCGB3A1	Secretoglobin, family 3A, member 1	Hs00369360_g1
SCGB2A2	Secretoglobin, family 2A, member 2	Hs00935948_m1
SCGB1D2	Secretoglobin, family 1D, member 2	Hs00255208_m1
TFF3	Trefoil factor 3 (intestinal)	Hs00902278_m1
HBZ	Hemoglobin, zeta	Hs00923579_m1
HBQ1	Hemoglobin, theta 1	Hs00362218_g1
HBD	Hemoglobin, delta	Hs00426283_m1
HBM	Hemoglobin, mu	Hs01392876_g1
HBG1 and *2*	Hemoglobin, gamma A and G	Hs00361131_g1
ALAS2	5'-aminolevulinate synthase 2	Hs00163601_m1
RNASpike	Sense 5'-TTCCCTTTAGTGAGGGTTAATTTCG -3'	
(Taqman)	Antisense 5'- GAGCGGATAACAATTTCACACA -3'	
	Probe FAM-AGCTTGGCGTAATCATGGTCATAGCTGT-BHQ-1	
	Amplicon size: 79 bp	

Supplemental Table 2: Summary of Functional Annotation Clusters derived from the DAVID Bioinformatics Resources 6.7: Control versus Heavy Menstrual Bleeding Group

Annotation Cluster*	Enrichment Score**	Number*** (and %¥) of genes in one or more annotations within a cluster	Genes included in one or more annotations within a cluster
1. Functional and cellular component cluster including the extracellular space with secreted and signal peptides, disulfide bond and glycoprotein	8.13	75 (45%)	**Up-regulated in Controls relative to HMB**: *ADAM10, AGR2, AMY2A, APOD, C20ORF114, CILP, CST4, CXCL6, DDR1, ERAP2, FAM3D, FCGBP, FOLR1, IL19, ITGA2B, ITGAX, LEFTY1, MMEL1, MUC4, MUC5AC, OR4K15, ORM1, PCDH20, PIGR, PIP, PTGDS, PVRL2, SAA1, SAA2, SCGB1D2, SCGB2A2, SCGB3A1, SLC34A2, SLC39A8, SLC4A4, SLC5A1, SOSTDC1, ST8SIA4, TFF3, TMEM178, TNFRSF18, TSPAN8, UTS2* **Down-regulated in Controls relative to HMB:** *APCDD1L, ARSI, BAGE, C3, CCL3L1, CCL3L3, CCL4L2, CNTN1, DEFA1, DST, GALNT9, HBEGF, HDLBP, HLA-A, IGF2, IL6ST, ITPRIPL2, KIRREL, LBP, NLGN4X, ODZ3, OPN1SW, OSM, PRRG3, PTPRA, PTPRC, RSPO3, SCTR, TMED6, TRPV1, VGF, WISP1*

2. Inflammatory and Immune Response	2.49	28 (17%)	**Up-regulated in Controls relative to HMB***: ADAM10, CXCL6, FAM3D, LBP, ORM1, PTGDS, SAA1, SAA2, TSPAN8* **Down-regulated in Controls relative to HMB:** *C3, CCL3L1, CCL3L3, CCL4L2, DEFA1, DSP, FOSB, HBEGF, IGF2, IL6ST, KAT5, LIG4, NLGN4X, NLRP3, OSM, PTPRC, TRPV1, TFF3, UHMK1*
3. Inflammatory and Immune Response	2.39	17 (10%)	**Up-regulated in Controls relative to HMB***: ADAM10, LBP, LIG4, PVRL2, SAA1, SAA2, TSPAN8* **Down-regulated in Controls relative to HMB:** *C3, HBEGF, IGF2, IL6ST, KAT5, NLRP3, OSM, PTPRC, TBX3, TRPV1*
4. Cell Adhesion	2.35	14 (8.5%)	**Up-regulated in Controls relative to HMB***: DDR1, FCGBP, ITGA2B, ITGAX, MUC4, MUC5AC, PCDH20,* PVRL2 **Down-regulated in Controls relative to HMB:** *CCL4L2, CNTN1, DST, NLGN4X, PTPRC, WISP1*
5. Cytokines	2.16	11 (6.7%)	**Up-regulated in Controls relative to HMB***: CXCL6, FAM3D, IL19, LEFTY1, SCGB3A1, TNFRSF18* **Down-regulated in Controls relative to HMB:** *CCL3L1, CCL3L3, CCL4L2, IL6ST, OSM*
6. Plasma Membrane	1.97	30 (18%)	**Up-regulated in Controls relative to HMB***: DDR1, DIRAS2, FOLR1, HSD17B2, ITGA2B,*

			ITGAX, LIG4, MUC4, PIGR, PVRL2, SLC34A2, SLC4A4, SLC5A1, TSPAN8 **Down-regulated in Controls relative to HMB:** *AKAP2, CDC42BPA, CNTN1, DSP, DST, HBEGF, HLA-A, IL6ST, NLGN4X, OPN1SW, OSM, PTPRA, PTPRC, SCTR, TRPV1, UBN1*
7. Cystine Knot	1.77	3 (1.8%)	**Up-regulated in Controls relative to HMB***: MUC5AC, SOSTDC1* **Down-regulated in Controls relative to HMB:** *WISP1*
8. Lipoprotein	1.70	3 (1.8%)	**Up-regulated in Controls relative to HMB***: SAA2, SAA1* **Down-regulated in Controls relative to HMB:** *HDLBP*
9. Lipocalin	1.66	3 (1.8%)	**Up-regulated in Controls relative to HMB***: ORM1, PTGDS, APOD*
10. Cell Proliferation and Migration	1.51	15 (9.1%)	**Up-regulated in Controls relative to HMB***: ADAM10, DDR1, KRT5, LIG4, MUC5AC, SCGB3A1* **Down-regulated in Controls relative to HMB:** *CCL3L1, CCL3L3, HBEGF, IGF2, IL6ST, OSM, PTPRC, TBX3, WISP1*
11. Apoptosis	1.50	13 (7.9%)	**Up-regulated in Controls relative to HMB***: EEF1A2, IL19, LIG4, MUC5AC, PRODH, PRUNE2, TNFRSF18* **Down-regulated in Controls relative to HMB:** *BCLAF1, IGF2, NLRP3, PTPRC, TBX3, VAV3*
12. Glycosaminoglycan Binding	1.48	5 (3.0%)	**Up-regulated in Controls relative to HMB***: CXCL6* **Down-regulated in Controls relative to HMB:** *HBEGF, NLRP3, PTPRC, RSPO3,*

*Annotation Cluster derived using a classification stringency of medium and an EASE threshold of 0.05 (modified Fisher Exact p-value)

**Enrichment Score: Negative log of the geometric mean of p-values (EASE values) for each annotation within a cluster. The EASE score determines whether the number of genes in an annotation is more than would be expected by random chance (relative to total genes in the annotation and the number of human genes overall), thereby providing an estimate of gene enrichment. We used a minimum EASE score/p-value of 0.05 for all analyses; a geometric mean of individual annotations in a cluster equal to 0.05 would equate to an Enrichment Score of 1.30.

***Count: Number of genes in the cluster (176 genes input into DAVID of which 164 genes were annotated overall).

¥Percent: Percent of genes annotated in this grouping relative to the 164 genes annotated overall.

Supplemental Table 3: Summary of Functional Annotation Clusters derived from the DAVID Bioinformatics Resources 6.7: Control versus Fibroid Group

Annotation Cluster*	Enrichment Score**	Number*** (and %¥) of genes in one or more annotations within a cluster	Genes included in one or more annotations within a cluster
1. Functional and cellular component cluster including the extracellular space with secreted and signal peptides, disulfide bond and glycoprotein	6.85	295 (52%)	**Up-regulated in Fibroids relative to Controls:** *ABCA6, ABCG1, AKAP2, AKAP7, ANGPTL4, ANKLE2, APCDD1L, ARC, ARSI, B3GNT6, BAI1, BAX, BCAN, C14ORF135, C14ORF165, C16ORF89, C19ORF26, C2CD2, C2ORF89, C6ORF105, CCR5, CCR7, CCRL1, CD19, CD34, CD3G, CD5, CD6, CD79A, CDC42BPA, CELA1, CERK, CHID1, CHST2, CTLA4, CYP1A1, CYP2A13, CYR61, D4S234E, DCLK1, DDX3Y, DEFA1, DEFA4, DENND1A, DHH, DHRS9, DIXDC1, DNASE1L1, DPB1, DRB1, DSC1, DSP, DST, ENAH, EPX, EPYC, FAIM3, FAM118A, FAM55B, FGA, FGFBP2, FLNC, FLVCR1, FN1, FOLR3, GAB2, GABBR1, GEM, GNAO1, GNG4, GPR183, GPR56, GPSM1, GRIN2B, GYPC, HBEGF, HDLBP, HLA-A, HLA-DPB1, HLA-DQA1, HLA-DQB1, HSD11B1, HTR7, IGDCC3, IGF2, IGFBP1, IGSF9B, IL1RL1, IL23A, IL24, IL2RA, IL6ST, IL7R, KCNH1, KIR2DS5, KIR3DL2, LBP, LDLRAD3, LHFPL1,*

LOC554223, LPL, LYSMD4, MAL, METRN, MLC1, MMP14, MMP3, MPZ, MSMB, MUC13, MYADML2, MYO7B, NPC1L1, NPIPL3, NPR3, OLFML1, OPN5, OPRL1, OR5P3, P2RY11, PDPK1, PI16, PIP5K1B, PLG, PLK3, POM121, PORCN, PTPRC, RASL10B, RELT, RRAD, RSPO3, SCTR, SDC1, SDCBP2, SH3KBP1, SHISA7, SLC16A10, SLC22A12, SLC22A3, SLC25A24, SLC29A4, ST3GAL1, SYTL2, TAS2R14, TAS2R41, TDGF3, TEX101, TGFB1I1, TIGIT, TMED6, TMEM183B, TMEM202, TNFRSF11B, TSGA10, TSPAN11, UBN1, ULBP2, VAMP1, VPREB3, VWCE, WISP1, XIRP1, XYLT1

Down-regulated in Fibroids relative to Controls: *ADAM10, AGR2, AGR3, AMY1A, AMY2A, APLN, ASPRV1, ATP6AP1L, B3GNT5, BBS7, BMP7, C20ORF114, C2ORF88, CCL4L2, CD2AP, CDH12, CDH3, CFB, CFTR, CHI3L1, CLCA4, CLCNKA, CPE, CXADR, CXCL6, CYP4X1, DDR1, DEFB4A, DMKN, EDN3, EFHA2, ERAP2, FAM3D, FCGBP, FCHO2, FGFR3, FOLR1, FUT3, GPBAR1, GPR128, GREM2, HAS3, HLA-C, HLA-DRB1, HP, HSPG2, IFNE, IL17C, IL19, IL33, ITGA2B, ITGB4, ITPRIPL2, KDELR3, LGALS7, LILRA3, LRRK2, LSR, LYNX1, MARVELD3, MAVS, MFF, MICA, MOBKL3, MSLN, MST1, MUC4, MUC5AC, NMU, NRCAM, NTN4, OLFM4, OR4K15, P2RY12, P2RY2, PCDH10, PCDHGC3, PIGR, PKD1L2, PROM2, PSTPIP2, RAB11FIP4, RELN, RGS17, ROCK1, SAA1, SAA2, SCGB1D2, SCGB2A2, SCGB3A1, SCNN1G, SEMA3C, SEMA4G, SERPINA3, SERPINB3, SERPINB4, SIGLEC14,*

			SLC16A14, SLC16A9, SLC1A2, SLC1A3, SLC25A27, SLC27A6, SLC2A11, SLC34A2, SLCO1B1, SOSTDC1, SSPO, SUMO1, SYT1, TAP2, TEDDM1, TFF3, TGM1, TMEM106B, TMEM170B, TMEM178, TMTC4, TNFSF13, TREML1, TSPAN12, TSPAN8, UGGT2, UGT2B7, UNC13A, UTS2, VCAM1, VPS16, WFDC2, XDH, ZG16B
2. Plasma Membrane	3.96	161 (28%)	**Up-regulated in Fibroids relative to Controls:** *ABCG1, ADAM10, AKAP2, AKAP7, ARC, BAI1, C2ORF89, CAM1, CCR5, CCR7, CCRL1, CD19, CD34, CD3G, CD5, CD6, CD79A, CDC42BPA, CTLA4, DCLK1, DDX3Y, DENND1A, DHH, DIXDC1, DSC1, DSP, DST, ENAH, FAIM3, FGA, FLNC, FLVCR1, FN1, GABBR1, GEM, GNAO1, GNG4, GPR183, GPR56, GPSM1, GRIN2B, GYPC, HBEGF, HDLBP, HLA-A, HLA-DPB1, HLA-DQA1, HLA-DQB1, HTR7, IGDCC3, IGSF9B, IL1RL1, IL2RA, IL6ST, IL7R, ITGA2B, ITGB4, ITPRIPL2, KCNH1, KIR2DS5, KIR3DL2, LDLRAD3, LOC554223, LPL, LYSMD4, MAL, MMP14, MPZ, MUC13, MYO7B, NPC1L1, NPR3, OPN5, OPRL1, OR5P3, P2RY11, PCDH10, PDPK1, PI16, PORCN, PTPRC, RASL10B, RELT, RRAD, SCTR, SDC1, SDCBP2, SH3KBP1, SHISA7, SLC16A10, SLC22A12, SLC22A3, SLC29A4, SYTL2, TAS2R14, TAS2R41, TDGF3, TEX101, TGFB1I1, TGM1, TIGIT, TMEM170B, TMEM178, TNFSF13, UBN1, ULBP2, VAMP1, XIRP1* **Down-regulated in Fibroids relative to Controls:** *ASPRV1, BBS7, CD2AP, CDH12, CDH3, CFB, CFTR, CLCA4, CLCNKA, CPE, CXADR, DDR1, FGFR3, FOLR1, GGT1, HAS3, HLA-C,*

			HLA-DRB1, LILRA3, LRRK2, LSR, LYNX1, MARVELD3, MICA, MSLN, MUC4, NRCAM, OR4K15, P2RY12, P2RY2, PCDHGC3, PIGR, PKD1L2, PROM2, SCNN1G, SEMA4G, SIGLEC14, SLC16A14, SLC16A9, SLC1A2, SLC1A3, SLC27A6, SLC2A11, SLC34A2, SLCO1B1, SYT1, TAP2, TREML1, TSPAN12, TSPAN8, UNC13A, VCAM1, VPS16
3. Oxygen Transport and Haemoglobin	3.70	15 (2.6%)	**Up-regulated in Fibroids relative to Controls:** *CYP1A1, CYP2A13, EPX, HBD, HBG1, HBG2, HBM, HBQ1, HBZ, PLG* **Down-regulated in Fibroids relative to Controls:** *ADH1A, CA8, CYP4X1, HP, KRT5*
4. Cell Adhesion	3.10	30 (5.3%)	**Up-regulated in Fibroids relative to Controls:** *BAI1, CD34, CD6, CYR61, DDR1, DSC1, DST, FN1, GPR56, MSLN, PCDH10, TGFB1I1, WISP1* **Down-regulated in Fibroids relative to Controls:** *CCL4L2, CDH12, CDH3, CXADR, FCGBP, ITGA2B, ITGB1BP1, ITGB4, MUC4, MUC5AC, NRCAM, OLFM4, PCDHGC3, RELN, SIGLEC14, SSPO, VCAM1*
5. Cystine knot and von Willebrand	2.81	8 (1.4%)	**Up-regulated in Fibroids relative to Controls:** *CYR61, VWCE, WISP1* **Down-regulated in Fibroids relative to Controls:** *FCGBP, GREM2, MUC5AC, SOSTDC1, SSPO*
6. Cell migration and motility	2.75	23 (4.0%)	**Up-regulated in Fibroids relative to Controls:** *BAX, CD34, DCLK1, FN1, GAB2, HBEGF, MMP14, NR4A2*

			Down-regulated in Fibroids relative to Controls: *BMP7, CCL4L2, CD2AP, EDN3, HOXA2, ITGB1BP1, NRCAM, RELN, ROCK1, SAA1, SAA2, SCNN1G, SEMA3C, TNFSF13, VCAM1*
7. Immune Response, MHC Protein Complex	2.67	41 (7.2%)	**Up-regulated in Fibroids relative to Controls:** *BCAN,CD34, CD6, CD79A, CTLA4, EPX, FAIM3, FN1, GPR183, HLA-A, HLA-DPB1, HLA-DQA1, HLA-DQB1, IGF2, IL23A, IL2RA, KIR2DS5, KIR3DL2, LOC554223, MPZ, PTPRC, SDC1, ULBP2* **Down-regulated in Fibroids relative to Controls:** *CDH3, CFB, CPE, CXADR, ERAP2, HLA-C, HLA-DRB1, ITGA2B, ITGB4, LILRA3, MAVS, MICA, NRCAM, PIGR, SIGLEC14, TAP2, TNFSF13, VCAM1*
8. Cell junctions	2.57	31 (5.4%)	**Up-regulated in Fibroids relative to Controls:** *ARC, BAI1, CDC42BPA, CDH3, DENND1A, DIXDC1, DSC1, DSP, DST, ENAH, FAIM3, GABBR1, GRIN2B, IL1RL1, PTPRC, SDC1, SH3KBP1, SLC16A10, TGFB1I1, UBN1, VAMP1, XIRP1* **Down-regulated in Fibroids relative to Controls:** *CFTR, CXADR, ITGA2B, ITGB4, P2RY2, SLC1A3, SYT1, TGM1, UNC13A*
9. Immunoglobulin	2.55	37 (6.5%)	**Up-regulated in Fibroids relative to Controls:** *BCAN, CD19, CD3G, CD79A, CTLA4, FAIM3, FLNC, HLA-A, HLA-DPB1, HLA-DQA1, HLA-DQB1, IGDCC3, IGSF9B, IL6ST, IL1RL1, KIR2DS5, KIR3DL2, MPZ, NFATC1, TIGIT, VPREB3* **Down-regulated in Fibroids relative to Controls:** *CXADR, FGFR3, HLA-C, HLA-DRB1,*

			HSPG2, LILRA3, LSR, MICA, NRCAM, PIGR, SEMA3C, SEMA4G, SIGLEC14, TGM1, TREML1, VCAM1
10. MHC Protein Complex	2.36	12 (2.1%)	**Up-regulated in Fibroids relative to Controls:** *HLA-A, HLA-DPB1, HLA-DQA1, HLA-DQB1, KIR2DS5, LOC554223, ULBP2* **Down-regulated in Fibroids relative to Controls:** *ERAP2, HLA-C, HLA-DRB1, MICA, TAP2*
11. Protein-lipid Complex	2.16	6 (1.1%)	**Up-regulated in Fibroids relative to Controls:** *HDLBP, LPL* **Down-regulated in Fibroids relative to Controls:** *HP, LSR, SAA2, SAA1*
12. Cytokine Activity	2.02	20 (3.5%)	**Up-regulated in Fibroids relative to Controls:** *CCR5, CCR7, IL23A, IL24, IL2RA, IL6ST, RELT, TNFRSF11B* **Down-regulated in Fibroids relative to Controls:** *BMP7, CCL4L2, CXCL6, FAM3D, GREM2, IFNE, IL19, IL33, IL17C, IL19, SCGB3A1, TNFSF13*
13. Leucocyte activation	1.98	21 (3.7%)	**Up-regulated in Fibroids relative to Controls:** *BAX, CD19, CD3G, CD79A, CRIP3, FGA, GPR183, IGF2, IL23A IL7R, PTPRC, ULBP2* **Down-regulated in Fibroids relative to Controls:** *ADAM10, LBP, MICA, P2RY12, SAA1, SAA2, TAP2, TREML1, VCAM1*
14. Cytoskeleton and Filament	1.97	8 (1.4%)	**Up-regulated in Fibroids relative to Controls:** *FLJ40504, KRT34, KRT72, KRT73, KRT83* **Down-regulated in Fibroids relative to Controls:** *KRT5, KRT6A, KRT6B*

15. Wound Healing and Coagulation	1.90	19 (3.3%)	**Up-regulated in Fibroids relative to Controls:** *DSP, FGA, FN1, FNTB, HBEGF, IGF2, IGFBP1, PLG, SDC1* **Down-regulated in Fibroids relative to Controls:** *AGR2, ANXA8, CDH3, MST1, P2RY12, SAA1, SAA2, SCNN1G, SERPINA3, TREML1*
16. Morphogenesis	1.87	9 (1.6%)	**Up-regulated in Fibroids relative to Controls:** *BAX, FLVCR1, HOXC11, MECOM, PITX1, TBX3* **Down-regulated in Fibroids relative to Controls:** *MBNL1, PRRX2, TP63*
17. von Willebrand factor	1.74	7 (1.2 %)	**Up-regulated in Fibroids relative to Controls:** *CYR61, VWCE, WISP1* **Down-regulated in Fibroids relative to Controls:** *FCGBP, MUC4, MUC5AC, SSPO*
18. Development of Female Primary Sexual Characteristics	1.71	10 (1.8%)	**Up-regulated in Fibroids relative to Controls:** *BAX, DHH, FANCA, FOXA1, FOXO3, MMP14, SDC1, TBX3* **Down-regulated in Fibroids relative to Controls:** *PLA2G4A, TP63*
19. Growth Factor Binding	1.64	9 (1.6%)	**Up-regulated in Fibroids relative to Controls:** *CYR61, FGFBP2, IGF2, IGFBP1, IL1RL1, IL2RA, IL6ST, IL7R, WISP1*
20. Inflammatory Response	1.63	17 (3.0%)	**Up-regulated in Fibroids relative to Controls:** *BAX, IGF2, IL2RA, IL6ST, PLG, TIGIT* **Down-regulated in Fibroids relative to Controls:** *ADAM10, BMP7, EDN3, LBP, MAVS, NMU, PLA2G4A, SAA1, SAA2, SLC1A3, TSPAN8*

21. Chemotaxis	1.62	14 (2.5%)	**Up-regulated in Fibroids relative to Controls:** *CCR5, CCR7, CCRL1, CYR61, DEFA1, GNAO1, NR4A2* **Down-regulated in Fibroids relative to Controls:** *CCL4L2, CXCL6, DEFB4A, EDN3, RELN, SAA1, SAA2*
22. Leucocyte Activation	1.59	18 (3.2%)	**Up-regulated in Fibroids relative to Controls:** *CD19, CD5, CD79A, CTLA4, IL2RA, IL6ST, IL7R, PTPRC, RORA, TIGIT* **Down-regulated in Fibroids relative to Controls:** *ADAM10, CFB, EDN3, LBP, MICA, TAP2, TNFSF13, VCAM1*
23. Glycosaminoglycan and Heparin Binding	1.56	17 (3.0%)	**Up-regulated in Fibroids relative to Controls:** *BCAN, CD34, CYR61, EPYC, FN1, HBEGF, LPL, PTPRC, RSPO3* **Down-regulated in Fibroids relative to Controls:** *BMP7, CHI3L1, CLC, CXCL6, LGALS7, PKD1L2, SIGLEC14, ZG16B*
24. Regulation of Cell Growth	1.52	21 (3.7%)	**Up-regulated in Fibroids relative to Controls:** *CYR61, DCLK1, FLVCR1, GAMT, GNG4, HBEGF, IGF2, IGFBP1, IL7R, ING5, SOCS4, WISP1* **Down-regulated in Fibroids relative to Controls:** *ADAM10, CDKN2C, CXADR, DDR1, FGFR3, NRCAM, ODF3B, SCGB3A1, TP63*
25. Purinoreceptor	1.44	4 (0.7%)	**Up-regulated in Fibroids relative to Controls:** *GPR183, P2RY11*

			Down-regulated in Fibroids relative to Controls: *P2RY12, P2RY2*
26. Fork-head	1.36	5 (0.9%)	**Up-regulated in Fibroids relative to Controls:** *FOXA1, FOXE3, FOXO1, FOXO3, FOXR1*

*Annotation Cluster derived using a classification stringency of medium and an EASE threshold of 0.05 (modified Fisher Exact p-value)

**Enrichment Score: Negative log of the geometric mean of p-values (EASE values) for each annotation within a cluster. The EASE score determines whether the number of genes in an annotation is more than would be expected by random chance (relative to total genes in the annotation and the number of human genes overall), thereby providing an estimate of gene enrichment. We used a minimum EASE score/p-value of 0.05 for all analyses; a geometric mean of individual annotations in a cluster equal to 0.05 would equate to an Enrichment Score of 1.30.

***Count: Number of genes in the cluster (641 genes input into DAVID of which 569 genes were annotated overall).

¥Percent: Percent of genes annotated in this grouping relative to the 569 genes annotated overall.

Supplemental Table 4: Summary of Functional Annotation Clusters derived from the DAVID Bioinformatics Resources 6.7: HMB versus Fibroid Group

Annotation Cluster*	Enrichment Score**	Number*** (and %¥) of genes in one or more annotations within a cluster	Genes included in one or more annotations within a cluster
1. Oxygen Transport and Haemoglobin	5.23	14 (7.6%)	**Up-regulated in Fibroids relative to HMB***: CA8, HP, XDH* **Down-regulated in Fibroids relative to HMB:** *CD8A, CYP26A1, CYP2C9, HBD, HBG1, HBG2, HBM, HBQ1, HBZ, SLC4A1, TCF7L2*
2. Functional and cellular component cluster including the extracellular space with secreted and signal peptides	4.44	68 (37%)	**Up-regulated in Fibroids relative to HMB:** *CCL2, CCL3L1, CCL3L3, CCL4, CCL4L2, CFTR, CLCA4, CLEC12A, CMTM1, CXCL5, DEFB103A, DEFB4A, GPR109A, GPR109B, GPR84, HLA-DRB1, HP, IL17RB, IL1RN, LILRA3, MICA, PVRL1, RAB27B, RNASE1, SAA2, SCNN1G, SSPO, SST, TREM1, TREML1, XDH* **Down-regulated in Fibroids relative to HMB:** *ADCY1, C5ORF46, CD8A, CHID1, CILP, CNTNAP2, DKK1, DNASE1L1, DSC1, FGA, FGFBP2, FOLR3, HAMP, HLA-DPB1, HLA-DQA1, HLA-DQB1, HLA-DRB4, IGFBP1, KCNC4, KIR2DS2, KIR3DL2, LEFTY1, LYSMD4, MMP26,*

			NPR3, OGN, P2RY11, PZP, RELT, SCGB3A1, SLC4A1, SLC5A1, TIMD4, TIMM13, TSPAN11, ULBP2, UPK1B,
3. MHC Protein Complex	2.96	9 (4.9%)	**Up-regulated in Fibroids relative to HMB:** *HLA-DQA1, MICA,* **Down-regulated in Fibroids relative to HMB:** *CD8A, HLA-DPB1, HLA-DQB1, HLA-DRB4, LOC554223, ULBP2*
4. Immune Response, MHC Protein Complex	2.71	24 (13%)	**Up-regulated in Fibroids relative to HMB:** *HLA-DRB1, LILRA3, MICA, PVRL1, TREM1, TREML1* **Down-regulated in Fibroids relative to HMB:** *ADCY1, CD8A, CILP, CNTNAP2, CYP2C9, DSC1, HLA-DPB1, HLA-DQA1, HLA-DQB1, HLA-DRB4, KIR2DS2, KIR3DL2, LOC554223, NPR3, SLC4A1, SLC5A1, TIMD4, ULBP2*
5. Chemokines, Chemotaxis and Viral Reproduction	2.37	17 (9.3%)	**Up-regulated in Fibroids relative to HMB:** *CCL2, CCL3L1, CCL3L3, CCL4, CCL4L2, CMTM1, CXCL5, DEFB4A, IL17RB, IL1RN, PLA2G4A, PVRL1, SAA2* **Down-regulated in Fibroids relative to HMB:** *ADCY1, LEFTY1, RELT, SCGB3A1*
6. Response to External Stimuli	2.09	8 (4.4%)	**Up-regulated in Fibroids relative to HMB:** *CCL2, DEFB103A, DEFB4A, MICA, PLA2G4A, SST* **Down-regulated in Fibroids relative to HMB:** *HAMP, HSPB7*
7. Immunoglobulin	1.96	15 (8.2%)	**Up-regulated in Fibroids relative to HMB:** *HLA-DRB1, LILRA3, MICA, PVRL1, TREM1,*

	TREML1 **Down-regulated in Fibroids relative to HMB:** *CD8A, CILP, HLA-DPB1, HLA-DQA1, HLA-DQB1, HLA-DRB4, KIR2DS2, KIR3DL2, TIMD4*

*Annotation Cluster derived using a classification stringency of medium and an EASE threshold of 0.05 (modified Fisher Exact p-value)

**Enrichment Score: Negative log of the geometric mean of p-values (EASE values) for each annotation within a cluster. The EASE score determines whether the number of genes in an annotation is more than would be expected by random chance (relative to total genes in the annotation and the number of human genes overall), thereby providing an estimate of gene enrichment. We used a minimum EASE score/p-value of 0.05 for all analyses; a geometric mean of individual annotations in a cluster equal to 0.05 would equate to an Enrichment Score of 1.30.

***Count: Number of genes in the cluster (213 genes input into DAVID of which 183 genes were annotated overall).

¥Percent: Percent of genes annotated in this grouping relative to the 183 genes annotated overall.

HMB: Heavy Menstrual Bleeding