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

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46 **ABSTRACT**

47 Heavy menstrual bleeding (HMB) is a significant social and public health issue for menstruating
48 women. Development of targeted treatments has been limited by poor understanding of local
49 mechanisms underlying HMB. We aimed to determine how gene expression differs in menstrual
50 phase endometrium from women with HMB. Menstrual phase endometrial biopsies were collected
51 from women with (n=7) and without (n=10) HMB (regular menstrual cycles, no known pelvic
52 pathology), as well as women with uterine fibroids (n=7, n=4 had HMB). Biopsies were analysed
53 using Illumina Sentrix Human HT12 arrays and data analysed using ‘Remove Unwanted Variation-
54 inverse’ (RUV-inv). Ingenuity Pathway Analysis (IPA) and the Database for Annotation, Visualization
55 and Integrated Discovery (DAVID) v6.7 were used to identify gene pathways, functional gene clusters
56 and upstream regulators specific to the clinical groupings. Individual genes of interest were
57 examined using quantitative PCR (qPCR). In total, 829 genes were differentially expressed in one or
58 more comparisons. Significant canonical pathways and gene clusters enriched in Controls relative to
59 both HMB and Fibroid groups suggest the mechanisms responsible for HMB include modifications of
60 the endometrial inflammatory or infection response. In contrast, differentially expressed genes in
61 women with fibroids suggest modifications of haemoglobin, antigen processing and the MHC
62 complex (Class II, beta chain) activity. In conclusion, HMB associated with fibroids may be regulated
63 by different endometrial mechanisms from HMB in women without fibroids and from normal
64 menstrual bleeding. These novel data provide numerous testable hypotheses that will advance our
65 understanding of the mechanisms responsible for HMB.

66
67 **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

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

98 Understanding of the mechanisms of normal and abnormal uterine bleeding is incomplete.¹⁰⁻¹³

99 Menstruation initiates after progesterone withdrawal from an estrogen-primed endometrium, with
100 a subsequent cascade of molecular events culminating in endometrial breakdown, bleeding and
101 repair. The process resembles an inflammatory response and includes leucocyte influx, cytokine
102 production, extracellular matrix degradation and vascular and tissue remodelling. Menstruation
103 involves multiple regulators, including prostaglandins; prostaglandin-mediated vasoconstriction of
104 spiral arterioles minimises blood loss and causes hypoxia in the functionalis. Abnormal
105 cyclooxygenase and prostaglandin production have been observed in women with HMB. Regulation
106 of haemostasis is a key feature of menstruation. Of particular importance is the fibrinolytic
107 pathway, which degrade fibrin deposits; key members of the fibrinolytic pathway (e.g. tissue
108 plasminogen activator) are dysregulated in HMB, consistent with the efficacy of anti-fibrinolytics in
109 reducing HMB.¹⁰⁻¹³ In this study, we have examined menstrual phase biopsies from women with (1)
110 regular menstrual cycles with self-reported HMB compared to those without HMB, and (2) women
111 with uterine fibroids. To our knowledge, our study is the first to provide detailed analyses of
112 endometrial gene expression from women with regular menstrual cycles and no known uterine
113 comorbidities. In addition, no previous studies have examined variations in menstrual phase gene
114 expression associated with the presence of uterine fibroids. We focused on carefully characterised
115 samples collected on days 1-5 of the menstrual cycle (during menstruation). As endometrial gene
116 and protein expression is highly dynamic with considerable heterogeneity among different phases of
117 the menstrual cycle and between women, we have used innovative bioinformatics methodology
118 called 'Remove Unwanted Variation-inverse' (RUV-inv) to examine gene array results.^{14,15} RUV-inv is
119 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.

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129 METHODS

130 Patient Samples and Ethical Approval

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

136 Data about menstrual history were collected and subjects completed a menstrual diary. All
137 subjects had a pelvic ultrasound performed by an experienced ultrasonologist (NW) within two
138 weeks of recruitment using a Phillips iU22 using a C10-3v curved array transvaginal probe and a C9-4
139 or C5-2 curved array transabdominal probe (standardised ASUM protocol for gynaecological
140 scanning¹⁶). The ultrasound reported the presence/absence, size and location of uterine fibroids and
141 any apparent distortion of the endometrial cavity. The ovaries were examined for any cysts or
142 masses and follicular dimensions and total number were recorded. Patients were classified as
143 having uterine fibroids if any fibroids were detected, irrespective of number, size or location.
144 Participants were excluded if they had other comorbidities (e.g. polyp, adenomyosis), unless
145 explicitly stated in Table 1, were taking sex steroids, or had known endocrine or haematological

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3 146 abnormalities that may cause HMB. We were not able to exclude endometriosis from this study as
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5 147 the women involved were not undergoing laparoscopic or other surgical procedures.
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7 148 Endometrial biopsies were collected on days 1-5 of the menstrual cycle during a bleeding
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9 149 episode. Day-of-cycle was verified using the patient’s menstrual diary (unless stated otherwise,
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11 150 Table 1). Endometrial biopsies were collected using a pipelle suction curette (Pipelle, Cornier,
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13 151 France) and stored in RNAlater (Ambion, Life Technologies, Carlsbad, CA, USA) overnight before
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15 152 being frozen and stored at -80°C.
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18 153 A total of 36 samples were arrayed, of which 24 carefully characterised samples were used in
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20 154 the final array analysis. The remaining 11 samples were excluded due to irregular menstrual cycles
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22 155 (n=1), no menstrual diary provided (n=8), and/or detection of additional comorbidities (e.g.
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24 156 adenomyosis) (n=5). The 24 samples were separated into three groups (Table 1):
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27 157 1. **Controls:** No heavy menstrual bleeding, regular menstrual cycles, no uterine
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29 158 comorbidities (n=7).
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31 159 2. **HMB:** Heavy menstrual bleeding, regular menstrual cycles, no uterine comorbidities
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33 160 (n=10).
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35 161 3. **Fibroids:** Mixed bleeding patterns and comorbidities (n=7).
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37 162 Groups 1 and 2 were restricted to those with regular menstrual cycles, defined using
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39 163 standardised international criteria as cycle to cycle variation of between 2 and 20 days^{6,7} and based
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41 164 on at least a 3 month menstrual diary (Table 1). Duration and frequency of menstruation were also
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43 165 noted, but not used as inclusion/exclusion criteria. Bleeding but not spotting days were used in
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45 166 calculations (Table 1).
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48 167 Due to the logistical difficulties of obtaining these valuable samples, we were not able to
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50 168 obtain a completely separate cohort of samples for validation analyses. Instead, an additional 9
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52 169 samples were included when individual genes of interest were examined using PCR. These included
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54 170 2 Control, 6 HMB and 1 Fibroid samples. Details about these participants are included in Table 1.
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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 package¹⁹ were used to remap the probes according to current knowledge of the human genome (hg19). Finally genes were declared Differentially Expressed (DE) if the Benjamini-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).

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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 $-(\log_{10})$ 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 BestKeeper²⁷ and delta CT²⁸ 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 $\Delta\Delta CT$ 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

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250 relative to Control and HMB groups (Figure 1). Four genes were significantly different in all
251 comparisons (chemokine (C-C motif) ligand 4-like 2 [CCL4L2; HMB > Control > Fibroid], serum
252 amyloid A2 [SAA2; Control > HMB > Fibroid], small Cajal body-specific RNA 27 [SCARNA27; Fibroid >
253 HMB > Control], secretoglobin, family 3A, member 1 [SCGB3A1; Control > Fibroid > HMB]). The top
254 20 most significantly up or downregulated genes for each comparison are provided in Table 2.
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256 **Functional Clusters and Pathway Analysis**

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

267 Of the differentially-expressed genes from the Control versus HMB groups, 164 genes (of 176
268 input) were annotated by the DAVID and 12 significant clusters were identified (Table 5,
269 Supplemental Table 2). Key function-related clusters included genes involved in the inflammatory
270 and immune response and, as with IPA analysis, included several acute phase proteins (*LBP*, *ORM1*,
271 *SAA1*, *SAA2* – all downregulated in HMB relative to Controls). A further cluster included several
272 cytokine-related genes (e.g. chemokine (C-C-motif) ligands: *CCL3L1*, *CCL3L3*, *CCL4L2*). Other clusters
273 included genes related cell adhesion, the plasma membrane, cystine knots, lipoproteins, lipocalin,
274 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

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3 301 between innate and adaptive immune cells. As with comparison between Control and HMB, the
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5 302 upstream regulators identified included STAT5A and interleukins, regulators suggesting the
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7 303 involvement of retinoic acid, and regulators associated with the immune response and pathogen
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9 304 recognition; Table 4). For SFTPA1 and TGFB1, analyses suggested that the activity of these
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11 305 regulators was inhibited and activated, respectively, in the Fibroid relative to Control groups.
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13 306 Of the differentially-expressed genes from the HMB versus Fibroid groups, 183 genes (of 213
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15 307 input) were also annotated by DAVID. We identified 7 significant clusters (Table 5, Supplemental
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17 308 Table 4), including haemoglobin subunits and other genes related to oxygen transport. Other
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19 309 clusters included genes related to secreted/signalling peptides and other extracellular components,
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21 310 the immune response and MHC complex and a cluster of chemokines (e.g. *CCL2*, *CCL3L1*, *CCL3L3*,
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23 311 *CCL4*, *CCL4L2*, *CMTM1*, *CXCL5*).
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31 314 A set of 10 genes were chosen to be examined using quantitative PCR. With the exception of
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33 315 one sample from the Fibroid group with insufficient RNA, gene expression was examined in the same
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35 316 samples used in the array as well as the additional new samples (Table 1). Genes examined included
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37 317 five haemoglobin genes (*HBG1/2*, *HBD*, *HBM*, *HBQ1* and *HBZ*) and a gene associated with heme
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39 318 production (*ALAS2*). In the array analysis, these genes were all significantly upregulated in the
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41 319 Fibroid group relative to both HMB and Control groups. We also examined four secreted proteins
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43 320 (three secretoglobins: *SCGB3A1*, *SCGB1D2* and *SCGB1A2*; previously known as uteroglobin-related
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45 321 protein 2 or cytokine high in normal-1, lipophilin-B, and mammoglobin-A, respectively; and trefoil
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47 322 factor 3 (intestinal) [*TFF3*]), which were all upregulated in Control relative to HMB samples in array
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49 323 analysis; *SCGB3A1* was also upregulated in Fibroids relative to the HMB group.
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52 324 As expected, there was considerable variation among mRNA expression values determined by
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54 325 quantitative PCR, even within a group. However, all genes were expressed in reasonable quantities
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56 326 by endometrial tissues (Figures 2 and 3). For the haemoglobin related genes (Figure 2), there was a
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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.

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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

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

374 It is also important to note that cluster analysis is based on differentially expressed genes
375 between the groups of interest and that our analyses included both up and downregulated genes
376 (see Tables 3-5 for genes that were up or down regulated in Control versus HMB groups). In
377 addition, the changes in gene expression between Control and HMB groups were not consistent
378 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 leucocytes³⁶ 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 (11 β HSD2) 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

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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 activator⁴²), 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

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

473 In summary, we have used gene array analysis and the bioinformatics technique RUV-inv to
474 identify genes and functional gene clusters that vary in menstrual phase endometrial biopsies from
475 women with regular menstrual cycles with and without HMB and in women with uterine fibroids.
476 Comparisons between women with regular menstrual cycles and with/without HMB suggest that the
477 excess bleeding may reflect deviations in the immune and inflammatory response. Our results also
478 suggest that different mechanisms are responsible for the HMB seen in women with/without uterine
479 fibroids with differentially expressed genes related to haemoglobin, antigen processing and the MHC
480 complex identified in women with these tumours. Overall, our results provide alternative genes and
481 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.

484

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492 **CONFLICT OF INTEREST**

The authors have no conflicts of interest to declare.

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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 $\Delta\Delta CT$ 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 $\Delta\Delta CT$ 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
<i>Extras for PCR</i>	2	29,36	3-4	No	No comorbidities detected
HMB (regular menstrual cycles*)	10	38 [31-44]	3 [2-5]	No	No comorbidities detected
<i>Extras for PCR</i>	6	32 [24-40]	4 [3-4]**	No	No comorbidities detected
Uterine fibroid	7	41 [34-47]	3 [1-4]***	Yes	<ul style="list-style-type: none">• 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.
<i>Extras for PCR</i>	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
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Downregulated in HMB versus controls (top 20 of 84 genes)

Gene Symbol	Gene Name	Adj. p value	Fold Change
BPIFB1	BPI fold containing family B, member 1	4.6E-3	9.3
(C20orf114)*			
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	TRHDE antisense RNA 1	5.0E-4	2.8
(LOC283392)			
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	1.8E-3	5.1
	alpha 1		
MIR1282*	MicroRNA 1282	1.7E-6	5.0
PRELID3B	PRELI domain containing 3B	2.1E-5	4.4
(SLMO2)			
SPRED*		8.3E-6	4.4
(LOC644701_)	Sprouty-related, EVH1 domain containing 1		
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	BPI fold containing family B, member 1	2.2E-4	21.8
(C20orf114)*			
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	2.7E-2	5.8
	1		
CCL4L2	Chemokine (C-C motif) ligand 4-like 2	7.9E-5	5.2
TEKT4P2	Tektin 4 pseudogene 2	2.7E-4	3.9
	(LOC100132288)		
CCL3L1	Chemokine (C-C motif) ligand 3-like 1	2.3E-3	3.3
CFTR	Cystic fibrosis transmembrane conductance	5.8E-5	3.0
	regulator (ATP-binding cassette sub-family C,		
	member 7)		
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),	5.8E-3	2.8
	member 7		
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	5.8E-5	2.7
	ligase 2		

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*		7.6E-5	4.4
(LOC644701_)	Sprouty-related, EVH1 domain containing 1		
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

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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 metalloproteinase 26	3.6E-3	2.8
HLA-DQB1	Major histocompatibility complex, class II, DQ beta	1.0E-2	2.8
	1		

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

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OX40 Signalling Pathway	1.4	1.46E-01	CD3G, HLA-A, HLA-C, HLA-DPB1, HLA-DQA1, HLA-
<i>(OX40 is involved in T cell activation)</i>		(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,
<i>(Allorecognition of non-self antigens, involves a T cell response.)</i>		(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,
<i>(Migration from vascular system to site of infection and inflammation)</i>		(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)	
<i>(IL-17A and IL-17F are interleukins linked to infection, inflammation and autoimmune disease)</i>			
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	HLA-DPB1, HLA-DQA1, HLA-DQB1, HLA-DRB1, HLA-DRB4
		(5/48)	
Granulocyte Adhesion and Diapedesis (neutrophils, basophils and eosinophils)	2.38	4.85E-02	CCL2, CCL3L1, CCL3L3, CCL4, CCL4L1/CCL4L2CXCL5, IL1RN, MMP26
		(8/165)	
Antigen Presentation Pathway	2.05	1.11E-01	HLA-DPB1, HLA-DQA1, HLA-DRB1, HLA-DRB4
		(4/36)	
Graft-versus-Host Disease Signalling	1.98	1.03E-01	HLA-DQA1, HLA-DQB1, HLA-DRB1, IL1RN
		(4/39)	
CDC42 Signalling	1.71	4.88E-02	HLA-DPB1, HLA-DQA1, HLA-DQB1, HLA-DRB1, HLA-DRB4, MYL3
		(6/123)	
B Cell Development	1.63	1.3E-01	HLA-DQA1, HLA-DQB1, HLA-DRB1
		(3/23)	

*-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.

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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, PI3R, 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,

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	synthetic mimic of double-stranded viral RNA			ITGAX, NLRP3, PIGR, ST8SIA4, TRPV1
Lipopolysacchari	LPS: found in the outer membrane of gram-	-0.045	1.26E-04	AKAP2, C3, CCL3L3, CREBBP, CXCL6, FOSB,
de ^{yy}	negative bacteria			HBEGF, HLA-A, IL19, IL6ST, ITGAX, LBP, MUC5AC,
				NFAT5, NFATC1, NLRP3, ORM1, OSM, PIGR,
				PTGDS, SAA1, SAA2, SLC39A8, SLC5A1, WISP1
Phorbol	Phorbol myristate acetate: chemical drug with	-0.096	1.29E-04	C3, CCL3L1, CCL3L3, CREBBP, FOSB, HBEGF,
myristate	potent tumour promoter activity, activates			HSD17B2, IGF2, ITGA2B, ITGAX, MUC4, MUC5AC,
acetate ^y	protein kinase C and NF-kB.			NFAT5, NFATC1, OSM, PTGDS, TNFRSF18, UTS2
Control versus Fibroid groups				
IL1B ^y	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,

Gene	Pathway	Enrichment Score	P-value	Genes
IL1 ^{***}	Group of interleukin genes including IL1B.	-0.694	2.69E-08	DEFB4A/DEFB4B, DLX1, FN1, FOSB, FUT3, GEM, GSTA1, HBEGF, HLA-A, HP, HSD11B1, HSPG2, IFNE, IGF2, IGFBP1, IL17C, IL19, IL1RL1, 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
STAT5A [¥]	Signal transducer and activator of transcription 5A (pleiotrophic transcription	-0.808	4.75E-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

	regulator)				PRR15L, RNF39, RORA, SERPINA3, SLC34A2,
					SYTL2, TFF3, TNNC1, TRAF4, TRIM31, TSPAN8
Resveratrol	A polyphenol produced by several plants 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,

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P2RY11, P2RY2, PDPK1, PLK3, PTPRC, RPL7,
SHROOM1, SMAD5, SUMO1, TGM1, TNFRSF11B,
VCAM1, VGLL3
AGR3, ANGPTL4, CYR61, FOSB, HBEGF, HBG1,
HBG2, IL1RL1, IL2RA, LGALS7/LGALS7B, SLC22A3,
ST3GAL1
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,

SFTPA1	Surfactant protein A1: Lung surfactant protein	Inhibited in	-2.887	4.33E-07
	that has a role in surfactant homeostasis and	Fibroid		
	in defence against pathogens.			
TGFB1	Transforming growth factor, beta 1:	Activated in	2.221	6.20E-07
	pleiotrophic cytokine	Fibroid		

					SLC1A2, SMTN, TBX3, TGFB1I1, TMOD3,
					TNFRSF11B, VCAM1, WISP1, XDH, XYLT1
	Dexamethasone [‡]	Synthetic glucocorticoid	0.277	7.13E-07	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	-0.682	7.21E-07	BAX, CCR5, CCR7, FOXO1, FOXO3, HBEGF, IL23A,
		transfer in cells.			IL2RA, IL33, MMP3, NFATC1, P2RY2, VCAM1

HMB versus Fibroid Groups					
Peptidoglycan	Bacterial cell wall constituent	Activated in	2.399	7.35E-08	BCL2A1, CCL2, CCL3L3, CCL4, CXCL5,
		HMB			DEFB4A/DEFB4B, IL1RN, NLRP3, PRAME
Lipopolysaccharide ^{yy}	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 ^{yyy}	Group of interleukin genes including IL1B.	Activated in	2.387	7.27E-07	CCL2, CCL4, CXCL5, DEFB103A/DEFB103B, DEFB4A/DEFB4B, HP, IGFBP1, IL1RN, PLA2G4A, S100A3, SAA2, UGT2B17, XDH
		HMB			
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	

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

* 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%)

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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%)

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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 $\Delta\Delta CT$ 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 $\Delta\Delta CT$ 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
RNA <i>Spike</i>	Sense 5'-TTCCCTTTAGTGAGGGTTAATTTTCG -3'	
(<i>Taqman</i>)	Antisense 5'- GAGCGGATAACAATTTACACACA -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%)	<p>Up-regulated in Controls relative to HMB: <i>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</i></p> <p>Down-regulated in Controls relative to HMB: <i>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</i></p>

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

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

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

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**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 % ^y) 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: <i>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, EPHYC, 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,</i>

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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, TGFB11I, 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,*

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

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				<i>HLA-DRB1, LILRA3, LRRK2, LSR, LYNX1, MARVELD3, MICA, MSLN, MUC4, NRCAM,</i>
				<i>OR4K15, P2RY12, P2RY2, PCDHGC3, PIGR, PKD1L2, PROM2, SCNN1G, SEMA4G,</i>
				<i>SIGLEC14, SLC16A14, SLC16A9, SLC1A2, SLC1A3, SLC27A6, SLC2A11, SLC34A2, SLCO1B1,</i>
				<i>SYT1, TAP2, TREML1, TSPAN12, TSPAN8, UNC13A, VCAM1, VPS16</i>
3. Oxygen Transport and	3.70	15 (2.6%)	Up-regulated in Fibroids relative to Controls: <i>CYP1A1, CYP2A13, EPX, HBD, HBG1,</i>	
Haemoglobin			<i>HBG2, HBM, HBQ1, HBZ, PLG</i>	
				Down-regulated in Fibroids relative to Controls: <i>ADH1A, CA8, CYP4X1, HP, KRT5</i>
4. Cell Adhesion	3.10	30 (5.3%)	Up-regulated in Fibroids relative to Controls: <i>BAI1, CD34, CD6, CYR61, DDR1, DSC1,</i>	
				<i>DST, FN1, GPR56, MSLN, PCDH10, TGFB111, WISP1</i>
				Down-regulated in Fibroids relative to Controls: <i>CCL4L2, CDH12, CDH3, CXADR,</i>
				<i>FCGBP, ITGA2B, ITGB1BP1, ITGB4, MUC4, MUC5AC, NRCAM, OLFM4, PCDHGC3, RELN,</i>
				<i>SIGLEC14, SSPO, VCAM1</i>
5. Cystine knot and von Willebrand	2.81	8 (1.4%)	Up-regulated in Fibroids relative to Controls: <i>CYR61, VWCE, WISP1</i>	
				Down-regulated in Fibroids relative to Controls: <i>FCGBP, GREM2, MUC5AC, SOSTDC1,</i>
				<i>SSPO</i>
6. Cell migration and motility	2.75	23 (4.0%)	Up-regulated in Fibroids relative to Controls: <i>BAX, CD34, DCLK1, FN1, GAB2, HBEGF,</i>	
				<i>MMP14, NR4A2</i>

				Down-regulated in Fibroids relative to Controls: <i>BMP7, CCL4L2, CD2AP, EDN3, HOXA2, ITGB1BP1, NRCAM, RELN, ROCK1, SAA1, SAA2, SCNN1G, SEMA3C, TNFSF13, VCAM1</i>
7. Immune Response, MHC Protein Complex	2.67	41 (7.2%)		Up-regulated in Fibroids relative to Controls: <i>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</i>
				Down-regulated in Fibroids relative to Controls: <i>CDH3, CFB, CPE, CXADR, ERAP2, HLA-C, HLA-DRB1, ITGA2B, ITGB4, LILRA3, MAVS, MICA, NRCAM, PIGR, SIGLEC14, TAP2, TNFSF13, VCAM1</i>
8. Cell junctions	2.57	31 (5.4%)		Up-regulated in Fibroids relative to Controls: <i>ARC, BAI1, CDC42BPA, CDH3, DENND1A, DIXDC1, DSC1, DSP, DST, ENAH, FAIM3, GABBR1, GRIN2B, IL1RL1, PTPRC, SDC1, SH3KBP1, SLC16A10, TGFB1I1, UBN1, VAMP1, XIRP1</i>
				Down-regulated in Fibroids relative to Controls: <i>CFTR, CXADR, ITGA2B, ITGB4, P2RY2, SLC1A3, SYT1, TGM1, UNC13A</i>
9. Immunoglobulin	2.55	37 (6.5%)		Up-regulated in Fibroids relative to Controls: <i>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</i>
				Down-regulated in Fibroids relative to Controls: <i>CXADR, FGFR3, HLA-C, HLA-DRB1,</i>

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

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

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

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

*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: <i>CA8, HP, XDH</i> Down-regulated in Fibroids relative to HMB: <i>CD8A, CYP26A1, CYP2C9, HBD, HBG1, HBG2, HBM, HBQ1, HBZ, SLC4A1, TCF7L2</i>
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: <i>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</i> Down-regulated in Fibroids relative to HMB: <i>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, LYSDM4, MMP26,</i>

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

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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