Changes in Gene Expression in Human Meibomian Gland Dysfunction

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Purpose. Meibomian gland dysfunction (MGD) may be the leading cause of dry eye syndrome throughout the world. However, the precise mechanism(s) underlying the pathogenesis of this disease is unclear. This study was conducted to identify meibomian gland genes that may promote the development and/or progression of human MGD.

Methods. Lid tissues were obtained from male and female MGD patients and age-matched controls after eyelid surgeries (e.g., to correct entropion or ectropion). Meibomian glands were isolated and processed for RNA extraction and the analysis of gene expression.

Results. The results show that MGD is associated with significant alterations in the expression of almost 400 genes in the human meibomian gland. The levels of 197 transcripts, including those encoding various small proline-rich proteins and S100 calcium-binding proteins, are significantly increased, whereas the expression of 194 genes, such as claudin 3 and cell adhesion molecule 1, is significantly decreased. These changes, which cannot be accounted for by sex differences, are accompanied by alterations in many gene ontologies (e.g., keratinization, cell cycle, and DNA repair). The findings also show that the human meibomian gland contains several highly expressed genes that are distinct from those in an adjacent tissue (i.e., conjunctival epithelium).

Conclusions. The results demonstrate that MGD is accompanied by multiple changes in gene expression in the meibomian gland. The nature of these alterations, including the upregulation of genes encoding small proline-rich proteins and S100 calcium-binding proteins, suggest that keratinization plays an important role in the pathogenesis of MGD. (*Invest Ophthalmol Vis Sci.* 2011;52:2727–2740) DOI:10.1167/iovs.10-6482

Meibomian glands play a critical role in the health and well-being of the ocular surface.[1–6] These glands secrete a lipid and protein mixture that provides a clear optical surface for the cornea, interferes with bacterial colonization, and retards tear overflow.[1–8] The glandular secretions also enhance the stability and reduce the evaporation of the tear film.[1–7] Conversely, meibomian gland dysfunction (MGD) destabilizes the tear film, increases its evaporation and osmolarity and is believed to be the key trigger for the induction of evaporative dry eye syndrome.[1–6,8–14]

The major cause of MGD appears to be excretory duct obstruction, due to hyperkeratinization of the ductal epithelium and an increased viscosity of meibum.[15–22] This obstruction may lead to cystic dilatation of glandular ducts, acinar cell atrophy, and a loss of secretory meibocytes.[15,23] The MGD may also facilitate bacterial growth on the lid margin[24–26] and promote inflammation in the adjacent conjunctiva.[27,28] The development of MGD has been linked to several risk factors, including aging,[29–34] androgen deficiency,[35–42] isotretinoin treatment,[43–45] and possibly postmenopausal estrogen therapy.[46–49] However, the molecular mechanisms that underlie the pathogenesis of MGD are unknown. This lack of information, in turn, has hampered the generation of safe and effective therapies for the treatment of MGD.

We hypothesize that alterations in gene expression promote the development and/or progression of human MGD. We also hypothesize that identification of such genetic changes will provide unique insight into the pathogenesis of MGD and will reveal novel glandular targets for possible therapeutic intervention. The purpose of this investigation was to begin to test these hypotheses by taking advantage of new advances in cDNA microarray technology, computational biology, and bioinformatics.

Methods

Human Subjects, Clinical Evaluation, and Tissue Collection

Human eyelid tissues were obtained from adult individuals with MGD (mean age, 73 ± 4 years) and from age-matched controls (mean age, 65 ± 9 years), after lid resection surgery at the Massachusetts Eye and Ear Infirmary (Table 1). Before surgery, normal or dysfunctional meibomian glands were diagnosed in the participants by two ophthalmologists (i.e., one evaluated two controls and two patients; the other four controls and four patients), according to a published classification system.[44] In brief, the grading scheme was 0 for clear excreta with or without small particles, 1 for opaque cloudy excreta with normal viscosity, 2 for opaque excreta with increased viscosity, and 3 for secreta that retained shape after digital expression. Subjects were excluded from the study if they had active infection, used topical antiglaucoma or anti-inflammatory medications, or wore contact lenses. Lid segments were placed in RNA stabilizer (RNAlater; Ambion, Austin, TX) and stored at −80°C. Meibomian glands (2–5 glands/tarsal plate) were then isolated under a dissecting microscope (Bausch & Lomb, Rochester, NY), by the removal of skin, subcutaneous tissue, muscle, and palpebral conjunctiva, and were processed in entirety for molecular biological procedures. The use of human eyelid samples that would otherwise have been discarded was approved by the Institutional Review Boards of Massachusetts Eye and Ear Infirmary and Schepens Eye Research Institute and adhered to the tenets of the Declaration of Helsinki.

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Supported by Grant EY05612 from the National Institutes of Health and by Alcon Research, Ltd.

Submitted for publication August 27, 2010; revised December 15, 2010, and February 13, 2011; accepted March 1, 2011.

Disclosure: **S. Liu**, P; **S.M. Richards**, None; **K. Lo**, None; **M. Hatton**, None; **A.M. Fay**, None; **D.A. Sullivan**, Alcon (F), P

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TABLE 1. Age, Sex, Meibomian Gland Secretion Quality, and Medication History of Subjects

Subject	Sex	Age (y)	Reason for Surgery	Secretion Quality	Medication
Controls					
1	F	80	Entropion	0	Lisinopril
2	M	79	Ectropion	0	Atorvastatin
3	F	85	Entropion	0	Atenolol
4	M	70	Lid laxity	0	Atorvastatin
5	F	32	Lid retraction	0	Levothyroxine sodium
6	M	44	Floppy eyelid syndrome	0	None
Patients					
1	M	77	Ectropion	2	None
2	F	59	Entropion	3	Alendronate sodium, escitalopram
3	F	72	Ectropion	2	None
4	M	78	Ectropion	3	Atenolol, hydrochlorothiazide
5	F	66	Ectropion	3	Clonazepam
6	F	88	Ectropion	2	None

The interval between tissue collection and storage at −80°C was less than 1 day for all samples, except for control tissue 4. This tissue was first stored in RNAlater at 4 °C for 7 days, a temperature and time period that protects RNA quality quite well.[50] The grades for meibomian gland secretion quality are explained in the Materials and Methods section. There was no significant difference between the ages of the control and patient populations.

Molecular Biological Procedures

Total RNA was extracted (RNAqueous Kit; Ambion) and analyzed (RNA Nano 6000 Series II Chip with a 2100 Bioanalyzer; Agilent Technologies, Palo Alto, CA) to verify RNA integrity. The RNA concentrations and 260/280-nm ratios were determined with spectrophotometer (a NanoDrop 1000; Thermo Scientific, Waltham MA).

The RNA (100 ng) samples were processed by Asuragen (Austin, TX) for the quantitation of mRNA levels by using microarray expression analysis (HumanHT-12 ver. 3 Expression BeadChips; Illumina, San Diego, CA). The chips target greater than 25,000 annotated genes with more than 48,000 probes derived from NCBI (National Center for Biotechnology Information, Bethesda, MD) reference sequences and the UniGene databases (http://www.ncbi.nlm.nih.gov/UniGene; NCBI). Briefly, biotin-labeled cRNA samples were prepared (MessageAmp II; Ambion, Inc.), quantitated by UV spectrophotometry and examined with a capillary electrophoresis system (2100 Bioanalyzer; Agilent). The labeled cRNAs were used to probe the chips. Hybridization, washing, and scanning of the arrays were performed according to the manufacturer's instructions. Data were processed with appropriate software (BeadStudio software ver. 3; Illumina), by using background subtraction and cubic spline normalization. Standardized hybridization intensity values were adjusted by adding a constant, such that the lowest intensity value for any sample was 16.[51] Hybridization intensity measurements are generally relied on in microarray experiments to infer transcript abundance levels for specific genes.[51]

Normalized data were evaluated with commercial software (GeneSifter.Net; Geospiza, Seattle, WA). This comprehensive program also produced gene ontology and *z*-score reports. Ontologies encompassed biological processes, molecular functions, and cellular components and were organized according to the guidelines of the Gene Ontology Consortium (http://www.geneontology.org/).[52] Gene expression data were analyzed, with and without log transformation, and statistical evaluation of these data was performed with Student's *t*-test (two-tailed, unpaired). Genes that were expressed in similar directions in different experiments were identified by using the intersector program (GeneSifter.Net; Geospiza; www.public.genesifter.net). All data from the microarray chips (BeadChips; Illumina) are accessible for download through the National Center for Biotechnology Information's Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) via series accession number (GSE17822).

Real-Time PCR Procedures

The differential expression of selected genes was confirmed by using quantitative real-time PCR (qPCR) procedures. The cDNAs were transcribed by using a transcriptase (SuperScript III Reverse Transcriptase; Invitrogen, Grand Island, NY) and random hexamer primers (Invitrogen). Real-time PCR reactions ($n = 3$ control and 5 MGD separate samples) were performed in triplicate (TaqMan Gene Assays; Applied Biosystems, Inc. [ABI], Foster City, CA) and specific primers and probes for small proline-rich protein 2A (Hs03046643_s1), keratin 10 (Hs00166289_m1), S100 calcium-binding protein A9 (S100A9; Hs00610058_m1), S100 calcium-binding protein A8 (Hs00374264_g1), and β-actin endogenous control (4326315E). Differential gene expression was calculated according to the comparative CT method (outlined in ABI User Bulletin 2; updated 2001).

RESULTS

Gene Expression

Analysis of gene expression in human meibomian glands ($n = 12$ samples) led to the identification of 11,173 genes that exceeded the manufacturer's quality value of 0.95 (Illumina). Of these genes, 423 were associated with lipid metabolic processes, 185 with lipid biosynthesis, 116 with fatty acid metabolic processes, 89 with phospholipid metabolic processes, and 66 genes with lipid transport.

The intensity range for gene expression spanned from 29 to 29,219 units, with the mean values of 7 genes averaging above 20,000 units, 47 genes over 10,000 units, 204 genes above 3,000 units, and 689 genes between 1,000 to 10,000 units. Examples of high-intensity genes are shown in Table 2. These include transcripts such as stearoyl-CoA desaturase, mucin-like 1, keratinocyte differentiation-associated protein, fatty acid synthase, and S100 calcium-binding proteins A8 and A9 (S100A8, S100A9). Several of these glandular transcripts have recently been discovered as proteins in human meibomian gland secretions,[8] including adenylate cyclase 7; dystrophin, Ellis-van Creveld syndrome protein; keratin-1, -5, -6, -7, -9, -10, -13, -14, and -16; the lacrimal proline-rich protein proline-rich 4 (similar to 1); lipocalin 1; seven transmembrane helix receptor mucin-like 1 (also termed small breast epithelial mucin); synaptonemal

TABLE 2. Gene Expression in Human Meibomian Glands

Accession Number	Gene	Intensity	Ontology
NM_021706	Leukocyte-associated immunoglobulin-like receptor 1	28,175 ± 2,172	Immune response
NM_024793	Clusterin associated protein 1	21,931 ± 1,679	Protein binding
NM_058173	Mucin-like 1	17,929 ± 3,223	Extracellular region
NM_005063	Stearoyl-CoA desaturase	16,792 ± 1,963	Fatty acid biosynthetic process
NM_001001851	Inter-α (globulin) inhibitor H5	13,233 ± 1,190	Peptidase inhibitor activity
NM_021109	Thymosin β4, X-linked	12,611 ± 1,361	Actin cytoskeleton organization
NM_021009	Ubiquitin C	11,721 ± 6,31	Protein ubiquitination
NM_031409	Chemokine (C–C motif) receptor 6	7,458 ± 813	Chemotaxis
NM_002652	Prolactin-induced protein	7,440 ± 3,029	Biological process
NM_000422	Keratin 17	7,084 ± 751	Epidermis development
NM_018955	Ubiquitin B	6,714 ± 305	Protein ubiquitination
NM_004104	Fatty acid synthase	6,408 ± 805	Fatty acid biosynthetic process
NM_002964	S100 calcium binding protein A8	6,116 ± 1,469	Inflammatory response
NM_000239	Lysozyme	5,379 ± 2,840	Defense response
NM_004048	β2-Microglobulin	5,306 ± 826	Antigen processing and presentation of peptide antigen via MHC class I
NM_001017369	Sterol-C4-methyl oxidase-like	5,121 ± 672	Sterol biosynthetic process
NM_001002858	Annexin A2	4,449 ± 292	Calcium-dependent phospholipid binding
NM_002407	Secretoglobin, family 2A, member 1	4,426 ± 2,738	Androgen binding
NM_006918	Sterol-C5-desaturase-like	4,174 ± 585	Fatty acid biosynthetic process
NM_003251	Thyroid hormone responsive SPOT14	3,882 ± 510	Lipid metabolic process
NM_006755	Transaldolase 1	3,837 ± 376	Carbohydrate metabolic process
NM_001042465	Prosaposin	3,610 ± 147	Glycosphingolipid metabolic process
NM_005554	Keratin 6A	3,582 ± 699	Ectoderm development
NM_018677	Acyl-CoA synthetase short-chain family member 2	3,553 ± 384	Acetyl-coa biosynthetic process
NM_006432	Niemann-Pick disease, type C2	3,539 ± 187	Phospholipid transport
NM_018099	Fatty acyl CoA reductase 2	3,503 ± 469	Lipid biosynthetic process
NM_002965	S100 calcium binding protein A9	3,416 ± 873	Inflammatory response
NM_021103	Thymosin β10	3,301 ± 351	Actin cytoskeleton organization
NM_004462	Farnesyl-diphosphate farnesyltransferase 1	3,165 ± 262	Cholesterol biosynthetic process
NM_207392	Keratinocyte differentiation-associated protein	3,049 ± 406	Cell differentiation
NM_005555	Keratin 6B	2,800 ± 374	Ectoderm development
NM_001012964	Kallikrein-related peptidase 6	2,787 ± 387	Proteolysis
NM_016142	Hydroxysteroid (17β) dehydrogenase 12	2,750 ± 319	Steroid biosynthetic process
NM_000421	Keratin 10	2,721 ± 641	Epidermis development
NM_002153	Hydroxysteroid (17β) dehydrogenase 2	2,720 ± 326	Steroid biosynthetic process
NM_006320	Progesterone receptor membrane component 2	2,713 ± 237	Steroid binding
NM_005194	CCAAT/enhancer binding protein, beta	2,644 ± 175	Acute-phase response
NM_006667	Progesterone receptor membrane component 1	2,582 ± 259	Steroid binding
NM_001006946	Syndecan 1	2,433 ± 212	Protein C-terminus binding
NM_001711	Biglycan	2,427 ± 398	G-protein coupled receptor protein signaling pathway
NM_005987	Small proline-rich protein 1A	2,276 ± 310	Keratinization
NM_002274	Keratin 13	2,225 ± 747	Epidermis development
NM_003064	Secretory leukocyte peptidase inhibitor	1,673 ± 483	Serine-type endopeptidase inhibitor activity
NM_005564	Lipocalin 2	1,663 ± 320	Transport

The accession number is the sequence identity of the gene fragment expressed on the gene microarray and appears in the NCBI nucleotide database. The intensities of selected genes were obtained after normalizing all meibomian gland data and calculating the mean (±SE) of all 12 samples without regard to disease status. Values were generated from nontransformed data.

complex protein 3; transcription elongation factor A (SII)-like 1; UDP-*N*-acetylglucosamine pyrophosphorylase 1; and zinc metalloproteinase.

Our data also demonstrate that human meibomian gland gene expression is not the same as that of another lid tissue, human conjunctival epithelium. We compared 300 sequences with the highest average intensity in our samples' raw data to those from human conjunctival epithelia (Gene Expression Omnibus Series accession number GSE5543).[53] By this process we found that 42.3% of the sequences matched and most were for ribosomal genes. If ribosomal genes were excluded, then only 24.1% of the remaining sequences were similar between the meibomian gland and conjunctiva. In effect, the most highly expressed genes in the human meibomian gland are unlike those in human conjunctival epithelia.

Effect of MGD on Gene Expression

Evaluation of non- and log-transformed data from controls subjects ($n = 6$) and MGD patients ($n = 6$) showed that

TABLE 3. Influence of MGD on Gene Expression

	Control ↑	MGD ↑	Total
No transformation	163	164	327
Log transformation	186	174	360
Total	194	197	391

Data were analyzed with and without log transformation. The numbers of common and nonoverlapping genes between analytical categories were quantitated, and then the total number was calculated. The expressions of listed genes were significantly ($P < 0.05$) upregulated (↑) in the control or patient (MGD) groups.

Table 4. Effect of MGD on Gene Expression Ratios within the Human Meibomian Gland

Accession No.	Gene	Ratio	*P*	Chromosome	Ontology
MGD > C					
NM_005416	Small proline-rich protein 3	19.48	0.0022	1	Epidermis development
NM_173086	Keratin 6C	12.42	0.0351	12	Cytoskeleton organization
NM_002963	S100 calcium binding protein A7	7.33	0.0476	1	Innate immune response
NM_001014450	Small proline-rich protein 2F	6.53	0.0039	1	Epidermis development
NM_005988	Small proline-rich protein 2A	6.3	0.0056	1	Epidermis development
NM_002964	S100 calcium binding protein A8	5.99	0.0123	1	Inflammatory response
NM_016190	Cornulin	5.44	0.0135	1	Cell-cell adhesion
NM_001024209	Small proline-rich protein 2E	5.42	0.0160	1	Epidermis development
NM_002965	S100 calcium binding protein A9	5.08	0.0092	1	Inflammatory response
NM_002638	Peptidase inhibitor 3, skin-derived	4.03	0.0288	20	Serine-type endopeptidase inhibitor activity
NM_005213	Cystatin A	3.66	0.0397	3	Keratinocyte differentiation
NM_000421	Keratin 10 (epidermolytic hyperkeratosis)	2.98	0.0321	17	Epidermis development
NM_199161	Serum amyloid A2	2.9	0.0473	11	Acute-phase response
NM_005555	Keratin 6B	2.57	0.0261	12	Ectoderm development
C > MGD					
NM_001306	Claudin 3	3.57	0.0418	7	Cell-cell adhesion
NM_080390	Transcription elongation factor A (SII)-like 2	2.47	0.0483	X	Transcription
NM_014333	Cell adhesion molecule 1	2.43	0.0176	11	Immune response
NM_022154	Solute carrier family 39 (zinc transporter), member 8	2.02	0.0448	4	Zinc ion transport
NM_006206	Platelet-derived growth factor receptor, α polypeptide	1.92	0.0271	4	Cell activation
NM_000561	Glutathione *S*-transferase M1	1.72	0.0157	1	Catalytic activity
NM_000848	Glutathione *S*-transferase M2	1.65	0.0370	1	Catalytic activity
NM_139029	CD151 molecule	1.58	0.0092	11	Cell adhesion
NM_004853	Syntaxin 8	1.54	0.0090	17	Transport
NM_019071	Inhibitor of growth family, member 3	1.51	0.0348	7	Regulation of cell growth
NM_001759	Cyclin D2	1.45	0.0473	12	G1/S transition of mitotic cell cycle

Relative ratios were calculated by comparing the degree of gene expression in meibomian glands from control subjects and MGD patients. Data were analyzed with and without log transformation. Genes were selected from the top 50 up- and downregulated genes. C, control.

significant differences exist in meibomian gland gene expression between these groups. As demonstrated in Table 3, MGD was associated with a significant increase in the expression of 197 genes and a significant decrease in the activity of 194 genes, compared with that of the control population. Particularly notable was the MGD-related upregulation of genes for small proline-rich (SPRR) proteins 1A, 2A, 2E, 2F, and 3; cornulin; cystatin A; S100A8; and S100A9 (Table 4).

Many of the genes upregulated in MGD, as shown in Table 4, are located on chromosome 1. Significantly increased gene expression in MGD was also found on chromosomes 12 and 14, whereas higher gene activity was observed on chromosomes 13 and 17 in control tissues (Table 5).

To confirm in part the microarray results, we analyzed selected genes by qPCR. This experimental approach verified the significant ($P < 0.05$) upregulation of MGD on all tested genes, including small proline-rich protein 2A (2.6-fold ↑),

Table 5. Impact of MGD on Chromosomal Gene Expression in Human Meibomian Glands

Chromosome	MGD Genes ↑	Control Genes ↑	MGD *z*-Score	Control *z*-Score
14	12	8	**3.26**	1.16
1	25	19	**2.94**	0.86
12	13	5	**2.03**	−1.17
8	1	8	**−2.16**	0.44
5	2	4	**−2.20**	−1.67
7	1	12	**−2.35**	1.57
13	1	9	−1.39	**2.61**
17	9	14	0.75	**2.33**

Chromosomes with the highest and lowest *z*-scores were selected after the analysis of log-transformed microarray data. A *z*-score is a statistical rating of the relative expression of genes and depicts how much they are over- or underrepresented in a given gene list.[54] Positive *z*-scores reflect a greater number of genes meeting the criterion than is expected by chance, whereas negative *z*-scores represent fewer genes meeting the criterion than expected by chance.[54] The *z*-scores with values >2.0 or < −2.0 are significant and are highlighted in bold. MGD Genes ↑, number of genes upregulated in MGD tissues; Control Genes ↑, number of genes upregulated in control tissues; *z*-score, specific score for the upregulated genes in the MGD and control tissues. High and low values for the MGD and control groups are highlighted in bold.

TABLE 6. Influence of MGD on the Expression of Gene Ontologies in the Human Meibomian Gland

Ontology	MGD Genes ↑	Control Genes ↑	MGD *z*-Score	Control *z*-Score
Biological Process				
Keratinocyte differentiation	8	0	**9.66**	−0.74
Keratinization	5	0	**8.23**	−0.53
Epidermis development	9	1	**5.85**	−0.41
DNA repair	9	3	**4.40**	0.71
Mitotic cell cycle	12	2	**4.09**	−0.89
Generation of precursor metabolites and energy	8	1	**3.17**	−0.99
Translation	9	2	**3.12**	−0.53
Regulation of nitrogen compound metabolic process	14	27	**−2.75**	0.62
Regulation of gene expression	14	23	**−2.80**	−0.36
Cell communication	20	23	**−3.12**	**−2.03**
Regulation of cellular process	43	49	**−3.18**	−1.25
Cell surface receptor linked signal transduction	2	11	**−3.93**	−1.25
Cellular nitrogen compound metabolic process	2	7	−0.28	**3.23**
Nucleotide metabolic process	7	7	**2.15**	**2.62**
Transport	21	32	−0.77	**2.27**
Proteasomal ubiquitin-dependent protein catabolic process	3	3	**2.09**	**2.26**
Establishment of localization	22	32	−0.59	**2.22**
Transcription from RNA polymerase II promoter	6	1	−0.44	**−2.14**
Regulation of RNA metabolic process	12	7	−1.40	**−2.21**
Molecular Function				
Deoxyribonuclease activity	4	2	**7.15**	**3.61**
Structural molecule activity	18	3	**5.76**	−0.81
Structural constituent of ribosome	6	0	**4.23**	−1.07
Protein kinase binding	5	1	**3.57**	−0.03
Enzyme binding	11	7	**3.46**	1.82
Nuclease activity	5	2	**3.39**	0.88
Receptor binding	0	6	**−2.73**	−0.05
Signal transducer activity	6	7	**−2.80**	**−2.13**
Receptor activity	3	10	**−3.11**	−0.79
Heme binding	2	4	1.01	**3.27**
Transferase activity	14	22	0.16	**3.17**
Iron ion binding	3	7	0.27	**3.10**
Tetrapyrrole binding	2	4	0.90	**3.09**
Manganese ion binding	3	4	1.57	**2.79**
Cellular Component				
Cornified envelope	5	0	**11.92**	−0.37
Intracellular non–membrane-bound organelle	43	20	**5.22**	0.28
Cytoskeleton	28	10	**5.22**	−0.03
Intracellular part	110	83	**4.96**	**3.19**
Cytoplasm	89	68	**4.82**	**2.41**
Macromolecular complex	47	21	**4.74**	−0.43
Intrinsic to membrane	21	44	**−4.31**	0.84
Intracellular membrane-bound organelle	77	81	1.87	**3.99**
Cytoplasmic vesicle	2	13	−1.50	**3.8**
Vesicle	2	13	−1.58	**3.62**
Endoplasmic reticulum membrane	4	7	1.17	**3.54**
Extracellular region	12	7	−1.14	**−2.13**

Specific ontologies, with some of the highest and lowest z-scores, were chosen after the analysis of non- and log-transformed microarray data. Criteria for inclusion in this table were an ontology containing ≥5 genes and having a *z*-score >2.0 or < −2.0. High and low values for the patient (MGD) and control groups in designated ontologies are highlighted in bold.

S100A8 (12.8-fold ↑), S100A9 (5.9-fold ↑), and keratin 10 (4.6-fold ↑).

Influence of MGD on the Expression of Gene Ontologies

The influence of MGD was quite striking in its stimulation of various gene ontologies (Table 6), such as keratinization (Table 7); cell cycle (Table 8); DNA repair (Table 9); and energy generation, translation, structural molecule activity, cytoplasmic vesicles, and cornified envelope components (Table 10). In contrast, meibomian glands of control subjects presented with a significant increase in ontologies associated with cellular nitrogen compound metabolic processes, transport, transferase activity, iron and manganese binding, and intracellular membrane-bound organelles (Table 6).

Examination of the data also showed that certain gene ontologies were overrepresented in both control and dysfunctional meibomian glands, including nucleotide metabolic processes, proteasomal ubiquitin-dependent protein catabolic processes, DNase activity and cytoplasm (Table 6). Other gene ontologies were underrepresented in both groups, such as cell communication and signal transducer activity (Table 6).

Impact of Sex on Gene Expression

Given that the control and patient groups were not sex matched (Table 1), it is possible that sex differences

TABLE 7. Effect of MGD on Genes Related to Keratinization

Gene	Keratinocyte Differentiation	Keratinization	Epidermal Cell Differentiation	Ectoderm Development	Epidermis Development	Tissue Development
Actin, α, cardiac muscle 1						↑
Activin A receptor, type 1B				↓	↓	↓
Cornifelin	↑	↑	↑	↑	↑	↑
Cyclin-dependent kinase inhibitor 2B						↑
Cystatin A	↑		↑	↑	↑	↑
E2F transcription factor 4						↑
Exostoses						↓
Keratin 10				↑	↑	↑
Keratin 6B				↑	↑	↑
LIM homeobox 1				↓		↓
Mitogen-activated protein kinase kinase 1	↑		↑	↑	↑	↑
Myogenic factor 6						↑
Pleiotrophin						↓
Protein kinase C, θ						↑
S100 calcium binding protein A7	↑		↑	↑	↑	↑
Small proline-rich protein 1A	↑	↑	↑	↑	↑	↑
Small proline-rich protein 2A	↑	↑	↑	↑	↑	↑
Small proline-rich protein 2E	↑	↑	↑	↑	↑	↑
Small proline-rich protein 2F	↑	↑	↑	↑	↑	↑
Small proline-rich protein 3	↑	↑	↑	↑	↑	↑
V-akt murine thymoma viral oncogene homolog 1				↓	↓	↓

Genes in a given ontology were significantly ($P < 0.05$) upregulated in meibomian glands of MGD patients (↑) or control subjects (↓). Results were generated by the analyses of non- and log-transformed data. The *z*-scores for log-transformed categories were 9.7 (keratinocyte differentiation), 9.0 (epidermal cell differentiation), 8.0 (keratinization), 6.3 (ectoderm development), 5.9 (epidermis development), and 3.7 (tissue development).

in meibomian gland gene expression may have contributed to the MGD-related results. More specifically, because more females were present in the MGD group, it may be that our data reflect female- rather than disease-associated genes. To explore this possibility, we compared meibomian gland gene expression between men ($n = 5$) and women ($n = 7$) and then compared these data to those between controls and patients.

Our analyses of non– and log-transformed data demonstrate that the sex of the individual has a significant impact on gene expression in human meibomian glands. We identified 257 genes that show sex-related differences, with 137 genes more highly expressed in men and 120 in women. Examples of these genes, which include prolactin-induced protein (male > female) and S100 calcium-binding protein A7 (female > male), are depicted in the Table 11. Sex also has a significant effect on the expression of gene ontologies in the human meibomian gland, such as processes involving Golgi vesicle transport, translation, amino acid ligase activity, protein binding, endoplasmic reticulum, and ribosomes (Table 12).

However, these sex-related differences do not account for the MGD-associated changes in gene expression. Comparison of these data sets showed that only four genes (e.g., zinc finger protein 658 and S100 calcium-binding protein A7) were upregulated, and only four genes were downregulated (e.g., der1-like domain family, member 2, and esterase D/formylglutathione hydrolase), in meibomian glands of both women (versus men) and patients (versus controls). Moreover, comparison of the ontologies listed in Tables 6 and 12 demonstrate that, of the 40 biological process (36 different, 4 the same), 19 molecular function (16 different, 3 the same), and 31 cellular component (25 different, 6 the same) ontologies, the majority ($n = 77$) were not the same ($n = 13$) in both women and patients.

DISCUSSION

Our study shows that MGD exerts MGD exerted a significant effect on gene expression in the human meibomian gland. Thus, MGD was found to be associated with significant alterations in the expression of almost 400 genes, including those encoding SPRR, S100A8,and S100A9. These changes, which cannot be accounted for by sex-associated differences between control and patient populations, are accompanied by the up- and downregulation of many ontologies (e.g., keratinization, cell cycle, DNA repair, translation, cytoplasmic vesicles, and cornified envelope components). Our study also demonstrates that the human meibomian gland contains several highly expressed genes that are distinct from those in an adjacent tissue (i.e., conjunctival epithelium). Overall, the findings support our hypothesis that alterations in gene expression promote the development and/or progression of human MGD. Our data also reveal novel glandular targets for possible therapeutic intervention in MGD.

We identified several SPRR, S100A8, and S100A9 genes that are significantly upregulated in human MGD. The SPRRs are precursor proteins of the cornified cell envelope of terminally differentiating stratified squamous epithelia and influence the flexibility, rigidity, or toughness of this envelope.[55–59] The increased expression of these SPRR genes reflect the stimulation of meibomian gland ontologies associated with keratinization; keratinocyte and epidermal cell differentiation; ectoderm, epidermis, and tissue development; and cornified envelope. Of particular interest, the

TABLE 8. Influence of MGD on Genes Associated with the Cell Cycle

Gene	Cell Cycle	Cell Cycle Phase	Mitotic Cell Cycle	Interphase of Mitotic Cell Cycle	Cell Cycle Process	Cell Cycle Checkpoint	M Phase of Mitotic Cell Cycle
Activin A receptor, type IB	↓	↓	↓	↓	↓		
Cell division cycle 73, Paf1/RNA polymerase II complex component, homolog	↓				↓		
Centromere protein E	↑	↑	↑		↑	↑	↑
Chromatin modifying protein 1B	↑				↑		
Chromosome 13 open reading frame 15	↓						
Cyclin B2	↑	↑	↑		↑		↑
Cyclin D2	↓				↓		
Cyclin-dependent kinase inhibitor 2B	↑	↑	↑	↑	↑	↑	
E2F transcription factor 4, p107/p130-binding	↑				↑		
General transcription factor IIH, polypeptide 1	↑				↑		
Growth arrest and DNA-damage-inducible, α	↑	↑	↑	↑	↑		
Karyopherin α2	↑	↑	↑	↑	↑		
Katanin p80 (WD repeat containing) subunit B 1	↑	↑	↑		↑		↑
Mitogen-activated protein kinase kinase 1	↑	↑	↑		↑		↑
M-phase phosphoprotein 6	↑	↑	↑		↑		↑
MRE11 meiotic recombination 11 homolog A	↓	↓			↓		
Opa interacting protein 5	↑	↑	↑		↑		↑
Potassium channel tetramerization domain containing 11	↑				↑		
Proteasome (prosome, macropain) 26S subunit, ATPase, 4	↑		↑		↑		
Proteasome (prosome, macropain) 26S subunit, non-ATPase, 10	↓		↓		↓		
Proteasome (prosome, macropain) activator subunit 2	↑		↑		↑		
Protein kinase C, θ	↑	↑	↑	↑	↑		
Protein phosphatase 1, catalytic subunit, α isoform	↑				↑		
Septin 4	↓				↓		
Translocated promoter region (to activated MET oncogene)	↑	↑	↑		↑	↑	↑
V-akt murine thymoma viral oncogene homolog 1	↓	↓	↓	↓	↓		
X-ray repair complementing defective repair in Chinese hamster cells 2	↑	↑			↑		

Genes in the designated ontologies were significantly ($P < 0.05$) upregulated in meibomian glands of MGD patients (↑) or control subjects (↓). Results were obtained by the evaluation of non- and log-transformed data. The *z*-scores for log-transformed categories were 4.1 (mitotic cell cycle), 3.3 (cell cycle), 3.1 (cell cycle phase), 3.1 (M phase of mitotic cycle), 3.0 (cell cycle process), 2.7 (cell cycle checkpoint), and 2.3 (interphase of mitotic cell cycle).

SPRRs, whether alone[55] or in combination with S100A8 and -A9,[60] are known to promote keratinization, such as in the conjunctiva[61,62] and elsewhere in the body.[63,64] Further, increased S100A8 and S100A9 expression induces proliferation of human keratinocytes.[65] If translated, these SPRR, S100A8, and S100A9 proteins may be responsible, at least in part, for the hyperkeratinization of meibomian gland ductal epithelium. This process is believed to be a primary cause of MGD.[1–6,8–14]

It is possible that SPRRs and S100A8 and S100A9 represent novel and unique targets for the potential topical treatment of MGD. First, downregulation of SPRRs may represent a mechanism to inhibit the keratinization process. Second, attenuation of S100A8 and S100A9 may permit suppression of both the keratinization and the possible conjunctival inflammation associated with MGD. The proteins S100A8 and S100A9 form a heterodimeric protein complex called calprotectin, which is a Toll-like receptor 4 agonist and a damage associated molecular pattern molecule (DAMP).[66] A DAMP can initiate and perpetuate an immune response in noninfectious inflammation.[66] Typically, calprotectin may play an important role in regulating cellular homeostasis (e.g., cytoskeleton), but after secretion may become a danger signal during inflammatory processes.[66] The secreted S100A8 and -A9 amplifies inflammatory responses[66,67] and could induce extravasation of leukocytes into the adjacent conjunctiva. Such conjunctival inflammation may occur during MGD.[27,28] For comparison, the S100A8 and -A9 proteins may be involved in the pathophysiology of pterygium,[68,69] as well as in Sjögren's syndrome, rheumatoid arthritis, inflammatory bowel disease, vasculitis, lichen planus, and lupus erythematosus.[70–73]

However, although calprotectin is commonly identified as a promoter and/or marker of inflammation,[67,74–76] it may also act, depending on its concentration (e.g., high), in an anti-inflammatory manner.[70,77] In fact, calprotectin may exert a variety of protective antioxidant, anti-invasive, and antimicrobial functions.[70,77,78] An anti-inflammatory action of calprotectin would be especially intriguing, given that the most highly expressed gene in the human meibomian gland is leukocyte-associated immunoglobulin-like receptor (LAIR)-1. The LAIR-1 protein is an inhibitory receptor that attenuates immune cell activation and suppresses proinflammatory cytokine production.[79,80] Working in tandem, calprotectin and LAIR-1 could theoretically be responsible for the typical absence of inflammation[20,23,81] within the meibomian gland in MGD. In addition, the ability of calprotectin to make epithelial cells more resistant to bacterial invasion[82] could be one reason why such bacterial infection is minimal or absent in obstructive MGD.[20] Overall, calprotectin could possibly protect the meibomian gland against inflammation and infection in MGD. Whether it exerts either or both of these activities or is instead proinflammatory remains to be determined.

TABLE 9. Impact of MGD on Genes Associated with DNA Repair

Gene	DNA Repair	Cellular Response to DNA Damage Stimulus	DNA Catabolic Process	DNA Metabolic Process
APEX nuclease (multifunctional DNA repair enzyme) 1	↑	↑		↑
Cryptochrome 2 (photolyase-like)	↓	↓		↓
DNA fragmentation factor, 45 kDa, alpha polypeptide			↑	
Flap structure-specific endonuclease 1	↑	↑		↑
General transcription factor IIH, polypeptide 1	↑	↑	↑	↑
Growth arrest and DNA-damage-inducible, α	↑	↑		↑
MRE11 meiotic recombination 11 homolog A	↓	↓		↓
MUS81 endonuclease homolog	↓	↓		↓
Nudix (nucleoside diphosphate linked moiety X)-type motif 1	↑	↑		↑
Thymine-DNA glycosylase	↑	↑	↑	↑
Topoisomerase (DNA) II α	↑	↑		↑
Tyrosyl-DNA phosphodiesterase 1	↑	↑		↑
X-ray repair complementing defective repair in Chinese hamster cells 2	↑	↑		↑
DNA fragmentation factor, 45 kDa, alpha polypeptide			↑	↑
Karyopherin α 2				↑
Nuclear factor I/B				↓
PC4 and SFRS1 interacting protein				↓
Platelet-derived growth factor, α polypeptide				↓

Genes in the selected ontologies were significantly ($P < 0.05$) upregulated in meibomian glands of MGD patients (↑) or control subjects (↓). Results were acquired by the examination of non- and log-transformed data. The *z*-scores for nontransformed categories were 4.4 (DNA repair), 3.8 (cellular response to DNA damage stimulus), 3.7 (DNA catabolic process), and 3.1 (DNA metabolic process).

Additional meibomian gland genes that are modulated in MGD may play a role in these keratinization, antimicrobial, and structural processes. For example, the upregulated genes cornulin and cystatin A may promote epidermal differentiation,[83,84] and the increased peptidase inhibitor 3, skin-derived, transcripts, if translated, may inhibit bacterial infection.[84] In addition, the genes encoding claudin 3, cell adhesion molecule 1, and CD151 molecule are downregulated in MGD, a response that may disrupt cell adhesion.[84] Our data also show that many of the most highly upregulated genes in MGD are from chromosome 1. In fact, the SPRR, S100A8, and S100A9 genes colocalize on chromosome 1 at q21, the region called the epidermal differentiation complex.[60] There are more than 850 diseases related to this chromosome, including deafness, Alzheimer's, glaucoma, and breast cancer.[85]

The influence of MGD is striking in its stimulation of various gene ontologies, such as cell cycle, DNA repair, generation of precursor metabolites, and translation. These responses may be analogous to those in other sebaceous glands, wherein stem cell niches are mobilized to repair injured or diseased tissue.[86,87] Of interest, a deficiency in the capacity to repair DNA may lead to neoplasia.[88,89]

A notable finding was our discovery that significant sex-related differences exist in gene expression in the human meibomian gland. Genes encoding such proteins as lysozyme and neurocalcin δ were upregulated in males (M), whereas leptin gene expression was increased in females (F). Analogous sex-associated differences have been observed for lysozyme in stimulated submandibular/sublingual saliva (M > F),[90] neurocalcin δ in the olfactory bulb (M > F),[91] and leptin in the plasma (F > M).[92] More importantly, sex-associated variations are also known to occur in the morphologic appearance, neutral and polar lipid profiles, and secretory output of the meibomian gland, as well as in the gene expression of murine meibomian tissues.[31,33–37,39,40,42,93–98] Many of these differences appear to be due to the effects of sex steroid hormones and sex-specific aromatase activities (Liu S, et al. *IOVS* 2007; 43:ARVO E-Abstract 5657).[49,98] It is possible that these sex-related differences in the meibomian gland contribute to the increased prevalence of dry eye disease in females.[11]

Our data demonstrate that the human meibomian gland contains several highly expressed genes that are associated with lipid dynamics, glandular structure, immunologic factors, and hormone action. For instance, these genes encode proteins involved in cholesterol transport (Niemann-Pick disease, type C2); cholesterol (farnesyl-diphosphate farnesyltransferase 1; sterol-C4-methyl oxidase-like; sterol-C5-desaturase-like), fatty acid (stearoyl-CoA desaturase; thyroid hormone responsive SPOT14; fatty acid synthase), and lipid (acyl-CoA synthetase short-chain family member 2; transaldolase 1) biosynthesis; fatty acyl-CoA reduction (fatty acyl CoA reductase 2) and elongation (hydroxysteroid (17β) dehydrogenase 12); and glycosphingolipid catabolism (prosaposin).[84,99] The primary product of stearoyl-CoA desaturase is oleic acid, which has been identified in meibomian gland secretions[100,101] and shown to be decreased in meibomianitis.[102] Of interest, androgens upregulate stearoyl-CoA desaturase mRNA levels in mouse male and female meibomian glands,[49,103] and the targeted disruption or this rate-limiting

TABLE 10. Effect of MGD on Genes Associated With Energy Generation, Translation, Structural Molecule Activity, Cytoplasmic Vesicles and Cornified Envelope Components in the Human Meibomian Gland

MGD Genes ↑	Control Subject Genes ↑
Generation of Precursor Metabolites and Energy	
Aldehyde dehydrogenase 5 family, member A1	V-akt murine thymoma viral oncogene homolog 1
ATP synthase, H+ transporting, mitochondrial F0 complex, subunit d	
Dihydrolipoamide S-succinyltransferase	
Glyceraldehyde-3-phosphate dehydrogenase	
Phosphoglycerate kinase 1	
Protein phosphatase 1, catalytic subunit, α isoform	
Succinate dehydrogenase complex, subunit A, flavoprotein	
Thioredoxin	
Translation	
Eukaryotic translation elongation factor 1 β2	HBS1-like V-akt murine thymoma viral oncogene homolog 1
Eukaryotic translation initiation factor 6	
Mitochondrial ribosomal protein L15	
Mitochondrial ribosomal protein S18B	
Ribosomal protein L29	
Ribosomal protein L41	
Ribosomal protein S27a	
Ribosomal protein S29	
Translocated promoter region (to activated MET oncogene)	
Structural Molecule Activity	
Cystatin A	Claudin 3
Keratin 10	Coatomer protein complex, subunit γ
Keratin 6B	Septin 4
Keratin 6C	
Mal, T-cell differentiation protein	
Mitochondrial ribosomal protein L15	
Mitochondrial ribosomal protein S18B	
Myosin binding protein C, slow type	
Myosin binding protein H	
Nebulin	
PDZ and LIM domain 3	
Plakophilin 1 (ectodermal dysplasia/skin fragility syndrome)	
Ribosomal protein L29	
Ribosomal protein L41	
Ribosomal protein S27a	
Ribosomal protein S29	
Small proline-rich protein 1A	
Small proline-rich protein 2E	
Small proline-rich protein 3	
Cytoplasmic Vesicle	
Annexin A2	Aquaporin 2 (collecting duct)
Melan-A	Brain-derived neurotrophic factor
	Cell adhesion molecule 1
	Chloride intracellular channel 4
	Coatomer protein complex, subunit γ
	Esterase D/formylglutathione hydrolase
	Fibronectin type III domain containing 3A
	Golgi associated PDZ and coiled-coil motif
	Heat shock protein 90kDa alpha (cytosolic), class A member 1
	Rab acceptor 1 (prenylated)
	Ribophorin I
	Synaptophysin-like 1
	Syntaxin binding protein 3
Cornified Envelope	
Cornifelin	
Cystatin A	
Small proline-rich protein 1A	
Small proline-rich protein 2A	
Small proline-rich protein 2E	
Small proline-rich protein 2F	

Genes in the designated ontologies were significantly ($P < 0.05$) upregulated in meibomian glands of MGD patients (↑) or control subjects (↑). Data were obtained by the analysis of non- and log-transformed data. The *z*-scores for increased categories (log-transformed) in MGD patients were 11.6 (cornified envelope), 5.8 (structural molecule activity), 3.2 (generation of precursor metabolites and energy), and 3.0 (translation). The *z*-score for the upregulated ontology in control subjects was 3.8 (cytoplasmic vesicle).

TABLE 11. Influence of Sex on Gene Expression Ratios within the Human Meibomian Gland

Accession No.	Gene	Ratio	*P*	Ontology
M > F				
NM_000239	Lysozyme	18.18	0.0422	Defense response to bacterium
NM_002652	Prolactin-induced protein	6.02	0.0422	Actin binding
NM_001040626	Neurocalcin δ	4.16	0.0424	Vesicle-mediated transport
NM_199462	Dual serine/threonine and tyrosine protein kinase	2.05	0.0011	Protein amino acid phosphorylation
NM_003033	ST3 beta-galactoside α2,3-sialyltransferase 1	2.0	0.0112	Protein amino acid glycosylation
F > M				
NM_006121	Keratin 1	8.41	0.0264	Epidermis development
NM_002963	S100 calcium binding protein A7	8.34	0.0353	Epidermis development
NM_006919	Serpin peptidase inhibitor, clade B, member 3	8.15	0.0289	Serine-type endopeptidase inhibitor activity
NM_000550	Tyrosinase-related protein 1	3.96	0.0317	Melanocyte differentiation
NM_001017920	Death associated protein-like 1	2.59	0.0182	Apoptosis
NM_002275	Keratin 15	2.42	0.0051	Epidermis development
NM_001069	Tubulin, β2A	2.33	0.0223	Microtubule-based movement
NM_001878	Cellular retinoic acid binding protein 2	2.32	0.0312	Signal transduction
NM_006390	Importin 8	2.15	0.0067	Intracellular protein transport
NM_031157	Heterogeneous nuclear ribonucleoprotein A1	2.07	0.0043	Nuclear mRNA splicing, via spliceosome
NM 000230	Leptin	2	0.0344	Glucose metabolic process

Relative ratios were calculated by comparing the degree of gene expression in meibomian glands from males ($n = 5$) and females ($n = 7$). Data were evaluated with and without log transformation. The listed genes have a known ontology, and the average intensities are ≥ 2-fold higher than those in the opposite sex.

enzyme causes meibomian gland atrophy.[104] Additional highly expressed genes are those related to extracellular matrix stabilization (inter-α (globulin) inhibitor H5), cell proliferation, migration and/or differentiation (clusterin associated protein 1; thymosin β4, X-linked; keratinocyte differentiation-associated protein; annexin A2), and proteasomal degradation (ubiquitin B and C).[84,105,106] One of these structure-related genes encodes biglycan, which, if overexpressed, leads to failure of meibomian gland formation in mice.[107] Immune-associated genes that present with high activities in the human meibomian gland are associated with antigen presentation (β2-microglobulin), dendritic and T-cell migration (chemokine (C-C motif) receptor 6), bacteriolytic action (lysozyme), and the inflammatory response (CCAAT/enhancer binding protein β, S100A8, S100A9, and LAIR-1).[84] Highly expressed genes related to the endocrine system include those that may bind androgens (secretoglobin, family 2A, member 1) and catalyze the transformation of estrone into estradiol (hydroxysteroid [17β] dehydrogenase 12).[84,108,109] Androgens are known to upregulate the prostatic levels of secretoglobin 2A1, a lipophilin that is also translated and strongly expressed in human meibomian gland epithelial cells.[110]

Several proteins encoded by meibomian gland genes have been identified in meibomian gland secretions.[8] In this regard, it is noteworthy that S100A8, S100A9, prolactin-inducible protein, lipocalin-1, lysozyme, β2-microglobulin, and the lacrimal proline-rich protein proline-rich 4 have all been termed tear film biomarkers in dry eye,[111,112] and some of these are proposed to be indicative of lacrimal gland aqueous deficiency.[112] However, given that the meibomian gland contains transcripts for all these proteins, it may be that alterations in their translation and secretion influence the tear film levels.

In this study, a number of the subjects were taking medications, including lisinopril (antihypertensive), atorvastatin (lowers blood cholesterol levels), atenolol (β1 receptor selective antagonist for cardiovascular disease), levothyroxine (hypothyroid treatment), alendronate (osteoporosis therapy), escitalopram (antidepressant), hydrochlorothiazide (diuretic), and clonazepam (antiepileptic). It is possible that some of these pharmaceutical agents influenced the secretion quality and/or gene expression in the meibomian gland. For example, atorvastatin inhibits a rate-limiting enzyme involved in the cholesterol synthetic pathway[113] and could theoretically alter the melting point and viscosity of meibomian gland secretions.[5] Similarly, thyroxine could possibly influence meibomian gland activity, given that this hormone has been reported to regulate the function of other sebaceous glands,[114] and antidepressant use is a risk factor for the development of dry eye syndrome.[42] However, none of these pharmaceutical compounds has been shown to have any effect on the meibomian gland, and neither statins nor antihypertensive medications promote dry eye.[42] Consequently, it remains to be determined whether any of these medications represented a confounding factor in this investigation.

Last, some limitations in this study should be considered. First, it is possible that very small pieces of adjacent lid tissue adhered to the meibomian glands during isolation under the dissecting microscope. However, such possible contamination would be similar for all tissues, given that the same individual removed glands from every lid segment in the same manner. Second, the number of tissue samples (i.e., five male and seven female) is relatively low for the analysis of sex-related differences in gene expression. Increasing the sample number is difficult, however, unless tissues are available from multiple lid surgeries. The tissue collection process for this study took more than 2 years to complete. Third, our data reflect mRNA levels. We have yet to determine whether these transcripts are translated into functional proteins.

Overall, our findings demonstrate that MGD is accompanied by multiple changes in gene expression in the meibomian gland. The nature of these alterations, including the upregulation of genes encoding SPRR and S100 calcium-binding proteins, suggest that keratinization plays an important role in the pathogenesis and/or progression of MGD.

TABLE 12. Effect of Sex on the Expression of Gene Ontologies in the Human Meibomian Gland

Ontology	M Genes ↑	F Genes ↑	M *z*-Score	F *z*-Score
Biological Process				
Golgi vesicle transport	5	1	**5.46**	0.38
Protein modification by small protein conjugation or removal	5	0	**4.37**	−0.99
Cellular macromolecule catabolic process	10	2	**3.08**	−1.14
Protein localization	11	3	**2.72**	−1.04
Protein transport	10	2	**2.71**	−1.27
Intracellular transport	8	4	**2.42**	0.10
Glycoprotein biosynthetic process	3	2	**2.28**	1.14
Vesicle-mediated transport	7	4	**2.19**	0.39
Proteolysis	10	3	**2.14**	−1.16
Cell surface receptor linked signal transduction	4	5	**−2.09**	−1.86
Response to stimulus	10	15	**−2.09**	−0.90
Anatomical structure development	6	15	**−2.14**	0.33
Regulation of biological quality	2	8	**−2.22**	−0.07
Multicellular organismal process	13	22	**−2.25**	−0.31
System process	1	4	**−2.59**	−1.60
Signal transduction	9	17	**−2.77**	−0.84
Cell communication	9	18	**−3.19**	−1.08
Epidermis development	0	6	−1.00	**4.99**
Translational elongation	1	4	0.62	**4.51**
Translation	2	8	−0.05	**3.95**
Glycoprotein metabolic process	3	4	1.84	**2.70**
Gene expression	19	31	−0.19	**2.54**
Nuclear mRNA splicing, via spliceosome	2	3	1.29	**2.25**
Blood vessel development	1	4	−0.39	**2.04**
Negative regulation of cellular process	5	3	−1.13	**−2.02**
Molecular Function				
Protein complex binding	4	1	**2.94**	−0.01
Acid-amino acid ligase activity	4	1	**2.86**	−0.04
Ligase activity, forming carbon-nitrogen bonds	4	2	**2.55**	0.76
Receptor activity	3	5	**−2.02**	−1.20
Structural constituent of ribosome	1	5	0.17	**4.69**
Structural molecule activity	1	9	−1.24	**3.45**
Protein binding	48	50	1.30	**2.02**
Transcription factor activity	5	0	0	**−2.29**
Cellular Component				
Cytoplasm	54	48	**2.76**	**2.49**
Endoplasmic reticulum	11	4	**2.56**	−0.33
Intracellular	72	68	**2.48**	**3.15**
Intracellular membrane-bound organelle	57	47	**2.69**	1.60
Intracellular organelle	60	56	**2.19**	**2.54**
Vesicle	8	2	**2.22**	−0.78
Golgi apparatus	11	5	**2.86**	0.30
Mitochondrion	1	6	**−2.09**	0.33
Extracellular region	4	7	**−2.24**	−0.96
Intrinsic to plasma membrane	1	6	**−2.32**	−0.05
Ribonucleoprotein complex	3	11	0.15	**5.58**
Ribosome	1	6	−0.08	**5.21**
Macromolecular complex	17	30	0.03	**4.33**
Nuclear part	12	21	0.64	**4.3**
Nuclear lumen	11	17	1.08	**3.87**
Intracellular organelle lumen	11	19	0.36	**3.66**
Non-membrane-bound organelle	14	24	0.01	**3.61**
Intracellular organelle part	24	33	0.29	**3.31**
Intracellular non-membrane-bound organelle	15	22	0.26	**2.86**
Nucleus	32	37	0.89	**2.83**
Nucleoplasm	4	10	−0.42	**2.79**
Nucleolus	7	8	1.61	**2.5**
Membrane	46	26	1.38	**−2.15**
Intrinsic to membrane	30	17	0.15	**−2.17**

Listed ontologies, with some of the highest and lowest *z*-scores, were selected after the analysis of non- and log-transformed data. Criteria for inclusion in the table were the same as in Table 6. High and low values for the male (M) and female (F) groups in designated ontologies are highlighted in bold.

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