Tear ^1H Nuclear Magnetic Resonance-Based Metabolomics Application to the Molecular Diagnosis of Aqueous Tear Deficiency and Meibomian Gland Dysfunction

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Keywords

Meibomian gland dysfunction · Dry eyes · Aqueous tear deficiency metabolomic fingerprint · Tears · Biomarkers

Abstract

Purpose: Meibomian gland dysfunction (MGD) is a major cause of signs and symptoms related to dry eyes (DE) and eyelid inflammation. We investigated the composition of human tears by metabolomic approaches in patients with aqueous tear deficiency and MGD. **Methods:** Participants in this prospective, case-control pilot study were split into patients with aqueous tear deficiency and MGD (DE-MGD [$n = 15$]) and healthy controls (CG; $n = 20$). Personal interviews, ocular surface disease index (OSDI), and ophthalmic examinations were performed. Reflex tears collected by capillarity were processed to ^1H nuclear magnetic resonance (NMR) spectroscopy and quantitative data analysis to identify molecules by spectra comparison to library entries of purified standards and/or unknown entities. Statistical analyses were

made by the SPSS 22.0 program. **Results:** Chemometric analysis and ^1H NMR spectra comparison revealed the presence of 60 metabolites in tears. Differentiating features were evident in the NMR spectra of the 2 clinical groups, characterized by significant upregulation of phenylalanine, glycerol, and isoleucine, and downregulation of glycoproteins, leucine, and $-\text{CH}_3$ lipids, as compared to the CG. The ^1H NMR metabolomic analyses of human tears confirmed the applicability of this platform with high predictive accuracy/reliability. **Conclusions:** Our key distinctive findings support that DE-MGD induces tear metabolomics profile changes. Metabolites contributing to a higher separation from the CG can presumably be used, in the foreseeable future, as DE-MGD biomarkers for better managing the diagnosis and therapy of this disease.

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Introduction

Dry eyes (DE) result from a variety of changes occurring in 1 or more of the ocular surface system (OSS) components. In fact, dry eye is a multifactorial disease of the OS characterized by a loss of homeostasis of the tear film, and accompanied by ocular symptoms, in which tear-film instability and hyperosmolarity, OS inflammation and damage, and neurosensory abnormalities play etiological roles. Ocular discomfort and visual disability occur in varying degrees through the disease [1, 2]. Recognized DE pathogenic mechanisms are increased tear osmolarity and OSS inflammation [3]. According to the etiology and clinical manifestations, the aqueous-deficient and evaporative DE forms need to be considered [4]. Main cause of evaporative DE is meibomian gland dysfunction (MGD), defined as the chronic, diffuse alteration of the gland by the occlusion of glandular orifices and/or quantitative-qualitative changes in glandular secretion. The MGD may lead to tear-film changes, clinical signs/symptoms of eye irritation, inflammation, and DE (DE-MGD) [5, 6]. Studies have shown analysis-method-dependent variations in tear composition, with a wide range of metabolites identified in these biosamples [7]. Changes in metabolite availability in the OSS appear to be associated with blepharitis, DE, or eye allergies [5, 6].

Metabolome is defined as the set of small molecules (i.e., intermediate metabolic products) that are present in biological tissues and fluids, the corresponding discipline being metabolomics [8]. Metabolomic profiling seeks to identify as many chemicals as possible to establish which metabolites (or metabolite patterns) discriminate samples from the cases and controls [8–10]. Metabolomic profile (MP) has become an important tool for addressing disease diagnostic/progression and for monitoring pharmacological substances [11]. High-resolution nuclear magnetic resonance (NMR) takes advantage of the magnetic properties of atomic nuclei to reveal a metabolite according to its chemical characteristics [8, 9, 11, 12]. Proton NMR (^1H NMR) spectroscopic analysis of tears can provide information on the structure/composition of low-molecular-mass compounds present in a sample. This is a fast and relatively low-cost technique for exploring metabolic processes. Major advantages include unbiased metabolite detection, quantitative nature, high reproducibility, and outstanding biomedical applications [8–12], including the pioneer ophthalmic studies [13–18].

It might be asked whether ^1H NMR spectroscopy is the most appropriate analytical technique for our purposes as

well as for processing our particular biosample, the human tears. Among the methods for analyzing human tears (high-pressure liquid chromatography, gas chromatography, mass spectrometry [MS], and NMR spectroscopy), the NMR may be less sensitive than MS (microgram vs. picogram, respectively) to separate/quantify the metabolites, but sufficient to detect changes in fluids. The NMR is based on the magnetic properties of some atoms, while the MS spectroscopy is based on the separation of the metabolites using chemical and physical properties. In contrast, major advantages of NMR spectroscopy include its unbiased metabolite detection, quantitative nature, and high reproducibility. Moreover, the MS needs more sample preparation and is a destructive technique [19, 20]. Tear-film and meibum composition have been largely studied [21–23], but comprehensive tear ^1H NMR spectroscopy metabolomic studies in DE-MGD are lacking.

Because of the poor correlation between subjective symptoms and diagnostic DE tests, as previously stated [1, 2, 22–25], major objective was to assess whether tear analysis – specifically analysis of the metabolome by NMR – could serve as a useful tool in the diagnosis and therapy of DE-MGD patients. We designed the present work using ^1H NMR spectroscopy metabolomics to analyze the capability of tear-metabolite profiling to identify metabolites that are differentially expressed in tears from the DE-MGD patient as compared to the controls.

Material and Methods

Study Design

This is a case-control study, approved by the Ethics Committee at the Hospital Clínico San Carlos (HCSC/2016; Madrid, Spain). The study fits all the principles of the Declaration of Helsinki for medical research. Risks/benefits were extensively explained, and written informed consent was signed by all subjects. We analyzed the composition of the human tears by ^1H NMR spectroscopy in order to determine differences in the MP that might distinguish between these 2 clinical groups: the DE-MGD patients and controls.

Subjects

According to the main inclusion/exclusion criteria (Table 1), the suitable participants were split into patients with DE-MGD ($n = 15$) and healthy controls (CG; $n = 20$). We have to emphasize that the finding of aqueous tear deficiency in many of the selected patients indicates that we have got a patient cohort with mixed pathology, if not primarily aqueous deficiency with additional MGD. Topical treatment may include only artificial tear instillation to help alleviate symptoms. Figure 1 summarizes the participants and protocols used in this study.

Table 1. Inclusion and exclusion criteria of the study participants

Inclusion criteria	Exclusion criteria
Both sexes 18–80 years old Informed consent to participate in the study	Systemic disorder (diabetes, hypertension, cancer, chronic or degenerative diseases, etc.) Allergy or hypersensitivity Contact lenses wearing from fifteenth days before and through the study course Toxic habits (alcohol, pharmacological substances, drugs) Ophthalmic surgery or laser treatments from 6 months before and through the study course Participation in other studies starting 1 month before the current study Physical/mental handicaps to participate in the study Other relevant exclusion criteria
Healthy control individuals OSDI < 12+ Oxford 0 BUT > 10 No MGD	
Patients with DE caused by MGD One or more of the following: Itchiness, dryness, foreign body sensation, photophobia, burning, stinging, grittiness, tearing, tired eyes, redness, blurred vision OSDI > 12 Oxford > 0 BUT < 10 MGD hyposecretion type	
OSDI, ocular surface disease index; BUT, breakup time; DE, dry eyes; MGD, meibomian gland dysfunction.	

Interview and Questionnaires

Personal interviews were performed regarding the DE signs/symptoms and the participants' subjective sensations. The ocular-surface disorder index (OSDI [copyright held by Allergan Inc., Irvine, CA, USA]) questionnaire [26] was used in all participants for gaining information concerning the OSS symptomatology, as before [17, 18, 21, 22, 25–27]. Based on previous reports [28], the group of patients with symptoms of discomfort of the OSS was confirmed by its OSDI scores.

Ophthalmic Examination

A systematized eye examination was sequentially performed: (1) the best-corrected visual acuity in each eye and (2) OSS examinations using a slit lamp. The eyelid-margin morphology (irregularities, hyperemia, telangiectasias, and/or posterior displacement of mucocutaneous interface) and the MG orifices and secretion were explored (see the Fig. 2). The orifices varied from normal (in opening and appearance) to pathologic (narrowing and obstruction). The meibum expression was assessed by a semiquantitative pattern by digitally pressing on the tarsus center, observing the MG secretion (as shown in the Fig. 2), and as reported elsewhere [29]. Meibomian gland hyposecretion is characterized by decreased meibomian lipid secretion without gland obstruction. This disorder is associated clinically with gland atrophy. Diagnosis was made by (1) decreased meibum secretion after lid pressure without meibomian gland plugging and (2) meibomian gland atrophy by meibography, as previously reported [5]. Tear production was evaluated by a Schirmer test with topical anesthesia (0.5% proparacaine hydrochloride eye drops). The strips <10 mm/5 min were diagnosed as positive, while ≤5 mm/5 min were

diagnosed as strongly positive; OSS of both eyes was stained with 2 µL of 1% fluorescein solution, which was instilled into the inferior fornix. The subject actively blinked for 3–5 s. Findings from the 4 corneal quadrants were progressively graded (no staining-to-severe staining, with confluent spots/filaments) and fluorescein breakup time (FBUT) was determined and considered positive when the final value was ≤10 s. Values were the mean ± SD of 3 examinations per everyone. Finally, the intraocular pressure was determined in each eye.

Sampling Procedures

The tear-collection method is described above. Patients were advised to stop using artificial tears at least 1–2 days before the tear collection, which was performed in the days following the baseline clinical visit. First, the eyelashes and the margins (inner/outer) of the eyelids were carefully dried with a soft towel to remove the remnant basal tears as much as possible. Then, reflex tear (the more watery type of tear produced in larger quantities whenever the eyes become gritted) was induced, this being the main tear constituent for our purposes. For reflex tear collection from all participants, the inferior meniscus of both eyes was gently rubbed with a Pasteur micro-pipette, collecting 20- to 30-µL tear samples (Fig. 3), as previously described [17, 18, 20, 30]. In 16% of our cases, it was necessary to repeat the collecting procedures the following day to obtain 2 sets of tears from the same patient. Tear-collecting procedures were performed between 10:00 and 10:30 h during the corresponding appointment. Tears were transferred to Eppendorf micro test tubes. Then, a sterile plastic Pasteur pipette was attached to the top of the micro-pipette, and the rubber bulb was pressed to release the total tear volume, transferring it to the

Fig. 1. Flowchart to document work-sampling procedures of the study participants. CG, healthy controls; DE-MGD, dry eyes meibomian gland dysfunction; OSDI, ocular surface disease index; BCVA, best corrected visual acuity; FBUT, flourescein breakup time; NMR, nuclear magnetic resonance.

Fig. 2. The slit lamp biomicrography shows mild inflammatory signs at the lid margin, no plugs, no putting, and no capping. Diagnosis was made after lid expression that was performed in the outer region of the inner eyelid by inducing gradually the pressure with the palpebral forceps. The observer checked that nothing or scarce meibum came out from the holes of the dysfunctional meibomian glands.

Color version available online

Fig. 3. Microtubes used for collecting tears and the reflex tear collection proceedings from the inner eyelid.

microtube. Then, tubes were marked and stored at -80° until processing.

Metabolomics of Human Tears

Tear samples were analyzed by ^1H NMR spectroscopy at the Faculty of Medicine and Odontology of the University of Valencia (Spain) and by the Institute of Research in Molecular Recognition and Technologic Development of the Polytechnic University of Valencia (Spain) in accordance with previous reports [13–18, 30–32]. For NMR analysis, 20 μL of tear was mixed with 2.5 μL of 90 mM sodium-3-trimethylsilylpropionate- 2,2,3,3- d_4 (TSP) in deuterium oxide. A total of 20 μL of the mixture from each subject was then transferred to a high-quality 1-mm NMR tube. All ^1H NMR spectra were determined using a standard one-dimensional pulse sequence with water suppression on a Bruker Avance 600 spectrometer operating at 600.13 MHz with a 1-mm $^1\text{H}/^{13}\text{C}/^{15}\text{N}$ TXI probe. A total of 256 free induction decays were grouped into 64 k data points with a spectral width of 14 parts per million (δ , ppm) and a recycle delay of 1 s. The water signal was saturated by weak irradiation during the recycle delay. Before Fourier transformation, the free induction decay was multiplied by a 0.3 Hz exponential line broadening. Proton chemical shifts cover a range of 30 ppm, but the vast majority appears in the region 0–10 ppm, where the origin is the chemical shift of TSP. Therefore, spectral chemical shift referencing on the TSP signal at 0 ppm was performed for all spectra. Tears were treated with water and strong organic solvent to separate the hydrophilic and hydrophobic compounds [11, 12] to achieve the proper compound isolation. Spectral regions from 0.5 to 4.4 ppm and from 5.5 to 9.5 ppm were binned in segments 0.01 ppm wide (6 Hz) for multivariate analysis. Binned data were normalized to total aliphatic spectral area. Available spectral databases and two-dimensional (2D) NMR experiments facilitated the structural identification of the most relevant tear chemical compounds [13–15, 31, 32]. Spectra were processed using MestRenova 6.2 software (Mestrelab Research S.L., Spain) and transferred to MATLAB 7.6 (The MathWorks Inc., Natick, MA, USA) for additional processing/analysis. Signals from specific chemical compounds were integrated/quantified using semi-automated in-house MATLAB peak-fitting routines based on Levenberg-Marquardt optimization procedures. Resolution of the NMR spectra and their signal-to-noise ratios (SNRs) (this latter defined as the ratio of signal power to the noise power which measures the original signal corruption in decibels) and the way this parameter may be corrupted during the process by undesirable noise. A higher SNR guarantees the clear acquisitions of the procedure. In this context, the resonances of the analytes present in the tear samples

have been revised, and the SNRs, being low, have been validated. We only considered peaks that appear in at least 60% of the spectra reducing the chance of these peaks belonging to the noise. Resonances were assigned according to the Human Metabolome Database (<http://www.hmdb.ca/>) and were based on the characteristic cross-peak from previously mentioned 2D spectra, as in earlier reports with human tears [13–15, 17, 18, 30–32]. The multiple variables analyzed during the metabolomic procedures make it necessary to ensure the accuracy of all obtained data [20, 33–35]. Despite the more limited sensitivity of proton NMR, the combination of the protocols for identifying the metabolites with using databases and possibly other ^1H NMR spectroscopy information makes possible and reproducible the assignment of the metabolites present in the tear samples.

Statistical Analyses

Chemometric statistical analyses were performed using in-house MATLAB scripts and PLS Toolbox 6.7 (Eigenvector Research Inc., Wenatchee, WA, USA). Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were applied to NMR spectra data matrix [8, 11, 32, 33, 36, 37]. Additional statistical procedures were employed. Higher values of these analyses indicated the presence of outstanding metabolites to carry out the discriminating classification. Data were processed to prepare a scatter plot combining the results from the statistics for the model variables with respect to model component scores. Data were expressed as mean $\pm$ SD. A difference was considered significant at $p < 0.05$. The statistical package for the social sciences (SPSS) (IBM Corp SPSS Statistics for windows version 22.0, Armonk, NY, USA) was used for statistics. The Mann-Whitney U test and the Spearman's rank correlation coefficient (ρ ; SR) were applied to establish the relationship between DE-MGD clinical manifestations, the morphologic/functional DE parameters, and the tear-film metabolites. Finally, $p < 0.05$ was considered statistically significant.

Results

Ocular data considered for the DE-MGD diagnosis were the baseline OSDI scores, eyelid evaluation, Schirmer, and FBUT test results (Table 2). Mean age of the study participants was 51 ± 19 years of the DE-MGD group and 46 ± 12 years of the CG. Participant distribution was 43%

Table 2. Characteristics of the study participants

Group	Participant distribution, %	Mean age, years	Gender men/women, %	OSDI scores	MG secretion semiquantitative evaluation [#]	Schirmer, mm	FBUT, s	IOP, mm Hg
DE-MGD (<i>n</i> = 15)	43%	51±19	66/34%	42±25**	2±1**	6±4**	4±2**	15±1
CG (<i>n</i> = 20)	57%	46±12	54/46%	4±3	0	19±9	14±3	14±1

DE-MED, dry eyes meibomian gland dysfunction; CG, control group; OSDI, ocular surface disease index; FBUT, fluorescein breakup time; IOP, intraocular pressure. ** Significant differences ($p \leq 0.001$). [#] Grading from absent to hypersecretion.

in the DE-MGD patient versus 57% of the CG. Regarding gender (men/women), our participants were distributed into 66/34% of the DE-MGD patient and 54/46% of the CG.

The DE-MGD patient reported a history of dryness before this study, while the CG showed no DE-related signs/symptoms. Moreover, it has also to be mentioned that some DE-MGD patients displayed very low Schirmer test data, as reflected in the Table 2, indicating the presence of aquodeficiency DE according to the TFOS-DEWS II report [2]. The Figure 2 shows a slit lamp image of hyposecretory non-obstructive MGD. None of the patients suffered from Sjögren's syndrome.

In both, the DE-MGD (SR: 0.745 [$p = 0.001$] and 0.712 [$p = 0.003$], respectively) and the CG (SR: 0.825 [$p = 0.000008$] and 0.860 [$p = 0.000001$], respectively), total tear lipids correlated positively and significantly with the Schirmer and the FBUT tests.

To build the tear metabolomic fingerprint, we first constructed the CG tear metabolome and next assigned the DE-MGD tear resonances. Tear-metabolite concentrations were within the millimolar range, which are several orders of magnitude lower than the water concentration. Therefore, ¹H NMR spectroscopic imaging needs the pre-saturation of the abundant water signals. Sixty tear chemical compounds were detected. Several unidentified compounds were excluded from our results (unknown peaks: UP-01 to UP-06 from the aliphatic region and UP-07 to UP-09 from the aromatic region of the same ¹H NMR spectral comparisons), 51 chemical compounds were properly identified in reflex tears (Table 3). The set of tear compounds found in both groups proved to be composed mainly of lipids and amino acids (aa), the latter being the most abundant. Figure 4 shows the sum of all spectra.

The highest concentrations from the controls corresponded to glycoproteins (acetyls), leucine, mobile lipids

(-CH₂-), lipid/cholesterol, isoleucine, lipids (LDL/VLDL), sphingomyelin, glutamic acid/glutamate, and glycerol. Other compounds identified included several lipids signals (-CH₃ lipid and, -CH₂ lipids), phospholipids, phenylalanine, valine, citrate, lactate, tryptophan, and glucose. Studying the aliphatic (0.5–4.7 ppm) and aromatic (5–10 ppm) regions, we assigned metabolites to each (Table 3).

Accurate comparison of tear compounds identified in both groups indicated that phenylalanine, glycerol, and isoleucine were upregulated, whereas glycoproteins, leucine, and -CH₃ lipids were downregulated in tears from the DE-MGD patients. Phenylalanine, isoleucine, arginine, cysteine, valine, and tryptophan reached significantly higher levels, whereas glycine and leucine registered significantly lower levels in tears from the DE-MGD patients as compared to the CG. Within the carbohydrate compounds, glucose was identified as significantly higher in the DE-MGD tears than in the CG (Table 3). A total of 10 lipid/protein compounds were identified in tears from both groups (mobile lipids, -CH₃ lipids, lipid/protein, lipid/cholesterol, -[CH₂]_{*n*}-lipids, sphingomyelins, glycoproteins, phospholipid, phosphoethanolamine, and glycerol). The most abundant molecule found in normal tears was glycoprotein (0.1699 ± 0.01) but significantly lower values were detected in the DE-MGD patient (0.1503 ± 0.009). Regarding the organic acid derivatives, acetate, lactate, creatine, 2-methyl-oxovalerate, pyruvate, citrate, choline, amino-*n*-butyrate, and 4-aminohippurate were found in tears from both groups of participants. However, only amino-*n*-butyrate significantly increased in concentration, while acetate significantly decreased in tear samples from the DE-MGD patient with respect to the CG. Main metabolites contributing to a wider separation between groups were monitored: phenylalanine, glycerol, isoleucine, glycoproteins, mobile lipids, leucine, -CH₃ lipids, and lipid/cholesterol, as reflected in the Figure 5.

Table 3. Comparison of “in vivo” tear metabolite ratios (absolute concentrations) between the DE-MGD versus the CG

No.	Metabolite	Region	CG		DE-MGD		<i>p</i> value ¹
			mean	SD	mean	SD	
Aliphatic compounds							
1	Mobile lipids	0.82–0.88	0.0790	0.008	0.0791	0.009	0.955
2	–CH ₃ lipids	0.84–0.90	0.0224	0.006	0.0202**	0.006	0.001
3	Lipid/protein	0.85–0.89	0.0452	0.006	0.0486**	0.006	0.0007
4	Lipid/cholesterol	0.88–0.92	0.0677	0.006	0.0660*	0.008	0.044
5	Valine	1.00–1.05	0.0211	0.002	0.0217	0.002	0.768
6		3.58–3.61	0.0131	0.001	0.0146**	0.006	0.0002
7	2-Metil-2-oxovalerate	1.08–1.10	0.0035	3e–4	0.0037	3e–4	0.811
8	Ethanol	1.15–1.20	0.0285	0.002	0.0290	0.006	0.766
9	Isoleucine	1.20–1.29	0.0544	0.005	0.0596**	0.01	0.0001
10	Sphingomyelin	1.26–1.32	0.0352	0.005	0.0371**	0.006	0.001
11	–(CH ₂) <i>n</i> lipids	1.26–1.42	0.0228	0.005	0.0216*	0.005	0.039
12	Alanine	1.46–1.49	0.0174	0.002	0.0169	0.002	0.648
13	Leucine	1.65–1.75	0.0797	0.004	0.0769**	0.004	0.001
14	Acetate	1.90–1.92	0.0174	0.002	0.0168*	0.002	0.027
15	Glycoprotein	1.99–2.07	0.1699	0.01	0.1503*	0.009	0.012
16	<i>N</i> -acetylglucosamine	2.0–2.08	0.079	0.02	0.052**	0.02	0.0002
17	Glutamic acid/glutamate	2.10–2.17	0.0329	0.003	0.0333	0.002	0.824
18	Pyruvate	2.36–2.37	0.007	5e–4	0.0069	6e–4	0.987
19	Carnitine	2.41–2.45	0.0108	0.002	0.0111	0.002	0.901
20	Citrate	2.50–2.55	0.0114	0.002	0.0118	0.0017e–4	0.557
21		2.64–2.70	0.0209	0.002	0.0210	0.001	0.926
22	Amino- <i>n</i> -butirate	2.95–3.02	0.0135	0.003	0.0157**	0.002	0.0004
23	P creatine	3.02–3.04	0.0073	8e–4	0.0076	0.001	0.753
24	Total creatine	3.02–3.05	0.0053	9e–4	0.0067*	0.001	0.026
25	Tyrosine	3.04–3.08	0.0106	0.001	0.0110	8e–4	0.629
26	Citrulline	3.10–3.16	0.0190	0.002	0.02	0.002	0.741
27	Phenylalanine	3.09–3.10	0.0011	1e–4	0.0012	2e–4	0.899
28	Phospholipid	3.17–3.20	0.0171	0.002	0.0183**	0.004	0.0009
29	Choline/acetylcholine	3.18–3.21	0.0089	0.002	0.010**	0.002	0.001
30	Arginine	3.21–3.28	0.0147	0.003	0.0189**	0.004	0.0005
31	Phosphoethanolamine	3.26–3.30	0.0150	0.002	0.0163*	0.002	0.036
32	Methanol	3.35–3.37	0.0038	0.001	0.0040	0.001	0.487
33	Glucose	3.40–3.42	0.0041	0.002	0.0045	0.002	0.608
34	Glycerol	3.52–3.58	0.0199	0.007	0.0219**	0.004	0.0002
35		3.62–3.68	0.0336	0.009	0.0496**	0.006	0.0008
36	Glycine	3.54–3.59	0.0208	0.006	0.0180**	0.003	0.0001
37	Cysteine	3.88–3.96	0.0210	0.002	0.0245**	0.002	0.001
38	Tryptophan	4.03–4.07	0.008	0.002	0.0084	0.002	0.901
39							
40	Choline	4.05–4.09	0.0036	0.001	0.0057**	0.002	0.0007
41	Lactate	4.07–4.13	0.0127	0.004	0.0131	0.006	0.388
42	Threonine	4.22–4.28	0.0107	0.003	0.0106	0.003	0.991
Aromatic compounds							
43	Glucose	5.20–5.25	0.0076	0.003	0.0084*	0.003	0.017
44	4-Aminohippurate	6.82–6.88	0.0113	0.001	0.0115	0.001	0.705
45	Methylhistidine	6.98–7.03	0.0083	0.001	0.0087	0.001	0.487
46		7.67–7.74	0.0064	9e–4	0.0067	1e–3	0.766
47	Tyrosine	6.86–6.92	0.0054	7e–4	0.0059	8e–4	0.552
48	Phenylalanine	7.20–7.40	0.0245	0.005	0.0333**	0.008	0.0009
49	Tryptophan	7.22–7.70	0.0182	0.004	0.0189	0.005	0.307
50	Formate	8.43–8.45	0.0026	7e–4	0.0031	8e–4	0.189
51	Histidine	8.60–8.68	0.0032	6e–4	0.0044**	7e–4	0.001

Marked in grey are the most abundant tear metabolites. CG, control group; DE-MGD, dry eyes meibomian gland dysfunction; SD, standard deviation.
¹ *p* obtained from the Student's *t* Test for independent samples.

Fig. 4. PCA and PLS-DA were applied to NMR spectra data matrix. The figure shows the PLS diagram with the assigned metabolic characteristics of the human tears from the DE caused by MGD and the CG. Red figures correspond to tears from the CG. Green squares correspond to tears from the DE-MGD patients. It is recommended to use LVs for the Y matrix to carry the variance of interest from a quantitative and reproducible analytical tool. It is also suggested that a limited number of LVs be used for the Y matrix in the regression, and they should carry less than the total variation in the corresponding spectra data. DE-MED, dry eyes meibomian gland dysfunction; NMR, nuclear magnetic resonance; PCA, principal component analysis; PLS-DA, partial least squares discriminant analysis; LVs, latent variables.

Discussion

This is the first study available dealing with the qualitative/quantitative characteristics of the metabolome in tears from DE-MGD patients. ^1H NMR metabolomics spectroscopy was used for these approaches. Our DE-MGD prediction model by using clinical measurements and tear metabolomics data revealed molecular fingerprints closely related to the OSS. Moreover, when clustering DE-MGD patients, a different MP of the healthy control subjects was detected. Because of this, the subjective symptoms assessed by personal interviews and questionnaires as well as the data gathered from the OSS clinical examination [1, 3, 5, 24–27, 29, 38] established the diagnosis of the DE-MGD, and the metabolomics evaluation confirmed the disease in terms of tear composition (related to OSS) (as reflected in Tables 2 and 3).

Exhaustive metabolomics data set corresponds to the estimated production of a metabolic system. The immense number of metabolic compounds makes it difficult to correctly decipher, assign, and biomedically inter-

pret the information provided by the specific sample processing [36]. There is a lack of consensus among the various works published on the tear-collection procedure [4, 7, 13, 15, 17, 18, 30, 39–41]. Here we collected the reflex tear samples by gentle rubbing and mild stimulation through capillarity from the inferior lachrymal meniscus of both eyes, as shown in Figure 3, and described elsewhere [17, 18, 30]. Authors described the effects of the reflex tear collection technique in detecting antioxidants by comparing the capillary-tube and the Schirmer-strip collection methods, and the less-invasive capillary-tube collection method was proposed as the choice for biochemical studies based on reflex tears [42], as we performed here.

The NMR spectroscopic approaches are rapid and reproducible, offering a standardized (relatively easy) technique for sample preparation and thus ensuring sample stability. When complemented with the principal component analysis, NMR spectroscopy can help to identify variations in cell metabolism in response to different stressors [43]. Our data from the tear chemometric analysis and ^1H NMR spectra comparison revealed the presence of 60 chemical compounds. Nine molecules (unidentified peaks: UP-01 to UP-09) were unable to establish proper identification with the Human Metabolome Database [44]. According to the literature search, the excellent analytical reproducibility and robustness of ^1H NMR metabolomic techniques with implications for the quantitative interpretation of metabolites in human tears [8–18, 31, 32, 45, 46] has been proved herein.

Figure 4 shows the PSL diagram with the assigned metabolomic fingerprint of the human tears from the DE caused by MGD and the CG. We can see how both groups are almost perfectly differentiated in this diagram. We were able to detect and perfectly identify chemical compounds in the tear samples; among them, 42 pertained to the aliphatic (0.5–4.7 ppm) and 9 to the aromatic regions. Most abundant compounds found in the CG tears were glycoproteins, leucine, mobile lipids, lipids/cholesterol, isoleucine, lipids/proteins, and $-\text{CH}_2$ lipids. Studying the aromatic region (5–10 ppm), we identified glucose, 4-aminohippurate, methylhistidine, tyrosine, phenylalanine, tryptophan, formate, and histidine. All the data gathered enabled us to build the human tear metabolome (Table 3).

By additional statistical procedures, as described above, we selected compounds that contributed heavily to the separation between groups (as shown in the Fig. 5). Six discriminant variables, 3 upregulated (phenylalanine, glycerol, and isoleucine), and 3 downregulated (glyco-

Fig. 5. The figure represents the sum of all spectra obtained from the tear-film metabolomics that are shown in a separate fashion. Representative spectra for the chemical standards for major metabolites (**a**), the control tears (**b**), the DE-MGD tears. The “lipids” identified in the spectra correspond to mobile lipids, $-\text{CH}_3$ lipids, lipid/protein and lipid/cholesterol regions (**c**). Finally, main metabolites contributing to a wider separation between groups in the PLS-DA diagram were phenylalanine, glycerol, isoleucine, glycoproteins, mobile lipids, leucine, $-\text{CH}_3$ lipids, and lipid/cholesterol. DE-MGD, dry eyes meibomian gland dysfunction; PLS-DA, partial least squares discriminant analysis.

proteins, leucine, and $-\text{CH}_3$ lipids) compounds were found. The above substances underwent statistically significant changes, suggesting the existence of differential metabolomic fingerprints for tears from the DE-MGD group with respect to the CG. Owing to the complexity of the tear metabolome, the main results of our study are discussed separately.

It has been reported that human tears contain 23 aa and that the availability and functionality of these may be altered by pathologic OSS processes [25]. Notably these authors also reported that aa profiles of the basal- and reflex tears gave similar results. Some free aa have been used to treat OSS disorders [47, 48].

We may suggest that some of the identified aa may play significant roles in carbohydrate synthesis in healthy tears. The observed tear upregulation of phenylalanine, isoleucine, arginine, cysteine, choline, valine, and tryptophan in the DE-MGD patients may be explained by the essential functions of these metabolites in several signaling pathways of OSS pathologies. It is also plausible that a major process of releasing the above aa into tears could occur in order to convert these molecules into others. Moreover, phenylalanine overexpression in the DE-MGD tears may reflect the pro-inflammatory response, as suggested elsewhere [4, 17, 18, 49]. It has been previously reported that reflex tears contain several antioxidants, including cysteine, ascorbate, glutathione, urate, and tyrosine in addition to other molecules [42]. Also, arginine has been proposed to modulate T-cell metabolism and cell survival via gene expression [50]. We detected noticeably higher levels of phenylalanine, arginine, cysteine, tyrosine, and methyl-histidine in the DE-MGD tears. These results may be useful to address OSS metabolic dysfunction as well as inflammatory and oxidative stress processes occurring in the DE-MGD eyes.

A mild increment of glucose was detected in the DE-MGD tears. It has been recently reported that 1/2 to 2/3 of diabetics suffer DE with the characteristic that the higher the Hb1AC values, the higher the severity of the OSS disease. In fact, there is a significant increase in the appearance of MGD in diabetics as compared to non-diabetics [51]. The precise levels of glucose in the tear-film from diabetics (and controls) remain controversial [52].

As widely recognized, meibum is a complex mixture of various lipids that are essential for the functionality of the tear-film/OSS integrity. Composition of the human tear-film and meibum includes hydrocarbons, wax, and cholesteryl esters and cholesterol, in addition to triacyl and diacyl glycerols, phospholipids, sphingomyelin, O-

acyl-omega-hydroxy fatty acids, and a variety of other molecules [53–62]. Studies using high-performance thin-layer chromatography semiquantitative methods of human tears reported an average global tear-lipid concentration of 0.13 g of lipids/L [59], in contrast to a later report of a total tear-lipid concentration of 1.8 ± 0.8 g/L [61], the most noteworthy being free cholesterol, free fatty acids, triacylglycerols, and polar lipids. Several lipid-associated signals are visible in ^1H NMR spectroscopy, such as the methyl ($-\text{CH}_2-$) signal at 0.84–0.90 ppm and the methyl ($-\text{CH}_3$) signal at 1.26–1.42 ppm. Among the lipid compounds found in tears of the DE-MGD study group were mobile lipids, $-\text{CH}_3$ lipids, lipid/protein, lipid/cholesterol, sphingomyelins, $-(\text{CH}_2)_n$ lipids, glycoprotein, phospholipid, phosphoethanolamine, and glycerol. Cellular lipid levels are affected by different stressors [47, 53, 54, 62].

It has recently been reported that sebum depletion from mouse-eye MGs negatively influenced the ability to repel water and increased the susceptibility to UVB-induced apoptosis [63]. Another noteworthy point in our findings was the presence of lipids/cholesterol in tears at 0.88–0.92 ppm, in agreement with previous reports pointing to cholesterol (and/or epi-cholesterol) as the most abundant sterol in the MGs lipids together with O-acyl-omega-hydroxy fatty acids [64]. We found sphingomyelins in tears, as well as phospholipids [54], suggesting that changes in uptake, utilization, and maintenance of these metabolites may help alter the tear-film composition of the DE-MGD patient. In this context, it has been reported [63] that tear-film instability can be considered the key point of DE disorders. It has been also speculated [64] that different tear-film components (meibomian lipids, ocular mucins, and proteins) may influence tear-film instability. Moreover, it was said [65] that MGD patients may have different compositions of meibomian lipids, specifically considering that the lipid profile could be an important indicator to diagnose the disease. It has also been conjectured that MGD is the result of a deficiency in meibum and/or abnormal lipids to constitute the tear-film lipid layer, leading to reduced tear-film stability [66]. In view of all scientific evidence on the role of lipids in the MGD [21, 22, 67, 68], and the differential MP found in DE-MGD tears, our data demonstrate that among the 8 tear compounds contributing to a sharper separation between the 2 study groups, 5 of these (62%) were lipids or were related to lipids (glycerol, mobile lipids, $-\text{CH}_3$ lipids, and cholesterol lipids). The role of other tear molecules found during the metabolomic approaches (2- methyl-2-oxovalerate, involving isoleucine biosynthesis; 4-ami-

nohippurate, an amide derivative of the carboxylic hippuric acid; 2 methylhistidine, an index of protein degradation) requires further research.

Current understanding of OSS at the cellular level has been noticeably enhanced by HR images from minimally invasive emerging technologies, such as the *in vivo* confocal scanning laser microscopy [68, 69] or the spectral-domain optical coherence tomography infrared meibography [70]. To determine the links between OSS clinical changes and molecular information, new challenging assays by analyzing human tears have arisen. Metabolomics of human tears using small sample volumes [8–18, 31, 32, 45, 71] offers a different level of information concerning the biological processes occurring in the OSS that are difficult to gain through other omics platforms. By maximizing the tear collecting and handling procedures, the chemical compounds extraction and SNR efficiency, we have evolved an optimized protocol for ¹H NMR human tear metabolomic analysis applicable to dry eye studies caused by MGD.

Regarding the study limitations, the following should be taken into consideration: (1) the relatively small number of participants (35 subjects) would benefit from the use of a larger sample in our future DE-MGD metabolomics research; (2) our preliminary results do not intend to be conclusive. But the possibility that the compounds contributing to a wider separation between the 2 study groups can be used as diagnostic biomarkers of the DE-MGD process should be validated through further research; (3) in general the biomedical potential of metabolomics is likewise hampered by the noticeable deviation in interpretation confidence; and (4) the small differences found between tear metabolites strongly suggest that the data will improve when validated by *omic* approaches at the protein level.

Conclusions

In view of the differential MP found in tears of both groups, we suggest that the most useful compounds for classification are phenylalanine, glycerol, isoleucine, glycoproteins, mobile lipids, leucine, –CH₃ lipids, and lipid/

cholesterol. These may be considered candidate molecules to be further validated and used for the diagnosis in this particular defined group, the DE-MGD patient.

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Statement of Ethics

The studies involving human participants were reviewed and approved by CEIC University Hospital San Carlos (HCSC/2016; Madrid, Spain). The patients/participants provided their written informed consent to participate in this study.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author Contributions

Benítez-del-Castillo and Pinazo-Durán share the first place with the authors. They both designed, recruited patients, obtained biosamples, obtained fundus, wrote, and edited; Sanz-González did much laboratory work, wrote, and edited; Muñoz-Hernández recruited patients and obtained biosamples; García-Medina recruited participants and obtained biosamples, wrote; Zanón-Moreno designed, wrote, edited, and reviewed the manuscript.

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