

A metabolomic approach to dry eye disorders. The role of oral supplements with antioxidants and omega 3 fatty acids

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Purpose: We used nuclear magnetic resonance spectroscopy of hydrogen-1 nuclei (¹H NMR S) to analyze the metabolic profile of reflex tears from patients with dry eye disorders.

Methods: We performed a prospective case-control study involving 90 participants: 55 patients diagnosed with dry eye syndrome (DESG) and 35 healthy subjects (control group, CG). From the DESG, two subgroups were formed: mild DES (n=22) and moderate DES (n=33). Participants were prescribed an oral nutraceutic supplementation containing antioxidants and essential polyunsaturated fatty acids to be taken as three capsules per day for 3 months. Reflex tears (20–30 µl) were collected from the tear meniscus of both eyes of each subject with a microglass pipette. Nuclear magnetic resonance (NMR) spectra were acquired with a standard one-dimensional pulse sequence with water suppression; 256 free induction decays were collected into 64,000 data points with 14 ppm spectral width.

Results: Basal tears showed a differential metabolomic profile between groups. Almost 50 metabolites were identified by H cholesterol, N-acetylglucosamine, glutamate, amino-n-butyrate, choline, glucose, and formate were detected before supplementation and choline/acetylcholine after supplementation. The metabolic profile of the tears was statistically different between groups, as well as before and after supplementation.

Conclusions: Our data indicate that DES induces changes in the tear metabolic profile that can be modified with appropriate oral supplementation with antioxidants and essential polyunsaturated fatty acids.

The lacrimal functional unit (LFU) is a structure that maintains the anatomic and physiologic properties of the anterior eye structures [1-5]. Ocular surface disorders represent a major cause of ophthalmological consultation worldwide [2]. These disorders involve various pathological changes of the conjunctiva and cornea, from minor issues (such as punctate keratitis) to extreme problems (such as symblepharon). Dry eyes result from LFU changes arising from a wide spectrum of factors that manifest through signs and symptoms that constitute dry eye syndrome (DES) [6]. The most frequent signs and symptoms are redness, itching, foreign-body sensation, tearing, pain, and blurred vision [4-6].

DES has two clinical forms caused by either deficient aqueous tear production (due to lacrimal gland dysfunction) or increased evaporative loss (due to meibomian gland disorder). However, combinations of both types are usually seen in the clinical practice. Dry eye disease was classified by the Subcommittee of the International Dry Eye Work

Shop (2007), which recommended a three-part classification system: etiopathogenic classification of DED (the multiple causes of dry eye), mechanistic classification (how each cause of dry eye may be involved through a common pathway), and severity of the DED (which is expected to provide a basis for therapy) [2,7,8].

DES usually affects people in their 60s [9]. Diverse LFU pathologies may be triggered by external or internal factors, such as light, environmental pollutants, air conditioning, computer use, hormonal changes with menopause, and topical or systemic medications [2,6].

Oxidative and antioxidant (AOX) activities, the levels of apoptotic mediators, antibodies, cytokines and chemokines, and hormones have been extensively studied in tears regarding the pathogenic mechanisms of DES [10-12]. These processes and their downstream effectors have also been suggested as presumptive biomarkers of DES [11]. Research is ongoing worldwide to identify genes and molecules involved in DES and to design new pharmacological strategies for treating DES and improving the vision-related quality of life for patients with DES.

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AOXs and anti-inflammatory compounds such as essential polyunsaturated fatty acids (EPUFAs) have been reported to be potentially useful for treating eye diseases [13]. Furthermore, EPUFAs such as omega-3 and omega-6 fatty acids have important effects on the body, particularly relevant to enhancing appropriate pre- and postnatal development (mainly of the central and peripheral nervous systems), lowering cholesterol and triglyceride levels, reducing acute and chronic inflammation, treating patients with neurodegenerative disorders, contributing to blood pressure regulation, and reducing the odds of developing cancer, heart disease, and stroke [14-18]. Omega-3-derived eicosanoids exert antioxidant and anti-inflammatory effects, whereas omega-6-derived eicosanoids are proinflammatory [16]. The biochemical regulation of oxidative stress and inflammation, performed in part by endogenous PUFA-derived autacoids (including proresolving mediators such as lipoxins, resolvins, protectins, and maresins) has recently been the subject of diverse studies [17-21].

The relevance of the complex array of metabolites present in our body has long been recognized for health and disease. Metabolites include not only the products and intermediates of metabolism but also carbohydrates, peptides, and lipids, many of which may be derived from the diet or altered in disease. Outstanding biotechnological advances, as in the application of nuclear magnetic resonance (NMR) spectroscopy with hydrogen-1 nuclei ($^1\text{H NMR S}$) within the molecules of a specific substance, help determine and identify the structure of the substance's components. With this technique, it is possible to acquire data sets from individuals by examining low-molecular-weight metabolites in tissue and fluid biosamples [22-24]. The global aim of metabolomics is to identify, determine, interpret, and quantify the complex time-related concentration, activity, and flux of endogenous metabolites in cells, tissues, and other biosamples such as blood, urine, and saliva. This approach is highly applicable to human studies and takes into account a spectrum of variables, including genetic background, environment, diet, and drug therapy, which collectively influence metabolism [25]. In the past 30 years, metabolomics has been used in clinical and animal studies for several diseases [26-28]. The metabolic consequences of ocular diseases have also been assessed by using the multiplexed analysis inherent in the metabolomic approach [25]. Specifically, rabbit corneas and lens extracts were processed with NMR spectroscopy [29-31]. These and other similar works emphasized the usefulness of metabolomics for monitoring patients with DES [31].

We aimed to improve our knowledge on human tear composition by using the $^1\text{H NMR S}$ -based targeted

metabolite profiling, followed by multivariate statistical analysis to explore metabolite imbalances. Additionally, DES pathogenic mechanisms are associated with the metabolite ensembles, if possible. Moreover, we assessed the effects of oral supplementation with a nutraceutical formulation containing antioxidants and omega-3 EPUFAs in relation to dry eyes.

METHODS

Study protocol and participants distribution: This prospective case-control study was approved by the Institutional Review Board of the University and Polytechnic Hospital La Fe (Valencia, Spain), as a non-significant risk investigational device study (Ref: 2013/0417). We observed all tenets of the Declaration of Helsinki for the protection of human subjects in medical research. The study also adhered to the ARVO statement on human subjects research.

Subjects managing: A total of 148 men and women aged 25–80 years were initially interviewed during the recruitment time frame for this study. During the preselection process (February 2013 to September 2013), patients successively attending the anterior eye segment section at the Ophthalmology Department of the study centers [the University and Polytechnic Hospital La Fe, Valencia (Spain) and the University Hospital Doctor Peset, Valencia (Spain)] were asked about having or not a previous diagnosis of dry eyes and their particularities.

A personal interview was conducted for all presumptive candidates regarding their characteristics, lifestyle, and personal and familial background. Special attention was paid to the nutritional facts of the study participants. In our Mediterranean area, participants were asked about nutritional aspects (adherence to consume vegetables, fresh fruit, legumes, whole grains, nuts, fish, olive oil, bread and wine—moderate intake) as well as following a particular preventive diet (hypocaloric, hyposodic, low fat, etc.), according to previous reports [32].

Patients who did not have a definitive DES diagnosis were carefully questioned about dry eye symptoms including sensations of dryness, irritation, grittiness, foreign-body feelings, light sensitivity, and/or tired eyes, and the intensity of these symptoms. The undiagnosed patients were also asked about vision fluctuations in conjunction or not with blinking, as well as any ocular symptoms that affect the ocular surface integrity. We also interviewed the individuals according to the data required for our study, as well as for the inclusion and exclusion criteria listed in Table 1 for the groups of participants.

TABLE 1. INCLUSION AND EXCLUSION CRITERIA.

Inclusion criteria	Exclusion criteria
Age 25–80 years	Aged <25 years or >80 years
Diagnosed with DES (DESG) <i>Schirmer</i> <10 mm/5 min were diagnosed + FBUT <10 s was considered +	Atopy and allergic disorders
Healthy subjects (CG)	Wearing contact lenses
Presence of DES symptoms	History of refractive surgery, ophthalmic laser treatment (<3 months)
Able to participate in the study	Systemic diseases and general treatments
Informed consent	Patients presenting with intermittent ocular surface signs and symptoms. Ocular disorders and eye drops other than artificial tears Not able to participate in the study

DESG: Dry Eye Syndrome Group; CG: Control Group

Specific ophthalmologic examination was performed for the study candidates, including detailed inspection of the anterior eye segment and adnexal structures, as well as the presence (or not) of lid disease. The ocular surface characteristics and eyelid margins evaluation under the slit lamp was also performed. Important components of the examination included the tear meniscus, Schirmer testing, tear film quality and tear break-up time, and any corneal surface staining with fluorescein.

A standard Schirmer's test without topical anesthesia was performed. A sterilized filter paper strip (5 mm × 35 mm) was placed in the external eye canthus and left in place for 5 min. Wetting of the paper after 5 min was recorded in millimeters according to the scale. The wetting strips <10 mm per 5 min were diagnosed positive, while ≤5 mm per 5 min were diagnosed as strongly positive.

The ocular surface of both eyes was stained with 2 μl of 1% fluorescein collyrium instilled in the inferior fornix. Then the patient was instructed to actively blink for 3–5 s to examine the cornea and conjunctiva. The fluorescein tear breakup time (FBUT) was determined as the time period between the last blink and the visualization of a random corneal black spot on the fluorescein stained tear film. In each participant, the FBUT was determined three times, and the value was expressed as the mean + standard deviation. FBUT was considered positive if the final value was less than 10 s.

Any clinical data and patients' or ophthalmologists' impressions of dry eye severity were recorded to better identify patients with various degrees of DES, for our study as well as to improve management of the ocular surface in these patients. The DED diagnosis and the severity of DED were finally achieved by integrating the patient's subjective sensations, clinical data, and impressions of the ophthalmologist

about dry eye severity according to the DEWS Subcommittee [4], as well as the reports from Murube et al. [8], and Sullivan et al. [33]. It was also taken into consideration that correlations between personal sensations and dry eye signs/symptoms are difficult to generalize.

After the ophthalmic examination, the ocular surface disorder index (OSDI; Allergan 1995, Irvine, CA), questionnaire was distributed to all participants to better differentiate normal, mild, moderate, or severe DES. The OSDI assesses the frequency of dry eye symptoms divided into the following sections: 1) Have you experienced photophobia, a gritty feeling, soreness, or blurred vision during the last week? 2) Have your eye problems limited you in reading, driving at night, computer work, or watching TV during the last week? 3) Have your eyes felt uncomfortable in windy or dry situations or because of air conditioning during the last week? The OSDI was calculated as previously reported by Schiffman et al. [34],

$OSDI = \text{summary of all scores} \times 25 / \text{number of answered questions}$: Overall OSDI score delineated the ocular surface from the normal eyes (0–12 points), mild level of disorder (13–22 points), moderate disorder (23–32 points), and severe stage of ocular surface disease (33–100 points). The OSDI and the clinical probes were done after the ophthalmic examination and were repeated at each of the two appointments.

At this point, 115 presumptive study participants (70 study candidates with DES and 45 healthy controls) were asked to participate in the study as volunteers. Then, the study was explained, and informed consent was signed by the participants and the investigators. Fifteen candidates with DED and ten healthy subjects declined to participate. Mostly declines in participating derived from working compatibilities, high use of medical care, or problems attending the study appointments. We also explained to all candidates that they

are always free to decline to participate in any aspect of the study at any time. Patients with obvious infection or significant eyelid inflammation were excluded from the study.

Therefore, a total of 95 participants were enrolled. The two main study groups were constituted of 60 patients with DES (DES) and 35 controls (CG).

A summary of all procedures is provided in the flow-chart in Figure 1. The DESG was subdivided into those with “mild” or “moderate” dry eyes, depending on the results of the personal interview, the clinical examination, the OSDI questionnaire, and the patient/ophthalmologist sensations on the dry eye characteristics and severity. The correlations between personal sensations and dry eye signs and symptoms are often difficult to generalize. Importantly, participants were required to discontinue use of nutritional supplements and treatments related to dry eyes as well as artificial tears containing vitamins and/or essential EPUFAs for at least 1 month after recruitment.

Tear sampling: Reflex tear samples were atraumatically obtained from the two main groups of participants (DES; n=60, and CG; n=35) by the gentle rubbing method (micro Pasteur pipette) from the tear meniscus of both eyes as previously described [35]. With this collecting method, a volume

of 20–30 µl was obtained whenever possible (given the difficulty of obtaining such amount of tears from the patients with DES). Tears were then transferred into a micro-Eppendorf conveniently marked and stored at –80° until processing. Special attention was taken for conserving and manipulating the tear samples. Two tear samples were obtained in this study: at baseline and at end of the study period. Tears from both eyes (18–35 µl) were deposited in one cryotube per participant, stored at –80 °C, and thawed before use (Figure 2). Five patients of the 60 participants with DES were definitively excluded from this study because it was impossible to obtain an adequate tear sample volume. Thus, the DESG totaled 55 patients (see Figure 1).

Nutraceutical oral supplementation: All participants were assigned to an oral nutraceutical formulation containing antioxidants (AOXs) and essential polyunsaturated fatty acids (EPUFAs; AOX/EPUFAs) to be taken as three capsules per day for 3 months. The formulation used was BrudySec 1.5 (Brudy Laboratories, Barcelona, Spain), and each capsule contained the following components: docosahexaenoic acid (350 mg), eicosapentaenoic acid (42.5 mg), docosapentaenoic acid (30 mg), vitamin A (133 mg), vitamin C (26.7 mg), vitamin E (4 mg), tyrosine (10.8 mg), cysteine (5.83 mg),

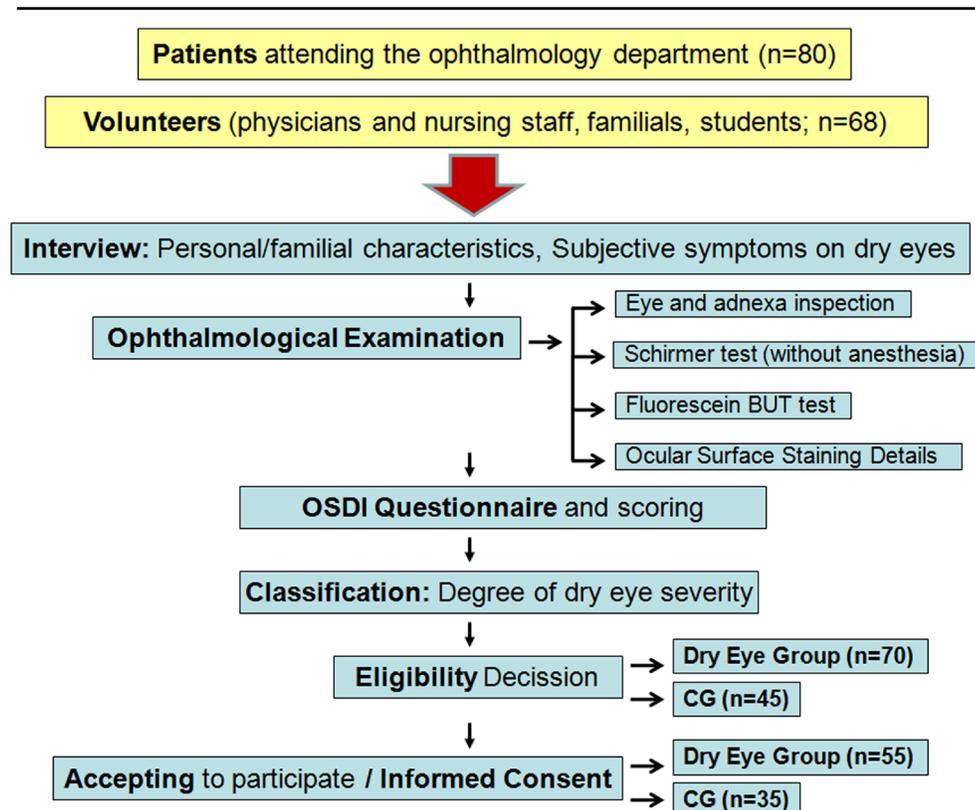


Figure 1. Schematic workflow for the study design.

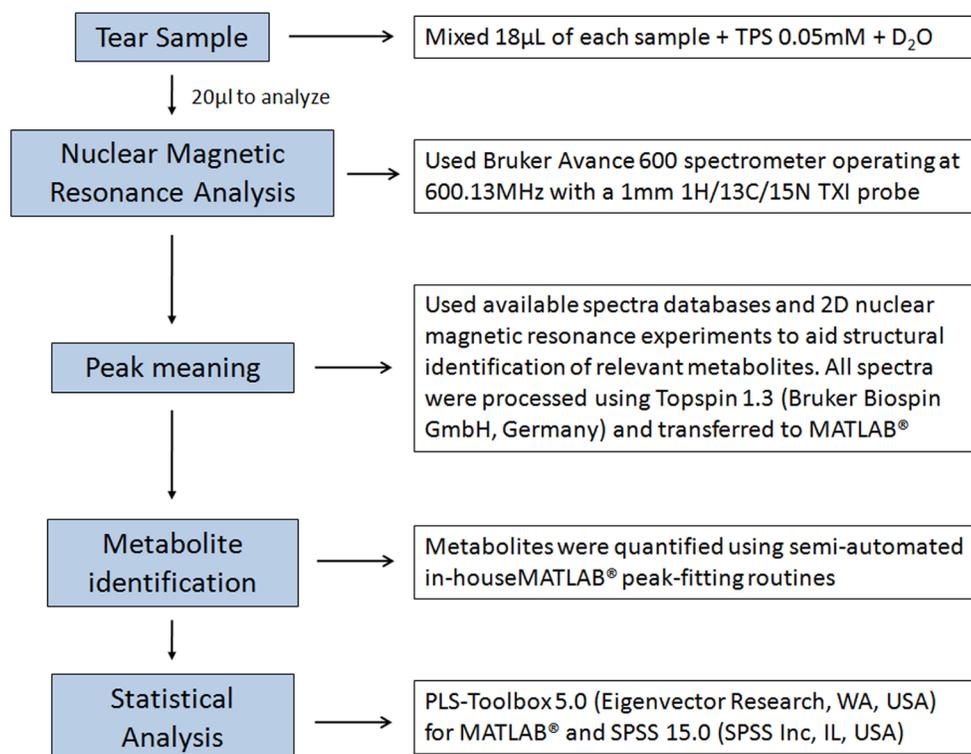


Figure 2. Schematic workflow for the metabolomic procedures.

glutathione (2 mg), zinc (1.6 mg), copper (0.16 mg), manganese (0.33 mg), and selenium (9.17 mg).

It has been demonstrated that compliance with long-term self-administered medication therapy is approximately 50% for those who remain in the study. Because of this, ophthalmologists were asked to pay special attention to measure the compliance threshold for participants during the personal interview. Therefore, the patients were telephoned regarding the supplements each month after the initial visit during the 3-month follow-up until the final appointment.

Metabolomic assays: We performed ^1H NMR S analysis in tear samples from study participants. From each participant, 20 μl of the prepared mixture was transferred into a 1 mm high-quality NMR tube, individually. All ^1H NMR spectra were acquired using a standard one-dimensional pulse sequence with water suppression. A total of 256 free induction decays (FIDs) were collected into 64,000 data points with a spectral width of 14 ppm and a recycle delay of 1 s. Water signal was saturated with weak irradiation during the recycle delay. Spectral chemical shift referencing the sodium-3'-trimethylsilylpropionate-2,2,3,3-d₄ (TSP) signal at 0 ppm was performed for all spectra. Spectral regions between 0.5 and 4.4 ppm and between 5.5 and 9.5 ppm were binned in segments of 0.01 ppm width (6 Hz) for multivariate analysis. We normalized the binned data to the total spectral area.

We used available spectral databases and two-dimensional NMR experiments to aid structural identification of relevant metabolites. Signals belonging to selected metabolites were integrated and quantified using semiautomated in-house MATLAB peak-fitting routines. The target function for the optimization included experimental spectra measured for standard solutions of selected metabolites with complex multiplet patterns. One-way ANOVA (ANOVA) was used to determine the statistical significance between the group means of the corresponding integrals (see Figure 2).

Comparison of the metabolic profiles between groups was performed using principal component analysis (PCA), which is an unsupervised test for homogeneity of the set of samples (detecting the existence of possible outliers). As part of this process, we excluded nine samples (two from the DESG and six from the CG) from further analysis. Because pooling of samples was not spontaneous, we performed a Partial Least Squares discriminant analysis (PLS-DA) that maximized the separation between the two groups.

Statistical procedures: All data are expressed as mean (SD). Differences between groups were evaluated with the Student t test. A significant difference was defined by $p < 0.05$. Pearson's correlation coefficients were used to examine the relations between quantitative variables. Statistical analysis was performed using the PLS_Toolbox 5.0 (Eigenvector Research,

Wenatchee, WA) and MATLAB in-house routines for all spectra. general statistics were performed with SPSS 15.0 for Windows (SPSS, Chicago, IL; see Figure 2).

RESULTS

The mean age of the participants was 52±4 years for the DESG and 36±5 years for the CG. The percentages of women were 24.41% (DESG) and 42% (CG). Due to the differences observed in age ($p=0.008$) and gender ($p=0.012$) between the DESG and the CG, all results were adjusted by these two variables.

Patients from the DESG complained of at least one of the following signs and symptoms: soreness ($n=39$), itchiness ($n=47$), grittiness ($n=23$), scratchy sensation ($n=24$), dryness ($n=41$), foreign-body sensation ($n=33$), burning ($n=14$), photophobia ($n=26$), redness ($n=21$), eye fatigue, or blurred vision ($n=48$). The ophthalmological examination and the LFU clinical tests performed reflected significant differences between the DESG and the CG.

Based on the personal interview, eye examination, OSDI questionnaire, and all referred symptoms, the DES cases were classified according to the degree of severity: 41.5% had mild DESG (17 patients), and 58.48% had moderate DESG (31 patients).

None of the study subjects (patients or healthy individuals) had an OSDI score of DES severity. Table 2 details the main clinical differences between the groups of participants with LFU disorders.

Tear samples obtained from the 35 healthy subjects in the CG allowed us to construct a preliminary set of 42 metabolites that were identified according to their spectra as compared with available spectral databases. Metabolites were classified into aliphatic and aromatics, the first accounting for up to 90% of the total number, as shown in Table 3. Then, data analysis from the tear ^1H NMR S at baseline permitted a comparison between the average spectrum of tears from the 55 patients of the DESG. Figure 3 shows the full spectrum between 0 and 10 ppm, including the most significant metabolite peaks.

The main signs and symptoms characterizing dry eyes significantly improved in the patients with DES (classified as mild DES and moderate DES) at 3 months from baseline. The main reported improvements were related to dryness, soreness, itchiness, foreign body sensation, photophobia, and eye fatigue (Figure 4). Interestingly, some patients (16% of the mild DES and 19% of the moderate DES) stated that their quality of life improved, and they felt better emotionally. This

improvement was noticeably reported by the patients in the DESG with moderate disease.

A clearly different distribution and significant differences in the metabolomic profile were shown to exist in patients with DES versus the healthy controls (Figure 5A). After supplementation with AOX/EPUFAs, noticeable changes in the metabolome were observed between the DESG and the CG (Figure 5B).

Table 3 shows the most important metabolites contributing to the discrimination profile between the patients with mild and moderate DES. A PLS-DA was performed to discriminate between these two DES subgroups (mild and moderate), and the metabolomic profile differed significantly as shown in Figure 6A. Figure 6B depicts the effects of the nutraceutical supplementation (BrudySec 1.5, three capsules per day) for 3 consecutive months. The metabolomic differences between the two study groups decreased significantly, and the arrangement of the PLS-DA spaces almost completely overlapped. This can be interpreted as that the tear baseline metabolic differences between groups noticeably changed, inducing moderate DESG to appear metabolically similar to mild DESG in the tear sample ^1H NMR diagrams.

DISCUSSION

Our findings demonstrated a different clinical pattern regarding the LFU and significant differences in the tear metabolomic profile for patients with DES compared to the healthy controls, and among the DES subgroups according to severity. We also observed significant improvement in the Schirmer and BUT scores and in the subjective sensations related to DES, as well as in the tear metabolome, after nutraceutical supplementation that contained AOX/EPUFAs.

The main goal of our work was to compare the tear film metabolite composition between the DESG and the CG. We also analyzed whether this metabolic profile changed with the severity of disease. We did not consider the major classes and subclasses of dry eye, according to their pathogenesis (“aqueous tear-deficient” dry eye and “evaporative” dry eye). However, the clinical signs of our patients with DES provided enough evidence of decreased aqueous tear production, decreased tear volume, increased tear evaporation rate, and increased tear osmolarity. To classify the severity of dryness in our study patients, we used the DEWS Subcommittee recommendations for the classification of disease based on severity with the modified basic scheme of the Delphi Panel Report [4]. We also followed the report from Murube et al. [8], regarding the triple classification of Madrid for dry eyes. We also utilized the recommendations from Sullivan et al. [33] about the definition on the severity of dry eye according to

TABLE 2. CLINICAL PROBES FOR DRY EYE DISEASE DIAGNOSIS AND MANAGING.

Groups	Subgroups	Schirmer's Test Score (mm/min)			BUT Score (seconds)			Subjective Sensations [#]		
		BS Mean (SD)	AS Mean (SD)	p	BS Mean (SD)	AS Mean (SD)	p	BS Mean (SD)	AS Mean (SD)	p
DESG	<i>Mild</i>	9.8 (1.7)	11.4 (0.5)	0.034*	7.5 (0.8)	8.1 (0.3)	0.007*	1.9 (0.3)	1.3 (0.1)	0.001**
	<i>Moderate</i>	9.5 (9.9)	13.3 (4.1)	0.039*	5.9 (0.6)	6.9 (0.0)	0.040*	2.8 (0.5)	1.8 (0.3)	0.000**
CG		18.9 (5.5)	25.1 (0.8)	0.002*	7.9 (0.1)	8.4 (0.0)	0.050*	0.1 (0.0)	0.0 (0.0)	0.214

DESG: dry eye syndrome group; CG: control group; BUT: breakup time test; BS: before supplementation; AS: after supplementation; SD: standard deviation # Subjective Sensations related to dry eyes (dryness, irritation, grittiness, foreign body feelings, light sensitivity, tired eyes, vision fluctuation and/or blurred vision) were classified considering both eyes (ranking from 0=none, 1=mild, 2=moderate, 3=severe, 4=very severe) * significant p value (<0.05) ** significant p value (<0.001)

TABLE 3. METABOLITES PRESENT IN MILD-DESG AND MODERATE-DESG TEARS PRIOR/AFTER TO SUPPLEMENTATION.

Metabolites	Region	Before Supplementation		p*	After Supplementation		p*
		Mild DESG	Moderate DESG		Mild DESG	Moderate DESG	
-CH ₃ lipids	0.84–0.88	0.0263 (0.005)	0.0233 (0.007)	0.007**	0.0208 (0.007)	0.019 (0.006)	0.47
Cholesterol/lipids	0.90–0.93	0.025 (0.004)	0.021 (0.006)	0.01**	0.021 (0.005)	0.018 (0.007)	0.11
N-acetylglucosamine	2.0–2.08	0.077 (0.01)	0.059 (0.02)	0.004**	0.063 (0.02)	0.055 (0.02)	0.25
Glutamate	2.325–2.415	0.031 (0.002)	0.027 (0.004)	0.02**	0.028 (0.005)	0.027 (0.005)	0.44
Amino-n-butyrate	2.95–3.025	0.0141 (0.001)	0.0142 (0.002)	0.04**	0.017 (0.005)	0.014 (0.003)	0.21
Choline/acetylcholine	3.18–3.21	0.010 (0.003)	0.010 (0.003)	0.9	0.011 (0.002)	0.009 (0.002)	0.03**
Choline	4.05–4.09	0.004 (0.001)	0.005 (0.002)	0.02**	0.005 (0.002)	0.006 (0.002)	0.61
Lactate	4.09–4.14	0.0064 (0.002)	0.0078 (0.003)	0.11	0.008 (0.003)	0.009 (0.004)	0.81
Glucose	5.17–5.29	0.0084 (0.005)	0.015 (0.009)	0.007**	0.019 (0.01)	0.023 (0.01)	0.48
Phenylalanine	7.20–7.40	0.028 (0.004)	0.034 (0.006)	0.08	0.031 (0.003)	0.034 (0.01)	0.54
Formate	8.45–8.475	0.0018 (0.0008)	0.003 (0.001)	0.02**	0.003 (0.0009)	0.003 (0.002)	0.50

DESG: dry eye syndrome group * p value after adjusting by age and gender; ** significant p value (<0.05).

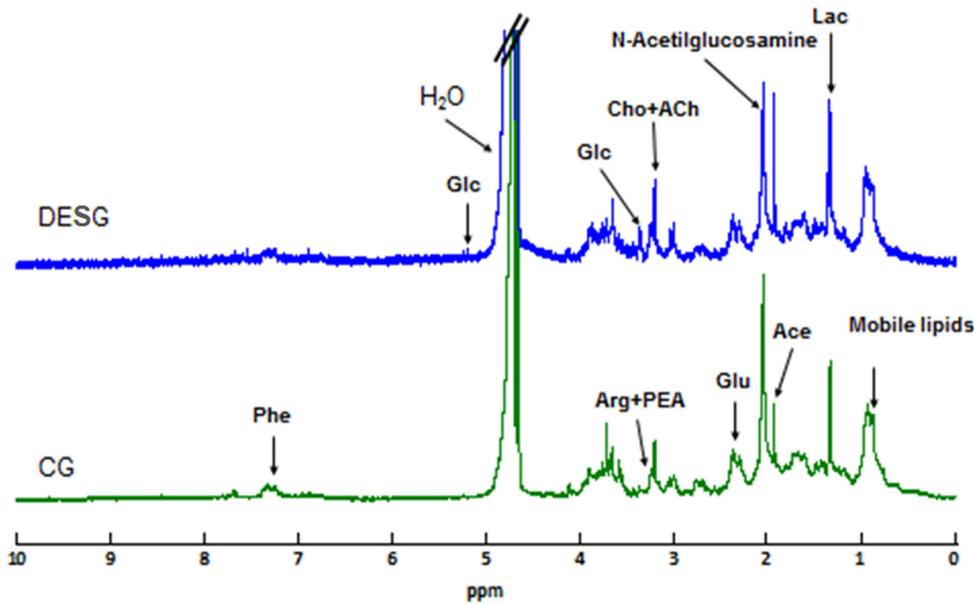


Figure 3. Average spectrum representation of the dry eye syndrome group tears compared to that of the control group before treatment.

a variety of diagnostic clinical signs and symptoms. Finally, we also considered the OSDI scores and the patient’s subjective sensations on ocular surface to appropriately classify the degree of severity of DES.

At the initial stage of recruitment, the participants in both groups were visited to complete data from the interview, to perform the ophthalmologic examination, and to collect the reflex tears. Then, the participants in the DESG and the CG were assigned to the daily intake of three pills of a nutraceutical formulation containing AOX/EPUFAs for 3

months. At the end of follow up, all participants again participated in the interview, clinical probes and the tear samples collection. Comparison of data from baseline and the end of study provided important information on the effects of the AOX/EPUFA supplementation in the study participants. The baseline Schirmer test and the FBUT scores increased significantly after supplementation in the two clinical severity DES subgroups, in agreement with other authors [1-6] and with our previous reports [11,35].

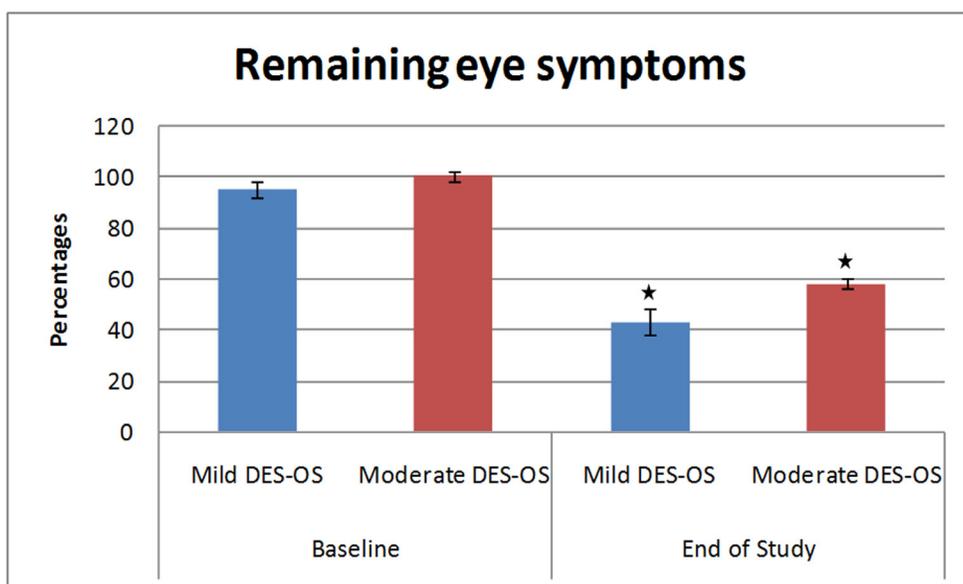


Figure 4. Improvement in subjective symptoms in patients with dry eyes after the oral nutraceutical supplementation.

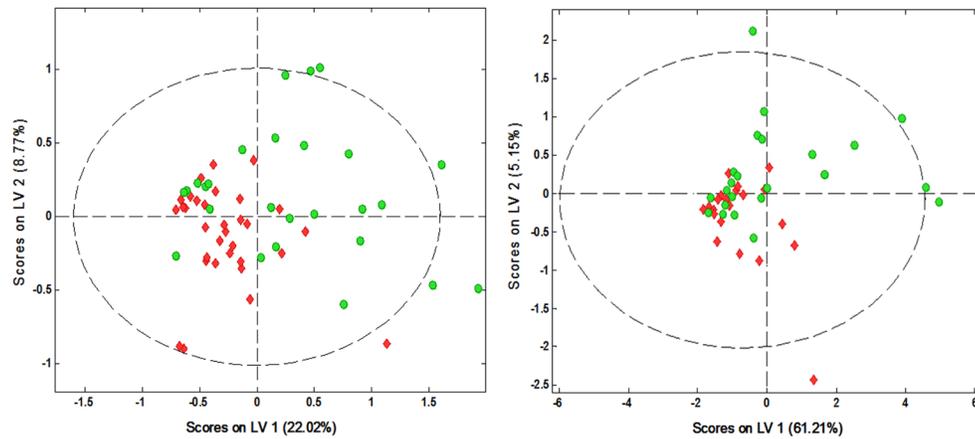


Figure 5. Scores diagram of partial least-squares discriminate analysis of the dry eye syndrome group (green) and the control group (red) before supplementation (A) and after supplementation (B).

The ¹H NMR S analysis of the tear samples from our participants showed significantly different metabolite peaks, according to previous assignment. The main metabolites found in the tear samples of the present study were lipids, amino acids, and carbohydrates. The most abundant molecules in the control tears were glycoprotein, mobile lipids, lipids/cholesterol, leucine, glycerol, and glutamate, among other metabolites shown in Table 2. However, the discriminating analysis of the DESG suggested a significant increase in glucose and lactate, whereas formate and N-acetylglucosamine noticeably decreased in the moderate DESG compared to the mild DESG. Our results are in agreement with other reports [36-44] in which the most outstanding tear metabolites were CH₃ lipids, cholesterol, lactate, glutamate, and glucose. In addition, lower levels of lactoferrin, lipocalin, and lipophilin AC-1 have been reported in patients with dry eyes, suggesting that these patients may be more susceptible to infections because the antimicrobial protein background is reduced [45]. Moreover, decreased levels of carnitine, which is derived from lysine and methionine, have also been reported in relation to the lower transport of fatty acids in patients with

DES compared to controls [46]. As shown, a wide variety of reports had been published in recent years about the composition of tears. However, discrepancies between the reports can be explained based on the collecting tear method, the technique used for the metabolomic analyses, and the bioinformatic process involved in identifying the metabolites.

Interestingly, in other metabolomic studies of body fluids such as the saliva, compounds similar to those reported here have been detected, such as glucose, lactate, choline, glutamine, formate, and N-acetyl groups [47,48]. One possible explanation for the presence of lactate in human fluids is that it is a consequence of anaerobic and aerobic metabolism of glucose. Another possibility involves a deficiency in the mitochondrial respiratory chain arising from the oxidative stress experienced by the LFU components exposed to the environment, causing decreased oxygenation and increased lactate production. In this context, lactate may presumptively serve as a biomarker to assess various eye diseases such as dry eye [49].

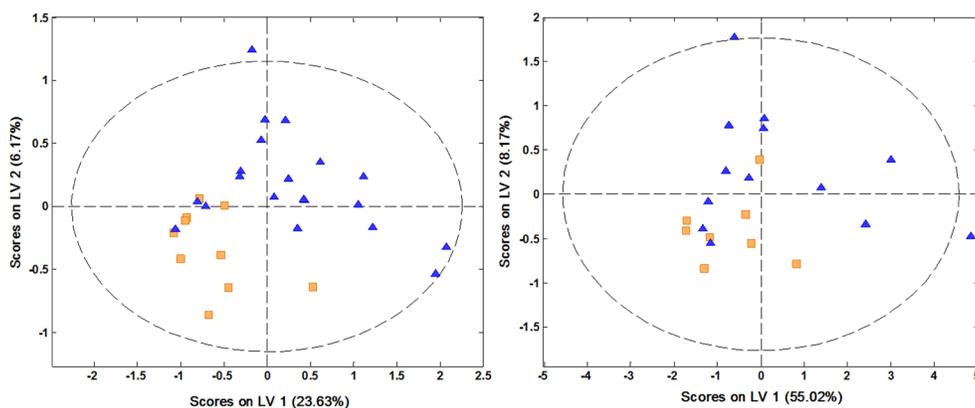


Figure 6. Load diagram model of partial least-squares discriminate analysis differentiating between mild dry eyes (orange) and moderate dry eyes (blue) before supplementation (A) and after supplementation (B).

Supplementation with AOX/EPUFAs induced a decrease in the baseline metabolic differential profile between groups (Figure 5B). The grouping of patients with DES by severity (mild, moderate) revealed a significant difference in metabolomic profiles. After supplementation, the baseline metabolomic differences between the three study groups decreased.

We also measured the PLS-DA regions to identify the most significant metabolites in the tears of all participants (Table 3). We analyzed these results, and after supplementation, we observed that the metabolome changed, and the main metabolites identified in each group were cholesterol, N-acetylglucosamine, glutamate, amino-n-butyrate, choline, glucose, and formate before supplementation, whereas major differences were found in the choline/acetylcholine after omega-3 EPUFA. The significance of each metabolite and its availability in the context of DES needs further research.

There are several limitations to this study. We were unable to account for all types of possible confounds. In addition, there were noticeable differences in age and sex between both groups of participants, but the appropriate statistical analyses were performed. Thus, due to the differences observed in age ($p=0.008$) and gender ($p=0.012$) between the DESG and the CG, all results were adjusted by these two variables. Finally, the study included only patients with mild and moderate DES. The precise statistical analyses was essential for reinforcing the consistency of our data. Notwithstanding these limitations, the data from this study may have important implications for better understanding the composition of the tear film in DES, which, in turn, may help in clinical practice by improving prognostic estimation and planning appropriate follow-up for the affected patients.

In summary, human tears contain valuable molecular information that can be isolated with metabolomics. This information can be useful for improving DES diagnosis, prognosis, and therapy. Our data demonstrate that the metabolomic profiles of healthy individuals and patients with dry eye differ and are positively modified toward normal with the use of supplementation with AOX and EPUFAs.

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