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Quantification of a panel for dry-eye protein biomarkers in tears: A comparative pilot study using standard ELISA and customized microarrays

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Purpose: This paper examines the tear concentration of cystatin S (CST4), calcyclin (S100A6), calgranulin A (S100A8), and matrix metalloproteinase 9 (MMP9), and the correlation between biomarker expression, clinical parameters, and disease severity in patients suffering from dry eye (DE). A comparison of the results is obtained via ELISA tests and customized antibody microarrays for protein quantification.

Methods: This single-center, observational study recruited 59 participants (45 DE and 14 controls). Clinical evaluation included an Ocular Surface Disease Index (OSDI) questionnaire, a tear osmolarity (OSM) test, the Schirmer test (SCH), tear breakup time (TBUT), fluorescein (FLUO) and lissamine green (LG) corneal staining, and meibomian gland evaluation (MGE). Tear concentrations of CST4, S100A6, S100A8, and MMP9 were measured using standard individual ELISA assays. The levels of CST4, S100A6, and MMP9 were also measured using customized multiplexed antibody microarrays. Correlations between variables were evaluated, and a significance level was p value <0.05.

Results: The quantification of tear protein biomarkers with ELISA showed that the concentration of CST4 was significantly (2.14-fold) reduced in tears of DE patients in comparison with control (CT) subjects ($p < 0.001$). S100A6 and S100A8 concentrations were significantly higher in the tears of DE patients (1.36- and 2.29-fold; $p < 0.001$ and 0.025, respectively) in comparison with CT. The MMP9 level was also higher in DE patients (5.83-fold), but not significantly ($p = 0.22$). The changes in CST4 and S100A6 concentrations were significantly correlated with dry eye disease (DED) severity. Quantification of CST4, S100A6, and MMP9, using antibody microarrays, confirmed the ELISA results. Similar trends were observed: 1.83-fold reduction for CST4 (p value 0.01), 8.63-fold increase for S100A6 (p value <0.001) and 9.67-fold increase for MMP9 (p value 0.94), but with higher sensitivity. The biomarker concentrations were significantly associated with the signs and symptoms related with DED.

Conclusions: S100A6, S100A8, and CST4 diagnostic biomarkers strongly correlate with DED clinical parameters. S100A6 and CST4 are also useful for grading DE severity. The multiplexed antibody microarray technique, used here for tear multi-marker quantification, appears more sensitive than standard ELISA tests.

Dry eye (DE) is one of the most frequent ocular surface diseases (OSD). The prevalence of dry eye disease (DED) ranges from 5% to 50% [1]. The DED prevalence is higher in women than in men, increases linearly with age, and appears to be higher in Asian populations than in Caucasian populations [1]. The DED incidence in Caucasian populations reported in the few available studies is 10.4%, 13.3%, and 21.6% over a period of two, five, and 10 years, respectively [2,3].

DED affects individuals' eyesight, quality of life (QoL), and work productivity. Patients with DED often report vision-related difficulties when performing daily activities. This results in a decreased QoL and is frequently linked to depression and anxiety [4]. This illness is the most common reason for seeking medical eye care and constitutes a significant cost burden due to direct and indirect health costs and reduced work productivity [5]. The total annual cost of its management has been estimated at USD $3.84 billion in the United States and USD $0.15 million in Singapore. In Europe, the annual total cost per 1000 DED patients managed by ophthalmologists ranges from USD $0.27 million in France to USD $1.10 million in the UK [1].

DED is a multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film and accompanied by ocular symptoms. Tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities contribute to the etiology of DED [6]. The definition updated in 2017 recognizes the multifactorial nature of DE as a disease in which the loss of

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homeostasis of the tear film is the central pathophysiological concept. The ocular symptoms, including discomfort, visual disturbance, or both, remain the central feature of the disease [6].

Traditionally, the etiopathogenic classification of DED has included the aqueous-deficient dry eye (ADDE) and the evaporative dry eye (EDE) forms. Depending on the stage of the illness, DED can be classified as mild, moderate, or severe [7]. However, recently, The Tear Film & Ocular Surface Society (TFOS) International Dry Eye Workshop II (DEWS II) Definition and Classification Report identified a wider spectrum in which different sub-categories of DED are recognized when the signs are predominantly evaporative or show increasing aqueous deficiency in the stages in between [6]. The severity of signs and the evaporative-to-aqueous bias also form a part of the sub-classification (diagnosis) designed to help manage DED [8].

Within this pathophysiological scenario, biomarkers have become important molecular and biochemical criteria and have been gradually incorporated into clinical practice in ocular surface pathologies. Tear biomarkers are of special interest because of the straightforward analysis of the tear samples and relatively easy and minimally invasive sample collection, which is painless and discomfort-free for patients [9]. The evaluation of tear biomarkers in an objective and appropriate management of the disease is of utmost importance. They can improve diagnosis, help assess disease severity, monitor response to therapy, and screen patients before ocular surgery (laser-assisted in situ keratomileusis [LASIK], photorefractive keratectomy [PRK], cataracts, cross-linking, and glaucoma procedures, among others). They can facilitate the choice of appropriate therapy, depending on the type and severity of DE, and help detect early defects induced by long-term treatments, such as antiglaucomatous, antiallergic, and antimicrobial eye drops with preservatives. However, before they can be used in clinical practice, the biomarkers must be well validated [10].

Many studies have compared healthy and pathological groups to identify specific DED biomarkers. In search of DE protein biomarkers, different proteomic approaches have been employed: Two-dimensional gel electrophoresis (2D-PAGE) [11–13] and mass spectrometry (MS)-based proteomic strategies, such as nano-liquid chromatography-tandem mass spectrometry [14,15], surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) [16], and isobaric tags for relative and absolute quantitation (iTRAQ) [17–19]. Some researchers have used techniques in which the candidate tear proteins are pre-selected for quantitation using immunodetection methods, e.g., ELISA assays [20–23], bead-based assays [24,25], and antibody arrays [26,27]. Others have employed a different approach, such as the LabChip protein bioanalyzer, using the standard protein ladder [28].

As a result, many new tear biomarkers have been proposed in the last decade. In the recent report of the Tear Film Subcommittee of the TFOS DEWS II, 122 candidate biomarkers (102 extracellular and 20 intracellular proteins) were referenced [29]. However, nearly half of the candidate biomarkers lacked immunological validation. Consequently, there is a real need for clinical validation studies of those candidate biomarkers; the DED protein levels and normal protein concentrations should be compared [9,29].

To determine the real value and significance of a biomarker (or a panel of biomarkers) to be translated into clinical practice, the evaluation of the correlation between biomarker concentration changes and clinical parameters is required [9]. Given the multifactorial etiology of DED, screening individual targets will result in an incomplete picture of the pathological state, and the evaluation of a single biomarker will not provide enough diagnostic power [13]. Proteomic studies have revealed that not just a single protein but a group of biomarkers are altered in the tear film of affected individuals. Panels of DED biomarkers with appropriate sensitivity and specificity have revealed changes in proline-rich protein 4 (PRP4), S100A8, serpin peptidase inhibitor clade A member 1 (SERPINA1), and submaxillary gland androgen-regulated protein 3B (SMR3B; sensitivity 90%, specificity 90%) [16]; enolase 1 (ENO1), prolactin-inducible protein (PIP), lipocalin-1 (LCN1), and S100 calcium binding protein A9 (S100A9; sensitivity 91%, specificity 90%) [30]; annexin A1 (ANXA1), annexin A11 (ANXA11), CST4, phospholipase A2-activating protein (PLAA), and S100A6 (sensitivity 96%, specificity 92%) [13]; and beta-2-microglobulin (B2M), PRP4, S100A8, secretoglobin family 1D member 1 (SCGB1D1), and secretoglobin family 2A member 1 (SCGB2A1; sensitivity 100%, specificity 100%) [31].

Our group has previously examined tear protein biomarkers for the diagnosis of DE and meibomian gland dysfunction (MGD), employing several proteomic techniques. We have shown, using unbiased two-dimensional difference gel electrophoresis (2D DIGE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) label-free quantitative proteomics, an increase in the expression of several proteins in the tears of DE patients: S100A6, S100A9, S100A8, S100A4, glutathione S-transferase-P (GSTP1), ANXA1, ceruloplasmin (CP), and apolipoprotein D (APOD). We have also observed a decrease in the levels of LCN1, lactotransferrin (LTF), dermcidin (DCD), CST4, and lacritin

(LACRT) in the DE group [13,27]. Our functional interaction network analyses have revealed a disease-related network of candidate proteins, including ANXA11 and PLAA, and identified five topological modules in which MMP9 was the best-represented protein. In the tears collected with a sponge and capillary from the eyes of DE patients, five candidate biomarkers, ANXA1, ANXA11, CST4, PLAA, and S100A6, were validated via standard ELISA assays [13], and S100A6, CST4, and MMP9, using customized microarrays [27].

In the current study, we focus on the correlation between changes in the concentration of selected biomarkers with clinical variables and endpoints to determine the suitability of candidate biomarkers for clinical practice and their potential as diagnostic and prognostic biomarkers for DED. We measured the concentration of S100A6, S100A8, CST4, and MMP9 using standard ELISA assays and examined the correlations with clinical parameters. We verified three candidate biomarkers, S100A6, CST4, and MMP9, using a novel customized antibody microarray system to compare the results of the individual and multiplexed quantification of biomarker concentrations. The correlations of the selected biomarkers with disease severity were also examined.

METHODS

Patients: We designed an observational, prospective, case-controlled study in which 59 subjects, 45 with DE and 14 healthy individuals, were enrolled. The research was conducted by medically qualified personnel after approval from the Institutional Review Board and Ethics Committee. Approval was obtained in strict accordance with the tenets of the Declaration of Helsinki on Biomedical Research Involving Human Subjects. Before sample collection, signed informed consent was obtained from all subjects once the nature and possible consequences of the study had been explained.

Patient enrollment was performed at the Ophthalmology Service Unit of the San Carlos Hospital (Madrid, Spain) during outpatient ophthalmologist consultations, and was based on the agreed inclusion and exclusion criteria. The inclusion criteria consisted of patients over 40 years of age, both male and female, who met the requirements for each group. Individuals with (or history of) any systemic or ocular disorders or conditions, individuals who have had eye surgery in the preceding three months, those with long-term medications (glaucoma) or a history of allergies, using any topical medication (other than artificial tears) or oral medication containing corticosteroids, and patients with atopy or with Sjögren's syndrome were excluded from the study. Contact lens users were also excluded to avoid any possible interference with the interpretation of the results.

Clinical examination included the Schirmer test (SCH), tear film osmolarity (OSM) measured with TearLab (TearLab Corporation, San Diego, CA), the tear breakup time (TBUT) test, the slit-lamp examination of the lid margin, and meibomian gland evaluation (MGE), fluorescein staining (FLUO) using the Oxford scale, and lissamine green staining (LG). Subjective symptoms were recorded, and each patient answered the ocular surface disease index (OSDI) questionnaire.

Patients assigned to the DE group presented symptoms of dryness worsening in dry environments and throughout the day (OSDI score greater than 13), SCH at 5 min <10 mm, fluorescein staining according to the Oxford grading scale (using a slit lamp and fluorescein stain, scale 0–5) [32], and TBUT <10 s. DE patients were classified as mild, moderate, or severe, following the DE severity grading scheme previously proposed [7].

Healthy subjects in the control (CT) group had no symptoms of DE or meibomian gland dysfunction (MGD), had an OSDI score below 13, SCH >10 mm, TBUT >10 s, wore no contact lenses, and did not suffer from atopy.

Clinical evaluation of patients: The order in which the clinical tests and sample collection were conducted was always the same. The clinical tests were performed in the following sequence, according to the DEWS criteria [33]: clinical history (diseases and medications), OSDI symptoms questionnaire, OSM, tear meniscus evaluation without fluorescein (MH), vital stains (FLUO and LG), TBUT, SCH test, evaluation of lid margin and meibomian glands, meibography to evaluate meibomian gland quality (MGQ), and meibomian gland expression (MGE).

A slit lamp was used to examine the lid margin for telangiectasia, irregularity, pouting, plugging, and retroplacement of the meibomian gland orifices. In MGD, the quality of expressed oil (MGQ) varies from cloudy fluid through viscous fluid containing particulate matter to a densely opaque, toothpaste-like material. There is a four-point system to register its quality, with scores of 0, clear (normal); 1, cloudy; 2, cloudy with particles; and 3, inspissated (like toothpaste). For MGE, we recorded the sum of the scores for each expressed gland to obtain a composite score. With eight glands expressed, the score range was 0–24 (8 × 3) [34]. The MGD phase or stage was assessed according to the International Workshop on Meibomian Gland Dysfunction Management and Treatment Report [35]. Meniscometry was performed at the central lower lid margin, just after blinking, using Keratograph 5 (OCULUS Optikgeräte GmbH, Wetzlar, Germany).

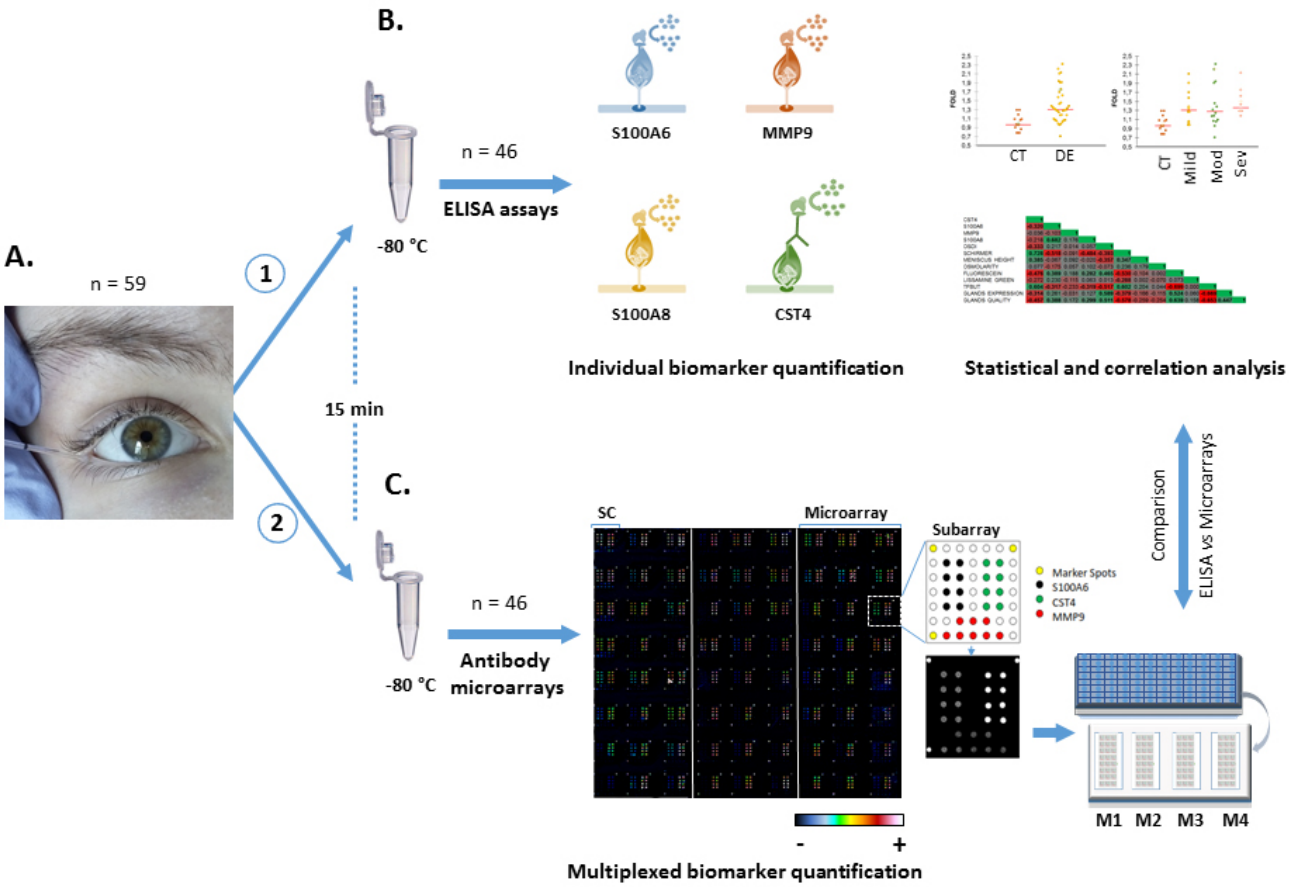

Figure 1. Workflow diagram of the experimental strategy used in the study. **A**: Tear samples were collected twice from each volunteer, with a 15 min interval between samplings. The study had two phases (see **B** and **C**). **B**: The first single biomarker quantification, using individual ELISA assays, followed by statistical and correlation analyses. **C**: The second simultaneous quantification of biomarkers, using multiplexed antibody microarrays. The results obtained using the two techniques were compared. SC: Standard curve.

On the first day, the OSDI questionnaire was conducted to assess the symptoms of ocular irritation and vision-related function. Then, the clinical tests were performed as indicated above. Tear samples were collected a day later to avoid interference between the clinical tests and the tear film biomarker quantitation.

Tear sample collection: All tear samples were collected using calibrated 10-µl glass microcapillary tubes (BLAUBRAND intraMark, Wertheim, Germany). Tear samples were obtained from the inferior temporal tear meniscus, minimizing the irritation of the ocular surface or lid margin, without anesthesia. The tear samples were collected from both eyes of each participant and placed immediately in separate precooled Eppendorf tubes. For comparative protein quantification analyses using ELISA and microarrays, the tears were collected twice with an interval of 15 min between collections (two tubes per eye). After tear collection, the samples were stored at −80 °C until analysis (Figure 1). The first tear samples collected were used in the ELISA assays, and the tears harvested in the second collection were used in the microarray assays. In both cases, samples from the left and right eyes of each individual were pooled before the analysis. The mean volume of the samples collected from DE patients and the CT group was 8.11 ± 7.04 µl and 15.44 ± 5.29 µl, respectively (per eye).

ELISA assays: Four candidate proteins were selected (Table 1). All commercial ELISA assays conducted in the study were performed following the instructions of the manufacturer. Commercially available sandwich-type ELISA kits were used for the quantification of three of the candidate biomarkers, S100A6, S100A8, and MMP9 (USCN Life Science Inc., Wuhan, China). The protein CST4 was examined using a customized ELISA immunoassay. For S100A6 and S100A8 quantitation, tear samples were diluted 2:100, and for MMP9 quantitation, tear samples were diluted 3:100. In the case of CST4, a standard protocol for antigen-coating immunoassay

Table 1. The panel of biomarkers selected for quantification in the study.

Biomarker	Function	Reference
S100A6 (calcyclin)	Epithelial integrity, calcium binding, proliferation	[13,17,27,29,44 -46]
S100A8 (calgranulin A)	Inflammation, immune response, inhibitor of casein kinase	[13,16,17,19,27,29,31,44, 46 -49]
CST4 (cystatin S)	Antimicrobial, defense, cysteine protease inhibitor	[13,27,41 -43]
MMP9	Inflammation, matrix collagen degradation	[20,29,46,52 -55]

was performed. The antigen (sample) was coated onto a 96-well polystyrene microtiter plate at a 1:100 dilution (with coating buffer, 0.2 M $NaHCO_3$, pH 9.6) and incubated for 3 h at room temperature. Washing was performed with phosphate buffered saline (PBS) supplemented with 0.05% Tween-20 (PBS-T; 3x), followed by blocking of the plate surface with 300 µl of PBS-T supplemented with 5% (w/v) dried milk. Then, the sample was incubated for 1 h with 5 µg/ml of a mouse anti-CST4 detection antibody in PBS (0.05% Tween, 5% dried milk, pH 7.2). It was washed with PBS-T (3x) and incubated for 1 h with 1 µg/ml of a goat anti-mouse secondary antibody in PBS with 0.05% Tween, 5% dried milk, and a pH level of 7.2. Five washes with PBS-T followed. Then, 90 µl of 3,3′,5,5′-tetramethylbenzidine (TMB) was added, and absorbance was measured at 450 nm in a Multiskan plate reader (Thermo Fisher Scientific, Uppsala, Sweden). In the ELISA assays, the final volume per well was 100 µl, and all biomarkers were analyzed in duplicate. Thus, 2 µl of each tear sample was needed for CST4, 6 µl for MMP9, and 4 µl for S100A6 and S100A8 (Table 2).

Antibody microarrays: Three biomarkers (S100A6, CST4, and MMP9) were selected for integration into customized sandwich-like antibody microarray slides. An indirect sandwich immunoassay scheme for protein quantitation in a microarray format was used (Figure 1C). The process of microarray customization for quantitative tear biomarker analysis involved several steps: i) identification of specific capture and detection antibodies for biomarkers; ii) determination of the affinity constant to ensure sensitive detection; iii) evaluation of cross-reactivity between antibodies; iv) determination of the detection limits and dynamic range; v) correlation of quantification with individual ELISA assays; and vi) optimization of the platform for high content multiplexing.

A set of customized microarrays were generated using previously published methods (IMG Pharma Biotech, Derio, Spain) [36]. Briefly, mouse monoclonal antibodies against the selected proteins and control antibodies (reference marker spots) were diluted in the printing solution to a final concentration of 0.4 mg/ml and 0.1 mg/ml, respectively. Twenty-four arrays (8 × 3), consisting of eight replicas of each sample surrounded by three replicas of the reference marker were spotted onto functionalized glass slides (Figure 1C) using a Nano-Plotter NP 2.1 microarrayer (GeSiM, Radeberg, Germany). The piezoelectric tips generate spots of approximately 100 microns in diameter. The slides were printed at room temperature at a relative humidity of 68%. Microarrays were stored at −20 °C until use.

The microarray assays were performed using a 4 × 24 Multi-Well Microarray Hybridization Cassette (Arrayit Corporation, Sunnyvale, CA) in a microplate-like format, on functionalized glass slides. The arrays were blocked with PBS-T supplemented with nonfat dry milk (5%) for 1 h. Antigens (S100A6, CST4, and MMP9) and samples were incubated overnight at 4 °C. The reaction volume was 70 µl for all steps in the immunoassay. Tear samples were diluted (1:70) in PBS-T. The rabbit detection antibodies (10 µg/ml) were added

Table 2. Tear sample volume required (per patient) for quantification with standard ELISA and multiplex antibody microarrays.

Biomarker	ELISA			Microarrays		
	Dilution	Final volume per well (µl)	Vol. sample needed* (µl)	Dilution	Final volume per well (µl)	Vol. sample needed** (µl)
CST4	1:100	100	2	1:70	70	1
MMP9	3:100	100	6			
S100A6	2:100	100	4			
S100A8	2:100	100	4	N/A	N/A	N/A
	Total volume (µl)		16	Total volume (µl)		1

*Each biomarker was examined in duplicate, ** each biomarker was examined in eight replicates. N/A, not analyzed

Table 3. Demographic and clinical data of the patients included in the study.

Demographic data	Group			
	Control	Mild	Moderate	Severe
Age	54.57 ± 10.31	57.26 ± 6.02	59.10 ± 12.97	56.85 ± 8.53
Sex % (F/M)	64.28/35.72	75/25	73.68/26.32	100/0
MH (mm)	0.33 ± 0.1	0.32 ± 0.08	0.32 ± 0.15	0.25 ± 0.07
TBUT	10.54 ± 0.88	5.72 ± 1.56*	4.14 ± 1.64*	2.75 ± 1.65*
MGQ	0.71 ± 2.19	8.25 ± 2.31*	8.97 ± 3.96*	11 ± 4.23*
MGE	0.07 ± 0.26	0.41 ± 0.56*	1.16 ± 0.82*	1.35 ± 0.59*
MGD STAGE	0.18 ± 0.48	1.59 ± 0.8*	2.34 ± 1.05*	2.55 ± 0.89*
FLUO	0	0.94 ± 0.25*	0.95 ± 0.32*	1*
LG	0	0.03 ± 0.18	0.18 ± 0.39	0
SCH	16.29 ± 5.76	8.63 ± 1.68*	8.18 ± 2.54*	4.20 ± 2.80*
OSM	304.04 ± 8.83	301.72 ± 10.12	301.11 ± 17.92	301.20 ± 16.44
OSDI	6.31 ± 4.03	16.06 ± 17.19*	30.50 ± 19.01*	35.92 ± 26.18*

MH, meniscus height; TBUT, tear breakup time; MGQ, meibomian gland quality; MGE, meibomian gland expression; MGD, meibomian gland dysfunction; FLUO, fluorescein; LG, lissamine green; SCH, Schirmer test; OSM, osmolarity; OSDI, Ocular Surface Disease Index. Asterisks indicate significant differences in comparison with controls. p-value < 0.001.

and the mixture was incubated for 1 h. Finally, after washing the slides with PBS-T, secondary Alexa Fluor 647 labeled anti-rabbit antibodies (5 µg/ml) were added and incubated for 1 h. Fluorescence intensity of the spots was measured using an Agilent G2565BA Microarray Scanner (Agilent Technologies, Santa Clara, CA) at 633 nm, and protein concentration was determined based on the standard curve intensity values. For the microarray (a multiplexed system allowing simultaneous quantification of several biomarkers with replicates examined in the same sub-microarray, eight replicates per biomarker), 1 µl of each tear sample was needed (Table 2).

Statistical analyses: After testing for normal distribution and homoscedasticity, the significant differences between the groups were determined using the Mann–Whitney U test for the comparison of two independent samples, the Kruskal–Wallis non-parametric test was used for more than two independent samples, and the Tukey post-hoc test was used for multiple pairwise comparisons. Non-parametric multivariate analysis of variance (MANOVA) or analysis of similarities (ANOSIM) was also performed for multivariate between-group comparisons.

Finally, hierarchical clustering using Ward's linkage and Pearson correlation analysis was performed to assess correlations between clinical parameters and protein levels. Statistical analysis was conducted using R statistical software [37].

RESULTS

Patients and clinical parameters: One hundred-eighteen eyes of 59 volunteers were included in the study. The mean age was 56.9 ± 10.5 years; this did not differ significantly between the CT and DE groups (p value = 0.21). Among the 45 patients with DED, 16 (35.6%) presented with the mild grade, 19 (42.2%) with the moderate grade, and 10 (22.2%) with the severe grade of the disease (Table 3).

No significant differences in gender were observed between the CT and DE groups (Fisher's exact test, p value = 0.0805). The female/male (F/M) ratio in the CT group was 64%/36%, whereas, in the DE group, the distribution differed, depending on the severity. For the mild and moderate DE grades, the F/M distribution ratio was 75%/25% and 74%/27%, respectively. In the severe DED group, all patients were women.

No significant differences in the MH (p value = 0.79), OSM (p value = 0.52), or LG test results (p value = 0.18) were observed between the CT and DE groups. The values for other clinical parameters, such as TBUT ($p < 0.001$) and the SCH test ($p < 0.001$), significantly decreased in the DE group in comparison to the CT group. However, the MGQ ($p < 0.001$), MGE ($p < 0.001$), MGD stage ($p < 0.001$), FLUO staining ($p < 0.001$), and OSDI ($p < 0.001$) values were significantly higher for DE patients.

Quantification of tear protein biomarkers: The concentrations of the four biomarkers (S100A6, S100A8, CST4, and MMP9) were individually measured in 14 CT subjects and

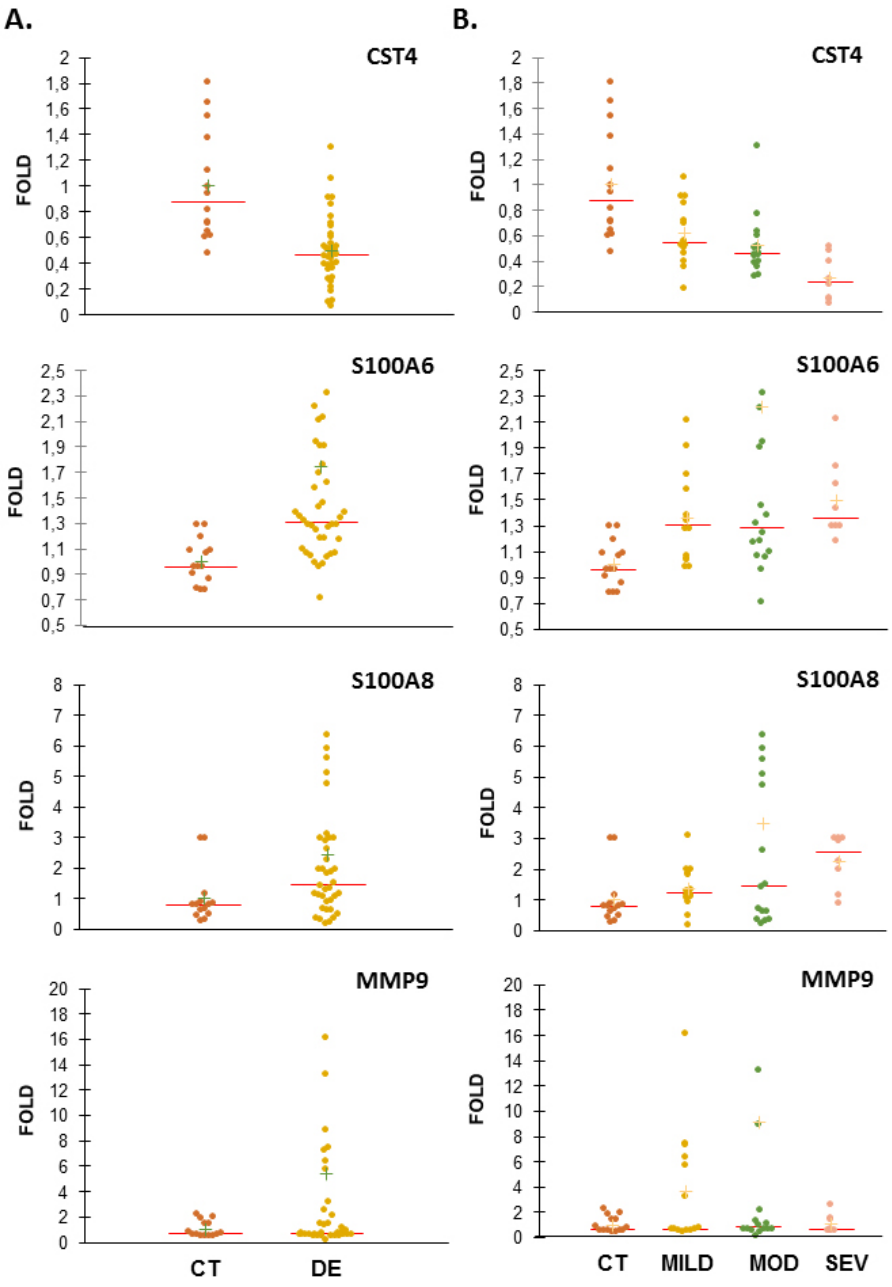

Figure 2. Dot plots depicting changes in the concentration of CST4, S100A6, S100A8, and MMP9 proteins. **A**: Comparison of the results for the entire DE group (n = 32) and CT group (n = 14). **B**: Changes in the DE group categorized by severity status: mild (MILD; n = 11), moderate (MOD; n = 14), and severe (SEV; n = 7), in comparison with the CT group. The (+) indicates the mean and the red line indicates the median. The significance of the results is shown in Table 4.

32 DE patients using ELISA assays (Figure 2A). It was not possible to measure tear biomarkers with ELISA for 13 of the 45 DE patients, as the sample volume for these patients was too small.

A significant decrease in the CST4 concentration was found in the DE group ($p < 0.001$) in comparison with the CT group. In contrast, the concentrations of S100A6 and S100A8 were significantly higher in this group ($p < 0.001$ and 0.025, respectively). An increase in MMP9 levels was also seen in some DE patients; however, it was non-significant ($p = 0.22$).

When the severity of DE was considered (Figure 2B), the CST4 concentration gradually and significantly decreased with the increase in severity ($p < 0.001$) in comparison with the CT group (Table 4). The concentration of S100A6 increased significantly in the mild ($p = 0.03$), moderate ($p = 0.02$), and severe ($p < 0.001$) DE groups. The concentration of S100A8 rose gradually as the severity increased; however, significant differences (in comparison with controls) were only observed for the severe group. The increased concentration of MMP9 was mostly observed in the mild and

Table 4. Statistical significance of proteins CST4, S100A6, S100A8, and MMP9 for the different dry-eye severity grades relative to control group.

DE	Control	Mild	Moderate
CST4			
Mild	0.0091*	-	-
Moderate	3.0e-05 *	0.2054	-
Severe	3.4e-06*	0.0162*	0.2054
S100A6			
Mild	0.02896 *	-	-
Moderate	0.02122*	0.98935	-
Severe	0.00087*	0.2832	0.2832
S100A8			
Mild	0.364	-	-
Moderate	0.546	0.573	-
Severe	0.047*	0.546	0.401
MMP9			
Mild	0.78	-	-
Moderate	1	1	-
Severe	1	1	1
NPMANOVA			
Mild	0.019*	-	-
Moderate	0.006*	0.174	-
Severe	0.006*	0.019*	0.019*

Statistical significance was determined by the Tukey test for multiple pairwise post-hoc comparisons. Non-parametric MANOVA or analysis of similarities (ANOSIM) was also performed for the multivariate-between-groups comparisons. *Significant differences at p-value < 0.05.

moderate DE groups, but it was not significant. In addition, a non-parametric MANOVA multivariate test, using the four biomarkers simultaneously, showed significant differences between the CT and mild DE ($p = 0.02$), CT and moderate DE ($p < 0.01$), CT and severe DE ($p < 0.01$), mild and severe DE ($p = 0.02$), and moderate and severe DE ($p = 0.02$) groups, but not between the mild and moderate DE group ($p = 0.17$; Table 4).

Correlation of tear biomarker concentrations with clinical endpoints: To determine the correlation of biomarker concentrations with clinical parameters, the hierarchical clustering of variables (Figure 3A) and the correlation matrix (Figure 3B) were used. In the hierarchical clustering analysis, the horizontal axis of the dendrogram represents the distance or dissimilarity between clusters. The analysis indicated that all biomarkers were associated, within the same cluster, with some of the clinical variables: MH, SCH, TBUT, OSM, and LG. The second cluster contained other clinical variables: OSDI, MGE, MGD stage, MGQ, and FLUO (Figure 3A).

A post-hoc Pearson correlation analysis was performed to determine individual associations with the tested clinical parameters (Figure 3B). Some significant correlations between the biomarkers were found: a negative correlation between CST4 and S100A6 and a positive correlation between S100A8 and S100A6. Significant correlations of the biomarkers with clinical variables were also revealed. CST4 showed a significantly positive correlation with SCH, MH, and TBUT and a significantly negative correlation with OSDI, FLUO, MGE, and MGQ. The biomarkers S100A6 and S100A8 were positively correlated with FLUO and MGQ and negatively correlated with SCH and TBUT. MMP9 did not show any significant correlations with any of the variables considered in the study.

For OSDI, we found a significantly positive correlation with the clinical variables FLUO, MGE, and MGQ and a negative correlation with SCH, MH, TBUT, and CST4. The SCH test results were significantly and positively correlated with MH, TBUT, and CST4, but showed a negative correlation

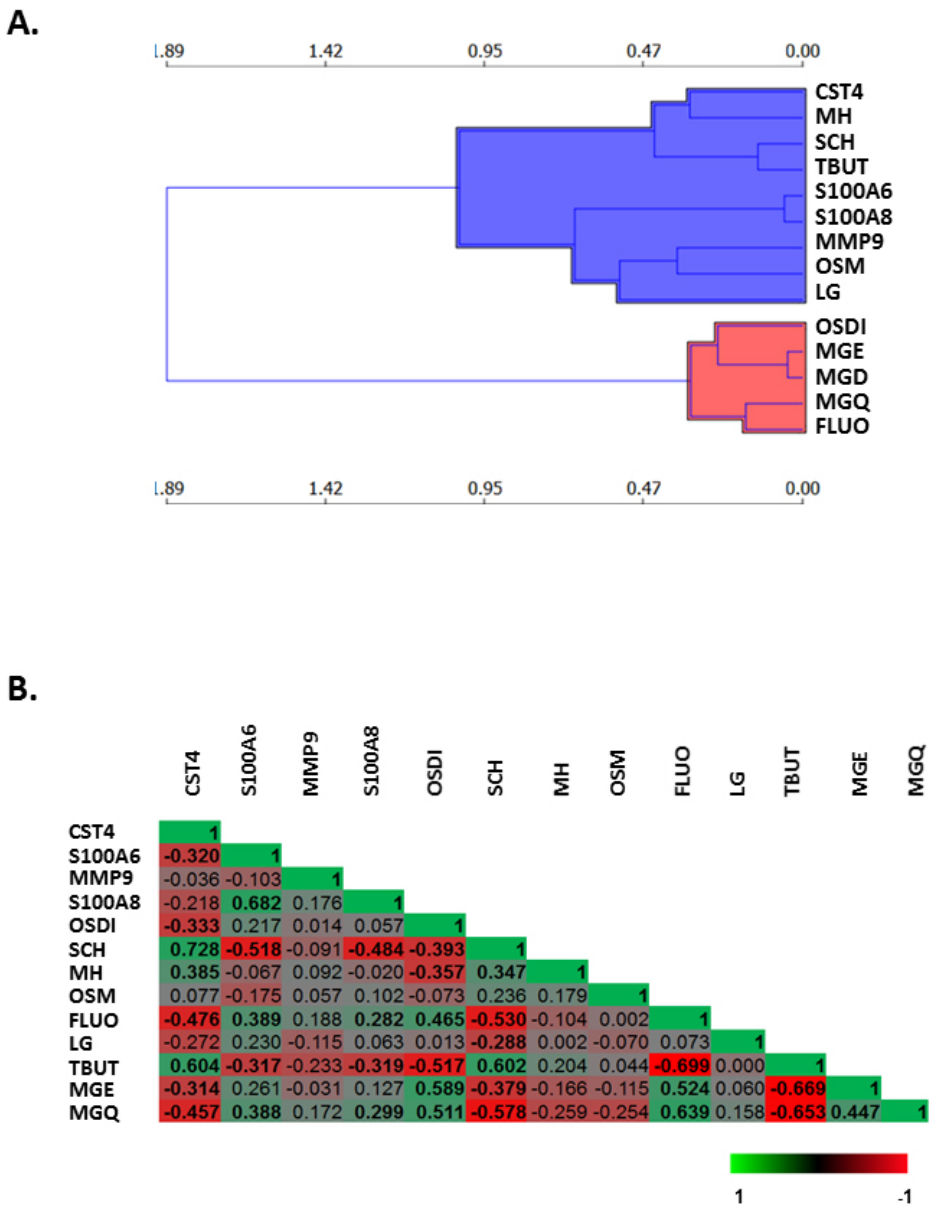

Figure 3. Correlation of validated tear biomarkers with clinical endpoints. **A**: Hierarchical clustering of variables. The horizontal axis of the dendrogram represents the distance or dissimilarity between clusters. **B**: Correlation matrix of biomarker concentrations and clinical parameters. The intense green and intense red represent significant positive and negative correlations, respectively. Significant correlations are indicated in bold (p value <0.05). OSDI: ocular surface disease index; SCH: Schirmer test; MH: meniscus height; OSM: osmolarity; FLUO: fluorescein staining; LG: lissamine green; TBUT: tear breakup time; MGE: meibomian gland expression; MGQ: meibomian gland quality.

with FLUO, LG, MGE, MGQ, OSDI, S100A6, and S100A8 levels. OSM did not show a significant correlation with any of the variables considered in the study. The FLUO results were positively correlated with MGE, MGQ, and OSDI results, and the levels of S100A6 and S100A8 were negatively correlated with TBUT, CST4, and SCH. LG staining showed a negative correlation with SCH. TBUT exhibited a positive correlation with CST4 and SCH and a negative correlation with MGE, MGQ, S100A6, and S100A8 concentrations, as well as OSDI and FLUO. MGE was positively correlated with MGQ, OSDI, and FLUO, and negatively correlated with CST4, SCH, and TBUT. MGQ showed a positive correlation with S100A6, S100A8, OSDI, FLUO, and MGE, and a negative correlation with CST4, SCH, and TBUT.

Verification of S100A6, CST4, and MMP9 concentration changes using multiplex antibody microarrays: To verify the results acquired using individual ELISA assays and to determine the possible effect of the analytical technique on obtained concentration values, customized multiplex antibody microarrays were employed. We measured CST4, S100A6, and MMP9 concentrations in the tear samples collected in the second round of sampling from the same cohort of patients. The standard curves for the microarrays showed a good limit

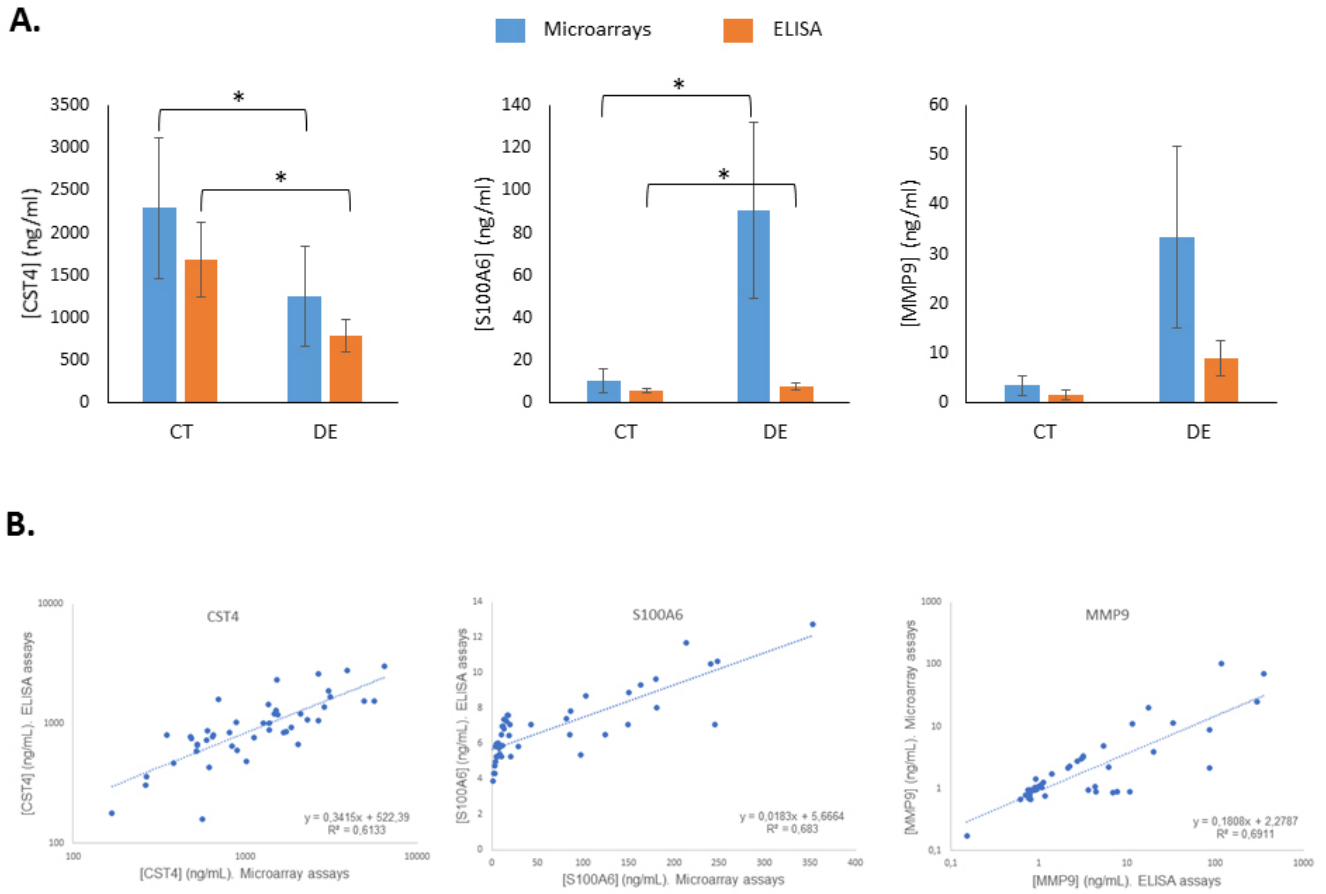

Figure 4. Comparison of mean biomarker concentrations obtained with ELISA and with antibody microarrays in the DE and CT groups. **A**: Comparison of the concentrations of CST4, S100A6, and MMP9 biomarkers in control (CT; n = 14) and dry eye (DE; n = 32) groups measured using the standard individual ELISA assays and customized multiplexed antibody microarrays. Concentration is expressed in ng/ml. The bars represent a comparison of mean biomarker concentration in the DE group relative to the CT group. Error bars show standard deviation, and * indicates statistical significance relative to control (p value <0.05). **B**: Regression analyses of the results obtained with ELISA and antibody microarray technology. In all three cases, the p values associated with the linear regressions were lower than 0.001.

of detection (LOD) and regression values for each biomarker (Table 5).

The CST4 concentration in tears, obtained using customized microarrays, appeared significantly lower in the DE group than in the CT group (p = 0.01). In contrast, the concentration of S100A6 was significantly higher in the DE group than in the CT group (p < 0.001). The abundance of MMP9 in DE patients was higher than in the CT group; however, the difference was not significant (p = 0.95). The results obtained with the antibody microarrays confirmed the data supplied by ELISA assays (Figure 4A).

Table 5. Limit of detection (LOD) and regression values for each biomarker in multiplexed antibody microarrays.

Biomarker	LOD (ng/ml)	R^2
S100A6	1,817	0,998
CST4	1,611	0,997
MMP9	4,476	0,999

Correlation between ELISA assay results and antibody microarray outcomes: The correlations between the concentrations of S100A6, CST4, and MMP9 biomarkers measured using standard ELISA assays and antibody microarrays were determined. In general, higher protein concentrations were obtained using antibody microarrays than in ELISA assays for all tested samples (Figure 4A). These results indicate that microarray technology has higher sensitivity and lower detection limits than ELISA assays. Nevertheless, in some cases, the standard deviations were larger for the antibody microarray results.

Linear regression analysis indicated a moderately high goodness-of-fit for the results obtained with the two techniques for the three biomarkers tested, with an adjusted R^2 of 0.61 for CST4, 0.68 for S100A6, and 0.69 for MMP9 (Figure 4B).

When the quantified samples from the DE patients were grouped according to the disease severity, 11 (34.4%) were classified as mild, 14 (43.8%) as moderate, and seven (21.9%) as severe DE, reflecting the original severity data (as shown in the "Patients and clinical parameters" of the "Results" section). The observed concentration changes were similar for the two quantification techniques; however, higher detection signals were obtained with the antibody microarrays than with the ELISA assays (Figure 5).

In the case of CST4, the apparent concentrations for mild and moderate DED in ELISA assays were lower than those obtained with microarrays. The two methods supplied similar concentration results for the severe DE group (Figure 5A). The gradual rise in the S100A6 protein concentration with increasing severity was more evident when the samples were examined using antibody microarrays than with the ELISA assays (Figure 5B). In the case of MMP9, the results were inconsistent in both ELISA and microarrays. ELISA tests showed growing MMP9 abundance for mild to moderate DED and a decrease in its concentration in severe DE samples. The microarray assays gave similar concentrations for mild and moderate DE and showed a drop in concentration in severe DED samples (Figure 5C). Stronger signals were observed with the microarrays; however, these differences were not statistically significant.

DISCUSSION

In the current study, we measured the protein concentration changes in the tears of patients with DED using a panel of biomarkers formed by CST4, S100A6, S100A8, and MMP9. We designed this study to address several key questions: i) are the selected biomarkers effective in DED diagnosis, ii) are the protein expression changes of these selected biomarkers associated with the severity of DED, iii) what are the clinical parameters most strongly correlated with the changes in the abundance of selected biomarkers, and iv) is the apparent biomarker concentration affected by using different quantitative techniques, such as the individual ELISA and multiplexed microarrays?

We selected the biomarkers based on the results of our previous research studies and considered the data reported by other groups. Our group found that three of the four examined biomarkers (CST4, S100A6, and S100A8) were dysregulated in the DE and MGD patients, as reported in two independent studies using different proteomic approaches [13,27].

CST4 is a natural cysteine protease inhibitor with antimicrobial activity. This is an extracellular tear protein secreted by the lacrimal glands (also detected in meibomian gland secretions) [38,39]. CST4 inhibits the endogenous, bacterial, and parasitic protozoa proteases, binds bacterial lipopolysaccharides, and seems to have some direct immunomodulatory effects [40]. We selected CST4 because its abundance has been reported as reduced in DED patients in several studies (Table 1). A drop in CST4 levels in tears has also been documented in patients with ADDE, blepharitis, Fusarium keratitis, and MGD [13,27,41–43].

Two proteins of the S100 calcium-binding protein family were also measured in the present study, S100A8 and S100A6. The expression of these two proteins were consistently upregulated in the DE patients in comparison with the healthy subjects (Table 1). S100A6 is a tear protein involved in several processes, such as calcium-binding/epithelial integrity and growth [29]. Its upregulation has been reported in ADDE [13,27], Sjögren syndrome dry eye (SSDE) [44], and MGD [13,27]. Furthermore, it was used to monitor the response of the patients when changing glaucoma treatment from preserved to preservative-free [45] and after accelerated corneal cross-linking (A-CXL) with pulsed ultraviolet light [46]. Thus, it could also have prognostic value when assessing the response to treatment in cases with a risk of iatrogenic, topical drug-induced DE, and the efficacy of surgical treatment to restore corneal homeostasis. In these monitoring studies, the initial increase in the concentration of this biomarker was reversed after a change in therapeutic treatment or surgical procedure.

S100A8 is an extracellular tear protein that acts as a cytokine and inhibitor of casein kinase associated with inflammation [29]. It is overexpressed in the tears of patients with ADDE [13,16,19,27,30,31,44,47–49]. This protein has been found upregulated in the conjunctiva of the DE and MGD patients [19,50] and in pterygium [51]. We included S100A8 in the panel of biomarkers as, among the extracellular S100A proteins, it has the largest number of replications in the unbiased mass spectrometric screening, and it has been included in biomarker panels of other groups.

The fourth selected biomarker is MMP9, whose levels are increased in the DED [29]. This is an enzyme involved in the matrix collagen IV and V degradation. MMP9 has not been detected in unbiased massive proteomics studies of DED tears; however, it has been evaluated in candidate immunodetection and activity detection studies. This protein is upregulated in ADDE, blepharitis, and conjunctivochalasis

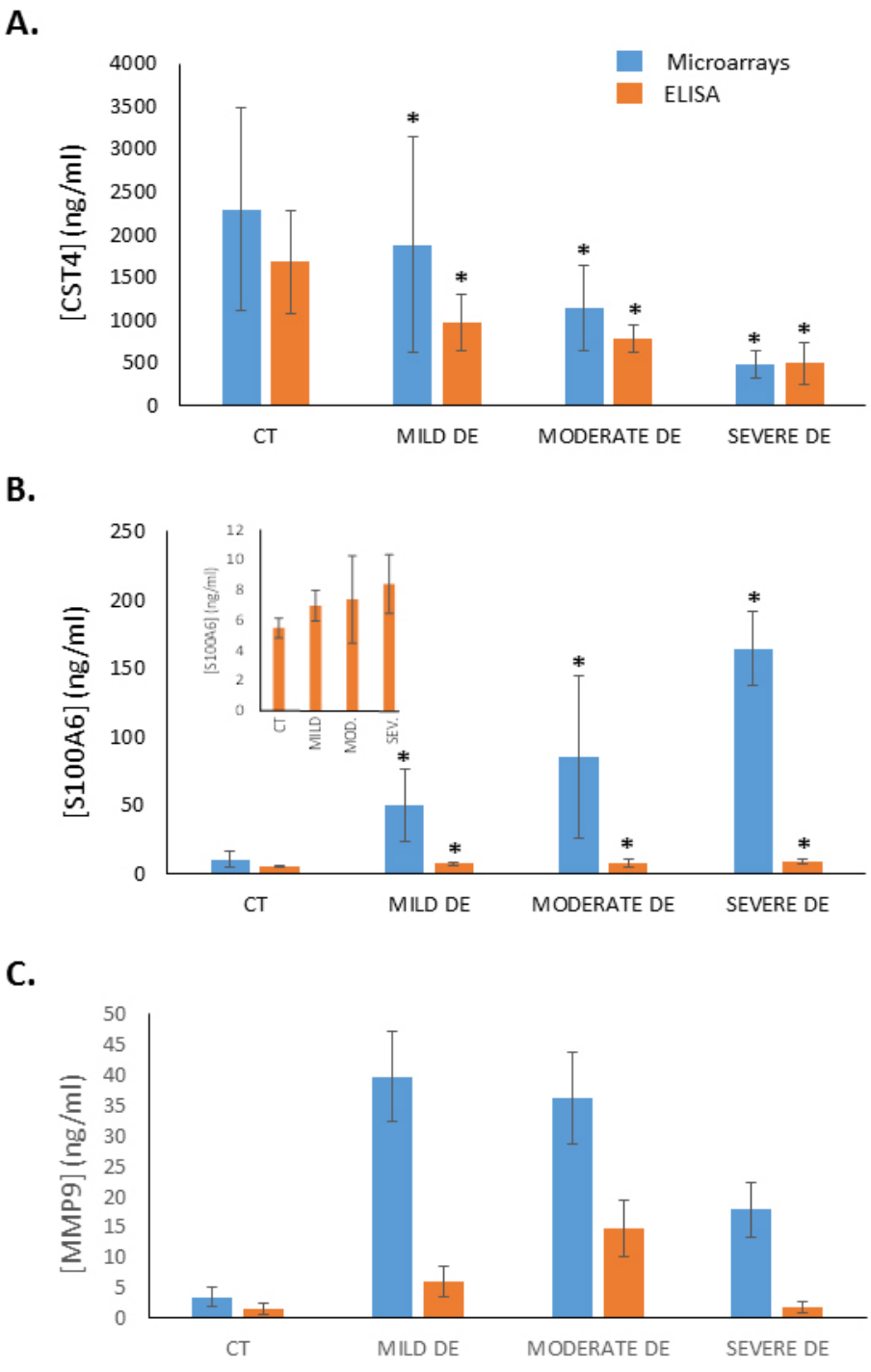

Figure 5. Comparison of the mean concentrations of biomarkers CST4 (**A**), S100A6 (**B**), and MMP9 (**C**) in control (CT; n = 14) and dry eye (DE) groups according to the severity (mild, n = 11; moderate, n = 14; and severe, n = 7), measured using standard individual ELISA assays and customized multiplexed antibody microarrays. The concentration is expressed in ng/ml. The insert in the S100A6 plot shows the details of the results obtained using ELISA assays. Error bars show standard deviation, and * indicates statistical significance relative to the corresponding control group (p value <0.05).

[20], MGD and SSDE [52], and post-LASIK DE [53] (Table 1). Although this biomarker is often studied, there is some controversy about its significance and the defined cutoff in DE patients. Even so, this is one of the few biomarkers that has been incorporated into a medical device, and it is currently used in clinical trials. It is also upregulated in the tears of keratoconus (KC) patients [54]. A reduction in this biomarker concentration has been documented in patients after corneal cross-linking (CXL) and conjunctivochalasis surgery [46,55], suggesting its role as a prognostic biomarker.

In the present study, the main criterion for patient grouping was severity. The gender ratio (F/M) was 75/25 in the mild and moderate groups but 100/0 in the severe group. These results agree with the study by Matossian et al. [56]. They recently reported that women are diagnosed with DED at an earlier age and that the progression to severe forms of the disease is more prevalent in women than men.

In general, the results obtained in this study agree with previous results, confirming the significant increase in S100A8 and S100A6 levels, as well as the reduction in CST4 concentration in DE patients. In the case of MMP9, an increase in its concentration was also observed in the DED group, but it was not significant in the cohort of patients analyzed in our study.

The reduction in the abundance of the CST4 protein might reflect the loss of tear homeostasis and possible dysfunction of the lacrimal and meibomian glands. As a result of the depletion in the levels of this protein, the tear viscosity, surface tension, and lipid transport can be altered, affecting tear film stability [57]. Since the CST4 protein has antimicrobial activity, a reduction in its concentration might increase the risk of infection in DE patients. In our previous studies, CST4 depletion was accompanied by a decrease in the concentration of other proteins with antimicrobial function, such as lipocalin-1 (LCN1), prolactin inducible protein (PIP), lactotransferrin (LTF), zinc-alpha-2-glycoprotein (AZGP1), galectin-7 (LEG7), cystatin-SN (CST1), actin B (ACTB), and mammaglobin B (SG2A1) in the DE and MGD patients in comparison with controls [13]. The observed reduction in the abundance of all these proteins suggests some association between the alterations in the levels of protective biomarkers in DED.

To the best of our knowledge, this is the first study in which alterations in the concentration of tear CST4 are associated with severity. Recently, Martini et al. [58] proposed CST4 as a biomarker for severity of submandibular gland involvement in Sjögren's syndrome. The authors measured the CST4 levels in saliva samples of patients with different subtypes of primary Sjögren's syndrome (pSS), classified on the basis of unstimulated salivary flow rate (USFR), minor salivary gland (MSG) focus score, and submandibular gland ultrasonography (SGUS) abnormalities (score < 2 or ≥ 2). The authors observed that the salivary CST4 level was significantly reduced in the patients with a submandibular gland ultrasonography score ≥ 2. These observations agree with our findings; thus, we can conclude that CST4 constitutes a new biologic tool for grading the severity of DED.

For S100A6, we found, using ELISA assays, a mean fold-increase of 1.4 in the DE group relative to the CT group. However, when we used microarray assays, we obtained an 8.6-fold increase for the whole DE group. This increase gradually changed with DE severity in both cases (Table 6). Here, for the first time, we report a correlation between S100A6 levels and DED severity. These results also reveal the effect of different quantitation techniques on the apparent significance of biomarkers, which will be discussed further.

S100A6 has been classified as an intracellular protein because it is found predominantly in the cytoplasm of the fibroblasts and conjunctival, corneal, and limbal epithelial cells [51,59,60]; however, it is also present in extracellular fluids [13,27,61]. Some functional network analyses performed by our group have indicated direct interactions of S100A6 with JNK, ANXA1, ANXA11, S100A10, and S100A11 [13,27], making this protein a key modulator in the pathogenesis of DED for several biologic processes, such as signal transduction, apoptosis, oxidation, and epithelial integrity.

Table 6. Comparison of fold-changes in DED, relative to CT, obtained in ELISA assays and using multiplexed antibody microarrays.

Group	CST4		S100A6		MMP9	
	ELISA	Microarrays	ELISA	Microarrays	ELISA	Microarrays
DE	−2.14	−1.83	1.36	8.63	5.83	9.67
Mild DE	−1.73	−1.22	1.27	4.75	3.95	11.52
Moderate DE	−2.15	−2.00	1.34	8.15	9.67	10.48
Severe DE	−3.39	−4.73	1.53	15.68	1.11	5.17

In the case of S100A8, we found a 2.29-fold concentration increase in the DE group relative to the CT group. Looking at the severity of the subgroups, we found no correlation between the gradual increase in its abundance and DED severity. We did not include this biomarker in the customized multiplexed microarrays; therefore, no microarray data are available to compare with the ELISA results.

The ELISA results for MMP9 showed a mean 5.8-fold concentration increase in the DE group in comparison with the CT group. However, the difference was not statistically significant. This lack of significance might have been caused by large variations in the concentrations of this biomarker in some DE patients. According to the ELISA results, in the CT group, the MMP9 concentrations varied from 0.67 ng/ml to 3.37 ng/ml. In the DE group, the concentrations varied from 0.17 ng/ml to 102.69 ng/ml. However, 30 DE patients (93.75%) presented concentrations lower than 40 ng/ml (the clinical cutoff established for immunoassay devices such as InflammaDry), and only two DE patients (6.25%) presented concentrations higher than 40 ng/ml.

Large comparable variations were also observed when the second round of samples obtained from the same cohort of patients was quantified using microarrays (with higher detection levels). We found a mean 9.7-fold concentration increase in the DE group (relative to the CT group). In the CT group, the MMP9 concentrations varied from 0.77 ng/ml to 10.81 ng/ml, and in the DE group, from 0.15 ng/ml to 355.80 ng/ml. In this last group, 27 patients (84.38%) presented with MMP9 concentrations <40 ng/ml and five (15.62%) with concentrations >40ng/ml.

These results increase the controversy about the levels of this biomarker in different cohorts of patients, thus suggesting that the problem deserves some special attention. A significant increase in MMP9 levels in the tears of DE patients has been observed in our previous studies using different sandwich-like microarrays [27]. An increased concentration of this protein has also been observed in the tears of keratoconus patients in comparison with controls. A significant drop from initial high concentration before CXL surgery in KC patients to low levels after surgery has been observed in a recent study using microarrays from the same batch employed here [46]. Thus, no technical errors arising from differences in microarray technology may be attributable to this result. However, some controversial results have also been reported by other groups [27,62–64].

The analysis of correlations with clinical parameters showed that CST4 was the only biomarker with a significant (negative) correlation with OSDI. This negative correlation suggests that the decrease in CST4 levels is linked to the symptoms and discomfort of the patients. CST4 showed the highest positive correlation with the SCH test results. In homeostasis, a high concentration of this protein in tears is associated with a stable tear film with an intact protective function. However, in DED, the reduction in its abundance indicates a possible dysfunction of the lacrimal or meibomian glands, destabilizing the tear film integrity even in the early stages of DED. S100A6 and S100A8 showed the highest positive correlations with the FLUO and MGQ results. In the case of S100A8, there is some evidence of upregulation of this protein in the conjunctival epithelium of DE patients [50]. There is also evidence of a positive correlation between the S100A6 tear levels and ocular surface staining in patients with keratoconus. Moreover, a reduction in S100A6 levels has been observed after accelerated corneal crosslinking [46]. Therefore, it is possible that the concentrations of these proteins in tears are negatively associated with epithelial integrity. In contrast, these two biomarkers showed the highest negative correlation with SCH test results (followed by TBUT), revealing an instability in tear film homeostasis beginning in the early stages of DED.

Remarkably, we did not observe statistically significant changes in the OSM in the DE group in comparison with the CT group. The OSM measurements were performed by medically qualified investigators following the manufacturer's instructions and DEWS recommendations for the order of diagnostic tests. Variability in tear osmolarity in DE patients has also been reported by other authors. Potvin et al. [65] reviewed the osmolarity data in DE studies and reported a positive impression of tear osmolarity in DED diagnosis in 72% of the articles, a neutral impression in 21%, and a negative impression in 7%.

As there is no "gold-standard" diagnostic test for DED, and the development of non-invasive, simple, reproducible, reliable, quantitative tools to measure biomarker levels is critical for the diagnosis, treatment, and follow-up of patients [9]. In general, increasing the number of biomarkers improves certainty and accuracy. However, we must consider the practicality of simultaneous measurements of several biomarkers in the same tear sample. There is an urgent need to translate the appropriate technology to clinical practice, which explains the growing interest in using multiplexed quantification systems. However, there are some serious limitations to quantifying and validating tear biomarkers in large populations: the small volume of tear samples (making it difficult to assess several biomarkers simultaneously), the high dynamic range of tear fluid where concentration differences of orders of magnitude are common (major protein concentrations are at the level of

mg/ml, whereas low abundance proteins, such as cytokines and chemokines, are found at ng/ml or pg/ml levels).

There have been many attempts to develop appropriate multiplexed technologies, including bead-based and multiplexed ELISA assays, which are now commercially available. However, these are all closed systems in which the preselected markers are already included in the platform. The available multiplexed systems are mainly limited to cytokine and chemokine analysis and do not allow the evaluation of other biomarkers of interest. Consequently, some groups have investigated different technologies to develop biomarker-multiplexed systems, including specific DE biomarkers [16,66–68].

Before implementing any multiplexed system for in vitro diagnosis (IVD), an appropriate validation using comparisons with standard quantification techniques must be performed (such as ELISA tests or other standard validated techniques). However, this step is not easy, as the same sample should be analyzed using both the gold standard and novel technology. Following this approach, we first analyzed the candidates using standard ELISA assays and, in the second part of the study, we also measured the biomarkers using customized multiplexed antibody microarrays.

We have already developed and tested customized sandwich-like antibody microarrays in a 12-subarray format (12 subarrays per slide, 10 replicates of each biomarker per subarray; Fraunhofer Institute technology, Germany) [27]. In the current work, we used a 24-subarray format (24 subarrays per slide, eight replicates of each biomarker per subarray; IMG Pharma, Spain) for the quantification of CST4, S100A6, and MMP9 biomarker proteins. We developed the new customized 24-subarray format microarrays to increase the number of tear samples per array with a similar number of replicates and to reduce the volume of samples required for analysis. The concentration ratios between the DE and CT groups were comparable for these two systems for the multiplexed quantification of biomarkers. However, a large difference was observed when comparing the results obtained using microarrays and traditional individual ELISA assays (Table 6).

The main limitations of this study are the relatively small number of patients (all from a Caucasian population) and the fact that the multiplexed antibody microarrays only used three biomarkers. More biomarkers should be included in the antibody microarrays to allow the quantification of larger biomarker panels. However, the microarrays constitute an intermediate tool, somewhere between complex proteomic technologies and medical devices. These microarrays are Point of Lab (PoL) but not Point of Care (PoC) tools, and their use requires a laboratory environment.

The future of DED biomarker research surely lies in the widespread use of multiplexed systems for the quantification of biomarkers of interest and establishing their clinical application. Well-defined, definitive diagnostic tests, to be employed in clinical practice and in clinical trials, are needed to determine the severity of the disease, efficacy of treatments, and the effects of topical treatments on ocular surface homeostasis [9]. The use of a panel of biomarkers, such as those reported here, should contribute to the proper classification of patients in similar studies. This should also help in the identification of the initial physiologic processes activated in mild forms of the disease. Our study presents several novel features in comparison with the already available similar publications.

We performed an in-depth characterization of clinical parameters to see how they correlate with the changes in the expression of tear biomarkers. We also analyzed the correlation between the studied biomarkers and DED severity; the results can be helpful in obtaining an accurate diagnosis. They also supply valuable information for selecting the best treatment for DED cases of different severities.

We developed customized 24-subarray antibody microarrays, which included eight replicates of each biomarker in the same subarray (well), and we analyzed up to 24 tear samples per array. These microarrays considerably reduced the required sample volume and increased the sensitivity and reproducibility of protein quantification.

Our findings demonstrated that the choice of quantification technique could affect the perceived biomarker significance. The results of the multiplexed microarray analysis showed that this technique is more sensitive than ELISA. As a result, we were able to detect some high-level fold-changes in protein abundance, such as those missed by ELISA.

In conclusion, this study found strong correlations between biomarker concentrations (CST4, S100A6, and S100A8), the clinical signs, and the results of diagnostic tests routinely used to evaluate patients with DED. CST4 and S100A6 concentrations also correlated with DED severity. Moreover, the newly developed microarray was verified by employing commercially available and in-house ELISA, using tear samples from patients with DED. However, a prospective masked study is necessary to properly validate this customized microarray assay.

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