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LABORATORY STUDY

Tear proteomics in evaporative dry eye disease

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Abstract

***Purpose*: To analyze tear protein variations in patients suffering from dry eye symptoms in the presence of tear film instability but without epithelial defects.**

***Methods*: Five microlitres of non-stimulated tears from 60 patients, suffering from evaporative dry eye (EDE) with a break-up time (BUT) <10 s, and from 30 healthy subjects as control (no symptoms, BUT >10 s) were collected. Tear proteins were separated by mono and bi-dimensional SDS-PAGE electrophoresis and characterized by immunoblotting and enzymatic digestion. Digested peptides were analyzed by liquid chromatography coupled to electrospray ionization quadrupole-time of flight mass spectrometry followed by comparative data analysis into Swiss-Prot human protein database using Mascot. Statistical analysis were performed by applying a *t*-test for independent data and a Mann–Whitney test for unpaired data ($P<0.05$).**

***Results*: In EDE patients *vs* controls, a significant decrease in levels of lactoferrin (data in % ± SD): 20.15 ± 2.64 *vs* 24.56 ± 3.46 ($P=0.001$), lipocalin-1: 14.98 ± 2.70 *vs* 17.73 ± 2.96 ($P=0.0001$), and lipophilin A–C: 2.89 ± 1.06 *vs* 3.63 ± 1.37 ($P=0.006$) was revealed, while a significant increase was observed for serum albumin: 9.45 ± 1.87 *vs* 3.46 ± 1.87 ($P=0.0001$). No changes for lysozyme and zinc α-2 glycoprotein ($P=0.07$ and 0.7, respectively) were shown. Proteomic analysis showed a downregulation of lipophilin A and C and lipocalin-1 in patients, which is suggested to be associated with post-translational modifications.**

***Conclusions*: Data show that tear protein changes anticipate the onset of more extensive clinical signs in early stage dry eye disease.**

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Keywords: proteomics; human tears; dry eye disease; mass spectrometry; western-blot; electrophoresis

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Introduction

'Dry eye' is now recognized as a disruption of the lachrymal functional unit,[1] a system comprising the ocular surface (cornea, conjunctiva, and limbus), the lachrymal and meibomian glands and the lids, interconnected by an integrated neural arc. The updated definition states that 'Dry eye is a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tear instability with potential damage to the ocular surface. It is accompanied by increased osmolarity of the tear and inflammation of the ocular surface'.[2] As postulated already in the previous report by Lemp *et al* in 1995,[3] the major classes of dry eye are mainly related to tear deficiency or increased tear evaporation.

This last form, in particular, is increasingly observed as a consequence of environmental factors such as forced air dry heat, wind, air pollution, or reduced blinking because of driving, TV watching, and computer work. Tear evaporation is considered the main contribution to tear thinning and break-up,[4] although neither the complete mechanism of stability nor the role of tear composition have been elucidated to date.

Proteomic methods have identified nearly 500 proteins in human tears,[5–8] but for only a minority of these proteins has the biological role in dry eye disease or, more generally, in ocular surface physiology been clarified. The most representative tear proteins have been analyzed either in Sjogren's and non-Sjogren's dry eye.[9,10] Abnormal changes in these protein profiles were suggestive of impaired lachrymal gland function, related to autoimmune disease or apoptotic events.

The purpose of this work was to analyze the behaviour of the main tear proteins in patients suffering from mild evaporative dry eye (EDE), the most frequent form of the disease, which consists in an excessive water loss from the ocular surface even in the presence of adequate tear production.

Materials and methods

Subjects

A total of 90 subjects, including 60 patients affected by EDE (18 men and 42 women; mean age 64.2 ± 22.3 years) and 30 healthy control subjects (8 men and 22 women; mean age 61.1 ± 17.8 years), were enrolled for this study.

Patients were classified as grade 1 dry eye severity according to DEWS[2] scheme (Table 1). For patients, the inclusion criteria were a Schirmer test I value higher than 10 mm/5 min, a tear film break-up time (TFBUT) value $\leqslant$10 s, mild subjective symptoms of ocular discomfort as evaluated by an OSDI questionnaire[11] (score 12–24), and use of tear substitutes only, for treatment, which were suspended for at least 3 days before tear collection.

Inclusion criteria for healthy control subjects were a Schirmer test I value higher than 10 mm/5 min, a TFBUT value $\geqslant$10 s, and absence of subjective symptoms of ocular discomfort (OSDI score $<$12).

In both groups, excluding criteria were the presence of punctuate keratopathy and/or autoimmune diseases, the use of contact lens, and having undergone ocular surgery in the last 6 months.

The study was conducted according to the declaration of Helsinki involving human subjects.

All the tear samples were provided by the Ophthalmic Unit at S Orsola-Malpighi University Hospital of Bologna (Italy) after obtaining informed consent from the subjects studied and according to DEWS guidelines.[2] From each subject, a minimum amount of 10 µl of unstimulated tears was collected using a micropipette with sterile tips, avoiding any reflex tearing. Patients were requested to position the head slightly reclined in such a way that tears are driven to the most outer side of the lower fornix. After 30 s, the tip of the micropipette was carefully positioned and tears aspired. Samples were centrifuged at 13 200 *g* for 15 min and stored frozen in plastic vials at −80 °C until use.

Monodimensional electrophoresis

Tear samples were diluted 1 : 2 with 0.125 M Tris-Cl pH 6.8, 4% sodium dodecyl sulfate (SDS), 20% glycerol, 10% 2-mercaptoethanol buffer, and boiled for 5 min. The proteins were separated using a 18% acryl amide -TRIS-HCl Ready-gel (Bio-Rad, Laboratories, Milano, Italia) applying a voltage of 200 V for 1 h at room temperature. A pre-stained standard of low molecular weight proteins (Bio-Rad) was used to monitor the electrophoretic separation. Gel staining was performed using Brilliant Blu G (Sigma-Aldrich, Milano, Italy) for 12 h at room temperature. The gel image was acquired with the densitometer scanner Umax (Amersham Biosciences-GE Healthcare, Milano, Italia) and analyzed for the percent abundance of each protein of interest in the samples using Gel-Pro Analyzer software (MediaCybernetics Inc., Bethesda, MD, USA).

Immunoblotting

Total protein from 1 µl of tears was subjected to SDS-polyacrylamide gel electrophoresis at 200 V constant using a Mini-Protean III (Bio-Rad) and transferred onto a nitrocellulose membrane (Hybond-C extra, Amersham) applying a voltage of 100 V (1 h, 4 °C) in buffer containing 0.3% Tris, 1.4% glycine, and 20% methanol using a Bio-Rad wet-blotting apparatus. The correct transfer of the proteins was evaluated using the reversible coloration with Red Ponceau. The nitrocellulose membrane containing the transferred proteins was saturated with 3% bovine serum albumin (Sigma) in phosphate-buffered saline (PBS) + 0.1% Tween 20 for 1 h at room temperature. For immunoblotting, anti-lactoferrin (sc-25622), anti-lipocalin (sc-34680), anti-lysozyme (sc-27956), anti-lipophillin A (sc-48324), and anti-lipophillin B (sc-48327) antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA; the anti- zinc-alpha-2-glycoprotein (ZAG) antibody was purchased from BioVendor GmbH, Heidelberg, Germany.

Table 1 DEWS dry eye severity grading system. Patients included in this study were classified as grade 1

Dry eye severity grade	*1*	*2*	*3*	*4*
Severity and frequency of discomfort	Mild and/or episodic	Moderate, episodic or chronic	Severe frequent or constant	Severe and/or constant
Conjunctival injection	None to mild	None to mild	+/−	+/++
Conjunctival staining	None to mild	Variable	Moderate to marked	Marked
Corneal staining	None to mild	Variable	Marked central	Severe punctuate erosions
Lid/meibomian glands	MGD variably present	MGD variably present	Frequent	Trichiasis, symblepharon
TFBUT (s)	Variable	$\leqslant$10 s	$\leqslant$5 s	Immediate
Schirmer score (mm/5 min)	Variable	$\leqslant$10 mm	$\leqslant$5 mm	$\leqslant$2 mm

Primary antibodies were diluted in PBS containing 0.1% Tween 20 and incubated overnight at 4 °C. The blots were washed three times with PBS + 0.1% Tween 20 and incubated with horseradish peroxidase-conjugated secondary antibodies diluted (1 : 10000) in PBS + 0.1% Tween 20 for 1 h at room temperature. All membranes were visualized by chemiluminescence using the ECL system (SuperSignal West Dura Extended Duration Substrate, Pierce Biotechnology Inc., Rockford, IL, USA).

Bidimensional electrophoresis

A total of 32 tear samples (10 healthy control subjects and 22 dry eye patients) were subjected to bi-dimensional electrophoresis followed by western blot analysis for the analysis of protein isoforms.

One microlitre of tears in TSU buffer (2 M Thiourea, 7 M Urea, 4% CHAPS, 0.8% ampholytes pH 3–10, benzonase, and protease inhibitors) was centrifuged at 21 000 *g* at 4 °C for 30 min. For the first electrophoretic dimension 7 cm IPG strips (pH 3–10) were used (Bio-Rad). The active rehydratation of the strip with the sample was performed at 50 V for 16 h, followed by step increase in voltage of 1000 V (1 h), 2000 V (1 h), 4000 V (2 h), and 4000 V (20 kV-h in total). Afterwards the strips were reduced using equilibration buffer (6 M urea, 2% SDS, 5 mM Tris-HCl pH 8.6, 30% glycerol, containing 125 mM dithiothreitol (DTT)) for 15 min at room temperature and alkylated with equilibration buffer containing 250 mM iodoacetamide (IAA) for 8 min. The strips were transferred to a 15%-polyacrylamide gel, and, for the electrophoretic separation, a Mini-Protean III apparatus (Bio-Rad) with a constant voltage (200V) was used. The proteins were stained with colloidal Coomassie Blue G-250 (Sigma) for 12 h at room temperature.

In-gel tryptic digestion

The monodimensional electrophoresis gel slices containing the proteins of interest were reduced with 10 mM DTT for 45 min at 56 °C, alkylated with 55 mM IAA for 30 min at room temperature in the dark and incubated overnight at 37 °C in a 50 : 1 (w/w) ratio with 12 ng/μl sequencing-grade-modified trypsin (Promega, Madison, WI, USA). The digested peptides were extracted from the gel slices using a solution of 5% trifluoroacetic acid and 50% acetonitrile. Peptides were lyophilized to dryness and resuspended with 10 μl of 0.1% formic acid for mass spectrometry (MS) analysis.

MS analysis and database search

The samples were analyzed by liquid chromatography MS using a CapLC (Waters, Manchester, UK), connected with a electrospray interface to a quadrupole-time of flight (QTOF) micro (Micromass, Manchester, UK). The peptide separation was performed on an Atlantis dC18 NanoEase column (150 × 0.3 mm, 3 μm) (Waters) with an Atlantis dC18 NanoEase precolumn (5 × 0.3 mm, 5 μm particle size) (Waters) using a flow rate of 4 μl/min (mobile phase A: H_2O/acetonitrile (95 : 5) 0.1% FA; B: acetonitrile/H_2O (95 : 5) 0.1% FA). The chromatographic gradient was set up to give a linear increase from 2% B to 80% B in 30 min, for a total run-time of 45 min. For identification experiments, QTOF was set to scan in survey mode the m/z 400–1800 range.

For protein identification Mascot (version 2.02.03, Matrix Science, London, UK; www.matrixscience.com) and the Swiss-Prot human database (version 52.2, 495929 in total) were used with the following settings: a 50 p.p.m. precursor and 0.3-Da fragment tolerance, carbamidomethylation of cysteine as a fixed modification, oxidation of methionine as a variable modification, and trypsin as enzyme (one miss cleavage allowed).

Statistic evaluations

Data were statistically evaluated by using the Statistical Package for the Social Sciences (SPSS) version 13.0 (SPSS, Chicago, IL, USA) and the MedCalc 9.3 software (MedcCalc Sofware bvba, Mariakerke, Belgium). Statistical analysis were performed by applying a *t*-test for independent data and a Mann–Whitney test for unpaired data ($P < 0.05$) to evaluate statistical difference between normal control groups *vs* EDE patients.

An evaluation was made of the linear relationship between the evaluated tear proteins and: break-up time, Schirmer test, OSDI subjective symptoms. This was performed by using Pearson's correlation coefficient *r* for parametric data.

Results

The separation of tear proteins by SDS-PAGE electrophoresis was optimized by using 18% acrylamide-TRIS-HCl gels, which allowed us to properly separate the major tear proteins within the same gel, facilitating their identification. Previous attempts with other types of gels were performed (data not shown).

Protein identification was carried out by means of two different complementary types of analysis: (i) excision of the bands of interest from the gel, followed by tryptic digestion and MS analysis; (ii) western blot analysis on the monodimensional electrophoretic gels. In Table 2, the results of the protein identification using MS and database searching are reported.

Table 2 Results of the protein identification by mass spectrometry and database search

Protein name	*Entry name*	*Protein score*	*Protein mass (Da)*	*No. of identified peptides*	*Protein coverage (%)*
Lactotransferrin	TRFL_HUMAN	735	80 014	12	20
Serum albumin	ALBU_HUMAN	699	71 317	17	38
Lipocalin-1	LCN1_HUMAN	243	19 409	7	31
Lipophilin-C	SG2A1_HUMAN	167	11 104	5	40
Extracellular glycoprotein lacritin	LACRT_HUMAN	133	14 237	6	29
Proline-rich protein 4	PROL4_HUMAN	119	15 116	2	10
Lysozyme C	LYSC_HUMAN	107	16 982	5	39
Lipophilin-A	SG1D1_HUMAN	86	10 234	2	12
Prolactin-inducible protein	PIP_HUMAN	76	16 847	2	15
Proline-rich protein 1	PROL1_HUMAN	65	22 970	1	6
Ig gamma-1 chain C region	IGHG1_HUMAN	62	36 596	1	3
Serotransferrin	TRFE_HUMAN	45	79 280	1	1
Zinc- α-2-glycoprotein	ZA2G_HUMAN	32	34 079	1	4

Table 3 Densitometric analysis of monodimensional electrophoresis gel stained by Coomassie blue (average %, SD)

	Controls		*Patients*		P-*Value*
	%	*SD*	*%*	*SD*	
Lysozyme	20.86	2.83	19.05	4.90	0.07
Lactoferrin	24.56	3.46	20.15	2.64	0.001
Serum albumin	3.46	1.87	9.45	3.03	0.0001
Lipocalin-1	17.73	2.96	14.98	2.70	0.0001
Lipophilin A-B	3.63	1.37	2.89	1.06	0.006
Zinc-α2-Glycoprotein	1.45	0.69	1.50	0.65	0.7

SD, standard deviation.
A statistically significant decrease in lactoferrin, lipophillin, and lipocalin abundances was found in patients *vs* controls; a statistically significant increase was shown for serum albumin; lisozyme and zinc-α2-glycoprotein amounts did not appear to change.

Protein quantification was assessed by means of computerized densitometric analysis using image analyzer software to calculate the relative percentage of each protein of interest within the proteins in the gel strip taken as the whole. For lactoferrin, lipophillin, and lipocalin, a statistically significant decrease in the protein abundances was found in patients *vs* controls, while for serum albumin a statistically significant increase could be observed. No significant changes for lisozyme and ZAG (Table 3) were noticed. The results were further confirmed by densitometric analysis on immunoblotted gels (Figure 1).

Bi-dimensional electrophoresis and western blot analysis were also performed on 10 controls and 22 patients, to analyze protein isoforms differently expressed as a consequence of pathological modifications. Alterations in the relative abundance of at least three differentially modified forms of lipocalin-1, as determined by variations in the isoelectric point (pI), was shown in patients *vs* control. (Figure 2).

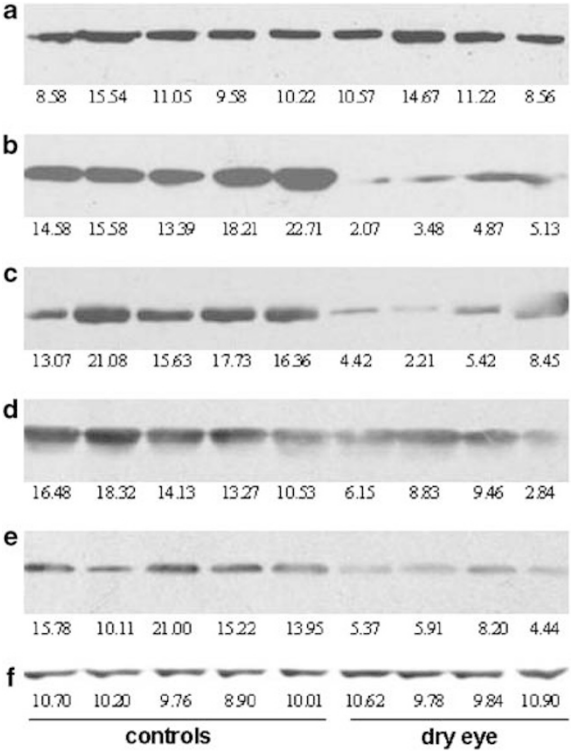

Figure 1 Monodimensional gel electrophoresis of human tear proteins; control subjects *vs* dry eye patients immunoblotting. The densitometric data in OD (optical density) unit are reported under each lane. (a) Decrease is shown in dry eye patients *vs* controls for lactoferrin (b), lipocalin-1 (c), lipophillin A (d) and lipophillin B (e), no significant changes for lisozyme (a), and ZAG (f) is evidenced.

Statistically significant correlations between the content of four proteins (lactoferrin, serum albumin, lipocalin-1, and lipophillin A–B) and clinical parameter values were found *vs* TFBUT and subjective symptoms score but not *vs* Schirmer test (Table 4 and Supplementary Figures 1–4). Lysozyme and ZAG did not show significant correlations *vs* any clinical parameter.

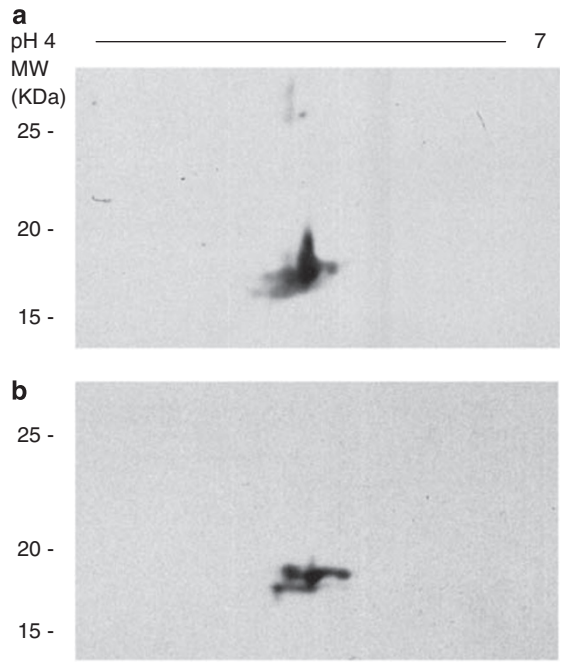

Figure 2 2-DE immunoblotting of human tear proteins; control subject (a) *vs* dry eye patient (b). For 2-DE western blotting, 1 µl of tears was separated on pH 4–7 strip (7 cm), followed by 10% constant SDS-PAGE. Tear proteins were transferred to nitrocellulose membranes for subsequent immunodetection with lipocalin-1 antibody (1 : 500), detected using a chemiluminescence method and visualized in a Kodak digital image station 2000R (Kodak, Rochester, NY, USA). Alterations in the relative abundance of at least three differentially modified forms of lipocalin-1 are shown.

Table 4 Pearson's correlation coefficient *r* between tear protein and clinical parameter (significance $P<0.05$)

	OSDI	*TFBUT*	*Schirmer test*
Lysozyme	$r=-0.2$, $P=0.05$	$r=0.24$, $P=0.06$	$r=0.005$, $P=0.95$
Lactoferrin	$r=-0.51$, $P<0.0001$	$r=0.61$, $P<0.0001$	$r=0.16$, $P=0.1$
Serum albumin	$r=0.63$, $P<0.0001$	$r=-0.65$, $P<0.0001$	$r=-0.09$, $P=0.39$
Lipocalin-1	$r=-0.361$, $P=0.0005$	$r=0.336$, $P=0.001$	$r=0.178$, $P=0.09$
Lipophillin A–B	$r=-0.288$, $P=0.005$	$r=0.336$, $P=0.001$	$r=-0.01$, $P=0.8$
Zinc-α2-glycoprotein	$r=0.04$, $P=0.6$	$r=0.04$, $P=0.6$	$r=-0.05$, $P=0.6$

OSDI, ocular surface disease index; TFBUT, tear film break-up time.

Discussion

The study of the tear proteome by electrophoretic separation and MS techniques has allowed for the evaluation of the pattern of proteins associated with specific pathological conditions[9,10,12] and the identification of over 500 proteins.[5,6]

Preliminary studies were performed by our group to optimize the separation of the main tear proteins in the same gel. Furthermore, tear aspiration by micropipette was compared with other methods previously published[13–15] and was found to provide the best protein release and protein expression in the gel. In our analytical approach, the 18% gels were used to achieve the best definition and separation of low molecular weight proteins and a sample size of only 1.5 µl of fluid were loaded, an amount of tears that can be collected in severe forms of dry eye as well.

Although MS can allow the identification and relative quantification of many proteins in a single analysis, monodimensional gel electrophoresis still remains one of the most used approaches for the preliminary evaluation of protein variations, although some limitations do exist. Indeed in this work some proteins were correctly identified, but their quantification could not be achieved because of lane overlapping. This is the case of prolin-rich protein 4, a lacrytin protein of emerging and increasing interest in ocular surface pathology.[16,17]

The following proteins were identified and quantified in the present work: lysozyme C, lactoferrin, serum albumin, lipocalin 1, lipophillin A and B, ZAG.

Lysozyme is a glycosidase produced in the acini of the main lachrymal gland, thus representing an indirect index of its function. For its quantification some methods have been adopted,[15,18,19] with inhomogeneous results being obtained. Lactoferrin is a single-chain polipeptide with important bacteriostatic activity,[20] which also represents an index of lachrymal gland function. Its determination in tears has been conducted in the past by techniques,[21,22] currently abandoned.

In this work, we have shown a low but significant lactoferrin decrease in mild EDE patients *vs* controls without observing changes in the lysozyme content; data would suggest an initial impairment of the lachrymal gland function, not yet detected by the Schirmer test.

Serum albumin indicates a passive exudation as a consequence of blood-ocular barrier failure in conjunctival vessels and its value rapidly increases under various physio–pathological conditions.[23] Our data showed a significant increase in tears of patients *vs* controls, suggesting that an early exudation is already present in EDE mild forms.

Lipocalins represent the greatest group of lipid affinity proteins in tears; they can associate with a wide variety of lipids;[24] the lipocalin–lipid bond favours lipid solubility bringing lipids to a rapid equilibrium during blinking. This event is thought to promote the formation of an homogeneous and compact outermost lipid layer,[25] suitable to control and limit tear evaporation rate. In addition, lipocalins are believed to serve as scavengers

for the removal of wasted lipid molecules that would contaminate the system.[26]

Lipophillines are present in tears with two isoforms (A–B),[27] belong to the Uteroglobin super family; small secretory molecules with anti-inflammatory properties that bind steroids and are subjected to their regulation.

In this study, both lipocalin-1 and lipophillin A–B were found to be reduced in EDE patients *vs* controls. Lipocalin decrease is in agreement with previous studies performed in patients suffering from Sjogren's Syndrome[28] and Meibomian Gland Dysfunction.[29] To our knowledge, the decrease in the abundances of lipophillin A–B observed in our study has never been described before and may constitute a further progress in the identification of mechanisms responsible for the stability of human tears.

In addition to this, lipocalin-1 and lipophillin variations were found to be moderately correlated with tear stability as measured by TFBUT and subjective symptoms of discomfort but not with tear secretion as evaluated by Schirmer test; thus confirming their role in stability.

It is important to note that a shift in the relative ratio of at least three forms of lipocalin-1 was evident between control subjects and EDE patients. The exact modifications that give rise to the different forms of lipocalin-1 as shown by two-dimensional western blot analysis have not been identified yet by MS because of their low abundance, but it could be speculated that they are due to the presence of both different lipocalin-1 isoforms and/or different post-translational modifications of the same isoform. This part of the work is still undergoing.

ZAG is a protein of interest because of its ability to have many important functions in the human body, including lipid mobilization and degradation.[30] The exact function of ZAG in tears and its possible role in lipid degradation have only been postulated and not completely clarified.[31,32] In our study, we detected ZAG in both patients and controls, with no statistically significant differences in the respective expression. Our data, do not suggest any significant role of ZAG in the tear stability mechanism, at least as far as mild dry eye is concerned.

Conclusive remarks

Our data showed a very early lachrymal gland dysfunction, the presence of a light inflammatory condition, and the altered distribution of tear lipid because of decreased expression of affinity lipid-proteins. These data confirm the importance of tear proteomic analysis and show that tear protein changes anticipate the onset of more extensive clinical signs.

In addition, our data are interesting when interpreted taking into consideration what recently was suggested[33] on the natural history of EDE. It is here suggested that in early EDE, a compensatory mechanism occurs and a stimulation of the lachrymal gland dilutes concentrated tears. In this study, the decrease showed in the amounts of certain proteins would account for a stimulation of the water phase only, and would further confirm the initial protein secretory function impairment of the gland.

Summary

What was known before

- The main body of the literature on tear protein variation in dry eye-related diseases deals with chronic or severe forms of dry eye.

What this study adds

- The purpose of this study was to analyze tear protein variations in a mild and initial form of dry eye disease. In this initial stage of dry eye disease, our data demonstrated a very early lachrymal gland dysfunction, the presence of a light inflammatory condition, and the altered distribution of tear lipid because of decreased expression of affinity lipid-proteins. These data demonstrate that tear protein changes anticipate the onset of more extensive clinical signs and confirm the importance of tear proteomic analysis for the identification of biological markers in lachrymal functional unit diseases.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Eye website (http://www.nature.com/eye)