

Identification of human tear fluid biomarkers in vernal keratoconjunctivitis using iTRAQ quantitative proteomics

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Abstract

Background: Understanding and treating vernal keratoconjunctivitis (VKC) has been a challenge because the pathogenesis is unclear and antiallergic therapy often unsuccessful. The aim of the study was to analyze peptide profiles in human tears using mass spectrometry to elucidate compositional differences between healthy subjects and patients affected by VKC.

Methods: Tears were collected from healthy subjects and VKC patients. Digested samples were treated with iTRAQ (isobaric tag for relative and absolute quantitation). Separation of tryptic peptides was realized using a MicroHPLC interfaced with a microfraction collector. MS and MS/MS mass spectra were performed using a MALDI TOF/TOF 4800 Applied Biosystem spectrometer. Protein PilotTM software with ParagonTM algorithm v4.1.46 or GPSTM with Mascot engine was used as search engines with SwissProt or IPI human as the databases.

Results: A significant number of peptides were examined, and 78 proteins were successfully identified. In all VKC samples, levels of serum albumin, transferrin, and hemopexin were found up to 100 times higher than control tear levels and correlated to the severity of disease. Hemopexin, transferrin, mammaglobin B, and secretoglobulin 1D were found significantly over-expressed in VKC samples compared with the control samples. Tear samples from patients treated with topical cyclosporine or corticosteroids showed a dramatic reduction in these protein levels.

Conclusions: LC MALDI MS and isobaric tag for relative and absolute quantitation technique may be useful in the quantitative and qualitative characterization of the peptidoma of human tears. These techniques may identify target proteins to be used in the diagnosis and management of VKC and other inflammatory ocular surface conditions.

The tear film is a highly complex entity that contains hundreds of proteins many of which are bioactive even in trace amounts (1, 2). Several studies focused on quantification of certain target proteins and investigated the relationship between normal subjects and patients with different ocular diseases (3). Proteomic analysis of tear fluids revealed that the composition of tear proteins is correlated with certain conditions such as dry eye (4, 5). Isobaric tag for relative and absolute quantitation (iTRAQ) quantitative proteomics has been successfully used for the identification of tear fluid

biomarkers in dry eye syndrome (5, 6), in climatic droplet keratopathy (7) and in glaucoma (8).

Vernal keratoconjunctivitis (VKC) is a severe ocular allergic disease that occurs predominately in children and young adults (9). It is characterized by intense ocular itching, photophobia, foreign body sensation, conjunctival hyperemia, and mucous secretion, typically accompanied by giant papillae formation on the upper tarsal conjunctiva and/or by limbal infiltrates and nodules. Corneal involvement is common, characterized by punctate keratitis or corneal ulcers, as a

result of the epitheliotoxic effects of proteins and enzymes released by activated eosinophils. The disease is accompanied by tissue remodeling, neovascularization, and scarring (9). To date, no specific laboratory test is suitable for VKC diagnosis and monitoring. Tear levels of inflammatory mediators have been extensively used in ocular allergy to find either a 'disease biomarker', to better understand the immune mechanisms involved, or to identify potential therapeutic targets (10, 11).

In this study, we analyzed quantitative and qualitative proteomic profiles in human tears of healthy subjects and VKC patients, using the 4-plex iTRAQ labeling (12) analysis to compare qualitative and quantitative peptide profiles of normal subjects to those of VKC patients and to identify potential biomarkers for disease diagnosis. Subsequently, using the 8-plex iTRAQ labeling, we monitored the effect of topical treatments on quantitative peptide profiles. Differently from previous studies, our analyses were performed on single and not pooled tear samples.

Materials and methods

Subjects and tear collection

Written informed consent was obtained from all subjects or, in the case of minors, from their parents after explanation of the nature and the possible consequences of the study. The research followed the Tenets of the Declaration of Helsinki. Institutional Review Board approval was obtained. Ten normal subjects (CT) and ten active VKC patients were included in the study (Table 1). In all VKC patients, a clinical activity score (0–4) was given considering the overall severity of the disease at the time of sample collection (13). All patients and normal volunteers were free of medication for at least 5 days before sample collection. Tear samples were collected from additional eight VKC patients (Table 1): one very mild VKC patient treated for 2 months with topical antihistamine (anti-H1) four times a day, one with topical clobetasone (Clb) 4 times/day for 7 days, 4 with topical cyclosporine A 1% (CsA) $\times$ 4 times/day for 2 months, and two recently diagnosed nontreated, active VKC patients. Ten to 20 μ l of tears were collected gently from the external canthus using a glass capillary micropipette. Tear samples were centrifuged and immediately frozen at -80°C before analyzed. Samples were not pooled from both eyes or from different patients.

iTRAQ sample preparation

Firstly, the iTRAQ 4-plex (AB Sciex, Foster City, CA, USA) was used to label simultaneously: two CT (CT1 and CT2) and 2 VKC samples (VKC1 and VKC2). We performed 5 sets of experiments (total 10 control and 10 VKC) using the standard protocol (see Supporting information). Isobaric tag for relative and absolute quantitation reagents with reporter groups 114 and 115 were added to the control samples, while iTRAQ reagents with reporter groups 116 and 117 were added to the VKC samples. Quantitative peptide analysis was performed comparing the ratio of reporter groups: 115/114 (quantitative ratio for each peptide in the two control

samples); 116/114 and 117/114 (quantitative ratio for the interest peptide in the VKC sample compared with the control one). Each experiment was repeated three times.

The iTRAQ 8-plex (AB Sciex) was used to analyze simultaneously the eight additional VKC patients: one treated with topical anti-H1 (reporter group 113), two with nontreated VKC (VKC1 and VKC2, 118 and 119, respectively), four with topical CsA (114, 115, 117, and 121), and one with Clb (116). Quantitative peptide analysis was performed using the ratio of reporter groups: 114/113 (CsA), 115/113 (CsA), 116/113 (Clb), 117/113 (CsA), 118/113 (VKC1), 119/113 (VKC2), and 120/113 (CsA).

MicroHPLC separation and collection

The modified peptides were eluted and separated by reversed-phase high-performance liquid chromatography (RP-HPLC) using an ULTIMATE™ (LC-Packings, Amsterdam, the Netherlands) system. (for column characteristic and HPLC conditions, see Supporting information).

The peptide fractions eluting from the column were mixed with a matrix solution (3 mg/ml of α -Cyano-4-hydroxycinnamic acid in acetonitrile : MilliQ-Water 3 : 1, with 0.15 mg/ml of diammonium citrate) and collected onto a MALDI plate by the MicroFraction Collector PROBOT™ (LC-Packings).

Mass spectrometry

Mass spectra were recorded in reflector mode using an AB 4800 MALDI TOF/TOF™ instrument (AB Sciex). External calibration was performed and mass accuracy was better than 50 ppm. The sequences of the peptide fragments were determined using Protein Pilot™ software with Paragon™ algorithm v3.0 (AB Sciex, Foster City, CA, USA).

Tear albumin and transferrin quantitative analysis

To validate the proteomic results, tears were collected from an additional cohort of 8 normal subjects and 14 VKC patients clinically similar to those used for the proteomic analysis. Commercial quantitative analysis (Dimension Vista System, Siemens, Erlangen, Germany) was used to measure tear concentrations of albumin and transferrin by immunochemical reaction with specific antibodies.

Statistics

Analyses of the frequencies of positive signals in tears of VKC and CT were evaluated and compared with the chi-square test followed by the exact Fisher's test using the SAS program (SAS Institute, Cary, NC, USA). To compare concentrations of albumin and transferrin between VKC and normal subjects (controls), statistical analyses were performed using the Prism 6 for Mac software package (GraphPad Software, La Jolla, CA, USA). Spearman correlation analysis was performed to determine the relationship between proteins and clinical severity of the disease. Stepwise linear regression

Table 1 Demographic and clinical data of 18 VKC patients

No.	Gender/age	Tarsal/limbal/ mixed	Activity score (0–4)	Serum IgE (KU/l)	Allergen (CAP-FEIA)	Associated allergic diseases	Topical treatment
1	M/5	L	2	57	Negative	–	–
2	M/8	T	2	1979	D, G, P, C	Rhinitis, asthma	–
3	M/25	Mx	4	25	Negative	–	–
4	M/9	Mx	3	31	Negative	Rhinitis	–
5	M/13	L	3	96	Negative	–	–
6	M/8	T	3	874	D, G, P, T	Rhinitis, asthma	–
7	M/7	Mx	1	304	Negative	–	–
8	M/8	T	2	88	Negative	–	–
9	M/15	T	2	95	Negative	–	–
10	M/11	T	3	41	D, C	–	–
11	F/7	L	1	74	D	Rhinitis	Anti-H1
12	M/8	T	3	1300	D, G, C	Rhinitis, asthma	Clb
13	M/15	T	3	1424	D, G, P	Rhinitis	CsA 1%
14	M/6	Mx	4	105	D, Cat	–	CsA 1%
15	M/10	T	3	59	G	Eczema	CsA 1%
16	M/7	T	2	144	Negative	Rhinitis	CsA 1%
17	F/12	L	2	256	D, G	–	–
18	M/7	Mx	3	77	D, G	Rhinitis, asthma	–

M, male; F, female; T, tarsal; L, limbal; Mx, mixed; D, dermatophagoides; G, graminaceae; P, parietariae; C, compositae; T, trees; Anti-H1, antihistamine; Clb, clobetasone; CsA, cyclosporine; VKC, vernal keratoconjunctivitis.

analyses, with the protein concentrations in tear samples as dependent variables, were then performed to determine the relative effect of clinical severity of VKC. A *P* value <0.05 was considered as significant.

Results

Tear samples from ten VKC patients and ten healthy controls were collected for the initial tear proteomic analyses and from six treated patients and two newly diagnosed, untreated patients for a pilot investigation of the potential effect of topical drugs on tear proteome (Table 1). Quantitative validation analysis was then performed in a second additional cohort of patients and age-matched normal subjects. All VKC patients were in their active phase classified according to the clinical severity score.

Tear protein identification and relative quantification of tear proteins from VKC patients and normal controls by iTRAQ technology

Seventy-eight proteins were identified (Table S1). Quantitative iTRAQ analysis was performed for the more abundant proteins: albumin, lactotransferrin, lysozyme, lacritin glycoprotein, secretoglobulin 1D, mammaglobin B, lipocalin-1, proline-rich protein-4, hemopexin, transferrin, and Ig α -1 chain C region. The ratio 114/115 that represents the quantitative ratio between the two control samples (see experimental) was close to 1 for most of the proteins analyzed, showing a similar expression of protein between controls. In VKC samples, all the proteins typical of the tear film (lacritin, lysozyme, lipocalin) were increased, compared with CT (Table 2); however, a statistically significant increase was shown only

Table 2 Quantitative iTRAQ for 13 tear proteins in normal subjects (CT) and vernal keratoconjunctivitis (VKC) patients

	CT Mean (min–max)	VKC Mean (min–max)	<i>P</i> -value
Serum albumin	1.3 (0.9–1.9)	29.75 (3–90)	0.0001
Lactotransferrin	0.9 (0.7–1.2)	3.79 (0.9–13.1)	0.5011
Lysozyme	1.03 (0.8–1.2)	2.1 (0.7–6.2)	0.5898
Lacritin	1.13 (0.8–1.5)	3.56 (0.7–8.4)	0.4982
Secretoglobulin 1D	0.98 (0.5–1.4)	6.09 (0.6–20.1)	0.0393
Mammaglobin B	1.03 (0.6–1.7)	4.75 (0.6–13)	0.0139
Lipocalin-1	1.1 (0.4–1.8)	4.4 (0.4–15.5)	0.3042
Proline-rich protein 4	0.93 (0.4–1.6)	2.12 (0.3–7.8)	0.6970
Cistatine-S	1.12 (0–0.9–2.95)	2.25 (0.14–6.32)	0.1222
Hemopexin	0.98 (0.9–1.1)	30.45 (1.1–85)	0.0049
Serotransferrin	1.1 (0.9–1.5)	26.6 (0.9–80)	0.0054
Ig α -1 chain	2.1 (0.8–6)	1.37 (0.8–3.6)	0.1631

for serum albumin, secretoglobulin, mammaglobin, hemopexin, and transferrin (Table 2).

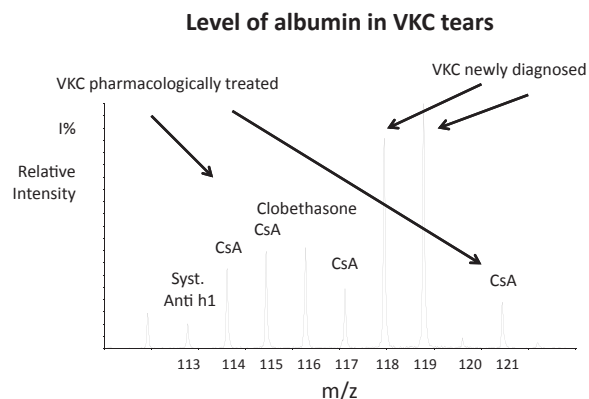
Relative quantification of tear proteins from treated and nontreated VKC by 8-plex iTRAQ technology

Nontreated VKC samples showed higher tear levels of albumin, transferrin, and hemopexin, than those found in tear of patients treated with CsA or corticosteroids (Table 3) with similar values between different samples. In these experiments, two additional proteins were quantified, apolipoprotein A-I and alpha 1-acid glycoprotein. Figure 1 shows the reporter groups for albumin: the two most intense peaks, at

Table 3 Quantitative analysis of 11 proteins identified in the iTRAQ 8-plex experiment using samples from pharmacologically topically treated and nontreated VKC patients

	Nontreated	Nontreated	CsA 1%	CsA 1%	CsA 1%	CsA 1%	Clb
Serum albumin	9.1	7.3	2.5	2.7	1.6	1.5	3.8
Lactotransferrin	1.5	1.1	0.6	0.8	1.1	0.4	1.1
Lysozyme	0.7	1.0	0.6	0.7	0.8	0.9	0.9
Lacritin	1.9	1.6	1.1	1.2	2.3	1.2	1.2
Mammaglobin B	1.5	1.2	0.8	1.1	1.6	0.8	0.9
Lipocalin-1	1.3	0.6	0.6	0.6	1.1	0.7	0.7
Proline-rich protein 4	1.2	1.4	0.5	0.7	1.6	1.3	1.1
Hemopexin	9.0	6.9	1.9	2.8	1.7	1.1	5.1
Serotransferrin	8.1	6.0	1.6	2.0	1.5	0.9	5.7
α -1 acid glycoprotein	9.7	16.1	1.9	2.9	2.2	1.4	2.5
Apolipoprotein a-1	3.0	2.9	1.6	1.1	0.8	0.9	1.1

CsA1%, cyclosporine A 1% eye drop; Clb, clobetasone eye drop; VKC, vernal keratoconjunctivitis.

**Figure 1** Magnification of a MS/MS spectrum showing relative intensities of the eight reporter groups for albumin. The two most intense peaks, at m/z 118 and 119 correspond to nontreated vernal keratoconjunctivitis (VKC) samples. The less intense peaks correspond to samples from pharmacologically treated VKC patients and show a decrease in the albumin level.

m/z 118 and 119, correspond to nontreated VKC samples, while the less intense peaks correspond to samples from pharmacologically treated VKC patients showing lower tear albumin levels due probably to the medication.

In the additional cohort of patients and normal subjects used for validation and quantification of tear proteins, both albumin and transferrin tear levels were significantly increased compared with normal subjects ($P < 0.05$) (Fig. 2) and correlated with the severity of the disease ($P < 0.05$). In the linear regression analysis, both tear albumin and transferrin concentrations were a significantly correlated ($P < 0.05$) to the severity score of the disease with an R^2 value of 0.432 and 0.678, respectively (Fig. 2).

Discussion

Patients with different diseases may have a different tear protein profile, and therefore, protein or peptide analysis in tears

can be used as a possible fingerprint for disease biomarkers and pathological molecule identification. The most abundant proteins in tears include lactoferrin, lysozyme, lipocalin, lipophilin, complement proteins, and various immunoglobulins. However, in severe inflammatory ocular surface conditions such as VKC, particular attention has been focused on cytokines, chemokines, growth factors, angiogenic modulators, histamine receptors using different technologies (10, 11, 14, 15). Using the iTRAQ quantitative method, 78 proteins were identified (Table S1). Of these, 11 were chosen for the iTRAQ quantification because of their particular high expression or because already described in tears of other pathologies.

Tear levels of albumin, transferrin, hemopexin, mammaglobin B, and secretoglobulin 1D1 were found significantly higher in VKC compared with normal subjects. In particular, albumin, transferrin, and hemopexin tear levels were 10–100 times higher than normal tears. By routine immunometric analysis, we have confirmed the significantly increased levels of albumin and transferrin in tears of VKC patients compared with normal tears. These levels were significantly correlated with the severity of the disease, suggesting that they could be considered potential biomarker for VKC diagnosis and monitoring.

The exact role of albumin in tears is not known; however, it may reflect the degree of integrity of the ocular surface and may be used as a biomarker of increased conjunctival capillary leakage from the serum. Serum albumin is also a carrier to the tears for cytokines and other inflammatory proteins related to the pathogenesis of VKC.

Transferrin is an iron-binding plasma glycoprotein found in many body fluids, primarily expressed by the liver but also by corneal epithelial cells (16). Increased oxidative stress status has been described in patients with allergy and allergic keratoconjunctivitis (17, 18). Transferrin may have antioxidant effects inhibiting iron-mediated oxidation through the capacity for binding free iron protecting against toxic radical oxygen species (17). Interestingly, urinary transferrin levels were increased in systemic lupus erythematosus patients and correlated with disease activity, suggesting that iron-binding

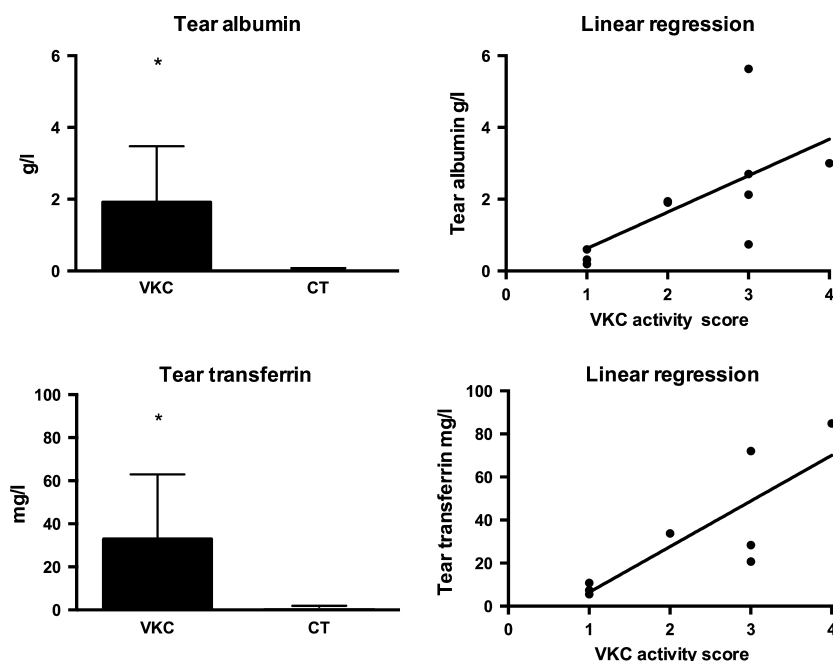


Figure 2 Tear levels of albumin and transferrin by commercial immunometric analysis in the additional cohort of vernal keratoconjunctivitis (VKC) patients and normal subjects (* $P < 0.05$). In VKC

patients, by linear regression analysis, tear albumin and transferrin levels were significantly correlated ($P < 0.05$) to the clinical severity score of the disease.

proteins could potentially play a role in regulating immunity (19). On the contrary, transferrin was found decreased while albumin increased in dry eye patients, suggesting that variations of these proteins in tears cannot be explained by a passive filtration mechanism only (20).

Hemopexin was already found increased in tears of VKC patients using a 2D-PAGE analysis (21) and in the cytoplasm of conjunctival epithelial cells and macrophages in impression cytology samples from VKC patients (22). It is a type II acute-phase reactant glycoprotein up-regulated by IL-6, playing a well-established biological role in sequestering heme. Hemopexin has also hyaluronidase, serine protease, pro-inflammatory, and anti-inflammatory activity and inhibits cellular adhesion and binding of divalent metal ions (23). Increased hemopexin levels were found in bronchoalveolar lavage fluid from individuals with asthma, suggesting that hemopexin may play a role in controlling inflammation in allergic diseases (24). An hemopexin-like domain is contained in MMPs, suggesting that increased tear levels of hemopexin may be related to the MMPs over-expression typical of VKC (25). Hemopexin has shown to have an anti-inflammatory property down-regulating secretion of TNF- α and IL-6 from murine macrophages (26). It is possible that the local micro-environment may influence the activity of hemopexin as pro- or anti-inflammatory protein (27, 28). Therefore, hemopexin in VKC may function to modulate the local inflammatory response and be involved in tissue remodeling.

Mammaglobin, a member of the uteroglobin family, is a protein of unknown function particular associated with breast cancer. Gene and protein expression of mammaglobin B was found in nasal polyps in patients with and without

allergic rhinitis and increased after glucocorticoid therapy, suggesting that mammaglobin might be either related to polyp growth or to the mucosal anti-inflammatory properties (29, 30). In fact, peptides derived from uteroglobin family are among the most potent anti-inflammatory agents identified to date (31). Similarly to nasal polyps, VKC is characterized by tissue remodeling and severe inflammation (32). However, in the present study, its expression was partially modified by the topical anti-inflammatory treatment.

Human secretoglobin 1D1 (also known as lipophilin A) is a small protein of unknown function that forms heterodimers with lipophilin C in tears (33). A down-regulation of lipophilin A and C, lipocalin-1, and lactoferrin has been found in tears of patients suffering from dry eye, suggesting that these changes anticipate the onset of more extensive clinical signs in dry eye disease (33). Secretoglobins were found expressed in human upper airway, and their expression can be differentially regulated by inflammatory cytokines (24). Reduced production of members of this protein family, which may be dependent to a local cytokine environment, has been associated with the hyperinflammation in chronic rhinosinusitis with nasal polyps (34). In the present study, tear secretoglobulin was increased compared with normal tears differently from dry eye (35) and apparently reduced by the anti-inflammatory treatment, thus the opposite of nasal polyps. Differences and similarities between VKC and nasal polyps were already described probably related to evident anatomical and physiological properties and different cytokine micro-environment (32).

Proteomic analysis with iTRAQ technology may also be useful also to evaluate the efficacy of topical treatments.

Although performed in a limited number of subjects, different protein expression was evident in treated and nontreated patients. What is definitely evident is the loss of ocular surface integrity in VKC patients with a dramatic change in tear components.

Here, identified proteins represent a wide range of biological categories including serum proteins such as albumin, apolipoprotein and transferrin, metabolic enzymes, binding proteins, intracellular signaling proteins, and a number of proteins whose properties and functions have not been well characterized. Interestingly, many of the over-expressed proteins may have anti-inflammatory properties, suggesting that regulatory mechanisms are up-regulated to limit and control the extent or effects of pro-inflammatory cytokine as a consequence of local immune reaction. Further studies are needed to evaluate the possible involvement of these mechanisms in the natural remission of VKC after puberty.

In conclusion, proteomic analysis of the tear film, particularly with iTRAQ technology, is a powerful tool for screening large numbers of proteins in tears. This analysis revealed previously undetected proteins that might participate in the pathogenesis of VKC, as well as demonstrating the profound changes in tear composition associated with prolonged allergic inflammation. This technology might be used to define tear mediator patterns specific to a single disease or disease phase, thus providing important diagnostic information for clinicians and researchers who study ocular allergic diseases.

References

- Zhou L, Zhao SZ, Koh SK, Chen L, Vaz C, Tanavde V et al. In-depth analysis of the human tear proteome. *J Proteomics* 2012;**75**:3877–3885.
- de Souza GA, Godoy LM, Mann M. Identification of 491 proteins in the tear fluid proteome reveals a large number of proteases and protease inhibitors. *Genome Biol* 2006;**7**:R72.
- Zhou L, Beuerman RW. Tear analysis in ocular surface diseases. *Prog Retin Eye Res* 2012;**31**:527–550.
- Grus FH, Podust VN, Bruns K, Lackner K, Fu S, Dalmaso EA et al. SELDI-TOF-MS ProteinChip array profiling of tears from patients with dry eye. *Invest Ophthalmol Vis Sci* 2005;**46**:863–876.
- Zhou L, Beuerman RW, Chan CM, Zhao SZ, Li XR, Yang H et al. Identification of tear fluid biomarkers in dry eye syndrome using iTRAQ quantitative proteomics. *J Proteome Res* 2009;**8**:4889–4905.
- Tong L, Zhou L, Beuerman RW, Zhao SZ, Li XR. Association of tear proteins with Meibomian gland disease and dry eye symptoms. *Br J Ophthalmol* 2011;**95**:848–852.
- Lei Z, Beuerman RW, Chew AP, Koh SK, Cafaro TA, Urrets-Zavalía EA et al. Quantitative analysis of N-linked glycoproteins in tear fluid of climatic droplet keratopathy by glycopeptide capture and iTRAQ. *J Proteome Res* 2009;**8**:1992–2003.
- Wong TT, Zhou L, Li J, Tong L, Zhao SZ, Li XR et al. Proteomic profiling of inflammatory signaling molecules in the tears of patients on chronic glaucoma medication. *Invest Ophthalmol Vis Sci* 2011;**52**:7385–7391.
- Leonardi A. Vernal keratoconjunctivitis: pathogenesis and treatment. *Prog Retin Eye Res* 2002;**21**:319–339.
- Leonardi A, Sathe S, Bortolotti M, Beaton A, Sack R. Cytokines, matrix metalloproteases, angiogenic and growth factors in tears of normal subjects and vernal keratoconjunctivitis patients. *Allergy* 2009;**64**:710–717.
- Shoji J, Inada N, Sawa M. Antibody array-generated cytokine profiles of tears of patients with vernal keratoconjunctivitis or giant papillary conjunctivitis. *Jpn J Ophthalmol* 2006;**50**:195–204.
- Li N, Wang N, Zheng J, Liu XM, Lever OW, Erickson PM et al. Characterization of human tear proteome using multiple proteomic analysis techniques. *J Proteome Res* 2005;**4**:2052–2061.
- Sacchetti M, Lambiasi A, Mantelli F, Deligianni V, Leonardi A, Bonini S. Tailored approach to the treatment of vernal keratoconjunctivitis. *Ophthalmology* 2010;**117**:1294–1299.
- Leonardi A, Curnow SJ, Zhan H, Calder VL. Multiple cytokines in human tear specimens in seasonal and chronic allergic eye disease and in conjunctival fibroblast cultures. *Clin Exp Allergy* 2006;**36**:777–784.
- Leonardi A, Di Stefano A, Vicari C, Motterle L, Brun P. Histamine H4 receptors in normal conjunctiva and in vernal keratoconjunctivitis. *Allergy* 2011;**66**:1360–1366.
- Kompella UB, Sundaram S, Raghava S, Escobar ER. Luteinizing hormone-releasing hormone agonist and transferrin functionalizations enhance nanoparticle delivery in a novel bovine ex vivo eye model. *Mol Vis* 2006;**12**:1185–1198.
- Bakkeheim E, Mowinckel P, Carlsen KH, Burney P, Carlsen KC. Altered oxidative state in schoolchildren with asthma and allergic rhinitis. *Pediatr Allergy Immunol* 2011;**22**:178–185.
- Wakamatsu TH, Dogru M, Ayako I, Takano Y, Matsumoto Y, Ibrahim OM et al. Evaluation of lipid oxidative stress status and inflammation in atopic ocular surface disease. *Mol Vis* 2010;**16**:2465–2475.
- Vanarsa K, Ye Y, Han J, Xie C, Mohan C, Wu T. Inflammation associated anemia and ferritin as disease markers in SLE. *Arthritis Res Ther* 2012;**14**:R182.
- Versura P, Bavelloni A, Grillini M, Fresina M, Campos EC. Diagnostic performance of a tear protein panel in early dry eye. *Mol Vis* 2013;**19**:1247–1257.

Author contributions

AL and DG contributed to conception and design of the article, analysis and interpretation of the data, and literature search and made provision of materials, patients, or resources. AL, DG, AP, and AM wrote the article. AL, DG, and AP performed critical revision of the article. AL, DG, EAM, and AM finally approved the article. MB, AP, EAM, AM, and EMSM collected the data. AP, AM, EAM, and EMSM are statistical expertise. AL and MB provided administrative, technical, or logistic support.

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Conflicts of interest

All the authors declare that they have no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Proteins identified in tear samples.

Data S1. Materials and methods.

21. Pong JC, Chu CY, Chu KO, Poon TC, Ngai SM, Pang CP et al. Identification of hemopexin in tear film. *Anal Biochem* 2010;**404**:82–85.
22. Pong JC, Chu CY, Li WY, Tang LY, Li L, Lui WT et al. Association of hemopexin in tear film and conjunctival macrophages with vernal keratoconjunctivitis. *Arch Ophthalmol* 2011;**129**:453–461.
23. Mauk MR, Smith A, Mauk AG. An alternative view of the proposed alternative activities of hemopexin. *Protein Sci* 2011;**20**:791–805.
24. Lee SH, Kim KH, Kim JM, Yoon SH, Kim TH, Park SW et al. Relationship between group-specific component protein and the development of asthma. *Am J Respir Crit Care Med* 2011;**184**:528–536.
25. Leonardi A, Brun P, Abatangelo G, Plebani M, Secchi AG. Tear levels and activity of matrix metalloproteinase (MMP)-1 and MMP-9 in vernal keratoconjunctivitis. *Invest Ophthalmol Vis Sci* 2003;**44**:3052–3058.
26. Liang X, Lin T, Sun G, Beasley-Topliffe L, Cavaillon JM, Warren HS. Hemopexin down-regulates LPS-induced proinflammatory cytokines from macrophages. *J Leukoc Biol* 2009;**86**:229–235.
27. Kapojos JJ, Poelstra K, Borghuis T, Banas B, Bakker WW. Regulation of plasma hemopexin activity by stimulated endothelial or mesangial cells. *Nephron Physiol* 2004;**96**:P1–P10.
28. Cheung PK, Klok PA, Baller JF, Bakker WW. Induction of experimental proteinuria in vivo following infusion of human plasma hemopexin. *Kidney Int* 2000;**57**:1512–1520.
29. Fritz SB, Terrell JE, Conner ER, Kurowska-Latallo JF, Baker JR. Nasal mucosal gene expression in patients with allergic rhinitis with and without nasal polyps. *J Allergy Clin Immunol* 2003;**112**:1057–1063.
30. Chusakul S, Phannaso C, Tongkobpetch S, Aemjaturapat S, Poovorawan Y, Suphapeetiporn K et al. Expression of mammaglobins A and B in nasal polyps is similar in patients with and without allergic rhinitis. *Am J Rhinol* 2008;**22**:135–138.
31. Miele L. New weapons against inflammation: dual inhibitors of phospholipase A2 and transglutaminase. *J Clin Invest* 2003;**111**:19–21.
32. Leonardi A, Brun P, Di Stefano A, Motterle L, Abatangelo G. Matrix metalloproteinases in vernal keratoconjunctivitis, nasal polyps and allergic asthma. *Clin Exp Allergy* 2007;**37**:872–879.
33. Lehrer RI, Xu G, Abduragimov A, Dinh NN, Qu XD, Martin D et al. Lipophilin, a novel heterodimeric protein of human tears. *FEBS Lett* 1998;**432**:163–167.
34. Lu X, Wang N, Long XB, You XJ, Cui YH, Liu Z. The cytokine-driven regulation of secretoglobins in normal human upper airway and their expression, particularly that of uteroglobin-related protein 1, in chronic rhinosinusitis. *Respir Res* 2011;**12**:28.
35. Versura P, Nanni P, Bavelloni A, Blalock WL, Piazzini M, Roda A et al. Tear proteomics in evaporative dry eye disease. *Eye (Lond)* 2010;**24**:1396–1402.

Supporting Information

Table S1. Proteins identified in tear samples

Accession (IPI)	Gene Symbol	Protein name	no. of peptides identified
IPI00298860	LTF	Lactotransferrin precursor	50
IPI00216773	ALB	Serum Albumin	50
IPI00019038	LYZ	Lysozyme C precursor	28
IPI00009650	LCN1	Von Ebner gland protein	22
IPI00022463	TF	Serotransferrin precursor	17
IPI00166729	AZGP1	Alpha-2-glycoprotein 1, zinc	5
IPI00020487	LACRT	Extracellular glycoprotein lacritin precursor	9
IPI00027019	PRR4	Proline-rich protein 4	9
IPI00022974	PIP	Prolactin-inducible protein	6
IPI00305477	CST1	Cystatin SN precursor	3
IPI00001468	SCGB1D1	Secretoglobulin family 1D member 1 (Lipophilin-A)	6
IPI00026126	SCGB2A1	Mammaglobin B precursor	3
IPI00641229	IGHA2	Ig alpha-2 chain C region	3
IPI00553177	SERPINA1	Alpha-1-antitrypsin	4
IPI00032294	CST4	Cystatin S precursor	7
IPI00423463	IGHG1	Ig gamma-1 chain C region	7
IPI00021841	APOA1	Apolipoprotein A-1	8
IPI00478003	A2M	Alpha-2-macroglobulin	4
IPI00009682	PROL1	Proline-rich protein 1	2
IPI00909649	IGKC	Ig kappa chain C region	2
IPI00783987	C3	Complement C3	8
IPI0002249	ORM1	Alpha-1-acid glycoprotein 1 precursor	2
IPI00004656	B2M	Beta-2-microglobulin	2
IPI00009792		Ig heavy chain V-I region V35	2
IPI00154742	IGLC	Ig lambda chain C region	2
IPI00387026		Ig kappa chain V-I region	2
IPI00299729	TCN1	Transcobalamin I precursor	2
IPI00004573	PIGR	Polymeric immunoglobulin receptor	2
IPI00953689	AHSG	Alpha-2-HS-glycoprotein	3
IPI00015180	SHROOM2	Protein Shroom 2	2

IPI00027846	MMP8	Neutrophil Collagenase precursor	2
IPI00006721	OPA1	Dynamin-like 120kDa protein, mitochondrial	3
IPI00008580	SLPI	Antileukoproteinase	2
IPI00022432	TTR	Transthyretin precursor	3
IPI00298497	FGB	Fibrinogen beta chain precursor	3
IPI00182304	TYW1	tRNA wybutosine-synthesizing protein 1	2
IPI00871174	MYCBP	C-Myc-binding protein	2
IPI00332633	RASSF5	Ras association domain-containing protein 5	3
IPI00796450	LYST	Lysosomal-trafficking regulator	3
IPI00299037	CACNA1G	Voltage-dependent T-type calcium channel subunit alpha-1G	3
IPI00007834	ANK2	Ankyrin-2	3
IPI00335589	RNMTL1	RNA methyltransferase-like protein 1	3
IPI00745556	NCAPH2	Condensin-2 complex subunit H2	3
IPI00788188	DYNC2H1	Cytoplasmic dynein 2 heavy chain 1	5
IPI00963992	CES4	Inactive carboxylesterase 4	2
IPI00010317	RRAGB	Ras-related GTP-binding protein B	2
IPI00156282	GPS1	COP9 signalosome complex subunit 1	2
IPI00514234	LAD1	Ladinin-1	2
IPI00329132	C6orf134	UPF0635 protein C6orf134	2
IPI00102313	SRA1	Steroid receptor RNA activator	3
IPI00002469	HCFC2	Host cell factor 2	2
IPI00026962	PLA2G2A	Phospholipase A2, membrane associated	3
IPI00418234	MECP2	Methyl-CpG-binding protein 2	2
IPI00024752	CLEC3A	C-type lectin domain family 3 member A	2
IPI00014845	DNAH8	Dynein heavy chain 8, axonemal	4
IPI00552279	ESCO1	N-acetyltransferase ESCO1	2
IPI00181824	VPS53	Vacuolar protein sorting-associated protein 53 homolog	2
IPI00007800	ANGPTL2	Angiopoietin-related protein 2	3

IPI00006433	DNAJC16	DnaJ homolog subfamily C member 16	2
IPI00884996	DNAH6	Dynein heavy chain 6, axonemal	4
IPI00017696	C1S	Complement C1s subcomponent	3
IPI00009207	NFE2L3	Nuclear factor erythroid 2-related factor 3	2
IPI00794207	SCHIP1	Schwannomin-interacting protein 1	2
IPI00550181	CHMP2B	Charged multivesicular body protein 2b	2
IPI00021987	RGP1	Retrograde Golgi transport protein RGP1 homolog	2
IPI00017617	DDX5	Probable ATP-dependent RNA helicase DDX5	2
IPI00010180	CES1	Liver carboxylesterase 1	2
IPI00164528	SNTA1	Alpha-1-syntrophin	3
IPI00294556	APBA1	Amyloid beta A4 precursor protein-binding family A member1	2
IPI00071360	A1CF	APOBEC1 complementation factor	2
IPI00023073	XRCC3	DNA repair protein XRCC3	2
IPI00218137	KLK11	Kallikrein-11	2
IPI00000203	LRP6	Low-density lipoprotein receptor-related protein 6	3
IPI00032187	NISCH	Nischarin	2
IPI00063408	DHTKD1	Probable 2-oxoglutarate dehydrogenase E1 component DHKTD1, mitochondrial	2
IPI00030285	SPINK4	Serine protease inhibitor Kazal-type 4	3
IPI00166296		Transmembrane protein FLJ37396	2
IPI00470596	ZNF638	Zinc finger protein 638	2

Supplementary Information

MicroHPLC separation and collection

The modified peptides were eluted and separated by reversed-phase high-performance liquid chromatography (RP-HPLC) using a LC-Packings system which consisted of a Capillary- and Nano- HPLC system ULTIMATE™, an UV-detector ULTIMATE™, a Well Plate Microautosampler FAMOS™ and an Advanced Microcolumn Switching Unit SWITCHOS II™ equipped with a C18 PepMap capillary columns, from LC-Packings (stationary phase: PepMap C18, 3µm particle size, 150 mm length, 300 µm i.d., 100 Å pore size). HPLC conditions: Solvent A: H₂O 0.05% TFA, solvent B: ACN 0.05% TFA, flow rate 4 µl/min, injection volume 5 µL, linear gradient of 120 min from 2% to 60% of solvent B. UV detection at 214 nm.

The peptide fractions eluting from the column were mixed with a matrix solution (3 mg/ml of α -Cyano-4-hydroxycinnamic acid in Acetonitrile:MilliQ-Water 3:1, with 0,15 mg/ml of diammonium citrate) and collected onto a 384-well MALDI plate (Applied Biosystems) by the MicroFraction Collector PROBOT™ (LC-Packings)

We have also checked a LC Packings Dionex Monolithic Capillary column PS-DVB, 50 mm length, 200 µm i.d, with the following HPLC conditions: Solvent A: H₂O 0.05% TFA, solvent B: ACN 0.05% TFA, flow rate 4 µl/min, injection volume 1 µL, column temperature 60°C, linear gradient of 25 min from 2% to 60% of solvent B. UV detection at 214 nm, but with lower performances in terms of chromatographic separations although the duration of the analysis was considerably decreased. Results reported in this paper refer to the C18 column.

Mass Spectrometry

1 *MALDI MS Calibration*

2 We performed external calibration using a peptides calibration mix with des-Arg¹-
3 Bradykinin (m/z 904.4681), Angiotensin 1 (1296.6853), Glu¹-Fibrinopeptide B (m/z
4 1570.6774), ACTH (1-17 clip) (m/z 2093.0867), ACTH (18-39 clip) (m/z 2465.1989).
5 MSMS spectra were calibrated with Glu1-Fibrinopeptide B fragments.

6
7 *MS/MS Data Analysis*

8 We used Protein PilotTM (AB Sciex, Foster City, CA USA) and MascotTM (Matrix
9 Science Inc Boston, MA USA) software for MS/MS data analysis. We found no
10 significative differences in the results of the two software used. Data presented here
11 were obtained with MascotTM for qualitative proteins identification (Table S1) and
12 with Protein PilotTM for relative quantitative proteins determination. Only proteins
13 identified by two or more peptides were reported in Table S1.

14
15 *Protein PilotTM with Paragon algorithm parameters*

16 Detected Protein Threshold cut-off>1.3 (95 Percent Confidence) for protein ID, cut-
17 off>2% (99 Percent Confidence) for relative protein quantitation.

18
19 *MASCOT MS/MS ion search parameters*

20 Peptide mass tolerance: 50ppm, fragment mass tolerance 0.3 Da, max missed
21 cleavages 1, MudPIT scoring, fixed modifications: MMTS (C), ITRAQ (K, N-term),
22 Decoy search performed, Protein ID with at least 2 peptides for a protein.