The Use of Conjunctival Swab for the Proteomic Characterisation of Dry Eye Syndrome

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Abstract

In this study we report the first gel based proteomic analysis of an inflamed dry eye utilising a clinically-based non-invasive methodology for collection of a specimen from the posterior lid and inferior conjunctival mucosa of the subject. This multidimensional technique allowed the identification of 592 proteins, having a MOWSE score of greater than 40, using the heuristic tool PROVALT. Automated curation of this list using an inbuilt randomised database searching tool with false discovery rate set at 1% significantly reduced this list to 86 proteins. Additional manual curation resulted in the final positive identification of 75 proteins. These identified proteins were functionally classified and physiochemically characterised. This led to the identification of a number of proteins involved in cell structure, inflammation, and the innate immune response. Contained within these proteins were a number of potential biomarkers of not only dry eye syndrome but also lacrimal gland acinar cell function such as lacritin, calgranulin A and lacrimal proline-rich protein 4.

Introduction

The front of the eye or 'ocular surface' comprises mucosal epithelium and various glandular structures that include lacrimal, accessory lacrimal and meibomian glands all of which are bathed in a thin fluid film. These structures are integrated together and function as a composite group designed to lubricate, nourish, protect and most importantly produce a clear, smooth optical surface to refract light (Stern et al.,1998; Rolando and Zierhut, 2001).

The overlying fluid tear film contains proteins, electrolytes and growth factors involved in maintaining the health of the ocular surface. The tear film is produced by lacrimal glands, meibomian glands, and the surface epithelium and is in close proximity to the systemic blood circulation via the highly vascularised submucosa. Various proteins within the tear film are recognised to play key roles in ocular defence including: lactoferrin, lysozyme, phospholipase A2, ceruloplasmin and immunoglobluins such as secretory IgA (Abe et al., 1999; Cullor et al., 1990; Glasson et al., 2002; Haynes et al., 1998; Haynes et al., 1999; O'Callaghan et al., 2003; Seal et al., 1986). Other defence proteins include trefoil peptides, SP-D (surfactant protein-D), gp340 (glycoprotein 340) and PLA2 and during inflammation the tear film quickly fills with inflammatory cells which leak from the adjacent blood vessels.

Recent research has demonstrated a plethora of proteins (>400) within the normal tear film (De Souza et al., 2006). The proteins present at any given time within the tears may be considered as a 'snapshot' of the status of the ocular surface. Alterations in such proteins undoubtedly contribute to the dynamic of the ocular surface in health and disease. Previous examination of the tear film in dry eye conditions such as Sogrens have demonstrated alterations in key proteins such as lysozyme, lactoferrin and EGF (Seal et al., 1986; Ohashi et al., 2003; Schoenwald et al., 1998).

The purpose of this study was to analyse the applicability of a posterior lid margin, inferior fornix mucosal swab to profile proteins present within the tear film and ocular surface. Most sampling of the tear film to date has been through the use of capillary tear collection, a laborious and technically difficult approach with limited clinical applicability (Zhou et al., 2006). An extensive search of the literature revealed limited investigation of the protein profile present in a conjunctival sample taken from the inferior conjunctiva of human subjects (Grus et al., 2005; Tsai et al., 2006). The use of a simple non-invasive swab technique to include both tears and conjunctival cells would simplify this clinical test and produce a more complete proteomic characterisation of the ocular surface environment to include non secreted cellular proteins as well as those present within tear film. Recent advances in genomics and proteomics offer huge potential to enable clinicians to view biomarker patterns in both healthy and diseased ocular surfaces. This study was designed to investigate the proteome of an eye

with dry eye syndrome clinically characterized by ocular surface inflammation, glandular dysfunction and an abnormal tear film.

Materials and Methods

Reagents

All reagents were purchased from Sigma-Aldrich (Poole, UK) with the exception of mass spectrometry grade water and acetonitrile, which were purchased from Romil (Cambridge, UK) and Trypsin, which was purchased from Promega (Southampton, UK).

Sample collection and patient assessment

A patient who presented with dry eye symptoms was clinically assessed using a slit lamp examination of meibomian glands; lids, lid margins, conjunctiva and tear film;(Foulks and Bron, 2003) impression cytological assessment of conjunctival goblet cells; (Anshu et al., 2001; Saini et al., 1990) tear break-up time using fluorescein (Kojima et al., 2004) and the Zone-Quick phenol red thread test (PRT) (Menicon, USA) (Hamano et al., 1983). The subject also completed the McMonnies dry eye questionnaire (McMonnies, 1986).

Sample collection was performed in a clean ophthalmic consulting room and the examining ophthalmologist wore sterile gloves in order to minimise contamination of test samples. In addition, negative control swabs were taken at the time and place of subject testing to confirm the lack of environmental contamination.

After instillation of topical anaesthetic, the ocular specimen for protein analysis was collected from the posterior lid margin and lower conjunctival sac using a sterile cotton swab (Bibby Sterilin Ltd., Stone, UK). The swab was immediately cut into a sterile lysing matrix extraction tube containing ceramic and silica beads (BIO 101 Anachem, UK) and 1ml of sodium phosphate buffer using sterile scissors.

Protein extraction and quantification

The extraction tube containing the swab was processed using a FastPrep Instrument (Anachem, UK) at a preset speed of 6.0 for 30 s with a 2 min resting period on ice before the procedure was repeated. The sample was the centrifuged for 30 minutes at 25,000 g to remove cellular debris, the supernatant was decanted and stored frozen at –70°C until required. The total soluble protein content was measured using the Bradford assay (Bradford, 1976).

One dimensional gel electrophoeresis

An aliquot of the supernatant (10μL) was added to 10μL Tris-Glycine SDS sample loading buffer (Invitrogen, Renfrewshire, UK) and boiled for 5 min. The sample (20μL; 50μg total protein) was loaded onto a 1 mm thick Nu-Page 4-12 % Bis-Tris gel (Invitrogen, Renfrewshire, UK). SeeBlue™ Plus 2 (Invitrogen, Renfrewshire, UK) was used as a protein molecular mass marker. The gel was electrophoresed, using MES SDS running buffer, in an X-Cell II mini gel system (Invitrogen, Renfrewshire, UK) at 200 V, 120 mA, 25 W per gel for 35 min. Proteins were visualised using SimplyBlue™ Safestain (Invitrogen, Renfrewshire, UK). The entire lane was excised from the gel and cut into 3 mm fractions.

In-gel tryptic digestion

Excised gel fractions were washed overnight in 200μL of a 50% (v/v) methanol and 5% (v/v) acetic acid solution. These fractions were then dehydrated by incubation for 5 min in 200μL acetonitrile. 30μL of 10 mM ditiothreitol was added and incubated for 30 mins at room temperature, followed by the addition of 30μL of 100 mM iodoacetamide for 30 min. 200μL acetonitrile was added and incubated for a further 5 min. Rehydration of gel fractions was carried out in 200μL of 100 mM NH_4HCO_3, pH 7.8 for 10 min at room temperature. Gel fractions were dehydrated as above usingacetonitrile and then dried in a rotary evaporator. 30μL of 20 ngμL trypsin in 50 mM NH_4HCO_3, pH 7.8 was added to each sample and incubated overnight at 37°C. 30μL of 50 mM NH_4HCO_3 was added to the samples and incubated for 10 min. The supernatant was subsequently recovered into microcentrifuge tubes and two further peptide extractions from these gel pieces were carried out with addition of 30μL 50% (v/v acetonitrile and 5% (v/v) formic acid) for 10 min. Peptide-containing liquid fractions were pooled, dried under vacuum and re-suspended in 20 μL 0.1% formic acid in 2% acetonitrile prior to storage at -70°C until required.

LC-MS analysis

Mass spectrometry was performed using a 3200 Q-TRAP Hybrid ESI Quadropole linear ion trap mass spectrometer, ESI-Q-q-Qlinear ion trap-MS/MS (Applied Biosystems/MDS SCIEX, Toronto, Canada) with a nanospray interface, coupled with an online Ultimate 3000 nanoflow liquid chromatography system (Dionex/LC Packings, Amsterdam, The Netherlands). A ∞-Precolumn™ Cartridge (300∞m × 5 mm, 5∞m particle size) was placed prior to the C_{18} capillary column (75 μm × 150 mm, 3 μm particle size) to enable desalting and filtering. Both columns contained the reversed phase material PepMAP™ 100 (C_{18} silica-based) with a 100 Å pore size (Dionex/LC Packings). The elution buffers used in the gradient were Buffer A (0.1% formic acid in 2% acetonitrile) and Buffer B (0.1% formic acid in 80% acetonitrile). The nanoLC gradient used was 60 min in length: 0 – 55% B in 45 min, 10 min at 90% B followed by 5 min at 100% A. The flow rate of the gradient was 300 nLmin^{-1}. The detector mass range was set at 400–1800 m/z. MS data acquisition was performed in positive ion mode. During MS acquisition peptides with $^{2+}$ and $^{3+}$ charge state were selected for fragmentation.

Database searching, protein identification and PROVALT analysis

Protein identification was carried out using an internal MASCOT server (version 1.9; Matrix Science, London, UK) searching against a human database extracted from the NCBI database (latest version at the time of processing). Peptide tolerance was set at ± 1.2 Da with MS/MS tolerance set at ± 0.6 Da and the search set to allow for 1 missed cleavage. Carbamidomethylation was set as a fixed modification and oxidation of methionine as a variable modification within the search parameter settings. In order to expedite the curation of the identified protein list from MASCOT, the result files were re-analysed against this extracted database using the heuristic method known as the protein validation tool PROVALT (Weatherly et al., 2005). This automated program takes large proteomic MS datasets and reorganises them by taking multiple MASCOT results and identifying those peptides that match. Redundant peptides are removed and related peptides are grouped together associated with their predicted matching protein, thus, the program dramatically reduces this portion of the curation process. For identification purposes the minimum peptide length was set at 6 amino acids, minimum peptide MOWSE score was set at 25 and the minimum high quality peptide MOWSE score was set at 40. PROVALT also uses peptide matches from a random database (in this case the extracted human database was randomised) to calculate false-discovery rates (FDR) for protein identifications as previously described by Weatherly et al. (2005). Briefly, identifications from searching the normal and random databases are used to calculate the FDRs and set score thresholds and thus identify as many 'actual' proteins as possible while encountering a minimal number of false-positive protein identifications. Rather than calculate error rates at the peptide level, the FDR calculations employed by PROVALT provide a reasonable balance between the number of correct and incorrect protein assignments. In this study the FDR was set at 1%, meaning that 99% of the reported proteins identified should be correct.

Code	Protein	MOWSE	Peptide No.	WoLF	Signalp SP Pred	SecreromeP
54607120	lactotransferrin	2093	38	E	CLA-GR [19-20]	n0
119395750	keratin 1	2093	29	N	none	n0
4502027	albumin precursor	1920	31	E	AYS-RG [18-19]	No
40354192	keratin 10	1903	26	N	none	n0
24430192	keratin 16	1462	21		none	n0
55956899	keratin 9	1449	21	N	none	n0
5031839	keratin 6A	1246	22	N	none	n0
47132620	keratin 1	986	16	N	none	n0
4502337	alpha-2-glycoprotein 1, zinc	761	14	**E**	AVP-QE [20-21]	no
58530840	desmoplakin isofrom 1	652	10	N	none	no
4504963	lipocalin 1 precursor	641	9	E	LQA-HH [18-19]	No
119395754	keratin 5	612	13	N	none	no
113141228	keratin 13 isoform b	517	6	CN	none	Yes 0.524
4557894	lysozyme precursor	462	7	E	VQG-KV [18-19]	No
4505171	lipophilin C (secretoglobin, family 2A, member 1) serine (or cysteine) proteinase inhibitor, clade B	443	6	E	CYA-DS [18-19]	No
5902072	(oavaIbumin), member 3 (SERPINB3)	430	6	**C**	none	no
4557701	keratin 17	420	6	M	none	Yes 0.658
31377806	polymeric immunoglobulin receptor	409	8	PM	IST-KS [18-19]	No
4505821	prolactin-iuduced protein	373	5	E	NKA-QD [28-29]	No
4506773	calgranulin B (S100 calcium-binding protein A9)	365	5	C	none	no
4503109	cystatin S precursor	310	5	E	ALA-SS [20-21]	no
15187164	lacritin precursor precursor	306	6	E	VYA-ED [19-20]	no
89357932	keratin 5b	126	2	N	none	no
4503107	cystatin C precursor	125	2	**E**	AAG-SS [26-27]	no
16933546	ribosomal protein P0	124	2	CN	none	no
9506669	keratin 24	115	2	M	none	no
42716297	clusterin isoform 1	115	3	N	none	Yes 0.646
7657532	calcyclin (S100 calcium-binding protein A6) serine (or cysteine) proteinase inhibitor, clade B	108	2	C	none	Yes 0.577
4505789	(oavalbumin), member 5 (SERPINB5)	106	3	C	none	no
28173554	histone H2B	102	1	N	none	no
6005802	proline rich 4 (lacrimal)	97	1	E	AQS-TD [18-19]	no
21489959	immunoglobuulin J chain	94	1	E	VKA-QE [22-23]	no
4503571	eaolase 1	90	1	C	none	Yes 0.536
89064523	similar to placophilin 1 isform 1a	90	1	N	none	no
4885063	fructose bisphosphate aldolase C	89	1	C	none	no
113419166	smilar to protein piccolo (Aczonin) inform 12	83	3	CN	N/A	N/A
20149594	heat shock 90kDa protein 1	76	1	C	none	no
10863927	peptid\\prolyl iscinerase A isoform 1	74	1	C	none	no
55741661	hypothetical protein LOC57653	73	2	ER	none	no
113410650	similar myomegalin	72	3	M	none	no
4504183	glutathione transferase	71	1	M	none	Yes 0.545
4885607	small proline rich protein 3 (cornifin)	63	1	C	none	Yes 0.812
53988378	mesothelin isoform 1 preprotein	60	1	E	TLA-GE [38-39]	no
5174693	small proline rich prctein 2a	59	1	C	none	Yes 0.879
4504315	H4 histone family member J	58	1	N	none	no

5454052	stratiin [tyrosine 3/ trypicphan -5 monooxygenase activation protein epsilon polypeptide] (14-3-3 protein)	290	4	CN	none	no
8393159	calmodulin-like skin protein	281	3	E	none	Yes 0.543
7669492	glyceraldehyde-3 -phosphate dehydrogenase	279	4	C	none	no
4501887	actin, gamma 1 propetide	277	4	Cysk	none	Yes 0.505
5729907	lipophilin A precursor	277	5	E	ANA-VV [21-22]	
21735625	tyrosine3/tryptophan 5-monooxygenase activation protein zeta polypeptide (14-3-3 protein)	262	4	C	none	no
116805344	proline-rich, lacrimal 1	242	4	E	SES-QR[21-22]	no
4557321	apolipoprotein A-I preproproyein	236	4	E	SQA-RH[18-19]	no
39777601	ransglutaminase 3 precursor	218	3	C	none	no
94536866	hypothetical protein (IgC domain)	214	3	M	none	Yes 0.778
4557581	fatty acid binding protein 5 (psoriasis-associated)	208	3	C	none	no
4504811	junction plakoglobin	174	3	CN	none	no
4504517	heat shock 27kDa protein 1	165	3	N	none	Yes 0.74
4557871	transferrin	156	4	E	CLA-VP[19]-[20]	no
4503471	eukaryotic translation elongation factor 1 alpha 1	152	2	C	none	no
91206438	hypothetical protein LOC649897 suppressed	146	3	N	none	Yes 0.644
42558279	tubulin, beta 8	145	3	C	none	no
4502101	annexin 1	142	2	C	none	Yes 0.511
21614544	calgranulin A (S100 calcium-binding protein A8)	140	2	M	none	no
110349719	titin isoformN2-A	136	6	N/A	N/A	N/A
4757826	beta-2-microglobiulin precursor (MHC class 1)	130	2	E	LEA-IQ[20-21]	no
6633801	deleted in malignant brain tumor 1 isoform b	127	1	**E**	VLS-TG[19-20]	no
60097902	filaggrin	54	1	**N**	N/A	N/A
4503483	eukaryctic tanslation elongadon factcr 2	53	1	**C**	none	no
4504241	H2A histone family, member D	53	1	**N**	none	Yes 0.536
7706635	comulin	53	1	**N**	none	no
55770844	catenin alpha 1	52	l	Cysk	none	no
50363219	serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1 (SERHNAl)	52	l	E	SLA-ED[24-25]	no
22758146	skin Asparic Protease	51	1	C	none	no
33286418	pynivate kinase 3 isofcrm 1	51	1	C	none	no

Footnote: Subcellular localisations; E – extracellular, N – nuclear, C- cytosol, ER – endoplasmic reticulum, M – mitochondrial, Cysk – cytoskeleton, PM – plasma membrane. Signal peptide prediction – SP Pred.

Table 1: Proteins identified from Eye swab proteome with sub-cellular localisation analysis by WoLF_PSORT, SignalP or SecretomeP

Results and Discussion

Eye examination

Examination of the eye demonstrated an abnormal tear film, inflamed ocular surface and abnormal lipid producing glands within the lid margins. Meibomian gland disease (MGD) was noted, characterised by significant plugging of more than 5 meibomian gland orifices, evidence of lid margin irregularity and inflammation, posterior meibomitis, palpebral and bulbar conjunctival inflammation and evidence of a mild capillary conjunctivitis. Tear volume as determined by the phenol red thread (PRT) test was not found to be significantly decreased. An overall reduced tear break-up time of 6 seconds, positive McMonnies questionnaire score of 18 (McMonnies et al., 1986), and presence of MGD indicated the presence of a dry eye syndrome secondary to abnormal tear film.
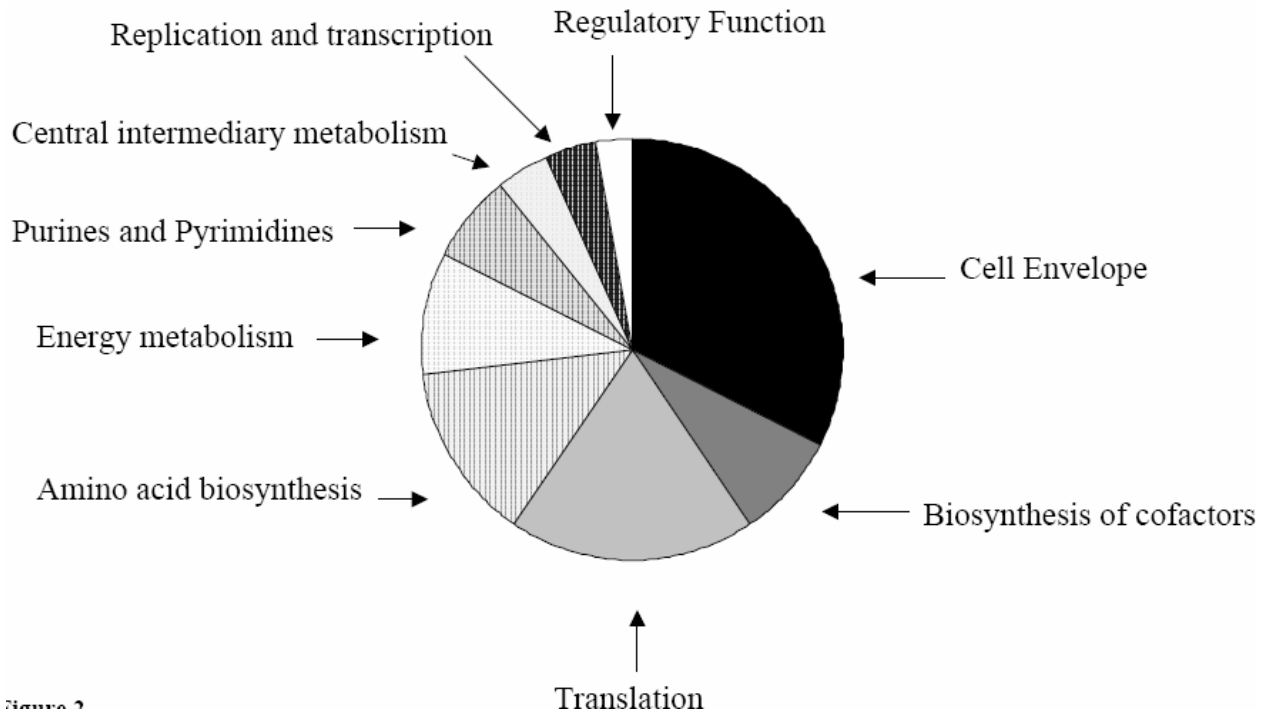
Figure 2: Chart showing the predicted functional categorisation of the proteins identified in the eye swab proteome utilising ProteinFun (Jensen et al., 2003).
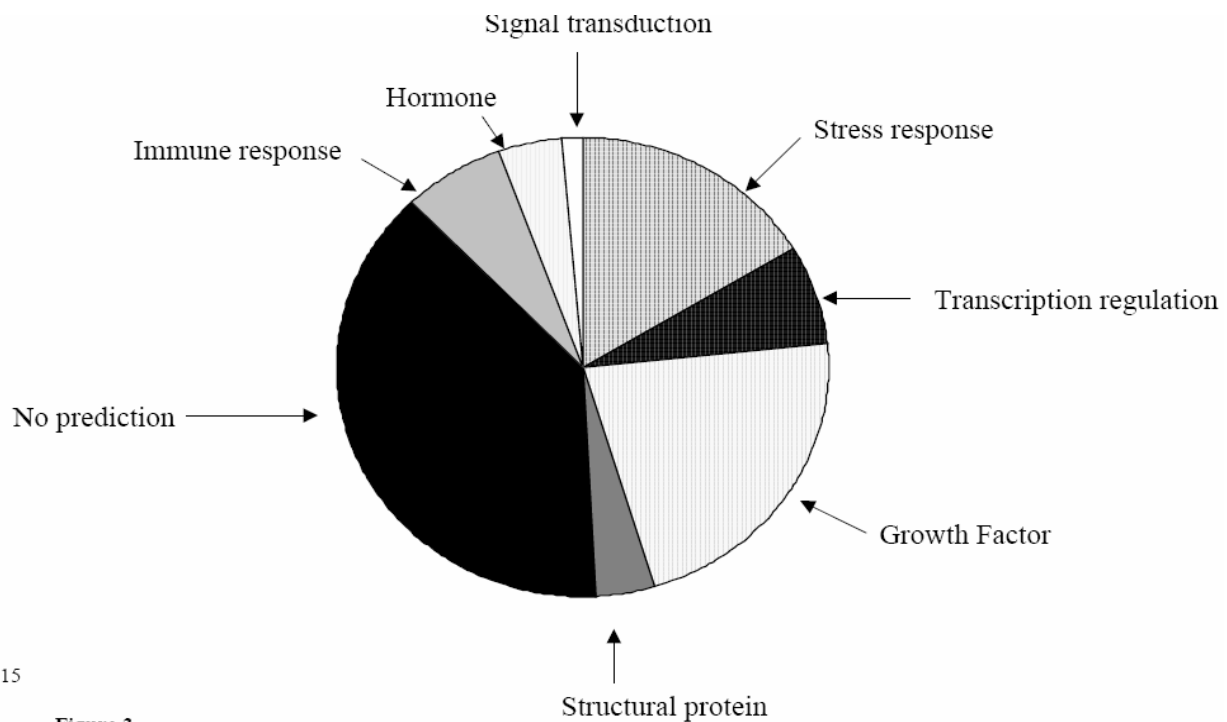
Figure 3: Chart showing the predicted ontology of the proteins identified in the eye swab proteome utilising ProteinFun (Jensen et al., 2003).

Comprehensive analysis of eye swab proteome

In this study we report the first gel based proteomic analysis of an inflamed dry eye specimen using a sterile cotton swab collected from the posterior lid and inferior conjunctival mucosa. This multidimensional analysis involved the eye swab proteome being first separated by one-dimensional gel electrophoresis. The resultant gel was then cut into 3 mm sections, each gel fraction was then trypsinized and the extracted peptides separated on a reversed phase C_{18} column over a 60 min time period prior to being introduced onto the mass spectrometer. This methodology allowed the identification of a total of 75 proteins (Table 1) from the eye swab utilising a false-discovery rate of 1% for protein detection.

As previously reported, (Graham et al., 2006) due to the complex nature of the peptide mixtures to be analysed, the separation capabilities of the LC-MS systems are often exceeded. In this study all peptide fractions were analysed in duplicate in order to increase overall peptide identifications. In the current study, automated curation of our initial dataset, by the heuristic bioinformatic tool PROVALT utilising random database searching (Weatherly et al., 2005) led to the identification of 86 proteins with further manual curation leading to the positive identification of 75 proteins. The average number of peptides per protein was 6 and the average MOWSE score was 357, with the minimum high quality peptide MOWSE score for this study set at 40.

The 75 identified proteins had a wide range of physiochemical properties in respect to pI and molecular mass (M_r) (Figure 1). This 2-D visualisation showed that the smallest protein identified was small proline rich protein 2a (M_r= 7965 Da). The largest protein identified was titin isoform N2-A (M_r= 3713727 Da). The most acidic protein identified was calmodulin-like skin protein (pI = 4.43) while the most basic was H4 histone family member J (pI = 11.36).

Of the 75 proteins detected in this study, functional roles for 72 proteins (96 %) were known or could be predicted from database analysis. Proteins within the eye swab proteome were assigned to functional categories utilizing the bioinformatics tool ProteinFun (Jensen et al., 2003). Figure 2 shows that the largest category of identified proteins was cell envelope (32.4 %), followed by those involved in translation (18.9 %), then those involved in amino acid biosynthesis (13.5 %). The remaining proteins were distributed amongst the other functional categories.

The gene ontology of this protein dataset could also be identified utilizing ProteinFun (Figure 3)(Jensen et al., 2003). Of the 75 identified proteins over 38% had no prediction for gene ontology; however, 22% were identified as growth factors, 16% as stress response proteins and 7% as immune response proteins.

The rapid increase in genomic data over the past decade has revealed many important aspects of cellular processes, however there are still a significant number of potential gene products for which we know nothing, save that they are classified as 'hypothetical proteins'. In previous work we have underlined the necessity to assign, where possible, an element of biological functionality to such gene products in order to develop both systems biology and our understanding of cellular processes within the system under investigation. Within the current study we have established the presence of three proteins that had previously been annotated as hypothetical conserved proteins (Table 1). The identification of such proteins establishes the biological functionality of these 'hypothetical' predicted protein coding sequences, and elegantly demonstrates the potential of proteomics to validate bioinformatics predictions.

Having established the presence of such proteins and wishing to understand how they contribute to functional processes we further examined them using NCBI BLASTp. Such an approach al-lows conserved domains within protein sequences to be identified and thereby enables a degree of inferred functionality. Using this methodology allowed us to assign putative function to one of these proteins, hypothetical protein LOC649897. This protein contained two IGc domains (CD00098) these are part of the immunoglobulin domain constant region subfamily; members of the IGc subfamily are components of immunoglobulins, T-cell receptors, CD1 cell surface glycoproteins, secretory glycoproteins A/C, and Major Histocompatibility Complex (MHC) class I/II molecules. This once again demonstrates the ability of proteomics to investigate and validate genomic analyses studies.

Sub-cellular protein localisation

Sub-cellular localization prediction tools have been used for many years to identify those proteins that are retained by and exported from cells. They may also have uses in identifying possible diagnostic and therapeutic targets as well providing information on the functionality of a protein (Gardy et al., 2005). In the current study a number of bioinformatics tools including WoLF_PSORT (Horton et al., 2007), SignalP (Bendsten et al., 2004) and SecretomeP (Bendsten et al., 2005) were utilized. These bioinformatics tools endeavour to assign a sub-cellular location for each protein. These tools use a set of descriptor rules and a variety of computational algorithms and networks to analyse a proteins' amino acid composition in an attempt to identify known motifs or cleavage sites.

All 75 proteins identified in this study were initially analysed using WoLF_PSORT; 20 were predicted to be cytoplasmic, 21 proteins were predicted to be extracellular, 18 were predicted to be nuclear with the remaining 16 having other sub-cellular localisations (Figure 4). These protein subsets were further analysed using SignalP, to predict amino-terminal signal peptides, and SecretomeP, which attempts to identify non-classically secreted proteins. Of those 20 proteins classified by WoLF_PSORT as being cytoplasmic, 15 were confirmed as non-secretory by this new analysis, while 5 were predicted to be potential secretory proteins (all non-classically secreted). Of the 21 proteins initially predicted by WoLF_PSORT to be extracellular all were predicted to be secreted, 20 had predicted signal peptide sequences, one was identified as non-classically secreted. Of the 18 proteins identified by initial analysis as nuclear, 13 were confirmed as non-secretory proteins, 4 were predicted to be non-classically secreted and one had no information available. Of those 16 proteins predicted to have localizations other than the three above, eight were predicted to be non-secretory, six were predicted to be secreted (5 non-classically) and two had no information available.

The 21 proteins identified, from the above analyses, as possessing an N-terminal signal peptide were grouped and analysed in order to ascertain whether they have the required domain architecture for eukaryotic signal peptides; namely an N-terminal region (n-region) that usually has a net positive charge, a hydrophobic core (h-region) containing six to fifteen hydrophobic amino acids and a C- terminal (c-region) which often contains helix breaking proline or glycine and small uncharged residues in position –3 and -1 (Table 2). As can be seen all the proteins predicted to contain signal peptides and therefore be secreted do indeed have the required architecture (Martoglio and Dobberstein, 1998).

Protein characterization

A goal of the current study was to ascertain the potential of this clinical sampling technique for the characterisation of dry eye syndrome proteome. As such it would be expected that our data set would incorporate proteins associated with tears and the ocular surface, whose functions would include roles in innate immunity, inflammation response and of course general cellular functions. Below we describe the categories of proteins detected in the current investigation outlining their cellular role and potential as biomarkers for dry eye syndrome.

NCBI-Code	Protein	Signal peptide
54607120	lactotransferrin	MKLVFLVLL FLG ALG LC LA
4502027	albumin precursor	MKWVT FISLLFLFSSAYS
4502337	alpha-2-glycoprotein 1, zinc	MVRMVPVLLSLLLLLGPAVP
4504963	lipocalin 1 precursor	MKPLLLAVSLGLIAALQA
4557894	lysozyme precursor	MKA LIVLGLVLLS VTVQG
4505171	lipophilin C (secretoglobin, family 2A, member 1)	M KL L M V L M LAALLL HCYA
31377806	polymeric immunoglobulin receptor	MLLFVLTCLLAVFPAIST
4505821	prolactin-induced protein	MRLLQLLFRASPATLLLVLCLQLGANKA
4503109	cystatin S precursor	MARPLCTLLLLMATLAGALA
15187164	lacritin precursor	MKFTTLLFLAAVAGALVYA
5729907	lipophilin A precursor	MRLSVCLLLLTLALCCYRANA
116805344	proline-rich, lacrimal 1	MKLTFFLGLLALISCFTPSES
4557321	apolipoprotein A-l preproprotein	MKAAVLTLAVLFLTGS QA
4557871	transferrin	MRLAV G A L L VC A VLG LC LA
4757826	beta-2-microglobulin precursor	M S R S V ALA VLA LLSLSGL EA
	deleted in malignant brain tumors 1 isoform b	MGISTVILEMCLLWGQVLS
6633801	precursor	
4503107	cystatin C precursor	MA G P L RA P LLLLAI LAVA LAVS P A A G
6005802	proline rich 4 (lacrimal)	M L LVLLSVVLLA LSSAQ S
21489959	immunoglobulin J chain	MKN H L L F WGVLA VFIKAVH VKA
53988378	mesothelin isoform 1 preprotein	MALPTARPLLGSCGTFALGSLLFLLFSLGWVQPSRTL
50363219	serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase. antitrypsin), member 1	MPSSVSWGILLLAG LCCLVPVSLA

Table 2: Proteins identified within the Eye swab proteome containing predicted export signals. Footnote: Putative signal peptides. The hydrophobic H-domain is coloured grey. The signal peptide cleavage sites are the last three aminoacid residues and are in bold. Positively charged amino acids are shown thus (K, R)

A number of cytoskeletal structural proteins could be identified including micro- and intermediate filaments (keratins, filaggrin, actin, titin); microtubules (tubulin); catenins (plakoglobin) and other cytoskeletal proteins (desmoplakin, myomegalin) (Table 1). Myomegalin is a novel protein that has been found to act as an anchor to localise components of the cAMP-dependent pathway to the Golgi/centrosomal region of the cell (Verde et al., 2001). Whilst keratin is known to be a contaminant in many proteomic investigations its presence here is not unexpected with Tsai et al. (2006) reporting some 10 keratin isoforms in their investigation of meibomian gland secretions. It is likely that the origin of keratin within the sample is the skin surrounding the eye, some of which will have been 'harvested' during eye swab preparation. A recent study of the tear fluid proteome by de Souza et al. (2006) identified all of these cytoskeletal structural proteins with the exception of keratin isoforms, filaggrin and myomegalin.

Four proline-rich proteins (PRPs) (Table 1) were also identified in this study. The biological importance of PRPs in the eye lies in their antimicrobial properties (Zhou et al., 2006). Fung et al. (2004) proposed that PRPs play a significant role in protection of the ocular surface and are involved in pathogenesis of inflammatory and autoimmune diseases; Grus et al. (2005) impli-cated PRPs in the modulation of the eye microflora. The significance of PRPs in eye health diagnosis was also highlighted by Grus and coworkers (Grus et al., 2005) who proposed that lacrial proline-rich protein 4, also detected in the current study, would be a useful biomarker for human lacrimal gland acinar cell function.

Within this investigation a number of calcium binding proteins were observed. These proteins are known to have a role in the innate immune response for example annexin is an important mediator of the anti-inflammatory actions of glucocorticoids, and calmodulin is also involved in inflammation response (Table 1) (Buckingham et al., 2006). In addition calprotectin (which is a complex of calgranulin A and B) was also identified; this myeloid-related protein complex is known to possess *in vitro* bacteriostatic and fungistatic properties and is found at high abundance in neutrophils, cells that are rapidly attracted to sites of inflammation (Herndon et al., 2003; Gaya and Mackenzie, 2002). Calprotectin is therefore a potential biomarker for a range of inflammatory disease states such as Crohns' disease (Gaya and Mackenzie, 2002). Indeed Grus et al. (2005) identified increased abundance of calgranulin A in dry eye patients and proposed this protein as a useful indicator for this condition.
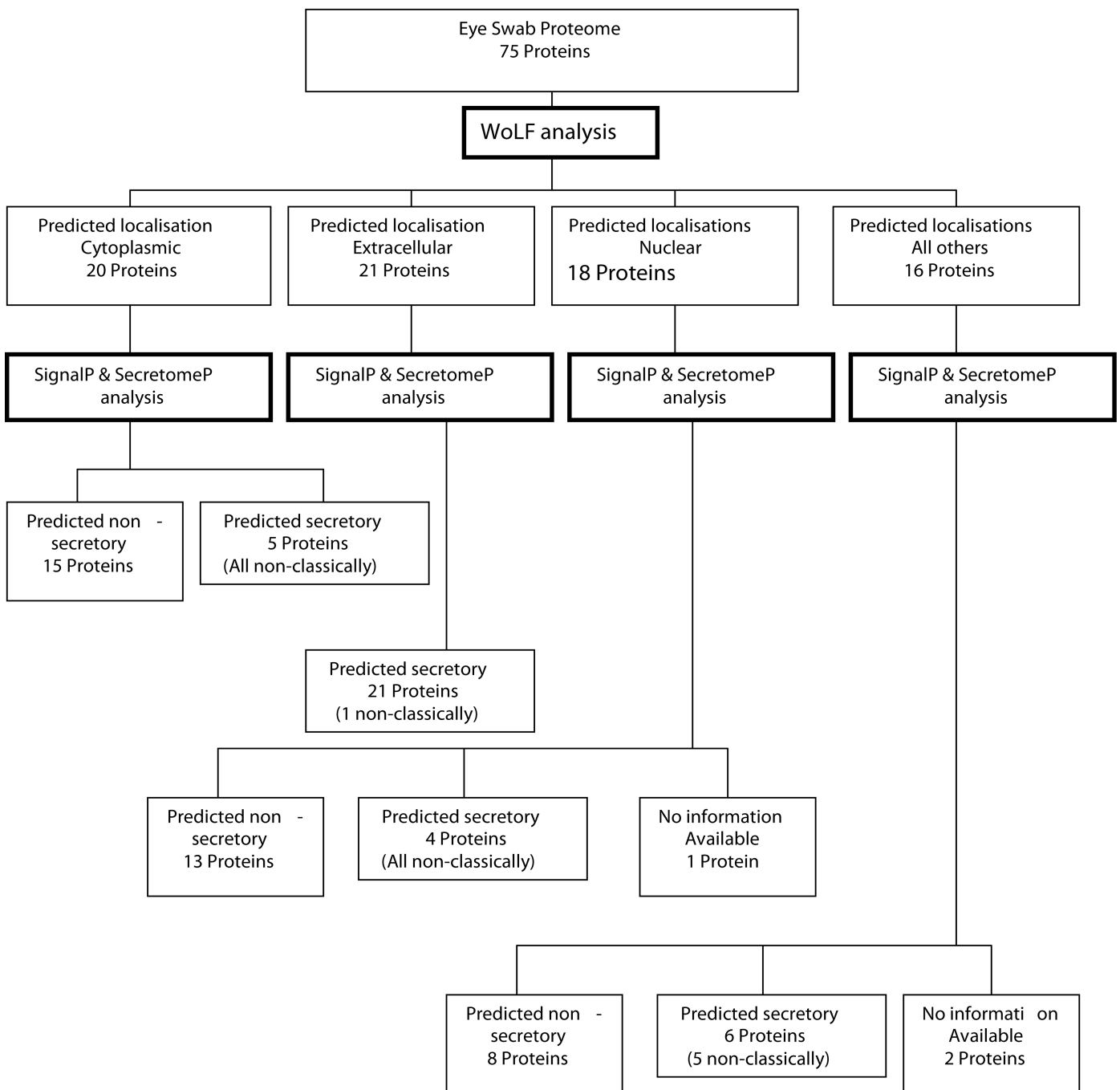
Figure 4: Overview of bioinformatics analysis of identified proteins from the eye swab proteome. Cellular localisation was predicted based upon the use of WoLF_PSORT (Horton et al., 2007), SignalP v3.0 (Bendsten et al., 2004) and SecretomeP v2.0 (Bendsten et al.,2004).

It has previously been shown (De Souza et al., 2006) that the proteome of tear fluid contains a number of proteins with protease inhibiting properties which was confirmed in our eye swab sample (Table 1). Such proteins included lipocalin 1 which in addition to its protease inhibiting activity, is known to bind a diverse range of molecules, functioning as a scavenger of physiologically damaging lipophilic ligands (Tsai et al., 2006). Cystatin C and S are extracellular proteins belonging to the family 2 cystatins that are ubiquitous in all human secretions and thought to be part of a non-immune protective system, functioning by inhibition of extra- or intracellular microbial cysteine proteases (Jasir et al., 2003).

Three serine proteinase inhibitors (SERPINs) were also identified in this study. SERPINA1 (alpha 1 antitrypsin) controls the activity of a diverse range of proteolytic enzymes and plays an important role in infection control by inactivating enzymes activated by bacteria and has been shown to play a role in regulating immune response by inhibiting the migration and transformation of lymphocytes (Sen et al., 1988). SERPINB3 and SERPINB5 are members of the Ov-serpins and have been shown to inhibit a number of proteinases. Their presence in epithelial cells is suggestive of a role in barrier/host defence against microbial or viral proteinases (Silverman et al., 2001).

It has been reported that 35-45% of normal human tears is comprised of a number of proteins with antimicrobial properties that form part of the innate immune response including lysozyme, lactotransferrin, transferrin, and IgA (Lehrer et al., 1998). Lysozyme is an enzyme present in human neutrophils, whose bacteriocidal activity is precipitated via the degradation of peptidoglycan, a major component of bacterial cell walls (Laible and Germaine, 1985). Transferrin and lactotransferrin interfere in microbial growth by effectively binding iron (Arnold et al., 1977; Arnold et al., 1982). Lactotransferrin is highly abundant in granules of human neutrophils and secretions from epithelial cells and can also be directly microbiocidal (Arnold et al., 1982). Both lactotransferrin and lysozyme are known to function as opsonins, effectively 'marking' the bacterial cells for phagocytosis by white blood cells (Jenssen, 2005). Lactotransferrin and lysozyme have also been shown to have synergistic activitiy (Travis et al., 1999). Secretory IgA is considered the first line of immune defense (Kaetzel et al., 1991) and has been reported to be present in those few tear proteomic investigations carried out to date (Zhou et al., 2006; Tsai et al., 2006). It is surprising therefore that IgA was not detected in the current study, however the polymeric immunoglobulin receptor (pIgR) which mediates the transfer of secretory IgA into external fluids was identified. In addition IgJ, the synthesis of which is critical for primary immune response, due to its function in assembling both IgA and IgM, was also present (Matsuuchi et al., 1986; Iwata et al., 2002). A possible explanation for our failure to detect IgA may be explained by the dimeric/polymeric nature of IgA during the immune response leading to the formation of a very high molecular weight protein that may be intractable by our gel-based method of analysis (Stubbe et al., 2000).

Also present within the swab sample was lipophilin A and C which are known to form a heterodimeric molecule *in vivo*. Whilst lipophilin C has been reported to have weak antimicrobial properties none have been recorded so far for lipophilin A. Indeed at present the role of this lipophilin heterodimer is unknown however it is part of the uteroglobin superfamily and has been found to be capable of transporting steroid and other nonpolar molecules. The ubiquity of lipophilins within human secretions suggests that they are likely to serve important functions in many different tissues and organs (Lehrer et al., 1998; Lehrer et al., 2000). A final protein detected in the swab that possessed antimicrobial properties was prolactin-induced protein (PIP) which binds to and thereby inhibits the growth of certain bacteria, such as *Streptococcus* (Schenkels et al., 1997). In addition to the antimicrobial role of PIP in secreted fluids it is also known to be a potent inhibitor of T lymphocyte programmed cell death induced by crosslinking of CD4 and T cell receptor (Gaubin et al., 1999; Kitano et al., 2006).

A number of proteins associated with inflammation and other disease processes were found (Table 1; apolipoprotein A1 (Burger and Dayer, 2002); alpha 2 glycoprotein 1 (Hale and Price, 2001); and DMBT1 (Mollenhauer et al., 2000). The protein lacritin, which was also detected is normally found in tear films and is known to stimulate new tear production but has also been shown to be deficient in dry eye syndrome and so may act as a useful biomarker for this disease (Koo et al., 2005).

Concluding Remarks

This proteomic study of a dry eye patient demonstrates the variety of known and unknown proteins/peptides present in this pathological state. This study highlights the possibility of using a simple non-invasive clinical swab technique in these patients rather than more complex clinical testing procedures in order to investigate dry eye syndrome and potentially other disease states of the eye. Further research is required to fully characterise relationships between clinical signs/symptoms and the proteome. The determination of protein changes which imply pathology and/or which are harbingers of pathology will enable focussed therapeutic treatments and may enable prophylaxis against dry eye conditions.

References

1. Abe T, Nakajima A, Matsunaga M, Sakuragi S, Komatsu M (1999) Decreased tear lactoferrin concentration in patients with chronic hepatitis C. Br J Ophthalmol 83: 684-687. » CrossRef » Pubmed » Google Scholar

2. Anshu, Munshi MM, Sathe V, Ganar A (2001) Conjunctival impression cytology in contact lens wearers. Cytopathology 12: 314-320. » CrossRef » Pubmed » Google Scholar

3. Arnold RR, Cole MF, McGhee JR (1977) A bactericidal effect for human lactoferrin. Science 197: 263-265. » CrossRef » Pubmed » Google Scholar

4. Arnold RR, Russell JE, Champion WJ, Brewer M, Gauthier JJ (1982) Bactericidal activity of human lactoferrin: differentiation from the stasis of iron deprivation. Infect Immun 35: 792-799. » CrossRef » Pubmed » Google Scholar

5. Bendtsen JD, Nielsen H, von Heijne G, Brunak S (2004) Improved prediction of signal peptides: SignalP 3.0. J Mol Biol 340: 783-795. » CrossRef » Pubmed » Google Scholar

6. Bendtsen JD, Kiemer L, Fausbøll A, Brunak S (2005) Non-classical protein secretion in bacteria. BMC Microbiol 5: 8. » CrossRef » Pubmed » Google Scholar

7. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 7: 248-254. » CrossRef » Pubmed » Google Scholar

8. Buckingham JC, John CD, Solito E, Tierney T, Flower RJ, et al. (2006) Annexin 1, glucocorticoids, and the neuroendocrine-immune interface. Ann N Y Acad Sci 1088: 396-409. » CrossRef » Pubmed » Google Scholar

9. Burger D, Dayer JM (2002) High-density lipoprotein-associated apolipoprotein A-I: the missing link between infection and chronic inflammation? Autoimmun Rev 1: 111-117. » CrossRef » Pubmed » Google Scholar

10. Cullor JS, Mannis MJ, Murphy CJ, Smith WL, Selsted ME, et al. (1990) In vitro antimicrobial activity of defensins against ocular pathogens. Arch Ophthalmol 108: 861-864. » CrossRef » Pubmed » Google Scholar

11. De Souza A, Godoy LMF, Mann M (2006) Identification of 491 proteins in the tear fluid proteome reveals a large number of proteases and protease inhibitors. Genome Biol 7: R72. » CrossRef » Pubmed » Google Scholar

12. Foulks GN, Bron AJ (2003) Meibomian gland dysfunction: A clinical scheme for description, diagnosis, classification and grading. Ocul Surf 1: 107-126. » CrossRef » Pubmed » Google Scholar

13. Fung KYC, Morris C, Sathe S, Sack R, Duncan MW (2004) Charachterization of the *in vivo* forms of lacramil-specific praline-rich proteins in human tear fluid. Proteomics 4: 3953-3959. » CrossRef » Pubmed » Google Scholar

14. Gardy JL, Laird MR, Chen F, Rey S, Walsh CJ, et al. (2005) PSORTb v.2.0: expanded prediction of bacterial protein subcellular localization and insights gained from comparative proteome analysis. Bioinformatics 21: 617-623. » CrossRef » Pubmed » Google Scholar

15. Gaubin M, Autiero M, Basmaciogullari S, Métivier D, Misëhal Z, et al. (1999) Potent inhibition of CD4/TCR-mediated T cell apoptosis by CD4-binding glycoprotein secreted from breast tumor and seminal vesicle cells. J Immunol 162: 2631-2638. » CrossRef » Pubmed » Google Scholar

16. Gaya DR, Mackenzie JF (2002) Faecal calprotectin: a bright future for assessing disease activity in Crohn's disease. Q J Med 95: 557-558. » CrossRef » Pubmed » Google Scholar

17. Glasson M, Stapleton F, Willcox M (2002) Lipid, lipase and lipocalin differences between tolerant and intolerant contact lens wearers. Curr Eye Res 25: 227-235. » CrossRef » Pubmed » Google Scholar

18. Graham RLJ, O Loughlin SN, Pollock CE, Ternan NG, Weatherly DB, et al. (2006) A Combined Shotgun and Multidimensional Proteomic Analysis of the Insoluble Subproteome of the Obligate Thermophile, Geobacillus thermoleovorans T80. J Proteome Res 5: 2465-2473. » CrossRef » Pubmed » Google Scholar

19. Grus FH, Podust VN, Bruns K, Lackner K, Fu S, et al. (2005) SELDI-TOF-MS ProteinChip Array Profiling of Tears from Patients with Dry Eye. Inv. Ophthal. Vis Sci 46: 863-876. » CrossRef » Pubmed » Google Scholar

20. Hale LP, Price DT (2001) Zinc alpha-2-glycoprotein as indicator of cancer. US Patent 20020155514. » CrossRef » Pubmed » Google Scholar

21. Hamano H, Hori M, Hamano T, Mitsunaga S, Maeshima J, et al. (1983) A new method for measuring tears. CLAO J 9: 281-289. » Pubmed » Google Scholar

22. Haynes RJ, Tighe PJ, Dua HS (1998) Innate defence of the eye by antimicrobial defensin peptides. Lancet 352: 451-452. » Pubmed » Google Scholar

23. Haynes RJ, Tighe PJ, Dua HS (1999) Antimicrobial defensin peptides of the human ocular surface. Br J Ophthalmol 83: 737-741. » CrossRef » Pubmed » Google Scholar

24. Herndon BL, Abbasi S, Bennett D, Bamberger D (2003) Calcium-binding proteins MRP 8 and 14 in a Staphylococcus aureus infection model: Role of therapy, inflammation, and infection persistence. J Lab Clin Med 141: 110-120. » CrossRef » Pubmed » Google Scholar

25. Horton P, Park KJ, Obayashi T, Fujita N, Harada H, et al. (2007) WoLF PSORT: Protein Localization Predictor. Nucleic Acids Res 35: W585-W587. » CrossRef » Pubmed » Google Scholar

26. Iwata A, Iwase T, Ogura Y, Takahashi T, Matsumoto N, et al. (2002) Cloning and expression of the turtle (*Trachemys scipta*) immunoglobulin joining (J) – chain cDNA. Immunogentics 54: 513-519. » CrossRef » Pubmed » Google Scholar

27. Jasir A, Kasprzykowski F, Kasprzykowska R, Lindström V, Schalen C, et al. (2003) New antimicrobial cystatin C-based peptide active against gram-positive bacterial pathogens, including methicillin –resistant Staphylococcus aureus and multiresistant coagulase-negative staphylococci. APMIS 111: 1004-1010. » CrossRef » Pubmed » Google Scholar

28. Jensen LJ, Stærfeldt HH, Brunak S (2003) Prediction of human protein function according to Gene Ontology categories. Bioinformatics 19: 635-642. » CrossRef » Pubmed » Google Scholar

29. Jenssen H (2005) Anti herpes simplex virus activity of lactoferrin/lactoferricin – an example of antiviral activity of antimicrobial protein/peptide. Cell Mol Life Sci 62: 3002-3013. » CrossRef » Pubmed » Google Scholar

30. Kaetzel CS, Robinson JK, Chintalacharuvu KR, Vaerman JP, Lamm ME (1991) The polymeric immunoglobulin receptor (secretory component) mediates transport of immune complexes across epithelial cells: a local defense function for IgA Proc Natl Acad Sci USA 88: 8796-8800. » CrossRef » Pubmed » Google Scholar

31. Kitano T, Tian W, Umetsu K, Yuasa I, Yamazaki K, et al. (2006) Origin and volution of gene for prolactin-induced protein. Gene 383: 64-70. » CrossRef » Pubmed » Google Scholar

32. Kojima T, Ishida R, Dogru M, Goto E, Takano Y, et al. (2004) A new non-invasive tear stability analysis system for the assessment of dry eyes. Invest. Ophthalmol Vis Sci 45: 1369-1374. » CrossRef » Google Scholar

33. Koo BS, Lee DY, Ha HS, Kim JC, Kim CW (2005) Comparative analysis of the tear protein expression in blepharitis patients using two-dimensional electrophoresis. J Proteome Res 4: 719-724. » CrossRef » Pubmed » Google Scholar

34. Laible NJ, Germaine GR (1985) Bactericidal activity of human lysozyme, muramidase-inactive lysozyme, and cationic polypeptides against *Streptococcus sanguis* and *Streptococcus faecalis*: inhibition by chitin oligosaccharides. Infect Immun 48: 720-728. » CrossRef » Pubmed » Google Scholar

35. Lehrer RI, Xu G, Abduragimov A, Dinh NN, Qu XD, et al. (1998) Lipophilin, a novel heterodimeric protein of human tears. FEBS Lett 432: 163-167. » CrossRef » Pubmed » Google Scholar

36. Lehrer RI, Nguyen T, Zhao C, Ha CX, Glasgow BJ (2000) Secretory Lipophilins: A tale of two species Ann N Y Acad Sci 923: 59-67. » CrossRef » Pubmed » Google Scholar

37. Martoglio B, Dobberstein B (1998) Signal sequences: more than just greasy peptides. Trends Cell Biol 8: 410-415. » CrossRef » Pubmed » Google Scholar

38. Matsuuchi L, Cann GM, Koshland ME (1986) Immunoglobulin J chain gene from the mouse Proc Natl Acad Sci USA 83: 456-460. » CrossRef » Pubmed » Google Scholar

39. McMonnies CW (1986) Key questions in a dry eye history. J Am Optom Assoc 57: 512-517. » CrossRef » Pubmed » Google Scholar

40. Mollenhauer J, Herbertz S, Holmskov U, Tolnay M, Krebs I, et al. (2000) DMBT1 encodes a protein involved in the immune defence and in epithelial differentiation and is highly unstable in cancer. Cancer Res 60: 1704-1710. » CrossRef » Pubmed » Google Scholar

41. Ohashi Y, Ishida R, Kojima T, Goto E, Matsumoto Y, et al. (2003) Abnormal protein profiles in tears with dry eye syndrome. Am J Ophthalmol 136: 291-299. » CrossRef » Pubmed » Google Scholar

42. O'Callaghan RJ, Girgis DO, Dao JJ, Sloop GD (2003) Host

defense against bacterial keratitis. Ocul Immunol Inflamm 11: 171-181. » CrossRef » Pubmed » Google Scholar

43. Rolando M, Zierhut M (2001) The ocular surface and tear film and their dysfunction in dry eye disease. Surv Ophthalmol 45: S203-210. » CrossRef » Pubmed » Google Scholar

44. Saini JS, Rajwanshi A, Dhar S (1990) Clinicopathological correlation of hard contact lens related changes in tarsal conjunctiva by impression cytology. Acta Ophthalmol 68: 65-70. » Pubmed » Google Scholar

45. Schenkels LC, Walgreen-Weterings E, Oomen LC, Bolscher JG, Veerman EC, et al. (1997) In vivo binding of the salivary glycoprotein EP-GP (identical to GCDFP-15) to oral and non-oral bacteria detection and identification of EP-GP binding species. Biol Chem 378: 83-88. » Pubmed » Google Scholar

46. Schoenwald RD, Vidvauns S, Wurster DE, Barfknecht CF (1998) The role of tear proteins in tear film stability in the dry eye patient and in the rabbit. Adv Exp Med Biol 438: 391-400. » CrossRef » Pubmed » Google Scholar

47. Seal DV, McGill JI, Mackie IA, Liakos GM, Jacobs P, et al. (1986) Bacteriology and tear protein profiles of the dry eye. Br J Ophthalmol 70: 122-125. » CrossRef » Pubmed » Google Scholar

48. Sen D, Sarin G, Mathur MD (1988) Alpha-1 antitrypsin and serum levels in tear fluid of healthy subjects and in persons with conjunctival diseases. Indian J Opthalmol 36: 22-26. » CrossRef » Pubmed » Google Scholar

49. Silverman GA, Bird PL, Carrell RW, Church FC, Coughlin PB, et al. (2001) The Serpins are an expanding superfamily of structurally similar but functionally diverse proteins. J Biol Chem 276: 33293-33296. » CrossRef » Pubmed » Google Scholar

50. Stern ME, Beurerman RW, Fox RI, Mircheff AK, Pflugfelder SC (1998) The pathology of dry eye: the interaction between the ocular surface and lacramil glands. Cornea 17: 584-589. » CrossRef » Pubmed » Google Scholar

51. Stubbe H, Berdoz J, Kraehenbuhl JP, Corthésy B (2000) Polymeric IgA is superior to monomeric IgA and IgG carrying the same variable domain in preventing *Clostridium difficile* toxin A damaging of T84 monolayers. J Immunol 164: 1952-1960. » CrossRef » Pubmed » Google Scholar

52. Travis SM, Conway BA, Zabner J, Smith JJ, Anderson NN, et al. (1999) Activity of abundant antimicrobials of the human airway. Am J Respir Cell Mol Biol 20: 872-879. » CrossRef » Pubmed » Google Scholar

53. Tsai PS, Evans JE, Green KM, Sullivan RM, Schaumberg DA, et al. (2006) Proteomic analysis of human meibomian gland secretions. Br J Ophthalmol 90: 372-377. » CrossRef » Pubmed » Google Scholar

54. Verde I, Pahlke G, Salanova M, Zhang G, Wang S, et al. (2001) Myomegalin is a novel protein of the Golgi/Centrosome that interacts with a cyclic nucleotide phosphodiesterase. J Biol Chem 276: 11189-11198. » CrossRef » Pubmed » Google Scholar

55. Weatherly DB, Atwood JA III, Minning TA, Cavola C, Tarleton RL, et al. (2005) A heuristic method for assigning a false-discovery rate for protein identifications from mascot database search results. Mol Cell Proteomics 4: 762-772. » CrossRef » Pubmed » Google Scholar

56. Zhou L, Beuerman RW, Foo Y, Liu S, Ang LPK, et al. (2006) Characterisation of Human Tear Proteins Using High-resolution Mass Spectrometry. Ann Acad Med Singapore 35: 400-407. » CrossRef » Pubmed » Google Scholar