RESEARCH ARTICLE

# Dog Tear Film Proteome In-Depth Analysis

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## Abstract

In this study, mass spectrometry was used to explore the canine tear proteome. Tear samples were obtained from six healthy dogs, and one-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (1D SDS-PAGE) was used as a first step to separate intact proteins into 17 bands. Each fraction was then trypsin digested and analysed by matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry (MALDI-TOF-MS/MS) to characterize the protein components in each fraction. In total, 125 tear proteins were identified, with MCA (Major Canine Allergen), Serum albumin, UPF0557 protein C10orf119 homolog, Collagen alpha-2(I) chain, Tyrosine -protein kinase Fer, Keratine type II cytoskeletal, Beta-crystallin B2, Interleukin-6 and Desmin occuring as the most confident ones with the highest scores. The results showed that the proteomic strategy used in this study was successful in the analysis of the dog tear proteome. To the best of our knowledge, this study is the first to report the comprehensive proteome profile of tears from healthy dogs by 1D SDS PAGE and MALDI-TOF. Data are available via ProteomeXchange with identifier PXD003124.

## Introduction

Proteome is a set of proteins expressed in a given time by a given tissue. Its name comes from a blend of proteins and genome. Proteomic analysis has become an important tool in biomedical and veterinary research [1,2,3]. The tear film covering the surface of the eye is a complex body fluid containing thousands of molecules with different structures and functions [3–7]. A molecular analysis of tear film composition is a useful source of information for the diagnosis, prognosis and treatment of diseases of the eye, as well as systemic diseases in humans [8–11]. In addition to its clinical utility, the identification of biomarkers in tear film may be useful in developing new pharmacologically active molecules and diagnostic tests [12–14]. Currently, few publications in the proteomics literature have evaluated the tear film of animals, especially dogs can be of particular interest, as they live in the same conditions and often suffer from diseases of similar aetiopathogenesis [15–17]. Despite well-developed veterinary ophthalmology research concerning dogs, reports on molecular studies of the tear film remains sparse, and in-

depth analyses of the protein composition of normal tear film is lacking. Most of the information related to protein profiles was obtained using less-accurate analytical methods [18]. Therefore, a systematic study applying the most advanced proteomic technology should begin with an analysis of the normal tear film protein profile of healthy subjects. This project introduces population studies to determine the correct levels of important tear film proteins in healthy individuals similar to that of haematological standards. The aim of this study was to examine the proteome profile of dog tear samples through one-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (1D SDS-PAGE) in combination with matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry (MALDI-TOF-MS/MS).

## Materials and Methods

Tear samples were collected from 6 healthy dogs using a special standard Schirmer's strip without local anaesthesia. Dogs of various breeds (2 German Shepherds, 1 Doberman, 1 Labrador and 2 mixed breeds) with ages ranging from 2 to 6 years were enrolled during routine admissions to clinics of the Faculty of Veterinary Medicine at the University of Life Sciences in Lublin. Informed consent was obtained from the owners prior to the clinical investigations and sample collection. Every animal used in this study was submitted to a comprehensive ophthalmic examination (anterior segment and fundus evaluation with introcular pressure measurement). Animals included in the study did not exhibit any ocular signs of disease. The exclusion criteria included the presence or history of any systemic or ocular disorder or condition (including ocular surgery, trauma, and disease) that could possibly interfere with the interpretation of the results. The current or recent use of topical ophthalmic or systemic medications that could affect tear status was also grounds for exclusion from this study. The results from blood-cell counts, sera biochemistry and urinalyses oscillated within the normal range. After collection, the Schirmer's strips were placed in elution buffer consisting of 50 mM phosphate-buffered saline (PBS) with protease inhibitors at 4°C for a maximum of 20 h. The total protein concentration was determined by Bradford's method at a wavelength of 280 nm (Picodrop, Cambridge, UK). The resulting protein solution was concentrated using SpeedVac at -4°C to a final protein concentration of 60 µg/10 µl.

### Electrophoresis

The protein samples were reduced in dithiothreitol (DTT) (Invitrogen, Carlsbad, CA, USA, cat. no. NP0004), and after mixing with loading buffer (Invitrogen, cat. no. NP0007) and heating to 70°C for 10 minutes, each sample containing 60 µg protein was loaded into a well and subjected to SDS-PAGE analysis using commercial 12% polyacrylamide gel (Invitrogen, NuPAGE® Novex® 12% Bis-Tris). Samples were electrophoresed at 150 V/50 mA/7.5 W until the stain reached 0.5 cm from the edge of the gel. Standard molecular weight markers ranging from 7.1 kDa to 209 kDa were run at the same time. Protein bands were detected by Coomassie Colloidal Blue staining according to the manufacturer's protocol (Novex, cat. no. LC 6025). In the next step, the lanes were divided into 17 bands, which were excised (Fig 1). Bands of the same molecular mass originating from 6 individual dogs were pooled. The bands that were cut from the 1D gel underwent washing followed by reduction and alkylation using DTT and iodoacetamide. Digestion with trypsin occurred in 50 mM ammonium bicarbonate buffer at 37°C for 12 hours (Promega, Trypsin Gold, Mass Spectrometry Grade, Technical Bulletin). The obtained peptides were sequentially eluted from the gel using a solution of 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50% acetonitrile in 5% trifluoroacetic acid (TFA) (v/v). The extracted peptides were purified using µ C18 Zip-TIP pipette tips in accordance with the
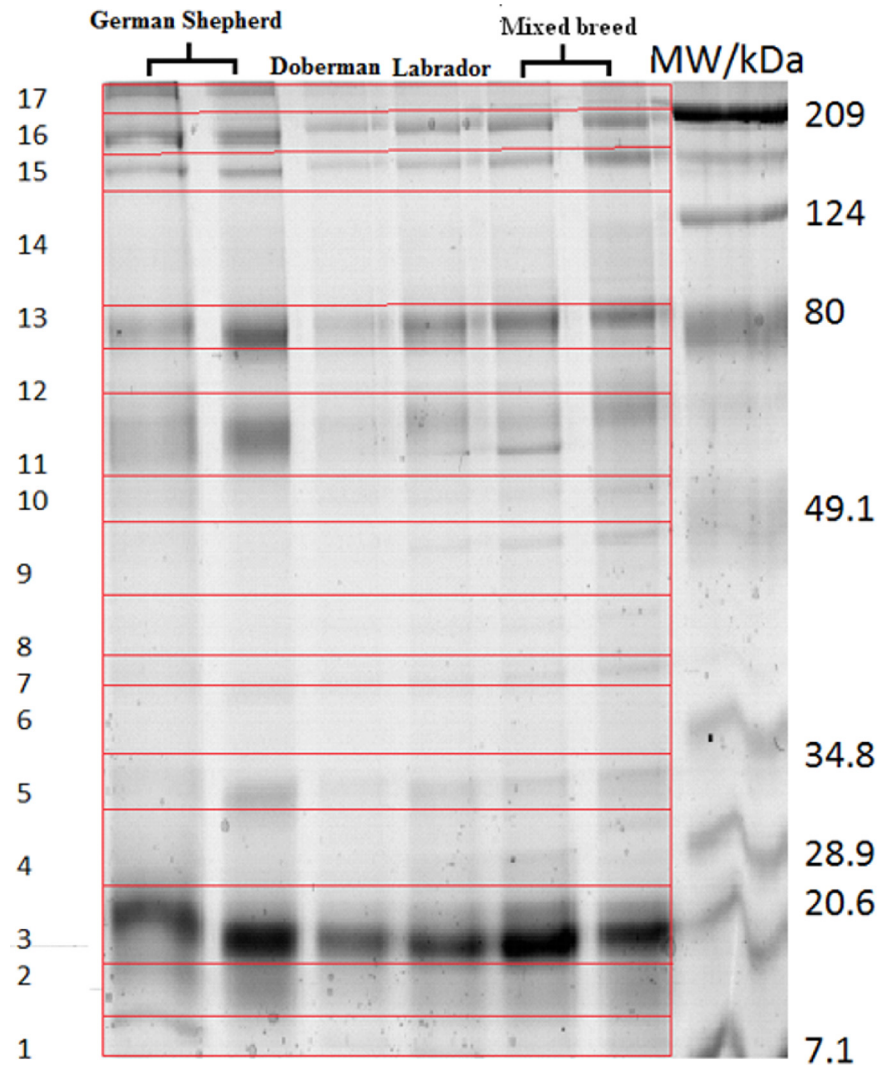
**Fig 1. 1D SDS–PAGE of Coomassie-stained proteins.** Lanes 1–6 are 20 µg of total tear film protein from individual dogs precipitated from tear film collected by a Schirmer strip. Lane 7 is the molecular weight marker.

manufacturer's procedure (Merck Chemicals, Billerica, MA, USA, PR 02358, Technical Note) and applied to the plate MTP AnchorChip 384 (Bruker, Bremen, Germany).

## Mass spectrometry

MALDI was used as a soft ionization method because it only produces a charge and does not cause fragmentation of the analysed compound. The experiment was conducted in an ultra-fleXtreme (Bruker) machine with a TOF/TOF detector to guarantee high accuracy and resolution of the measurements. All of the spectra were collected within the 800–3500 Da range in the active reflection mode, and this mass range was used to acquire the MS/MS spectra. HCCA (alpha-cyano-4-hydroxycinnamic acid, portioned; Bruker) was used as the matrix in the dried droplet method (0.5 µl sample + 0.5 µl matrix) following the standard manufacturer's protocol for peptide analysis. An MTP AnchorChip 384 (Bruker) with hydrophilic spots was used as the holder for sample preparation. Each sample was spotted onto 3 different active spots, and the

profiled spectra were calibrated using the peptide mixture Peptide Calibration Standard I (Bruker). The flexControl program 3.3 (version 108) was used for mass spectra collection, flexAnalysis 3.3 (version 80) was used for analysis, and finally, SwissProt database was searched using the software BioTools 3.2 (version 4.48). All spectra were systematically processed as follows: smoothing was performed by the Savitsky-Golay method; baseline subtraction was performed by the Top Hat baseline algorithm; peak geometry was characterized by the Stanford Network Analysis Platform (SNAP) algorithm; and all peaks with a signal ratio above 4 were qualified for further analysis. The parameters for the Mascot database search were as follows: errors in both MS and MS/MS mode at 0.3 Da [19]; global modification of carbamidomethyl (C); possible modification and oxidation (M) [20]; partials at 1; and trypsin enzyme. Spectra with peptide matches above 5 peaks were considered statistically significant, and only 5 proteins were identified with a single peptide match. All of the peptide mass fingerprint spectra were analysed again in MS/MS mode to confirm their exact amino acid sequence.

## Results and Discussion

For over two decades tear film has become an intesivly investigated material due to its assets, like ease to obtain and handle, unlike the other body fluids, ie plasma. The greatest limitation is yet to be the small volume of sample, and low protein concentration. In veterinary field, Hemsley et al were one of the first to investigate tear film for certain proteins, and succeeded to find 6 reproducible HPLC protein peaks in dogs, coming to conclusion that they do not correspond in all respects to human tears [18]. With the development of the proteomic approaches like MALDI-TOF, new opportunities arised [21]. De Freitas et al. performed 2-D electrophoresis analysis combined with MALDI-TOF protein identification to find potential cancer biomarkers in dog tear film. They identified some most abundant proteins like MAC, and pointed at albumin and actin elevated levels in dogs with cancer [16]. However there has been no exact identification of each protein present in the electrophoresis, and the samples were collected using microcapillaries, contrary to our Schirmer strips which has been proved to provide more proteins into analysis [22]. Differences with human proteome, similar to those described by de Freitas, like absence of zinc-alpha-2-glycoprotein, were also observed in our study.

MS has become the detection method of choice in proteomics analysis. The majority of studies use a bottom-up approach in which proteins are proteolytically digested into peptides and then subjected to multidimensional protein identification technology. Acquired MS (peptide masses) and MS/MS (sequence information) spectra are used to identify the corresponding proteins via database search algorithms [14]. Based on this proteomics approach, normal human tear fluid was observed to contain almost 500 proteins, although the recent work of Zhou et al. reported that the total number of proteins can reach over 1500 [21]. In our study, we separated intact tear proteins by 1D SDS-PAGE prior to detection using the MALDI-TOF technique. A total protein amount of 60 µg was loaded in each lane of the gel to standardize the sample and ensure that the differences noted in the gel patterns were caused by differences in the presence/absence of proteins rather than other reasons. From the 17 bands, a total of 125 distinct/unique proteins were identified (Tables 1 and 2). Several proteins were observed multiple times in different molecular weight regions of the gel. For example, desmin has a molecular weight of 53 kDa, but it was also observed in bands 15, 16 and 17 between approximately 140 and 210 kDa. Such multiple appearances most likely represent posttranslational modifications or the formation of homopolymers (e.g., dimers, trimers, and multimers of a protein) of lower molecular weight proteins, although they could also represent protein complexes that were not denatured. Higher molecular weight proteins were also observed at lower molecular weight regions in the gel; for example, the collagen alpha-2(I) chain was observed in band 13 at

**Table 1. Summary of protein identification.**

| No. | Protein | Accession no. | Score | Matches | Gene name | Molecular function | Biological process |
|---|---|---|---|---|---|---|---|
| 1 | Tuftelin-interacting protein 11 | Q29RR5 | 35 | 12 | TFIP11 | DNA binding | mRNA processing |
| 2 | Selenocysteine insertion sequence-binding protein 2-like | Q93073 | 34 | 4 | SECISBP2L | RNA binding | unclassified |
| 3 | Visual system homeobox 2 | P58304 | 51 | 6 | VSX2 | DNA binding | transcription |
| 4 | Tensin-1 | Q9HBL0 | 61 | 11 | TNS1 | RNA binding | unclassified |
| 5 | Zinc finger protein 780A | O75290 | 34 | 4 | ZNF780A | DNA binding | transcription |
| 6 | Putative homeodomain transcription factor 1 | Q9UMS5 | 32 | 5 | PHTF1 | DNA binding | transcription |
| 7 | Transcription factor TFIIIB component B'' homologue | A6H8Y1 | 40 | 14 | BDP1 | DNA binding | transcription |
| 8 | Hepatoma-derived growth factor | Q8VHK7 | 37 | 5 | Hdgf | DNA binding | transcription |
| 9 | Metastasis-associated protein MTA3 | Q924K8 | 32 | 9 | Mta3 | DNA binding | cell cycle |
| 10 | Nuclear protein 14 | Q8R3N1 | 33 | 13 | Nop14 | RNA binding | rRNA processing |
| 11 | Zinc finger protein 582 | Q96NG8 | 34 | 9 | ZNF582 | DNA binding | transcription |
| 12 | Zinc finger protein 2 | P08043 | 61 | 6 | Zfp2 | DNA binding | transcription |
| 13 | Eukaryotic translation initiation factor 3 subunit H | Q91WK2 | 54 | 7 | Eif3h | RNA binding | protein biosynthesis |
| 14 | 78 kDa glucose-regulated protein//Heat shock 70 kDa protein 5 | Q0VCX2 | 62 | 10 | HSPA5 | nucleotide binding | unclassified |
| 15 | Splicing factor, proline- and glutamine-rich | P23246 | 37 | 4 | SFPQ | nucleotide binding | unclassified |
| 16 | Putative fidgetin-like protein 2 | A6NMB9 | 41 | 4 | FIGNL2 | nucleotide binding | unclassified |
| 17 | UPF0557 protein C10orf119 homolog | A5PJM5 | 51 | 10 | MCMBP | chromatin binding | cell cycle |
| 18 | Guanine nucleotide-binding protein G(t) subunit alpha-2 | P38400 | 30 | 5 | GNAI2 | transducer | cell cycle |
| 19 | Protein Spindly | Q08DR9 | 54 | 13 | SPDL1 | kinetochore binding | cell cycle |
| 20 | Cell division cycle protein 27 homolog | P30260 | 41 | 7 | CDC27 | phosphatase binding | cell cycle |
| 21 | Kinesin-like protein KIF11 | P52732 | 55 | 9 | KIF11 | motor protein | cell cycle |
| 22 | Parafibromin | Q6P1J9 | 44 | 5 | CDC73 | RNA polymerase binding | cell cycle |
| 23 | G2/mitotic-specific cyclin-B3 | Q659K0 | 19 | 5 | CCNB3 | cycline | cell cycle |
| 24 | Transcription factor 4 | P15881 | 28 | 4 | TCF4 | activator | transcription |
| 25 | Cysteinyl-tRNA synthetase, cytoplasmic//Cysteine—tRNA ligase, cytoplasmic | Q9ER72 | 38 MS/MS | 10 | Cars | ligase | protein biosynthesis |
| 26 | Pinin | P79149 | 30 | 4 | PNN | activator | transcription |
| 27 | C-C motif chemokine 25 | Q68A93 | 47 | 4 | CCL25 | cytokine | inflammatory response |
| 28 | Interleukin-18 | Q9XSR0 | 27 | 3 | IL18 | cytokine | immune response |
| 29 | Interleukin-12 subunit alpha | Q28267 | 41 | 4 | IL12A | cytokine | immune response |
| 30 | Interleukin-1 family member 8//Interleukin-36 beta | Q9NZH7 | 46 | 4 | IL36B | cytokine | immune response |
| 31 | Interleukin-6 | P79341 | 79 | 7 | IL6 | cytokine | immune response |
| 32 | Ig heavy chain V-II region COR | P01815 | 37 | 3 | N/A | antigen binding | immune response |
| 33 | Scavenger receptor cysteine-rich type 1 protein M130 | Q2VLG6 | 23 | 5 | CD163 | scavenger receptor | inflammatory response |

(Continued)

**Table 1.** (*Continued*)

| No. | Protein | Accession no. | Score | Matches | Gene name | Molecular function | Biological process |
|---|---|---|---|---|---|---|---|
| 34 | Ig heavy chain V region GOM | P01784 | 23 MS/MS | 1 | N/A | antigen binding | unclassified |
| 35 | Zinc finger BED domain-containing protein 5 | A4Z944 | 33 | 6 | ZBED5 | DNA and metal ion binding | unclassified |
| 36 | Desmoglein-1 | Q9GKQ8 | 31 | 5 | DSG1 | ion binding | cell adhesion |
| 37 | Calcium uptake protein 1, mitochondrial | Q8VCX5 | 48 | 7 | Micu1 | ion binding | calcium transport |
| 38 | Cysteine and glycine-rich protein 2 | Q32LE9 | 49 | 5 | CSRP2 | ion binding | differentiation |
| 39 | Alpha-fetoprotein | Q8MJU5 | 25 | 3 | AFP | ion binding | transport |
| 40 | Ankyrin repeat domain-containing protein 5// Ankyrin repeat and EF-hand domain-containing protein 1 | Q9NU02 | 42 | 5 | ANKEF1 | ion binding | unclassified |
| 41 | Zinc finger UFM1-specific peptidase domain protein | Q3T9Z9 | 50 | 7 | Zufsp | ion binding | unclassified |
| 42 | N-acetylglucosamine-1-phosphotransferase subunits alpha/beta | P60529 | Protein autopho | 4 | HBA | oxygen binding | oxygen transport |
| 43 | Haemoglobin subunit gamma | P02099 | 60 | 6 | HBG | oxygen binding | transport |
| 44 | Myosin-Ic | Q63355 | 56 | 6 | Myo1c | motor activity | transport |
| 45 | Drebrin-like protein | Q9UJU6 | 48 | 6 | DBNL | actin binding | transport |
| 46 | Serum albumin | P49822 | 60 | 9 | ALB | transport/carrier protein | unclassified |
| 47 | Major allergen Can f 1 | O18873 | 95, 200 MS/MS | 8 | N/A | transport protein | unclassified |
| 48 | Gastrin/cholecystokinin type B receptor | F1Q0L4 | 40 | 3 | CCKBR | gastrin receptor | unclassified |
| 49 | Growth hormone receptor | Q9TU69 | 25 | 4 | GHR | receptor | endocytosis |
| 50 | Transferrin receptor protein | Q9GLD3 | 36 | 8 | TFRC | receptor | endocytosis |
| 51 | Gastrin-releasing peptide receptor | P30550 | 15 MS/MS | 1 | GRPR | receptor | cell proliferation |
| 52 | Desmin | Q5XFN2 | 87 | 4 | DES | muscle protein | cell structure |
| 53 | Beta-crystallin B2 | P02522 | 76 | 6 | CRYBB2 | eye lens protein | cell structure |
| 54 | Keratin, type I microfibrillar 48kDa, component 8C-1 | P02534 | 52 | 8 | N/A | structural | cell structure |
| 55 | Collagen alpha-2(I) chain | O46392 | 58 | 10 | COL1A2 | matrix protein | cell structure |
| 56 | Adipocytes plasma membrane-associated protein | Q3T0E5 | 55 | 5 | APMAP | structural | membrane protein |
| 57 | DnaJ homologue subfamily member 18 | Q5EA26 | 54 | 7 | DNAJC18 | structural | membrane protein |
| 58 | Keratin, type II cytoskeletal 8 | Q28810 | 37 | 4 | KRT8 | structural | cell structure |
| 59 | Keratin, type I cytoskeletal 9 | P35527 | 115 | 17 | KRT9 | structural | cell structure |
| 60 | Myosin-binding protein C cardiac-type | O70468 | 43 | 13 | Mybpc3 | structural | cell structure |
| 61 | Collagen alpha-2(IV) chain | P08122 | 39 | 6 | Col4a2 | matrix protein | angiogenesis |
| 62 | Arylsulfatase K | Q32KH0 | 35 | 5 | ARSK | hydrolase | unclassified |
| 63 | Glycogen debranching enzyme | Q2PQH8 | 25 | 9 | AGL | hydrolase | glycogen biosynthesis |
| 64 | Cystic fibrosis transmembrane conductance regulator | Q5U820 | 29 | 7 | CFTR | hydrolase | ion transport |
| 65 | Lysozyme C, spleen isozyme | P81709 | 32 | 3 | N/A | hydrolase | antimicrobial |
| 66 | Coagulation factor IX | P19540 | 33 | 5 | F9 | hydrolase | haemostasis |
| 67 | Proteasome subunit beta type-3 | P33672 | 54 | 6 | PSMB3 | hydrolase | unclassified |
| 68 | Endonuclease 8-like 3 | Q6IE77 | 57 | 10 | NEIL2 | hydrolase | DNA repair |

(*Continued*)

**Table 1.** (*Continued*)

| No. | Protein | Accession no. | Score | Matches | Gene name | Molecular function | Biological process |
|---|---|---|---|---|---|---|---|
| 69 | Gamma-glutamyl hydrolase | A7YWG4 | 30 | 6 | GGH | hydrolase | unclassified |
| 70 | Multidrug resistance-associated protein 1 | Q6UR05 | 31 | 11 | ABCC1 | hydrolase | transport |
| 71 | Ubiquitin carboxyl-terminal hydrolase 15 | Q9Y4E8 | 32 | 6 | USP15 | hydrolase | transcription |
| 72 | 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2 | O60825 | 45 | 5 | PFKFB12 | hydrolase | unclassified |
| 73 | Probable ATP-dependent RNA helicase DDX58 | Q9GLV6 | 43 | 10 | DDX58 | hydrolase | immune response |
| 74 | ATP-dependent RNA helicase DDX3X | O00571 | 40 | 10 | DDX3X | hydrolase | unclassified |
| 75 | Endonuclease 8-like 3 | Q3MHN7 | 57 | 10 | NEIL3 | hydrolase | DNA repair |
| 76 | Werner syndrome ATP-dependent helicase | Q14191 | 68 | 17 | WRN | hydrolase | DNA repair |
| 77 | 6-Phosphofructokinase, muscle type | P52784 | 21 | 2 | PFKM | kinase, transferase | glycolysis |
| 78 | Tyrosine-protein kinase Fer | Q9TTY2 | 36 | 6 | FER | kinase, transferase | unclassified |
| 79 | Kalirin | O60229 | 40 | 14 | KALRN | kinase | unclassified |
| 80 | Cell division protein kinase 3//Cyclin-dependent kinase 3 | Q80YP0 | 43 | 4 | Cdk3 | kinase | cell cycle |
| 81 | Ribosomal protein S6 kinase delta-1 | Q8BLK9 | 14 MS/MS | 1 | Rps6kc1 | kinase | unclassified |
| 82 | Dual specificity protein kinase CLK3 | P49761 | 23 MS/MS | 5 | CLK3 | kinase | unclassified |
| 83 | Amine oxidase (flavin-containing) | Q7YRB7 | 16 | 4 | MAOB | oxidoreductase | unclassified |
| 84 | Peroxiredoxin-5, mitochondrial | Q9BGI1 | 57 | 4 | PRDX5 | oxidoreductase | unclassified |
| 85 | Cytochrome P450 1A2 | P56592 | 40 | 5 | CYP1A2 | oxidoreductase | unclassified |
| 86 | Lysine-specific demethylase 5C | Q38JA7 | 37 | 12 | KDM5C | oxidoreductase | transcription |
| 87 | Lysine-specific demethylase 2B | Q8NHM5 | 46, 38 MS/MS | 12 | KDM2B | oxidoreductase | transcription |
| 88 | Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 | O00469 | 40 | 5 | PLOD2 | oxidoreductase | unclassified |
| 89 | Hydroxysteroid dehydrogenase-like protein 2 | Q2TPA8 | 42 | 7 | Hsdl2 | oxidoreductase | unclassified |
| 90 | Adenine phosphoribosyltransferase | P08030 | 37 | 4 | Aprt | transferase | purine salvage |
| 91 | F-box only protein 4 | Q9UKT5 | 58 | 7 | FBXO4 | transferase | cell cycle |
| 92 | Fukutin | O75072 | 41 | 5 | FKTN | transferase | unclassified |
| 93 | Chondroitin sulphate synthase 3 | Q70JA7 | 36 | 5 | CHSY3 | transferase | unclassified |
| 94 | Poly[ADP-ribose] polymerase 12 | Q9H0J9 | 33 | 7 | PARP12 | transferase | unclassified |
| 95 | Alkyldihydroxyacetonephosphate synthase, peroxisomal | O00116 | 31 | 5 | AGPS | transferase | lipid metabolism |
| 96 | Heparan sulphate glucosamine 3-O-sulfotransferase 6 | Q5GFD5 | 55 | 6 | Hs3st6 | transferase | unclassified |
| 97 | Rhophilin-2 | Q8HXG3 | 22 | 3 | RHPN2 | signal transduction | unclassified |
| 98 | F-actin capping protein subunit alpha-2 | Q09YN4 | 56 | 5 | CAPZA2 | actin capping | unclassified |
| 99 | Adenylate cyclase type 5 | P30803 | 32 | 7 | ADCY5 | cyclase | cAMP biosynthesis |
| 100 | Calnexin | P24643 | 28 | 5 | CANX | chaperone | protein folding |
| 101 | Rho GTPase-activating protein 7 | B9VTT2 | 46 | 10 | DLC1 | GTPase | signal transduction |
| 102 | Signal recognition particle 68 kDa protein | Q00004 | 27 | 6 | SRP68 | ribonucleoprotein | unclassified |
| 103 | Endothelin-1 | P13206 | 34 | 5 | EDN1 | vasoactive | unclassified |
| 104 | Signal recognition particle 54kDa protein | P61010 | 26 | 7 | SRP54 | ribonucleoprotein | unclassified |
| 105 | Oxygen-regulated protein 1 | Q8MJ04 | 27 | 13 | RP1 | microtubule binding | sensory transduction |

(*Continued*)

**Table 1.** (*Continued*)

| No. | Protein | Accession no. | Score | Matches | Gene name | Molecular function | Biological process |
|---|---|---|---|---|---|---|---|
| 106 | Arf-GAP with SH3 domain ANK repeat and PH domain-containing protein 1 | O97902 | 42 | 7 | ASAP1 | GTPase activation | cilium biogenesis/ degradation |
| 107 | E3 ubiquitin-protein ligase RNF115 | Q9Y4L5 | 43 | 4 | RNF115 | ligase | unclassified |
| 108 | Collectrin | Q0VCT4 | 34 | 4 | TMEM27 | metalopeptidase | unclassified |
| 109 | EGF-like module-containing mucin like hormone receptor-like 2 | Q2Q421 | 25 | 4 | 2EMR2 | unclassified | inflammatory response |
| 110 | Gastrin-releasing peptide | P47851 | 55 | 5 | GRP | unclassified | neuropeptide signalling |
| 111 | Protein SDA1 homolog | Q9NVU7 | 34 | 5 | SDAD1 | unclassified | transport |
| 112 | Sorcin | P30626 | 28 | 3 | SRI | unclassified | unclassified |
| 113 | Bcl-2-like protein | Q9HB09 | 31 | 4 | BCL2L12 | unclassified | apoptosis |
| 114 | Girdin | Q3V6T2 | 40, 34 MS/MS | 6 | CCDC88A | unclassified | DNA replication |
| 115 | Leucine-rich repeat-containing protein 16C | Q6F5E8 | 31 | 6 | RLTPR | unclassified | immune response |
| 116 | Coiled-coil domain-containing protein 148 | Q8HZY8 | 58 | 9 | CCDC148 | unclassified | unclassified |
| 117 | COMM domain-containing protein 6 | Q3V4B5 | 34 | 4 | Commd6 | unclassified | unclassified |
| 118 | Breast cancer anti-oestrogen resistance protein 3 | Q9QZK2 | 40 | 5 | Bcar3 | unclassified | unclassified |
| 119 | SH2 domain-containing protein 3C | Q9QZS8 | 48 | 12 | Sh2d3c | unclassified | unclassified |
| 120 | Keratin, type II cytoskeletal 1 | P04264 | 112 | 15 | KRT1 | unclassified | unclassified |
| 121 | Sestrin-1 | Q4R6P7 | 55 | 9 | SESN1 | unclassified | unclassified |
| 122 | Myelin transcription factor 1-like protein | P70475 | 45 | 6 | Myt1l | unclassified | transcription |
| 123 | Coiled-coil domain-containing protein 125 | Q5U465 | 54 | 6 | Ccdc125 | unclassified | unclassified |
| 124 | Phosducin | O77560 | 37 | 5 | PDC | unclassified | sensory transduction |
| 125 | Growth arrest-specific protein 6 | Q14393 | 8 MS/MS | 1 | GAS6 | unclassified | growth regulation |

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approximately 80 kDa, which might have resulted from protein degradation caused by storage or tear proteases. The human tear protein profile revealed similar variation when analysed by fractionation in 1D electrophoresis or high-performance liquid chromatography (HPLC) in combination with MS [22,23]. Studies of tear film proteins among domestic animals have also shown significant variations [24,25]. Therefore, the accurate and sensitive characterization of tear components in individual species to establish normal tear profiles is crucial for interpreting disease-induced changes, and characterising differences between normal and diseased animals should enhance our understanding of host responses to numerous agents and improve diagnoses, treatments and prognoses. To the best of our knowledge, this study is the first to report the comprehensive proteome profile of tear film from healthy dogs based on 1D SDS-PAGE and MALDI-TOF. In Table 1, we present 125 identified proteins, including the accession number, score, peptide matches, gene name, molecular function and biological process. Certain proteins are described as unclassified because of a lack of information or multiplicity of function. The proteins identified here were classified using the Uniprot.org database according to biological processes and molecular functions. Several abundant tear proteins, such as MCA (Major Canine Allergen), Serum albumin, UPF0557 protein C10orf119 homolog, Collagen alpha-2(I) chain, Tyrosine -protein kinase Fer, Keratine type II cytoskeletal, Beta-crystallin B2, Interleukin-6 and Desmin, as the most confident ones with the highest scores were observed. Some of them hold potential to be used in future as a biomarkers for given

**Table 2. Proteins found in each band of electrophoretic pattern.**

| Band | Protein | Mass (kDa) | Score |
|---|---|---|---|
| 1 | Collectrin | 25 | 34 |
| | Major alergen Can 1 | 19 | 90 |
| | Sorcin | 22 | 28 |
| | Bcl-2-like protein | 22 | 31 |
| | E3 ubiquitin-protein ligase RNF115 | 34 | 43 |
| | Ig heavy chain V-II region COR | 13 | 37 |
| 2 | COMM domain-containing protein 6 | 10 | 34 |
| | Zinc finger UFM1-specific peptidase domain protein | 67 | 50 |
| | Hepatoma-derived growth factor | 27 | 37 |
| | Major allergen Can f 1 | 19 | 95 MS/MS |
| | Phosducin | 29 | 37 |
| | C-C motif chemokine 25 | 17 | 47 |
| | Cysteine and glycine-rich protein 2 | 22 | 35 |
| | Adenine phosphoribosyltransferase | 20 | 37 |
| | Signal recognition particle 68 kDa protein | 70 | 26 |
| | Endothelin-1 | 23 | 34 |
| | Adipocyte plasma membrane-associated protein | 46 | 55 |
| | DnaJ homolog subfamily C member 18 | 42 | 54 |
| 3 | Ankyrin repeat domain-containing protein 5 | 87 | 42 |
| 4 | Friend of PRMT1 protein | 27 | 10 MS/MS |
| | Heparan sulphate glucosamine 3-O-sulfotransferase 6 | 40 | 55 |
| | Keratin, type II cytoskeletal 8 | 35 | 37 |
| | Cell division protein kinase 3 | 34 | 43 |
| | Visual system homeobox 2 | 66 | 47 |
| | Transcription factor TFIIIB component B" homolog | 300 | 40 |
| | 6-phosphofructo-2-kinase/fructose-2,6-biphosphatse 2 | 59 | 42 |
| 5 | Ankyrin repeat domain-containing protein 5 | 87 | 42 |
| | Zinc finger protein 2 | 54 | 61 |
| 6 | UPF0557 protein C10orf119 homolog | 74 | 70 |
| | Calcium uptake protein 1, mitochondrial | 55 | 48 |
| 7 | Cysteine and glycine-rich protein 2 | 22 | 49 |
| 8 | | | |
| | Coagulation factor IX | 53 | 33 |
| | Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 | 86 | 40 |
| | Ubiquitin carboxyl-terminal hydrolase 15 | 114 | 32 |
| 9 | Dual specificity protein kinase CLK3 | 74 | 23 MS/MS |
| 10 | | | |
| | Parafibromin | 61 | 44 |
| | ATP-dependent RNA helicase DDX3X | 74 | 40 |
| | Haemoglobin subunit gamma | 16 | 60 |
| | Myosin-Ic | 120 | 56 |
| | Zinc finger protein 582 | 62 | 34 |
| | Nuclear protein 14 | 100 | 33 |
| | Hydroxysteroid dehydrogenase-like protein 2 | 55 | 42 |
| | Kinesin-like protein KIF11 | 120 | 55 |
| | Metastasis-associated protein MTA3 | 68 | 32 |
| | 6-Phosphofructokinase, muscle type | 86 | 21 |

(*Continued*)

**Table 2.** (*Continued*)

| Band | Protein | Mass (kDa) | Score |
|------|---------|------------|-------|
| | Tyrosine-protein kinase Fer | 95 | 22 |
| | Keratin, type I microfibrillar 48kDa, component 8C-1 | 48 | 52 |
| | Gamma-glutamyl hydrolase | 36 | 30 |
| | Arf-GAP with SH3 domain ANK repeat and PH domain-containing protein 1 | 126 | 42 |
| | Ig heavy chain V region GOM | 13 | 23 MS/MS |
| 11 | Ribosomal protein S6 kinase delta-1 | 117 | 14 MS/MS |
| | Sestrin-1 | 57 | 55 |
| | Myelin transcription factor 1-like protein | 135 | 45 |
| | Serum albumin | 70 | 60 |
| | Beta-crystallin B2 | 23 | 76 |
| | Tyrosine-protein kinase Fer | 95 | 23 |
| | Calnexin | 68 | 28 |
| | Rhophilin-2 | 78 | 22 |
| | Collagen alpha-2(I) chain | 130 | 58 |
| | Arylsulfatase K | 61 | 28 |
| | Adenylate cyclase type 5 | 142 | 24 |
| | Peroxiredoxin-5, mitochondrial | 23 | 57 |
| | Lysozyme C, spleen isozyme | 15 | 32 |
| | Interleukin-18 | 22 | 27 |
| | Guanine nucleotide-binding protein G(t) subunit alpha-2 | 41 | 30 |
| | Gastrin/cholecystokinin type B receptor | 50 | 40 |
| 12 | Growth hormone receptor | 72 | 25 |
| | Coiled-coil domain-containing protein 125 | 57 | 54 |
| | Werner syndrome ATP-dependent helicase | 164 | 68 |
| | Eukaryotic translation initiation factor 3 subunit H | 40 | 54 |
| | Pinin | 88 | 30 |
| | G2/mitotic-specific cyclin-B3 | 153 | 19 |
| | Collagen alpha-2(I) chain | 130 | 44 |
| | Scavenger receptor cysteine-rich type 1 protein M130 | 127 | 23 |
| | Gastrin-releasing peptide | 15 | 55 |
| | F-actin capping protein subunit alpha-2 | 33 | 56 |
| | Desmoglein-1 | 115 | 31 |
| | Cytochrome P450 1A2 | 58 | 40 |
| | Endonuclease 8-like 3 | 62 | 57 |
| | Interleukin-6 | 24 | 79 |
| 13 | Interleukin-1 family member 8 | 49 | 48 |
| | Amine oxidase (flavin-containing) B | 59 | 16 |
| | Transcription factor 4 | 69 | 18 |
| | Fukutin | 54 | 41 |
| | Growth arrest-specific protein 6 | 82 | 8 MS/MS |
| | Interleukin-1 family member 8 | 19 | 46 |
| | Amine oxidase (flavin-containing) B | 59 | 16 |
| | Selenocysteine insertion sequence-binding protein 2-like | 123 | 34 |
| | Splicing factor, proline- and glutamine-rich | 76 | 37 |
| | Putative fidgetin-like protein 2 | 67 | 40 |
| | Protein SDA1 homolog | 80 | 34 |
| | N-acetylglucosamine-1-phosphotransferase subunits alpha/beta | 142 | 30 |

(*Continued*)

**Table 2.** (*Continued*)

| Band | Protein | Mass (kDa) | Score |
|------|---------|-----------|-------|
| | Zinc finger protein 780A | 77 | 34 |
| 14 | Putative homeodomain transcription factor 1 | 88 | 32 |
| | Rho GTPase-activating protein 7 | 126 | 46 |
| | Signal recognition particle 54kDa protein | 56 | 26 |
| | Alpha-fetoprotein | 70 | 25 |
| | Interleukin-12 subunit alpha | 25 | 41 |
| | Lysine-specific demethylase 5C | 177 | 37 |
| | Pleckstrin | 40 | 32 |
| | Tuftelin-interacting protein 11 | 96 | 35 |
| | Transferrin receptor protein 1 | 87 | 36 |
| | Protein Spindly | 70 | 54 |
| | SH2 domain-containing protein 3C | 95 | 48 |
| | Keratin, type II cytoskeletal 1 | 66 | 112 |
| | Myosin-binding protein C cardiac-type | 142 | 43 |
| | Gastrin-releasing peptide receptor | | 15 MS/MS |
| 15 | Desmin | 53 | 87 |
| | 78 kDa glucose-regulated protein | 73 | 62 |
| | Desmin | 53 | 20 |
| | Multidrug resistance-associated protein 1 | 173 | 31 |
| | F-box only protein 4 | 46 | 58 |
| | Lysine-specific demethylase 2B | 155 | 46, 38 MS/MS |
| | Kalirin | 343 | 40 |
| | Girdin | 217 | 40, 34 MS/MS |
| | Tensin-1 | 187 | 61 |
| | Chondroitin sulphate synthase 3 | 101 | 36 |
| | Leucine-rich repeat-containing protein 16C | 156 | 31 |
| | 6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 2 | 65 | 43 |
| | Coiled-coil domain-containing protein 148 | 73 | 58 |
| | Probable ATP-dependent RNA helicase DDX58 | 109 | 43 |
| | Cysteinyl-tRNA synthetase, cytoplasmic //Cysteine—tRNA ligase, cytoplasmic | 95 | 38 MS/MS |
| | Collagen alpha-2(IV) chain | 108 | 39 |
| | Breast cancer anti-oestrogen resistance protein 3 | 93 | 40 |
| 16 | Oxygen-regulated protein 1 | 243 | 27 |
| | EGF-like module-containing mucin like hormone receptor-like 2 | 94 | 25 |
| | Desmin | 53 | 36 |
| | Zinc finger BED domain-containing protein 5 | 80 | 28 |
| | Tyrosine-protein kinase Fer | 95 | 36 |
| | Arylsulfatase K | 61 | 35 |
| | Transcription factor 4 | 69 | 28 |
| | Adenylate cyclase type 5 | 142 | 32 |
| | Glycogen debranching enzyme | 177 | 25 |
| | Cystic fibrosis transmembrane conductance regulator | 169 | 29 |
| 17 | Proteasome subunit beta type-3 | 23 | 54 |

diseases, i.e serum albumin in tears is usually weakly expressed, but in human patients with cancer it tends to be highly elevated due to the plasma leakage. One of the most important biological functions of tear proteins is its antimicrobial activity against pathogens because the ocular surface is constantly exposed to the environment [14]. This function is reflected by the substantial representation of immune response proteins, such as cytokines, hydrolases, lysozyme and IgG heavy chains, and a number of these proteins, such as IgG, may be involved in microorganism aggregation rather than death or inhibition. According to Zhou at al., there are top four, well-known human tear film proteins: lysozyme, lactoferrin, secretory IgA and lipocalin [21]. Interestingly, apart from proteins such as lysozyme or serum albumin, many proteins are similar in dogs and humans, including MAC, a main protein found in dog tear film that is most likely analogous to lipocalin, which is found in human tears. However in our study there has been neither presence of sIgA nor lactoferrin in dog tear film. Nevertheless, a number of other proteins are similar or identical in both tear films, like scavenger receptor cysteine-rich type 1 protein M130, which appears to be involved in pattern recognition receptors (PRRs) in humans. All in all, we have revealed that 25 out of 125 proteins identified in our study are common for dogs and humans (Table 3)

This shows that animal tear film is similar to human, yet there are some significant differences that have to be taken under consideration during analysis.

These findings may be useful for investigations using dogs as an animal model for certain natural diseases that mimic human disorders.

The analysis method used to determine the mass spectra of the major allergen *Canis familiaris*, which was also used for the remaining proteins identified in this study, is described

**Table 3. Proteins common for human and dog.**

| No | Protein | Gene |
|---|---|---|
| 1 | Hepatoma-derived growth factor | Hdgf |
| 2 | 78 kDa glucose-regulated protein//Heat shock 70 kDa protein 5 | HSPA5 |
| 3 | Splicing factor, proline- and glutamine-rich | SFPQ |
| 4 | Guanine nucleotide-binding protein G(t) subunit alpha-2 | GNAI2 |
| 5 | Cysteinyl-tRNA synthetase, cytoplasmic//Cysteine—tRNA ligase, cytoplasmic | Cars |
| 6 | Interleukin-18 | IL18 |
| 7 | Ig heavy chain V-II region COR | N/A |
| 8 | N-acetylglucosamine-1-phosphotransferase subunits alpha/beta | HBA |
| 9 | Drebrin-like protein | DBNL |
| 10 | Serum albumin | ALB |
| 11 | Keratin, type I microfibrillar 48kDa, component 8C-1 | N/A |
| 12 | Keratin, type II cytoskeletal 8 | KRT8 |
| 13 | Keratin, type I cytoskeletal 9 | KRT9 |
| 14 | Keratin, type II cytoskeletal 1 | KRT1 |
| 15 | Glycogen debranching enzyme | AGL |
| 16 | Lysozyme C, spleen isozyme | N/A |
| 17 | Proteasome subunit beta type-3 | PSMB3 |
| 18 | 6-Phosphofructokinase, muscle type | PKFM |
| 19 | Peroxiredoxin-5, mitochondrial | PRDX5 |
| 20 | Adenine phosphoribosyltransferase | Aprt |
| 21 | F-actin capping protein subunit alpha-2 | CAPZA2 |
| 22 | Calnexin | CANX |
| 23 | Sorcin | SRI |
| 24 | Growth arrest-specific protein 6 | GAS6 |

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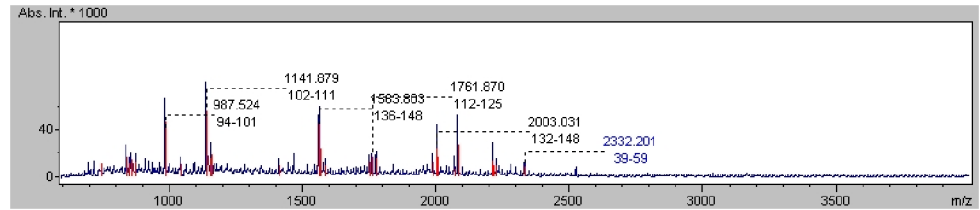
**Fig 2. Peptide mass spectra of the major allergen *Canis familiaris* protein.**

doi:10.1371/journal.pone.0144242.g002
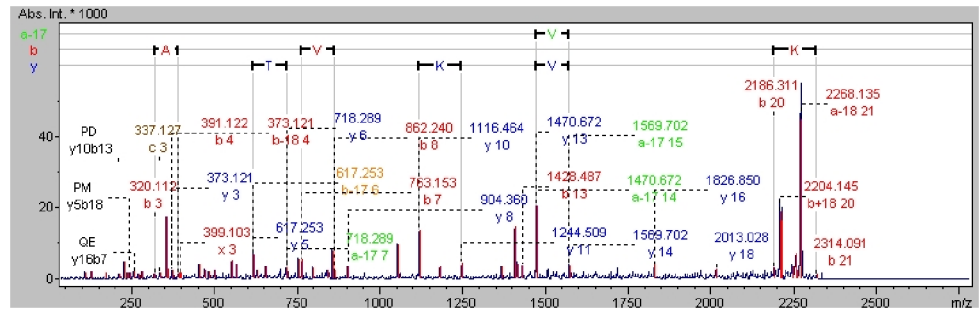


**Fig 3. MS/MS peptide mass spectra the 2332.201 m/z peak, which has been identified and confirmed in MS/MS mode as unique for the major allergen *Canis familiaris* protein.** The amino acid sequence can be observed on the graph.

doi:10.1371/journal.pone.0144242.g003

below. After acquisition and computation, the protein obtained a score of 78.5 with a statistical significance factor value of 54, and seven peaks with the following masses were assigned to this protein: 987.524 m/z; 1141.879 m/z; 1563.803 m/z; 1586.810 m/z; 1761.870 m/z; 2003.031 m/z; and 2332.201 m/z (Fig 2). The sequence coverage in MS mode was 43.7%. The MS/MS analysis score was equal to 199.87 (987.524 m/z score: 38; 1141.879 m/z score: 22; 1563.803 m/z score: 90; 1586.810 m/z score: 0; 1761.870 m/z score: 43; 2003.031 m/z score: 67; 2332.201 m/z score: 49) with 6 peptide matches and statistical significance factor value 24. (The peak at 1586.810 m/z was rejected as a characteristic of the *Canis familiaris* protein) (Fig 3).

Based on these results, our future work will include two-dimensional (2D) electrophoresis and HPLC in combination with MALDI-TOF-MS and LC-MS/MS with a quadrupole detector for protein identification and sequence characterization. Glycosylation, phosphorylation, and other posttranslational modifications of proteins will be considered during further in-depth analyses.

In summary, we have identified 125 proteins in the tear film of healthy dogs, and to the best of our knowledge, this is the first comprehensive study published thus far. Additional proteomic analysis has been performed by 2D electrophoresis [16]; however, previous studies have not presented a coherent proteome map. Tear film is easily collected non-invasively, and its proteome delivers a rich source of information that may be used for various diagnostics. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium [26] via the PRIDE partner repository [27] with the dataset identifier PXD003124.

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## Author Contributions

Conceived and designed the experiments: MSW DMW SW. Performed the experiments: MSW DMW TB DPO. Analyzed the data: MSW DMW LA JeM JaM SW. Contributed reagents/materials/analysis tools: TB DPO. Wrote the paper: MSW DMW SW TB.

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