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Clusterin from human clinical tear samples: Positive correlation between tear concentration and Schirmer strip test results

Valerie Yu
University of Southern California

Dhruva Bhattacharya
University of Arizona

Andrew Webster
University of Southern California

Aditi Bauskar
University of Southern California

Charles Flowers
University of Southern California

See next page for additional authors

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Abstract
Purpose: To investigate the relationship between tear concentration of the homeostatic protein clusterin (CLU) and dry eye signs and symptoms, and to characterize tear CLU protein.

Methods: Two independent studies were conducted, one in Tucson (44 subjects), the other in Los Angeles (52 subjects). A cohort study design was employed to enroll patients without regard to dry eye diagnosis. Dry eye signs and symptoms were assessed using clinical tests. Tear samples were collected by Schirmer strip, and also by micropipette at slit lamp when possible. CLU from both sample types was quantified by immunoassay. The relationship between CLU concentration and clinical test scores was determined by Pearson's correlation coefficient (for individual eyes) and multiple linear regression analysis (including both eyes). CLU was also evaluated biochemically by western blotting.

Results: In the Tucson cohort, a positive correlation was observed between tear CLU concentration and results of the Schirmer strip test, a measure of tear flow ($p = 0.021$ includes both eyes). This result was corroborated in the Los Angeles cohort ($p = 0.013$). The mean tear CLU concentration was 31 ± 14 μg/mL ($n = 18$ subjects, 33 eyes; range = 7-48 μg/mL). CLU from clinical tear samples appeared biochemically similar to CLU from a non-clinical tear sample and from blood plasma.

Conclusions: Results support the hypothesis that an optimal concentration of tear CLU is important for ocular surface health, and that this drops below the effective threshold in dry eye. Tear CLU measurement might identify patients that could benefit from supplementation. Information about concentration will aid development of therapeutic dosage parameters.

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Original Research

Clusterin from human clinical tear samples: Positive correlation between tear concentration and Schirmer strip test results

Valerie Yu[a,1], Dhruva Bhattacharya[b,1], Andrew Webster[c,1], Aditi Bauskar[d], Charles Flowers[e], Martin Heur[e], Shravan K. Chintala[c], Tatsuo Itakura[c], Mark R. Wilson[f], Joseph T. Barr[g], Shinwu Jeong[h,1], Mingwu Wang[b,1], M. Elizabeth Fini[c,*,1]

[a] MD Program, Keck School of Medicine of USC, University of Southern California, Los Angeles, CA, USA
[b] Department of Ophthalmology & Vision Science, University of Arizona College of Medicine, Tucson, AZ, USA
[c] USC Institute for Genetic Medicine, Keck School of Medicine of USC, University of Southern California, Los Angeles, CA, USA
[d] PhD Program in Medical Biology, Keck School of Medicine of USC, University of Southern California, Los Angeles, CA, USA
[e] USC Roski Eye Institute and Department of Ophthalmology, Keck School of Medicine of USC, University of Southern California, Los Angeles, CA, USA
[f] Illawarra Health and Medical Research Institute, School of Biological Sciences, University of Wollongong, Wollongong, New South Wales, Australia
[g] The Ohio State University College of Optometry, Columbus, OH, USA
[h] USC Institute for Genetic Medicine, USC Roski Eye Institute and Department of Ophthalmology, Keck School of Medicine of USC, University of Southern California, Los Angeles, CA, USA

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ABSTRACT

Purpose: To investigate the relationship between tear concentration of the homeostatic protein clusterin (CLU) and dry eye signs and symptoms, and to characterize tear CLU protein.

Methods: Two independent studies were conducted, one in Tucson (44 subjects), the other in Los Angeles (52 subjects). A cohort study design was employed to enroll patients without regard to dry eye diagnosis. Dry eye signs and symptoms were assessed using clinical tests. Tear samples were collected by Schirmer strip, and also by micropipette at slit lamp when possible. CLU from both sample types was quantified by immunoassay. The relationship between CLU concentration and clinical test scores was determined by Pearson's correlation coefficient (for individual eyes) and multiple linear regression analysis (including both eyes). CLU was also evaluated biochemically by western blotting.

Results: In the Tucson cohort, a positive correlation was observed between tear CLU concentration and results of the Schirmer strip test, a measure of tear flow ($p = 0.021$ includes both eyes). This result was corroborated in the Los Angeles cohort ($p = 0.013$). The mean tear CLU concentration was 31 ± 14 μg/mL ($n = 18$ subjects, 33 eyes; range = 7–48 μg/mL). CLU from clinical tear samples appeared biochemically similar to CLU from a non-clinical tear sample and from blood plasma.

Conclusions: Results support the hypothesis that an optimal concentration of tear CLU is important for ocular surface health, and that this drops below the effective threshold in dry eye. Tear CLU measurement might identify patients that could benefit from supplementation. Information about concentration will aid development of therapeutic dosage parameters.

1. Introduction

Dry eye syndrome is a common affliction associated with aging that affects 5% to 34% of all people globally [1]. According to the definition, recently revised by the Tear Film and Ocular Surface Society Dry Eye Workshop II [2], dry eye is "a multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film, and accompanied by ocular symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play etiological roles". Reduced tear flow

Abbreviations: Beta-MeOH, beta-mercaptoethanol; CI, Confidence Interval; ELISA, enzyme-linked immunosorbent assay; IGA, immunoglobulin A; MMP, Matrix Metalloproteinase; SC-CTSI, Southern California Clinical and Translational Science Institute; SD, standard deviation; USC, University of Southern California

* Corresponding author. Current Address: Tufts Medical Center, Tupper Building, 2nd Floor, 15 Kneeland Street, Boston, MA 02111, USA.
E-mail address: mefini@tuftsmedicalcenter.org (M.E. Fini).

[1] The first three authors contributed equally as first authors and the last three authors contributed equally as senior authors.

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and/or increased tear evaporation causes tear hyperosmolarity and desiccating stress, which stimulates inflammation and expression/activity of matrix metalloproteinases (MMPs). This can lead to ocular surface disease characterized by increased apoptosis, desquamation and barrier disruption, which is visualized as punctate staining with clinical dyes [3].

Clusterin (CLU[2]) is a secreted glycoprotein that serves as a "molecular chaperone". As such it is part of an extracellular quality control system maintaining proteostasis by binding to misfolded proteins, inhibiting their precipitation and participating in their clearance from bodily fluids as high molecular weight soluble complexes [4–6]. Experimental immunodepletion of CLU from human blood renders other plasma proteins susceptible to stress-induced precipitation [7]. CLU is found in insoluble protein deposits in diseases of amyloid deposition such as pseudoexfoliation glaucoma and Alzheimer's disease, perhaps representing an aborted attempt to fulfill the molecular chaperone role [8]. However, studies show that if CLU attains a critical concentration threshold, it potently inhibits deposit formation and provides substantial cytoprotection [8]. CLU is also proteostatic by virtue of its ability to serve as a potent inhibitor of several MMP family proteinases [9,10].

An early study characterizing CLU revealed a striking level of expression in mucosal epithelial cells, as well as several non-epithelial secretory cell types from a broad range of tissues that form cellular interfaces with fluid compartments [11]. The results suggested that localized CLU synthesis might be particularly important for protection of fluid barrier tissues. CLU protein is found in the apical cell layers of the ocular surface epithelia [12–14] and mass spectrometric analyses have consistently identified CLU in human tears (e.g., [15–21]). CLU in the ocular surface epithelia decreases dramatically in inflammatory disorders that manifest as severe dry eye [22–24]. Using a mouse model for desiccating stress that mimics human dry eye, we recently provided the first causal evidence in support of the idea that tear CLU protects the ocular surface. We showed that CLU prevent and ameliorates ocular surface signs of dry eye when present in sufficient amounts in the tears [25], either endogenously, or when supplemented by topical application. Tear CLU concentration was reduced to about 2/3rds of normal in mice subjected to desiccating stress, dropping below the critical threshold. This suggested that there is an optimal concentration of tear CLU needed for ocular surface health, and that this concentration might drop below the effective threshold in dry eye. If so, supplementation could be therapeutic.

In this report, we describe the results of two independent but coordinated studies conducted with the common goal to determine for the first time, the concentration and biochemical characteristics of CLU in human tears, and to investigate whether changes in these parameters are correlated with clinical signs and symptoms of dry eye.

2. Methods

2.1. Ethics statement and human subjects

The two studies described herein were compliant with the Declaration of Helsinki, the Health Insurance Portability and Accountability Act (HIPAA), and the Institutional Review Boards of the University of Arizona Tucson and the University of Southern California (USC; Los Angeles). Informed consent was obtained from all research subjects after explanation of the nature of the study and possible consequences.

In the first study, patients were recruited in Tucson as part of a larger investigation ongoing at the University of Arizona headed by Dr. Mingwu Wang. Patients were enrolled into the study in Dr. Wang's cornea clinic under the parent protocol, and all patient data and specimens were collected at this site. After results were obtained from the Tucson study, a second study was conducted in Los Angeles with the goal to independently corroborate results of the first. Patients were enrolled from the cornea clinic at the USC Roski Eye Institute on the USC Health Sciences Campus under Drs. Martin Heur and Charles Flowers.

2.2. Study design

We utilized a cohort study design to enroll subjects across the spectrum of dry eye signs and symptoms. Subjects over 18 years of age were enrolled as they came into the eye care clinic for a scheduled appointment. Subjects were enrolled without regard to sex, gender, race or ethnic origin. Children were not included, since dry eye is a disease of aging. Chart records of inflammatory or autoimmune disorders of the ocular surface were recorded for each enrolled subject.

To assess dry eye signs in enrolled subjects, several standard clinical tests were used (procedures described below). Tests were performed for both the left and right eyes separately. All test variables are presented as the mean ± SD. To assess dry eye symptoms, all enrolled subjects were asked to fill out the Ocular Surface Disease Index, a set of 12 multiple choice questions [26,27].

Tears were collected on Schirmer test strips from both the left and right eyes separately, and CLU was extracted for analysis (method described below). As discussed more in the next sections, these samples allowed us to obtain a relative measure of CLU concentration. To directly measure the actual CLU concentration, tear samples were also collected in Tucson by micropipette, if possible, from both the left and right eyes separately (discussed more below).

We estimated sample size for our study using data from mouse experiments and from the Tucson clinic. In the published mouse study, CLU tear concentration was reduced by ~30% in mice subjected to desiccating stress [25]. Prior Tucson clinic data for fluorescein staining on the standard clinical scale of 1–15 was as follows: mean = 6; standard deviation (SD) = 3. Assuming a 30% reduction in means relative to healthy subjects, this translates to an effect size of 0.6 ((6–4.2)/3). Thus, to detect a reasonable group mean difference in CLU, testing at a 2-sided alpha = 0.05, with 80% power, and incorporating a 0.5 correlation between eyes (within subject), 34 subjects (68 eyes) per group would be required. This sample size would also provide 80% power to detect within-group correlations of 0.40 and higher with 34 subjects (68 eyes), and 0.29 and higher in a combined sample of 68 (128) subjects (eyes).

2.3. Clinical tests for dry eye signs

In the Tucson study, four clinical tests were used to assess signs of dry eye. Three are tests of tear dysfunction: 1) Schirmer strip test, 2) tear break-up time and 3) tear osmolarity. The fourth test was fluorescein staining, which assesses ocular surface damage.

The Schirmer strip test uses calibrated filter paper strips to measure tear flow [28]. Test strips were purchased from Alcon Laboratories, Inc. (Fort Worth, TX). To administer the test, the "head" portion of the strip was placed with gloved hands over the subject's lid margin, at the junction of the lateral and middle thirds of the lower eyelids, and the eyes were closed for 5 min. The strip was then removed and the progress of fluid flow onto the body of the strip by passive capillary action was recorded from the calibration markings. The test was performed without anesthetic according to the parent protocol, the rationale being to avoid any possible dilution of the tears with the anesthetic. (Dilution was a concern of the investigators at the Tucson site since concentration measurements were planned; whether tear dilution actually occurred was not specifically investigated.)

[2] For each known human gene, the Human Genome Organisation (HUGO) Gene Nomenclature Committee (HGNC) (http://www.hugo-international.org/HUGO-Gene-Nomenclature) approves a unique gene name and symbol. HUGO nomenclature is used for all genes and their products discussed herein.

Tear break-up time is a measure of tear quality and vulnerability to evaporation [3]. Briefly, fluorescein was instilled onto the ocular surface and the patient was asked not to blink while the tear film was observed under a broad beam of cobalt blue illumination. The tear break-up time was recorded as the number of seconds that elapsed between the last blink and the appearance of the first dry spot in the tear film. Tear osmolarity, a measure of salt concentration (due to reduced tear aqueous volume), utilized the TearLab device [29,30].

Fluorescein staining was quantified using the standard National Eye Institute grading system [31]. Briefly, the cornea is divided into 5 areas (central, superior, nasal, inferior and temporal); punctate fluorescein staining in each area is graded on a scale of 0–3, with 3 being the most severe. The scores from all five areas are then summed, for a final score of 0–15.

In the Los Angeles study (informed by experience obtained with the Tucson study), a more streamlined set of tests for dry eye signs was used: 1) the Schirmer strip test and 2) corneal grading for ocular surface damage assessment. Some minor modifications were made in the Schirmer strip test to fit the practices of the Los Angeles clinic. First, the clinic purchased Schirmer strips from a local supplier (HUB Pharmaceuticals, LLC, Rancho Cucamonga, CA). In addition, the clinic insisted on use of a local anesthetic for patient comfort (0.5% Proparacaine Hydrochloride). Corneal grading was used instead of fluorescein staining, also because this was the practice of the Los Angeles clinic. Briefly, this involved careful inspection of the ocular surface for punctate epithelial erosions without the use of stain. Eyes were graded on a scale of 0–5, with 5 being the most severe.

2.4. Tear sample collection procedures

Following the Schirmer strip test, which was performed on all subjects enrolled in both the Tucson and Los Angeles clinics, tear-saturated test strips from each eye of a given subject were saved for extraction and analysis of collected tear proteins by placing them individually into sterile 2-mL centrifuge tubes.

Efforts were made at the Tucson site to also collect tear samples on all participants by micropipette. This was done at the slit lamp from each eye, using a fine 5 μL pipette tip point, precisely at the tear lake at the medial canthal area. In this way, the ocular surface tissues were not touched or stimulated. Each time, based on the accumulation at this location, 1 or 2 μL of tears were aspirated from each eye. Some eyes had an inadequate tear lake for aspiration and hence failed to generate any tear samples. In general, if it were possible to collect a sample from one eye of a given individual, it was also possible to collect a sample from the other eye. However, in some cases, we could obtain samples from only a single eye of a specific individual. Samples from each eye were placed in separate 2-mL centrifuge tubes and diluted with PBS to increase stability.

All sample tubes were set on dry ice, then stored at −80 °C until they were sent on dry ice to the Fini research laboratory at USC. Samples were stored at −80 °C until analysis.

2.5. Extraction of proteins from Schirmer strips

The measurement of tear volume using the calibration on the Schirmer test strip is a relative (not an absolute) measurement of tear volume, meaning it was not possible to calculate the actual concentration of CLU. Thus, we chose to compare the amount of CLU collected from equal volumes of tears by extracting only the “head” of each strip, each head being equal in size and completely saturated by tears in all cases. Extraction was performed according to a method previously described [32]. Briefly, the head was removed from the strip and placed in a new 2-mL centrifuge tube containing 100 μL of elution buffer (100 mM ammonium bicarbonate, 0.25% NP-40) with addition of proteinase inhibitors cocktail (Roche, Indianapolis, IN). Proteins were eluted by incubating the tubes on a rotator overnight at 4 °C, followed by micro-centrifugation (10,000 rpm, 5 min). Protein was precipitated in acetone, then dissolved in diluent buffer provided with the Human Clusterin Quantikine ELISA kit (R&D Systems, Minneapolis, MN).

2.6. CLU quantification

CLU was quantified for each sample (protein solution eluted from a Schirmer strip head or a sample collected by micropipette) individually by enzyme-linked immunosorbent assay (ELISA) using the Human Clusterin Quantikine ELISA kit (R&D Systems). To prepare samples for analysis, 50 μL (half) of the protein solution eluted from each Schirmer strip head, or 0.5 μL of each micropipette-collected sample, was supplemented with assay diluent to 100 μL, as specified by the manufacturer. Raw ELISA quantification results were compared to a CLU standard curve, newly constructed each time a set of samples was analyzed. The resulting values were described in ng/strip head (Schirmer strip) or ug/mL (micropipette). The former is a comparative concentration measure; the latter is an actual concentration measure.

2.7. Correlation analysis

De-identified data were analyzed by statisticians from the Southern California Clinical and Translational Science Institute (SC-CTSI) to determine the association between tear CLU concentration and clinical test scores. Measurements from the two eyes of a single subject are often positively correlated, resembling each other more than they do measurements from the eyes of other subjects. This inter-eye correlation must be accounted for in analysis [33]. Thus, the Pearson correlation coefficient, r, was used to assess the degree of linear association between the independent variable, CLU amount per strip head, and the dependent variable, the clinical test score (clinical outcome), for the right eye and left eye respectively. Then, linear regression with a random effect model (specifying a random effect for subject) was used to assess the CLU versus clinical test correlation for both eyes, accounting for repeated measurement from the same individual (i.e., both left and right eyes from the same participant). All significance tests were two-sided, and statistical significance was set at $P < 0.05$. Data were analyzed using SPSS for Windows (Version 12.0, SPSS Inc., Chicago, IL).

2.8. Western blotting

Proteins in Schirmer strip extracts and tear samples were separated by SDS-PAGE and transferred to plastic membranes. Membranes were probed with primary antibody against CLU and developed by chemiluminescence with Luminol enhancer solution and peroxide solution (GE Healthcare UK limited, Buckinghamshire, UK). Images were captured with a Fujifilm imaging system (LAS-4000; Fujifilm, Tokyo, Japan).

CLU from clinical samples was compared with CLU derived from two different non-clinical sources. Natural secreted human serum CLU was purified in the Wilson lab from discarded human blood according to an immunoaffinity protocol previously described [34]. In addition, non-clinical tears were collected from a male subject enrolled in the USC research laboratory; this individual did not report any dry eye symptoms. A published tear wash method was utilized to collect this sample [35]. Briefly, both eyes were washed 6 times (1 drop/time) with Refresh (Allergan, Irvine, CA) and a total of 110 μL was collected from the inferior fornix by micropipette and transferred into a sterile polypropylene tube.

Two different CLU antibodies were used to probe the western blots. One was a rabbit polyclonal antibody (Catalog no. Ab69644, from Abcam, Cambridge, MA) raised against a synthetic peptide matching a sequence near the C-terminus of the human CLU beta-chain. The second antibody was a goat polyclonal antibody raised against mouse CLU

Table 1
Characteristics and clinical test scores of study subjects.

Number of Subjects	Tucson Cohort			Los Angeles Cohort
	$n = 44$			$n = 52$
	Group A	Group B		
	$n = 16$	$n = 28$	Subgroup B1	
			$n = 18$	
Tear Sampling Method	Schirmer strip	Schirmer strip	Micropipette in addition to Schirmer strip	Schirmer strip
Mean Age (range)	58.7 years (29–81)	65.9 years (39–86)	68.2 years (53–81)	61.9 years (24–95)
Sex (Male/Female)	2/14	7/21	6/12	21/31
Autoimmune Diagnosis	0	1	1	11
Dry Eye Signs (Mean ± SD)				
Schirmer (mm wetting, 5 min)	12.9 ± 11.9	14.1 ± 11.1	15.8 ± 11.9	11.16 ± 9.3
Tear Break-Up (seconds)	5.9 ± 3.3	5.6 ± 3.3	6.0 ± 3.5	n/a
Osmolarity (mOsm/L)	302.4 ± 14.3	302.4 ± 15.7	301.5 ± 10.3	n/a
Fluorescein (0–15 scoring scale)	5.0 ± 4.5	5.1 ± 3.0	5.1 ± 3.1	n/a
Corneal Grade (0–5 scoring scale)	n/a	n/a	n/a	0.5 ± 0.76
Dry Eye Symptoms (Mean ± SD)				
Questionnaire (1–100 scoring scale)	41.3 ± 21.3	31.8 ± 26.4	26.0 ± 25.3	31.4 ± 25.2

SD = standard deviation from the mean.
Autoimmune diagnoses included Sjögren's syndrome, rheumatoid arthritis, lupus erythematosus, psoriatic arthritis, and non-specified autoimmune disease.
The following test scores for dry eye signs are considered as "normal": Schirmer test > 15 mm (with or without anesthetic), tear break-up time > 10 s, tear osmolarity of < 296 mOsm/L, fluorescein staining score < 2, corneal grade = 0.
Higher scores on the questionnaire represent greater disability.

(catalog no. sc-6420, from Santa Cruz Biotech, Dallas, Texas), recommended for detection of mouse, rat and human CLU (Santa Cruz Biotechnology, Dallas, TX). The epitope recognized by the antibody maps at the C-terminus of the CLU alpha-chain. (See Fig. 2A for a graphic of CLU structure showing the two protein chains).

3. Results

3.1. Characteristics and clinical test scores of study subjects

Characteristics and clinical test scores of the subjects from the two different studies are compiled in Table 1.

A total of 44 subjects were enrolled in the Tucson cohort. One laboratory worker quantified CLU collected on individual Schirmer test strips for the first 16 patients enrolled (group A), while a second laboratory worker performed this task for samples from the rest of the patients (group B). Subjects for which it was possible to also obtain tear samples were a subset of the second set of patients (subgroup B1).

A total of 52 subjects were enrolled in the Los Angeles cohort. This time, only a single laboratory worker quantified CLU collected on the individual Schirmer test strips, with determinations for all samples done at the same time. Thus, there was no need to split the cohort into groups.

3.2. Tear CLU concentration and clinical test outcome correlation

The mean CLU protein amount extracted from individual Schirmer strip heads for both the Tucson cohort and the Los Angeles cohort is compiled in Table 2. The results for Tucson group A and group B were systematically different (partially because of use of two different standard curves) and it was realized that they could not be pooled. This was the reason for maintaining the two groups as separate.

Table 2
Quantification of CLU protein extracted from Schirmer strip heads.

	Tucson Cohort		Los Angeles Cohort
	Group A	Group B	
Number of Subjects (Eyes)	$n = 16$ [32]	$n = 28$ [55]	$n = 52$ (104)
Mean CLU per Strip Head (ng)	1.8 ± 0.7	13.7 ± 3.9	15.2 ± 5.1

Tucson group A was too small ($n = 16$) for a valid statistical analysis, thus analyses were performed using data from group B only, which was close in size ($n = 28$) to our power estimate. There was no significant correlation between CLU amount and the Schirmer test score for the right eye in Tucson group B ($r = 0.163$, slope = 0.28, $p = 0.406$). However, there was a significant positive correlation between CLU amount and the Schirmer test score for the left eye ($r = 0.62$, $p = 0.013$). In addition, a significant positive correlation was observed when the eyes were analyzed together by linear regression (slope estimate = 0.45, 95%CI = 0.07–0.82, $p = 0.021$). Correlations of CLU amount to other clinical test results were not significant.

Analysis of data from the Los Angeles cohort was done in the same way as the Tucson cohort. There was no significant correlation between the CLU amount per strip head and Schirmer test scores for the right eye ($r = 0.172$, $p = 0.224$). However there was a statistically significant positive correlation for the left eye ($r = 0.362$, $p = 0.008$). In addition, a significant positive correlation was observed when the eyes were analyzed together by linear regression (slope estimate = 0.42, 95%CI = 0.09–0.75, $p = 0.013$).

The compiled data for corneal grading was quite discontinuous, which we attribute to the short scoring range of this test; thus statistical analysis was not performed. Correlation of the CLU amount per strip head to questionnaire test results was not significant.

An example of the Pearson correlation analysis scatter chart results is shown in Fig. 1. Results for the linear regression analysis of both the Tucson and Los Angeles studies are compiled in Table 3.

These results, corroborated in two independent studies, indicate that CLU concentration is positively correlated with tear flow, so that a decrease in CLU concentration indicates a decrease in tear flow, a clinical outcome that is a sign of dry eye disease.

3.3. Actual concentration of tear CLU

The actual volume of tear samples collected by micropipette from Tucson group B1 patients was measured, making it possible to determine the actual CLU tear concentration. Table 4 summarizes the ELISA data. The mean ± SD CLU concentration was 31 ± 14 μg/mL

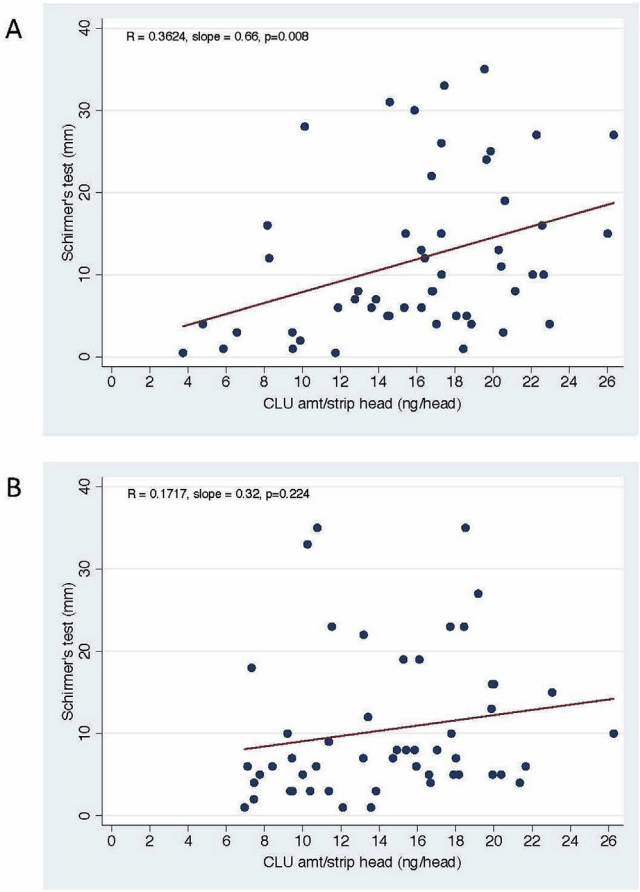

Fig. 1. Correlation Analysis. An example of a correlation analysis result is shown. Pearson correlation coefficient, r, was used to assess the degree of correlation between Schirmer test results and the amount of clusterin protein on the Schirmer strip head for each patient in the Los Angeles cohort.

A) The scatter graph illustrates the range of data distribution for the left eye. The correlation is statistically significant (R = 0.362, p = 0.008).
B) The scatter graph illustrates the range of data distribution for the right eye. The correlation is not statistically significant (R = 0.172, p = 0.224).

(n = 18 subjects; 33 eyes; range = 7–48 μg/mL; in one subject, tear collection was possible from only one eye).

3.4. Tear sample collection method correlation

The fact that tear samples were collected by both Schirmer test strip and micropipette for subgroup B1 of the Tucson B cohort provided an opportunity to compare the two methods to determine their equivalence. The correlation for the right eye was not significant (r = 0.33, slope = 0.17, p = 0.192); however the correlation for the left eye was significant (r = 0.62, slope = 0.31, p = 0.013). There was also a significant correlation between CLU amount collected by the two methods analyzing across both eyes with adjustment for repeated measures as shown in Table 5 (slope estimate = 0.18, 95% CI = 0.02–0.35, p = 0.032).

These results indicate that measurement of tear CLU concentration by the two tear collection methods correlate with one another.

3.5. Biochemical Characterization of Tear CLU

Western blot analysis was performed on tear samples to assess biochemical characteristics of tear CLU. Representative results are shown in Fig. 2. First, a schematic of CLU structure is provided as an aid

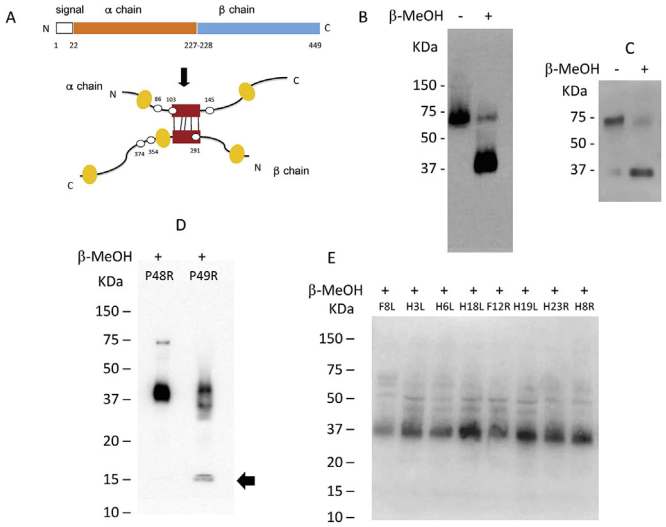

Fig. 2. Biochemical characterization of tear CLU.
A) Predicted structure of secreted human CLU (adapted from Refs. [6,76,77]). Secreted CLU exhibits an apparent mass of 75–80 kDa by SDS-PAGE, although the actual mass is ~58–63 kDa. It is composed of two polypeptide chains derived from an intracellular precursor. In the first processing step, the 22-mer secretory signal peptide is cleaved from the 449-amino acid precursor. Subsequently the chain is cleaved again between Arg227-Ser228 to generate an alpha-chain and beta-chain of approximately equal length. These are assembled in anti-parallel fashion and linked at the cysteine-rich center by five disulfide bridges (black lines) to generate a heterodimeric molecule with four "arms". Amphipathic alpha-helices (yellow ovals) located near the ends of each of the otherwise disordered arms are thought important for binding to hydrophobic regions exposed on denatured proteins and for insertion into lipid structures. Sites for N-linked glycosylation (white spots) are located around the cysteine-rich center of each chain resulting in a molecule that is 17–27% carbohydrate by weight. Amino acid numbering for the N- and C-termini, the cleavage sites, and the sites for N-linked glycosylation are indicated, as in Ref. [78]. Schematic originally published in Ref. [36], used with permission.
B to E) Western blots of purified human blood serum CLU and human tear CLU samples probed with CLU antibodies are shown. The blots in panels **B, D**, **E** were probed with a polyclonal antibody raised against a synthetic peptide matching a sequence near the C-terminus of the CLU beta-chain (see schematic, panel **A**). The blot in panel **C** was probed with a polyclonal antibody that recognizes an epitope mapping to the C-terminus of the CLU alpha-chain (see schematic, panel **A**; more details on the antibodies in the Methods section). Beta-MeOH = beta-mercaptoethanol. R = right eye, L = left eye; CLU amounts loaded on gels were determined by ELISA; The electrophoretic position of molecular size standards (in kDa) is indicated.
B) Purified human blood serum CLU, 20 ng loaded in each lane.
C) Tears from a non-clinical subject without dry eye symptoms, collected by the eye wash method described in the Methods section. Ten uL was loaded in each lane.
D) Tear samples collected by pipette from two of the Tucson cohort subgroup B1 patients with dry eye signs and symptoms. The arrow indicates the 15 kDa doublet. Patient 48R: questionnaire score = 35; Schirmer score = 5, CLU loaded = 31 ng.
Patient 49R: questionnaire score = 37; Schirmer score = 8, CLU loaded = 26 ng.
E) Schirmer strip elution samples from the Los Angeles cohort patients with a range of OSDI questionnaire and Schirmer strip test scores. Clinical test scores and expected CLU amounts loaded per lane according to ELISA assay are indicated below.
Patient F8L: questionnaire = 14.6; Schirmer = 25, CLU = 6 ng.
Patient H3L: questionnaire = 3.6; Schirmer = 15, CLU = 8 ng.
Patient H6L: questionnaire = 68.2; Schirmer = 4, CLU = 7 ng.
Patient H18L: questionnaire = 27.1; Schirmer = 27, CLU = 7 ng.
Patient F12R: questionnaire = 75.0; Schirmer = 4, CLU = 6 ng.
Patient H19L: questionnaire = 4.2; Schirmer = 19, CLU = 6 ng.
Patient H23R: questionnaire = 11.4; Schirmer = 16, CLU = 6 ng.
Patient H8R: questionnaire = 22.2; Schirmer = 16, CLU = 6 ng.

Table 3
Association between amount of CLU protein extracted from Schirmer strip heads and clinical test results for dry eye, adjusted for repeated measures.

Clinical Test	Cohort Group	Slope estimate (95% CI)	*P*-value
Schirmer	Tucson B	0.45 (0.07–0.82)	0.021*
	Los Angeles	0.42 (0.09–0.75)	0.013*
Fluorescein	Tucson B	0.03 (0.06–0.12)	0.499
Tear Break-Up	Tucson B	−0.07 (−0.18–0.04)	0.219
Osmolarity	Tucson B	−0.04 (−0.77–0.68)	0.903
Questionnaire	Tucson B	0.09 (−0.23–0.42)	0.573
	Los Angeles	−0.08 (−0.51–0.34)	0.699

* denotes a statistically significant association.

Table 4
CLU concentration in tear samples from tucson cohort subgroup B1.

Number of Subjects (Eyes)	n = 18 [33]
Mean CLU Concentration (ug/mL)	31 ± 14

Table 5
Association between CLU protein amount extracted from Schirmer strip heads and CLU protein concentration in tears.

Slope estimate (95% CI)	*P*-value
0.18 (0.02–0.35)	0.032*

* denotes a statistically significant association.

to understanding the analysis and results (Fig. 2A). CLU derived from the tears of a non-clinical subject exhibited an apparent size of ~75 kDa (Fig. 2B), the apparent size typical for CLU obtained from various bodily fluids [36]. When the sample was reduced by addition of beta-mercaptoethanol to break the disulfide bonds in the CLU molecule, tear CLU appeared as a single band migrating at about 37 kDa, consistent with dissociation into two polypeptide chains of approximately equal size (only one of which was recognized by the antibody used to probe the western blot) (Fig. 2B). This electrophoretic behavior was indistinguishable from human serum CLU (Fig. 2C). The small amount of CLU that did not dissociate into two polypeptide chains after treatment with beta-mercaptoethanol is typically observed, and represents unprocessed protein. CLU from one of the two micropipette-collected samples analyzed from Tucson cohort subgroup B1 appeared to be cleaved at the C-terminus of the beta chain, generating two small fragment of ~15 kDa that were bound by the CLU antibody (Fig. 2D). This would remove one of the C-terminal amphipathic helices, raising the intriguing possibility that it is an inactivating cleavage. However, this cleavage was not identified in 8 different tear samples eluted from Schirmer strips collected from the Los Angeles cohort (Fig. 2E, thus, may not be of general significance. In addition, no higher molecular weight aggregates that might represent CLU bound to denatured client proteins were evident in any of the samples.

These results indicate that tear CLU is biochemically similar to the form of CLU found in blood, and is not substantially different in the tears of patients with signs and symptoms of dry eye.

4. Discussion

CLU is an important component of the quality control system in bodily fluids that maintains proteins, also called “proteostasis” [4–6]. We recently demonstrated, using a mouse model for desiccating stress, that CLU prevents and ameliorates ocular surface signs of dry eye disease if present in sufficient amounts in the tears. However, when CLU tear concentration dips below a critical threshold, the ocular surface became vulnerable [25]. Our results suggested that tear CLU needed for health of the ocular surface might become limiting in human dry eye disease, and that topical supplementation might be therapeutic. The current study investigated whether tear CLU concentration correlates with dry eye signs and symptoms in people. Consistent with our hypothesis, we found that CLU concentration is positively correlated with Schirmer strip test results. Lower CLU concentration indicates a decrease in tear flow, a clinical outcome that is a sign of dry eye disease. This result was corroborated in two independent studies. We further report a tear CLU concentration of 31 ± 14 μg/mL (n = 18 subjects; 33 eyes; range = 7–48 μg/mL), determined by ELISA using tear samples from patients with a broad range of test scores for dry eye signs and symptoms. Finally, we show that tear CLU is biochemically similar to CLU from blood plasma. CLU present in clinical tear samples is also biochemically similar to non-clinical samples, suggesting that the protein in clinical tears is functional, even when concentration is reduced.

Table 6A
Concentration of CLU in human bodily fluids.

Concentration (ug/mL)	Reference
Seminal plasma	
250–500	[62]
438 ± 235	[63]
Blood serum	
35–105	[64]
111 ± 50	[63]
340	[65]
325 ± 100.3	[66]
101 ± 42	[37]
52.8 ± 0.8 (men)	[67]
49.3 ± 0.5 (women)	
Blood plasma	
72	[64]
50–100	[62]
Cerebrospinal fluid	
1.6–3.6	[68,69]
Aqueous humor	
0.8 ± 0.5	[66]

CLU concentration varies widely in different human bodily fluids. Table 6A summarizes information about this, derived from the scientific literature. The value determined here by ELISA of ~30 μg/mL for human tear CLU is about 3-fold lower than the most recently reported value of 101 ± 42 μg/mL for human blood serum, also determined by ELISA [37]. CLU has previously been identified in tear proteomics profiles of normal subjects [15,17–19,38], dry eye subjects [39], and subjects with pterygium, Sjögren's syndrome and diabetes [16,40,41], however its concentration has never been determined. Table 6B summarizes information taken from the scientific literature that provides context to other tear proteins. A small number of highly abundant proteins are estimated to comprise more than 90% of the total tear protein by weight, including lysozyme (LYZ), lactoferrin (LTF) tear lipocalin (LCN1) and lacritin (LACRT) [42]. However, the remaining 10% is highly complex; in the most comprehensive mass spectrometry list, 1543 tear proteins were identified [17]. At ~30 μg/mL, CLU abundance is substantially lower than that of the major tear proteins (e.g., ~50 fold less than LCN1 and ~10-fold less than LACRT), but near the upper end of abundance for the other proteins. This is consistent with the hypothesis that there is an optimal concentration of tear CLU needed for ocular surface health.

Both reduced and elevated levels of CLU are associated with disease states. The former likely represents dysfunction, and the latter reflects a compensatory stress response, which may or may not be sufficient to bring CLU to the necessary level [43]. While we report reduced CLU concentration associated with reduced tear flow here, elevated CLU in the saliva has been associated with Sjögren's syndrome [44,45], a disease that also manifests as dry eye [46]. Low plasma CLU concentration is associated with an adverse prognosis in patients with chronic heart failure independent of traditional cardiovascular risk factors and potential confounders [47]. An increased CLU concentration is associated

Table 6B
Relative CLU abundance in tears.

Tear Protein	Concentration (ug/mL)	Reference
Total tear proteins	6000-11,000	[70]
Highly Abundant (~70–85% of total)		
LYZ	1678	[71]
LTF	~1500	[72]
LCN1	~1500	[72]
Abundant (combined with highly abundant, ~90% of total)		
Secretory IGA	~300	[42]
LACRT	~300	[73]
Proline rich proteins	~300	[42]
Others (10% of total)		
CLU	~30	This paper
TIMP1	~3	[74,75]
Growth factors and cytokines	pg/mL range	[42]

The total protein concentration in human tears was recently estimated at 6–11 mg/mL [70]. A few highly abundant proteins, including lysozyme (LYZ), lactoferrin (LTF) and tear lipocalin (LCN1), comprise 70 to 85% of this total [72]. Expanding to the next level of abundance to include a small group of additional proteins, such as secretory immunoglobin A (IGA), lacritin (LACRT), and proline-rich proteins (PRRL4, PROL1, etc.), increases this value to more than 90% [42]. In the most comprehensive mass spectrometry list, 1543 different tear proteins were identified [17], and this list includes the remaining 10%. At ~30 μg/mL, CLU is at the top end. The value of 30 μg/mL is consistent with the ranking of CLU on mass spectrometry protein abundance lists (e.g., [17]).

with severity, pathology, and progression of Alzheimer's disease [48] and cognitive impairment [49]. However, functional analyses suggest reduced secretion of CLU protein as the mode of action for three of the examined CLU mutations associated with Alzheimer's disease [50]. The fact that CLU concentration in cerebrospinal fluid is quite low, suggested that the levels could be easily overwhelmed in disease and prompted the proposal that CLU supplementation might be of therapeutic value in Alzheimer's disease [50]. Our findings here support the idea that supplementation might be of value for dry eye disease as well. The range of tear CLU concentrations measured here of 7–48 μg/mL provides a starting point around which to design a dose range for topical supplementation in clinical trials.

An interesting finding of this study was the consistent observation that a reduction in the Schirmer test for tear flow correlated to a reduction in tear CLU concentration for the left eye, but not for the right. Perhaps of relevance is the report that cataracts and skin cancers appear on the left side of the face in North America at a greater frequency than on the right. Since the opposite correlation is observed in countries that drive motor vehicles on the other side of the road, it has been theorized that greater sun exposure through the driver-side window accelerates the correlated pathologies [51]. The ocular surface epithelia can also be damaged by sun exposure [52], thus perhaps something similar can explain our results. While results in the right eye were not statistically significant in our study, we observed the same correlative trend as in the left eye. We suspect that the right eye data would also become statistically significant in a study with a larger sample size.

Why would CLU concentration be lower in patients with reduced tear flow? To answer this question, it is helpful to consider where CLU is synthesized. CLU mRNA appears in RNA profiles of the human lacrimal glands [55,56], meibomian glands [56] and accessory lacrimal glands of Wolfring [57] and CLU protein is localized to CD31-positive endothelial-like cells in the lacrimal gland of mice [58]. Lacrimal gland dysfunction leading to aqueous-deficient dry eye might also cause a reduction in CLU protein secretion, as has been suggested for LCN1 and LACRT [38].

Shedding and lysis of apical epithelial cells also contribute to the tear protein profile [42]. CLU mRNA is expressed throughout the corneal epithelium, but the protein product is found in humans [12–14,59] and mice [10] only in the apical cell layers. When these mucosal epithelial cells undergo squamous metaplasia in dry eye, CLU protein is depleted [60]. This would translate into a lower concentration of CLU in the tears.

There has been much interest in developing a tear protein biomarker profile for dry eye and other ocular surface diseases [38,42]. Two recent studies compare relative CLU levels in subjects with and without dry eye. The first suggested that the tear CLU concentration was reduced in type 2 diabetic patients with dry eye [41]. The second study suggested that tear CLU could serve as a biomarker for Sjögren's syndrome patients with dry eye [40]. However, in both cases, a very small number of samples were pooled and analyzed as a group, and the analyses were not quantitative. Tear CLU was reported as non-detectable in the normal samples from the second study [40], which is not consistent with the findings reported here and in other publications [15,17–19,38]. Our current study is the first that is sufficiently powered statistically to achieve a meaningful comparison of tear CLU levels in normal and dry eye subjects. The range of CLU concentrations (as apparent in Table 2 and Fig. 1) is typical of biological samples; however, if CLU reduction corresponds only with one type of dry eye (such as aqueous deficient), this would increase variability. Thus, tear CLU concentration could probably not be used to diagnose dry eye (unless it becomes possible to stratify for dry eye subtype), but still might serve as a proxy for assessing efficacy of investigational new drugs in clinical trials powered with sufficient number of subjects for statistical significance. It might also be used to identify patients that could benefit from CLU supplementation therapy.

In this study, samples were collected without topical anesthesia (Tucson cohort) and *with* topical anesthesia (Los Angeles cohort). The question of topical anesthetic use has been investigated in numerous publications and although tear secretion generally decreases, there are many associated variables [61]. In our study, the mean Schirmer test scores were quite similar (Table 1) and in both cases, a significant correlation with CLU concentration was observed. In addition, the correlation between CLU concentration measurement by Schirmer strip sampling without anesthesia, and pipette sampling (Table 5), which would not stimulate reflex tearing as performed in this study, suggests that anesthesia was not a confounding factor in our results. The use of anesthesia is more comfortable for the patient, providing for a better experience. Sampling of tears by Schirmer test strip is more acceptable to the patient than the pipette method, and has the advantage of being part of a routine clinical work-up.

Why was a significant correlation observed only with the Schirmer test, and not the other tests performed for signs and symptoms of dry eye? The Schirmer strip test measures tear flow, which is reduced in “aqueous-deficient” dry eye, characterized by a reduction in the secretions from the lacrimal glands that produce the aqueous component of the tears [53]. Thus, a reduction in CLU concentration may correspond best with aqueous deficient dry eye. Since no reliable tests exist to diagnose dry eye subtype at this time, this can only be conjecture [3,54].

5. Conclusions

Our previous work in a mouse model for dry eye disease suggested that reduction of tear CLU below a critical threshold results in ocular surface vulnerability to stress [25]. Results reported here support the hypothesis that an optimal concentration of tear CLU is important for ocular surface health, and that this concentration drops below the effective threshold in dry eye. Tear CLU measurement might identify patients that could benefit from supplementation. Information about concentration, reported here for the first time, will be valuable for developing therapeutic dosage parameters.

Disclosures

SKC, JTB, MRW, SJ, and MEF, have equity interest and/or serve as

employees/consultants of Proteris Biotech, Inc., a company engaged in development of CLU as a therapeutic for dry eye, and/or are named as inventors on patents related to CLU commercialization. The other authors have no commercial or proprietary interest in any concept or product described in this article.

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Co-author contributions

Author	Research design	Data acquisition and/or research execution	Data analysis and/or interpretation	Manuscript Preparation
Yu		X	X	X
Bhattacharya		X	X	X
Webster		X	X	X
Bauskar		X	X	X
Flowers		X	X	X
Heur		X	X	X
Chintala		X	X	X
Itakura	X		X	X
Wilson	X	X	X	X
Barr	X		X	X
Jeong	X	X	X	X
Wang	X	X	X	X
Fini	X		X	X

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References

[1] TFOS. Report of the international dry eye workshop (DEWS). Ocul Surf 2007;5:65–204.
[2] Craig JP, Nichols KK, Akpek EK, Caffery B, Dua HS, Joo C-K, et al. TFOS DEWS II report: definition and classification 2017 Available from: http://www.tfosdewsreport.org/report-definition_and_classification/48_36/en/.
[3] Bron AJ, Tomlinson A, Foulks GN, Pepose JS, Baudouin C, Geerling G, et al. Rethinking dry eye disease: a perspective on clinical implications. Ocul Surf 2014;12(2 Suppl):S1–31.
[4] Carver JA, Rekas A, Thorn DC, Wilson MR. Small heat-shock proteins and clusterin: intra- and extracellular molecular chaperones with a common mechanism of action and function? IUBMB Life 2003;55(12):661–8.
[5] Humphreys DT, Carver JA, Easterbrook-Smith SB, Wilson MR. Clusterin has chaperone-like activity similar to that of small heat shock proteins. J Biol Chem 1999;274(11):6875–81.
[6] Wilson MR, Easterbrook-Smith SB. Clusterin is a secreted mammalian chaperone. Trends Biochem Sci 2000;25(3):95–8.
[7] Poon S, Rybchyn MS, Easterbrook-Smith SB, Carver JA, Pankhurst GJ, Wilson MR. Mildly acidic pH activates the extracellular molecular chaperone clusterin. J Biol Chem 2002;277(42):39532–40.
[8] Yerbury JJ, Poon S, Meehan S, Thompson B, Kumita JR, Dobson CM, et al. The extracellular chaperone clusterin influences amyloid formation and toxicity by interacting with prefibrillar structures. Faseb J Off Publ Fed Am Soc Exp Biol 2007;21(10):2312–22.
[9] Matsuda A, Itoh Y, Koshikawa N, Akizawa T, Yana I, Seiki M. Clusterin, an abundant serum factor, is a possible negative regulator of MT6-MMP/MMP-25 produced by neutrophils. J Biol Chem 2003;278(38):36350–7.
[10] Jeong S, Ledee DR, Gordon GM, Itakura T, Patel N, Martin A, et al. Interaction of clusterin and matrix metalloproteinase-9 and its implication for epithelial homeostasis and inflammation. Am J Pathol 2012;180(5):2028–39.
[11] Aronow BJ, Lund SD, Brown TL, Harmony JA, Witte DP. Apolipoprotein J expression at fluid-tissue interfaces: potential role in barrier cytoprotection. Proc. Natl. Acad. Sci. U.S.A 1993;90(2):725–9.
[12] Kinoshita S, Adachi W, Sotozono C, Nishida K, Yokoi N, Quantock AJ, et al. Characteristics of the human ocular surface epithelium. Prog Retin Eye Res 2001;20(5):639–73.
[13] Nishida K, Adachi W, Shimizu-Matsumoto A, Kinoshita S, Mizuno K, Matsubara K, et al. A gene expression profile of human corneal epithelium and the isolation of human keratin 12 cDNA. Invest Ophthalmol Vis Sci 1996;37(9):1800–9.
[14] Wong P, Pfeffer BA, Bernstein SL, Chambers ML, Chader GJ, Zakeri ZF, et al. Clusterin protein diversity in the primate eye. Mol Vis 2000;6:184–91.
[15] 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(6):2052–61.
[16] Zhou L, Beuerman RW, Foo Y, Liu S, Ang LP, Tan DT. Characterisation of human tear proteins using high-resolution mass spectrometry. Ann Acad Med Singapore 2006;35(6):400–7.
[17] Zhou L, Zhao SZ, Koh SK, Chen L, Vaz C, Tanavde V, et al. In-depth analysis of the human tear proteome. Journal of proteomics 2012;75(13):3877–85.
[18] 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(8):R72.
[19] Green-Church KB, Nichols KK, Kleinholz NM, Zhang L, Nichols JJ. Investigation of the human tear film proteome using multiple proteomic approaches. Mol Vis 2008;14:456–70.
[20] Perumal N, Funke S, Wolters D, Pfeiffer N, Grus FH. Characterization of human reflex tear proteome reveals high expression of lacrimal proline-rich protein 4 (PRR4). Proteomics 2015;15(19):3370–81.
[21] Tong L, Zhou XY, Jylha A, Aapola U, Liu DN, Koh SK, et al. Quantitation of 47 human tear proteins using high resolution multiple reaction monitoring (HR-MRM) based-mass spectrometry. Journal of proteomics 2015;115:36–48.
[22] Hogasen K, Mollnes TE, Harboe M, Gotze O, Hammer HB, Oppermann M. Terminal complement pathway activation and low lysis inhibitors in rheumatoid arthritis synovial fluid. J Rheumatol 1995;22(1):24–8.
[23] Newkirk MM, Apostolakos P, Neville C, Fortin PR. Systemic lupus erythematosus, a disease associated with low levels of clusterin/apoJ, an antiinflammatory protein. J Rheumatol 1999;26(3):597–603.
[24] Nishida K, Kawasaki S, Kinoshita S. Clusterin may be essential for maintaining ocular surface epithelium as a non-keratinizing epithelium. Adv Exp Med Biol 1998;438:629–35.
[25] Bauskar A, Mack WJ, Mauris J, Argueso P, Heur M, Nagel BA, et al. Clusterin seals the ocular surface barrier in mouse dry eye. PLoS One 2015;10(9):e0138958.
[26] Ozcura F, Aydin S, Helvaci MR. Ocular surface disease index for the diagnosis of dry eye syndrome. Ocul Immunol Inflamm 2007;15(5):389–93.
[27] Amparo F, Schaumberg DA, Dana R. Comparison of two questionnaires for dry eye symptom assessment: the ocular surface disease index and the symptom assessment in dry eye. Ophthalmology 2015;122(7):1498–503.
[28] Nichols KK, Mitchell GL, Zadnik K. The repeatability of clinical measurements of dry eye. Cornea 2004;23(3):272–85.
[29] Wolffsohn JS, Arita R, Chalmers R, Djalilian A, Dogru M, Dumbleton K, et al. TFOS DEWS II diagnostic methodology report. Ocul Surf 2017;15(3):539–74.
[30] Yoon D, Gadaria-Rathod N, Oh C, Asbell PA. Precision and accuracy of TearLab osmometer in measuring osmolarity of salt solutions. Curr Eye Res 2014;39(12):1247–50.
[31] Lemp MA. Report of the national eye institute/industry workshop on clinical trials in dry eyes. CLAO J Off Publ Contact Lens Assoc. Ophthalmol. Inc 1995;21(4):221–32.
[32] Green-Church KB, Zhang L, Nichols KK. Comparison of protein extraction techniques from tears collected by schirmer strips for proteomic analysis. Investig Ophthalmol Vis Sci 2010;51(13):4315.
[33] Ray WA, O'Day DM. Statistical analysis of multi-eye data in ophthalmic research. Invest Ophthalmol Vis Sci 1985;26(8):1186–8.
[34] Wilson MR, Easterbrook-Smith SB. Clusterin binds by a multivalent mechanism to the Fc and Fab regions of IgG. Biochim Biophys Acta 1992;1159(3):319–26.
[35] Gipson IK, Spurr-Michaud SJ, Senchyna M, Ritter 3rd R, Schaumberg D. Comparison of mucin levels at the ocular surface of postmenopausal women with and without a history of dry eye. Cornea 2011;30(12):1346–52.
[36] Fini ME, Bauskar A, Jeong S, Wilson MR. Clusterin in the eye: an old dog with new tricks at the ocular surface. Exp Eye Res 2016;147:57–71.
[37] Morrissey C, Lakins J, Moquin A, Hussain M, Tenniswood M. An antigen capture assay for the measurement of serum clusterin concentrations. J Biochem Biophys Meth 2001;48(1):13–21.
[38] Karnati R, Laurie DE, Laurie GW. Lacritin and the tear proteome as natural replacement therapy for dry eye. Exp Eye Res 2013;117:39–52.
[39] 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(11):4889–905.
[40] Li B, Sheng M, Li J, Yan G, Lin A, Li M, et al. Tear proteomic analysis of Sjogren

syndrome patients with dry eye syndrome by two-dimensional-nano-liquid chromatography coupled with tandem mass spectrometry. Sci Rep 2014;4:5772.
[41] Li B, Sheng M, Xie L, Liu F, Yan G, Wang W, et al. Tear proteomic analysis of patients with type 2 diabetes and dry eye syndrome by two-dimensional nano-liquid chromatography coupled with tandem mass spectrometry. Invest Ophthalmol Vis Sci 2014;55(1):177–86.
[42] Zhou L, Beuerman RW. Tear analysis in ocular surface diseases. Prog Retin Eye Res 2012;31(6):527–50.
[43] Wyatt AR, Yerbury JJ, Ecroyd H, Wilson MR. Extracellular chaperones and proteostasis. Annu Rev Biochem 2013;82:295–322.
[44] Cuida M, Legler DW, Eidsheim M, Jonsson R. Complement regulatory proteins in the salivary glands and saliva of Sjogren's syndrome patients and healthy subjects. Clin Exp Rheumatol 1997;15(6):615–23.
[45] Delaleu N, Mydel P, Kwee I, Brun JG, Jonsson MV, Jonsson R. High fidelity between saliva proteomics and the biologic state of salivary glands defines biomarker signatures for primary Sjogren's syndrome. Arthritis Rheum 2015;67(4):1084–95.
[46] Akpek EK, Klimava A, Thorne JE, Martin D, Lekhanont K, Ostrovsky A. Evaluation of patients with dry eye for presence of underlying Sjogren syndrome. Cornea 2009;28(5):493–7.
[47] Koller L, Richter B, Winter MP, Sulzgruber P, Potolidis C, Liebhart F, et al. Clusterin/apolipoprotein J is independently associated with survival in patients with chronic heart failure. J Clin Lipidol 2017;11(1):178–84.
[48] Thambisetty M, Simmons A, Velayudhan L, Hye A, Campbell J, Zhang Y, et al. Association of plasma clusterin concentration with severity, pathology, and progression in Alzheimer disease. Arch Gen Psychiatr 2010;67(7):739–48.
[49] Thambisetty M, An Y, Kinsey A, Koka D, Saleem M, Guntert A, et al. Plasma clusterin concentration is associated with longitudinal brain atrophy in mild cognitive impairment. Neuroimage 2012;59(1):212–7.
[50] Bettens K, Vermeulen S, Van Cauwenberghe C, Heeman B, Asselbergh B, Robberecht C, et al. Reduced secreted clusterin as a mechanism for Alzheimer-associated CLU mutations. Mol Neurodegener 2015;10:30.
[51] Boxer Wachler BS. Assessment of levels of ultraviolet a light protection in automobile windshields and side windows. JAMA ophthalmology 2016;134(7):772–5.
[52] Bashir H, Seykora JT, Lee V. Invisible shield: review of the corneal epithelium as a barrier to UV radiation, pathogens, and other environmental stimuli. J Ophthalmic Vis Res 2017;12(3):305–11.
[53] Milner MS, Beckman KA, Luchs JI, Allen QB, Awdeh RM, Berdahl J, et al. Dysfunctional tear syndrome: dry eye disease and associated tear film disorders - new strategies for diagnosis and treatment. Curr Opin Ophthalmol 2017;27(Suppl 1):3–47.
[54] Nichols KK, Nichols JJ, Mitchell GL. The lack of association between signs and symptoms in patients with dry eye disease. Cornea 2004;23(8):762–70.
[55] Ozyildirim AM, Wistow GJ, Gao J, Wang J, Dickinson DP, Frierson Jr. HF, et al. The lacrimal gland transcriptome is an unusually rich source of rare and poorly characterized gene transcripts. Invest Ophthalmol Vis Sci 2005;46(5):1572–80.
[56] Sullivan DA, Jensen RV, Suzuki T, Richards SM. Do sex steroids exert sex-specific and/or opposite effects on gene expression in lacrimal and meibomian glands? Mol Vis 2009;15:1553–72.
[57] Ubels JL, Gipson IK, Spurr-Michaud SJ, Tisdale AS, Van Dyken RE, Hatton MP. Gene expression in human accessory lacrimal glands of Wolfring. Invest Ophthalmol Vis Sci 2012;53(11):6738–47.
[58] Mishima K, Inoue H, Nishiyama T, Mabuchi Y, Amano Y, Ide F, et al. Transplantation of side population cells restores the function of damaged exocrine glands through clusterin. Stem Cell 2012;30(9):1925–37.
[59] Reeder DJ, Stuart WD, Witte DP, Brown TL, Harmony JA. Local synthesis of apolipoprotein J in the eye. Exp Eye Res 1995;60(5):495–504.
[60] Nakamura T, Nishida K, Dota A, Kinoshita S. Changes in conjunctival clusterin expression in severe ocular surface disease. Invest Ophthalmol Vis Sci 2002;43(6):1702–7.
[61] Senchyna M, Wax MB. Quantitative assessment of tear production: a review of methods and utility in dry eye drug discovery. J Ocul Biol Dis Infor 2008;1(1):1–6.
[62] Jenne DE, Tschopp J. Molecular structure and functional characterization of a human complement cytolysis inhibitor found in blood and seminal plasma: identity to sulfated glycoprotein 2, a constituent of rat testis fluid. Proc. Natl. Acad. Sci. U.S.A 1989;86(18):7123–7.
[63] Choi NH, Tobe T, Hara K, Yoshida H, Tomita M. Sandwich ELISA assay for quantitative measurement of SP-40,40 in seminal plasma and serum. J Immunol Meth 1990;131(2):159–63.
[64] Murphy BF, Kirszbaum L, Walker ID, d'Apice AJ. SP-40,40, a newly identified normal human serum protein found in the SC5b-9 complex of complement and in the immune deposits in glomerulonephritis. J Clin Invest 1988;81(6):1858–64.
[65] Hogasen K, Mollnes TE, Tschopp J, Harboe M. Quantitation of vitronectin and clusterin. Pitfalls and solutions in enzyme immunoassays for adhesive proteins. J Immunol Meth 1993;160(1):107–15.
[66] Dota A, Nishida K, Quantock AJ, Kinoshita S. Clusterin in human corneal endothelium and aqueous humor. Exp Eye Res 1999;69(6):705–8.
[67] Kujiraoka T, Hattori H, Miwa Y, Ishihara M, Ueno T, Ishii J, et al. Serum apolipoprotein j in health, coronary heart disease and type 2 diabetes mellitus. J Atherosclerosis Thromb 2006;13(6):314–22.
[68] Choi-Miura NH, Ihara Y, Fukuchi K, Takeda M, Nakano Y, Tobe T, et al. SP-40,40 is a constituent of Alzheimer's amyloid. Acta Neuropathol 1992;83(3):260–4.
[69] Calero M, Rostagno A, Matsubara E, Zlokovic B, Frangione B, Ghiso J. Apolipoprotein J (clusterin) and Alzheimer's disease. Microsc Res Tech 2000;50(4):305–15.
[70] Ng V, Cho P, To C. Tear proteins of normal young Hong Kong Chinese. Graefes Arch Clin Exp Ophthalmol 2000;238(9):738–45.
[71] Aine E, Morsky P. Lysozyme concentration in tears–assessment of reference values in normal subjects. Acta Ophthalmol 1984;62(6):932–8.
[72] Baier G, Wollensak G, Mur E, Redl B, Stoffler G, Gottinger W. Analysis of human tear proteins by different high-performance liquid chromatographic techniques. J Chromatogr 1990;525(2):319–28.
[73] Seifert K, Gandia NC, Wilburn JK, Bower KS, Sia RK, Ryan DS, et al. Tear lacritin levels by age, sex, and time of day in healthy adults. Invest Ophthalmol Vis Sci 2012;53(10):6610–6.
[74] Robert MC, Arafat SN, Spurr-Michaud S, Chodosh J, Dohlman CH, Gipson IK. Tear matrix metalloproteinases and myeloperoxidase levels in patients with Boston keratoprosthesis type I. Cornea 2016;35(7):1008–14.
[75] 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(7):3052–8.
[76] Bailey RW, Dunker AK, Brown CJ, Garner EC, Griswold MD. Clusterin, a binding protein with a molten globule-like region. Biochemistry 2001;40(39):11828–40.
[77] Jones SE, Jomary C. Clusterin. Int J Biochem Cell Biol 2002;34(5):427–31.
[78] Kapron JT, Hilliard GM, Lakins JN, Tenniswood MP, West KA, Carr SA, et al. Identification and characterization of glycosylation sites in human serum clusterin. Protein Sci Publ Protein Soc 1997;6(10):2120–33.