Human tear protein analysis using a quantitative microfluidic system: a pilot study

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Running title: Tear protein analysis using on-chip microfluidic system

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Number of figures – 5, number of tables -3

Conflict of interest: This work is original, has not been published and is not being considered for publication elsewhere. The authors disclose no commercial relationship associated with this research.

Acknowledgement: Nil

Key words: Tear film, dry eye, tear analysis, lysozyme, lactoferrin, biomarker

Transparency Declaration

The corresponding author affirms that this manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and there is no discrepancy from the study as planned.

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Abstract

Objectives: Human tears have the potential to be used as biomarkers to aid in the diagnosis and management of dry eye disease (DED). This prospective, controlled pilot study aimed to investigate the hypothesis that a panel of tear protein profiles can be detected and are repeatable when analysed using a miniaturised quantitative microfluidic system.

Methods:

Ten participants were recruited following institutional ethics committee approval. Participants attended two visits one week apart when the following measurements were taken in a sequence: tear meniscus height (TMH), non-invasive breakup time (NIBUT), ocular redness, tear collection, corneal and conjunctival staining. Basal tears (>4µl) were collected using glass microcapillary tubes. Tears were processed to analyse a panel of proteins (14-230 kDa) following manufacturer guidelines using a miniaturised quantitative microfluidic system (Protein 230 LabChip® with Agilent 2100 Bioanalyzer). Demographics of the clinical measurements and a comparison of the panel of identified proteins and their repeatability were made.

Results: Mean age of the participants was 20.8 ± 1.6 years, nine were females, three fulfilled the TFOS DEWS-II diagnostic criteria for DED. The total protein concentration across participants was 6.72 ± 3.56 mg/ml. Seven proteins (Lysozyme-C, Lipocalin-1, IgA- light chain, Zinc-α2- glycoprotein, IgA – heavy chain, Albumin, and Lactoferrin) were identified at both visits for seven or more participants. There were no significant differences ($p>0.05$) in individual protein concentrations between the two visits. A high correlation was found between the two visits for all proteins where correlation coefficient ranged between 0.63-0.98 ($P<0.05$).

Conclusion:

The protein profiles measured by the quantitative microfluidic system are repeatable, thus validating it as a reliable method for investigating a panel of tear proteins. This method is quick, affordable, requires only 4µL of tear and is relatively easy to perform which can be incorporated in a clinical setting. Further studies in larger clinical setting may be beneficial exploring the usability of this method in various patient groups.

Keywords: tear analysis; proteins; IgA; lysozyme; lactoferrin

Introduction:

The tears are composed mainly of lipids, proteins, mucins, and electrolytes, which provide a regulated environment that is critical to the health of the ocular surface. The tear film provides a mechanical, lubricating and antimicrobial barrier to the ocular surface, and helps to ensure a smooth optical refractive surface. It is approximately 3µm thick[1] and 8±3 µl in volume[2], and its production by the lacrimal and meibomian glands is regulated by parasympathetic and sympathetic innervation. The aqueous/mucin layer makes up the bulk of the tears with the outer lipid layer being around 50-100nm thick[1]

Alteration of healthy tear proteins and upregulation of pro-inflammatory small proteins (cytokines) are the hallmark of ocular surface diseases such as dry eye disease (DED). Tear proteins can be classified according to their abundance.[3] The bulk of the tear protein concentration is made up of a few high abundance lacrimal proteins (mg/ml to µg/ml): lysozyme, lipocalin, lactoferrin, secretory immunoglobulin A (IgA), plasma-derived albumin and lipophilin.[4, 5] Proteins secreted by the ocular tissue or cell signalling molecules tend to be of moderate abundance (µg/ml to ng/ml) and cytokines and growth factors in low abundance (ng/ml to pg/ml)[6]. A reduction in the total tear protein content and a decrease in proteins with antibacterial and protective functions in early DED have been reported previously[5]. A decrease in proteins with lipid-binding functions and an increase in some pro-inflammatory proteins was also found.

The Agilent 2100 Bioanalyzer is a microfluidics-based platform for the simultaneous quantification of proteins, DNA/RNA or cells, by miniaturised capillary gel electrophoresis in conjunction with an appropriate LabChip kit. Three protein kit assays are available: Protein 80 kit, Protein 230 kit and High Sensitivity protein kit, for sizing and quantifying protein samples from 5 to 80kDa, 14 to 230kDa and 5 to 250kDa, respectively.[7] Each chip contains an interconnected set of microchannels that sieve proteins by size as they are driven through it by means of electrophoresis. During gel electrophoresis, the proteins to be separated are pushed by an electrical field through the gel and detected with laser-

induced fluorescence. The bioanalyzer software allows the data for each individual sample to be displayed as a gel-like image in bands, as in SDS-PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis), or as electropherogram peaks. Peaks in the protein profile can be identified based on their molecular weight/mass (kDa) identity. Peak height, area, relative concentration and percentage of the overall protein content, can also be investigated. Comparisons between tear samples taken at different times are possible by overlaying the electropherograms, allowing for any changes in the profile to be identified.

The Bioanalyzer rapidly produces results in 30-40 mins (in addition to 30 minutes of preparation time) and only uses a 4µl sample for analysis of proteins between 14 to 230 kDa. The purpose of the pilot study was to characterise the panel of human tear proteins and their repeatability, using this chip-based capillary gel-electrophoresis device.

Methods:

This was a prospective, controlled pilot study to investigate the repeatability of the tear protein profile found by on-chip electrophoresis utilising the Agilent 2100 Bioanalyzer. The study received ethics approval from the Aston University Research Integrity and Ethics Committee (ref# AUREC 1375 (21-04)) and followed the tenets of the declaration of Helsinki.

Participant enrolment

Ten consecutive participants with no ocular or systemic disease except DED were recruited from the patient population. All investigations were conducted at the baseline (visit 1) and after 1 week ± 2 days (visit 2). A thorough ocular and systemic history were taken, contact lens wearers were allowed to participate and participants were allowed to use rewetting drops at least two hours before the study visits.[8] The 12-item Ocular Surface Disease Index (OSDI) questionnaire was administered to all participants at visit 1 to assess any symptoms of dry eye disease. A score of ≥13 combined with one or more following signs of a loss of homeostasis of the tear film; non-invasive tear breakup time (NIBUT) <10 seconds or ocular surface staining (>5 fluorescein corneal spots, >9 lissamine green spots or lid wiper staining ≥2 mm length & ≥25 % widths) were used to diagnose DED.[9]

Clinical processes:

Clinical evaluations were made at both visits using the Keratograph 5M (Oculus, Wetzlar, Germany) in a non-invasive to an invasive sequence. These included tear meniscus height (TMH; average of three measurements taken at the lower limbus), NIBUT (average of three readings), ocular redness, tear collection, and corneal and conjunctival staining with fluorescein (Bio Fluoro Biotech, Ahmedabad, India) illuminated with blue light and a yellow observation filter, and lissamine green (Green Glo Ophthalmic Strips, HUB Pharmaceuticals, Himachal Pradesh, India) staining. Corneal and conjunctival staining was graded according to the Oxford Grading Scale.[10] Ocular redness (bulbar and

limbal areas both nasally and temporally, a total of four measurements) was graded objectively by the automated Keratograph 5M using JENVIS grading scale of 0 to 4 in 0.1 steps.

Tear collection:

Glass microcapillary tubes (10 µl, Sigma-Aldrich, USA) were used for the collection of non-stimulated tears from participants. To avoid possible diurnal variations in secreted proteins,[11] all the tear samples were collected at the same time of the day (between 2 and 4pm) except for two, where both their visits had to be scheduled in the late morning based on participant availability.

Participants were asked to tilt their heads towards the side of the collection and look up and away, at least 4 µL of basal tears were collected for a maximum of 5 minutes. The microcapillary tube was placed carefully at the lateral tear meniscus (figure 1), with minimal touch to the bulbar conjunctiva or lid margin to avoid stimulation of reflex tearing. Participants were able to blink during the procedure and the collection was paused for 30 minutes if any reflex tearing was suspected. Collected samples were transferred from the microcapillary tubes with a plunger into 0.5ml microcentrifuge tubes (Eppendorf, Germany), centrifuged at 5000 g for 10 minutes at 4˚C, the supernatant collected, labelled and stored at -80˚C until use.

Tear analysis using Agilent bioanalyzer quantitative microfluidic system:

At first, tear samples (samples) were pre-treated according to the protocols provided with the Protein 230 LabChip® kit. This involved the addition of 2 µl of denaturing solution to 4 µl of the sample. The denaturing solution was prepared by combining 200 µl of sample buffer with 7µl of the reducing agent β-mercaptoethanol. The sample buffer contained upper and lower markers of known molecular weight, identical to those in the standard ladder and were therefore incorporated into each sample allowing direct comparison against it. The samples and ladder (6µl) were denatured by placing the tubes in a hot water bath (95-100°C) for 5 min, cooled immediately and centrifuged for 15s (Fisherbrand™ Fisher Scientific, MA, USA, 2000g at room temperature). Eighty-four microlitres of

deionized water was then added to each sample and the ladder. As deionized water contains no charge, only denatured proteins, which have a net negative charge, conduct electricity.

A 6 µl aliquot of each sample (into wells 1 to 10) and the ladder (into ladder well) were loaded onto the chip, which was initially filled with 12 µl of a gel/dye mix into wells marked 'G' and a de-staining solution into well 'DS' (figure 2). It was necessary to keep all the reagents covered as they contained dye which decomposes when exposed to light.

Once loaded the chip was placed carefully into the bioanalyzer. Each chip run took 30 to 40 minutes, during which time the ladder and then each sample was processed in turn. Results are displayed as gel-like image bands or as electropherogram peaks for each sample. Peaks in the protein profile were identified based on their molecular weight/mass (kDa) and the findings from previous studies[4, 5, 7, 12-15], and concentrations were obtained the result page of the software.

Statistical analysis:

Data analysis was performed using Microsoft® Office Excel® and GraphPad InStat version 3.00 for Windows, GraphPad Software, San Diego California USA. A comparison of the main proteins identified and the protein concentrations at each visit was made for each participant. Quantitative data are expressed as mean ± standard deviation (SD). Frequency and percentage were used for the categorical data and clinical parameters. The coefficient of repeatability (1.96 x standard deviation of the mean of the differences) was calculated for the percentage of the total concentration for each major protein identified at both visit 1 and 2. The Kolmogorov- Smirnov test was used to test for normality. As some data was not normally distributed the non-parametric Wilcoxon Matched Pairs t-test (Wilcoxon signed ranks test) was used where proteins were present in both visit samples. The strength of any correlation between the two visits (Spearman's Rank correlation coefficient r) was estimated [16]. A high correlation was considered to be 0.5-1.0, moderate 0.5-0.3, and 0.3 to 0.1 a small if any correlation [17]. Given the pilot nature of this study, sample size calculation was not conducted. A p value of <0.05 was considered statistically significant.

159

Results:

All participants completed both visits, among them 9 were females and 1 male with a mean age of 20.8 ± 1.6 years. Participant demographics, ocular surface and tar film characteristics of the study participants are detailed in Table 1. Three participants fulfilled the TFOS DEWS II diagnostic criteria for DED. Four participants wore soft contact lenses, two of whom were classified as DED by TFOS DEWS II diagnostic criteria. Contact lens wearers were asked to remove contact lens and tear was collected immediately after following the same procedure for all study visits. Attention was given to make sure contact lens wear schedule and regimen didn't change during the study visits. Four participants reported occasional use of rewetting or lubricating drops, but not within 2 hours to the visit.

Total protein content and concentrations:

In this study, the mean total protein concentration collected by microcapillary tubes during both visits was 6.72 ± 3.56 mg/ml. Figure 3A shows the total protein concentrations per visit for each participant. Participant 1 (RS01) visit 2 had a substantially higher tear concentration of 19.64mg/ml, which is evident in Figure 3B box plot shown as an outlier, further outlier analysis was not done. Median and mean protein concentrations during visit 1 were 5.91 mg/ml and 6.15±2.16 mg/ml and during visit 2 were 6.08 mg/ml and 7.28±4.62 mg/ml respectively. The box and whisker plot show that the mean for both the visits were in similar range, inter-quartile distribution was wider for the visit 1 compared to visit 2, however the median was higher for visit-2 compared to visit-1. After removing the outlier data of Participant 1 visit 2, the mean total protein concentration for visit 1 and 2 combined was 6.04 ± 1.90 mg/ml. There was no significant difference ($P<0.05$) in the total protein concentrations found between visit 1 and 2.

Protein analysis and interpretation:

The results from the ladder were scrutinised carefully where, a successful ladder featured 7 well resolved ladder peaks, a flat baseline and readings of at least 20 sequential fluorescence units ranging between 4.5 kDa and 240 kDa. The standard protein ladder contained proteins of known concentrations and molecular weights allowing for semiquantitative analysis.

A range of 6 to 12 protein peaks were found, the mean being 9.65 ± 1.70 proteins identified. Based on protein identification provided by previous studies, [5, 7, 14, 18, 19] seven of the most abundant proteins were identified which are detailed in table 2, including their theoretical size, the study size range, mean concentrations and confidence intervals.

To assess the repeatability of the tear analysis method, gel-images for visit-1 and visit-2 protein bands were placed alongside each other (figure 4). The darker bands correspond to the electropherogram peaks. The upper and lower markers of known molecular weight contained in the sample buffer appear at 4.5 kDa and 240 kDa respectively. Individual protein concentrations as a percentage of total protein concentration are shown in figure 5.

Table 3 shows an analysis of eight proteins that were present at both visits for five or more participants. Considering each protein as a percentage of the total protein concentration, the coefficient of repeatability was high ranging between 0.85 to 11.83. The Wilcoxon t-test indicated that no significant differences were found for each protein as a percentage of the total concentrations between the two visits. The Spearman's correlation coefficients for the percentages of the total concentration between the two visits were between 0.5-1.0, which are considered to be high or very high.

Discussion

This study showed that the panel of tear protein assessment by on-chip electrophoresis with the Agilent 2100 Bioanalyzer are repeatable, and able to determine a range of key tear proteins such as lysozyme, lactoferrin, lipocalin, albumin and lgA. This microfluidic on-chip approach is quick (taking 30-40 minutes), easy to use and particularly suitable for ocular surface and dry eye research where collection of a large volume of basal tear is challenging.[4] The potential impact of wide scale use of this technique could be easy monitoring of tear protein constituents which may unfold underlying pathophysiology and progression of various ocular surface diseases as well as acting as a biomarker for effective treatments.

Given the nature of ocular surface disease such as DED, the collection of sufficient tears could be challenging, particularly when the patient had an element of aqueous deficiency. The current method examined a panel of tear proteins and their concentrations, the total protein concentration, the relative concentration of each protein, and the percentage of the total tear protein concentration. It is important to emphasise that the percentage of each peak contributes to the whole profile is an important relative measure could be found by this method, which could also be used for the comparison of protein profiles over time or pre and post treatment observations. In comparison, the Westernblot or ELISA (enzyme-linked immunosorbent assay) can only measure one or a handful of samples at a time and detect one or two proteins. Other customizable multiplex arrays which allow quantification of multiple proteins are expensive, require a well-validated antibody pair, and involve multiple complex steps. Therefore, the Bioanalyzer provides a relatively quick analysis of tear samples, requiring only 4 µL, compared to the typical 25–50 µL sample volume required for multiplex immunoassays or >10 µL per target required for ELISA.[20]

Sample treatment prior to electrophoresis has been shown to lead to different migration behaviour of proteins dependent on whether they have undergone non-reducing or reducing conditions. Denaturing or reducing the proteins prior to analysis gives a more accurate measure of molecular

weight and can separate subunits in multimeric proteins. In reduced tears, (as per the protocol for the Bioanalyzer), immunoglobulin heavy and light chains with molecular weights of 64 and 28 kDa, respectively, have been detected.[13]

Tear proteins have been shown to be sensitive to changes in sample buffer temperature and incubation time. Intrasubject analysis of tear protein patterns using the Agilent 2100 Bioanalyzer has shown differences dependent on whether the samples were analysed immediately following addition of a sample buffer or incubated with the sample buffer for 30 minutes at 37 °C;[21] two additional protein bands were identified between 31-33 kDa after prolonged incubation.

Distinct diurnal variations in the total protein concentration of tear fluid have also been shown, from 6.0 mg/ml in reflex tears, to 9.0 mg/ml in open eye tears and 18.0 mg/ml in closed eye tears. Closed eye tears were not collected in this study.

A protein peak between 144.7-149.2 kDa was present at both visits in five of the participants and at a single visit in 3 participants. Using the same method, Mann et al. also found a protein peak between 146.2-148.5kDa which they suggested to be secretory immunoglobulin A (sIgA) whole molecule. [15] However, the total size of sIgA is 385 kDa, composed of a light-chain and a heavy-chain, which are linked by a J-Chain and a glycoprotein called a secretory component. [22] The secretory component (~85 kDa) has been reported to migrate with lactoferrin.[13] Mann et al. also reported fragments of secretory IgA at ~26 kDa (light-chain) and ~73 kDa (heavy chain). [15] Other studies have reported similar findings, with an IgA-light chain at 28 kDa and an IgA-heavy chain at 64 kDa.[13, 23] However, using SDS-gel monodimensional electrophoresis in parallel to the Bioanalyzer, Giannaccare et al later confirmed that IgA-heavy chain in tears is found between 47 to 62 kDa which is similar finding to the current study.[24] Therefore, the peak present in this study between 28.1-30.5 kDa could be identified as IgA-light chain. The study by Sack et al. investigating tear samples separated on 12.5% SDS-PAGE under reducing conditions, indicated a significant increase in the gel band corresponding to this molecular weight in closed eye tears, when compared to open or reflex tears, suggesting its correct identification.[23]

This study found that the coefficient of repeatability for were high for most proteins, which may be due to the large measurement range and the relatively small number of repeats performed. As the concentration of albumin is more likely to be variable than the main indigenous proteins, it is contrary to the expectation that this should have the smallest coefficient of repeatability. Wilcoxon t-test showed no significant differences to the percentages of the total protein concentrations between the two visits. The Spearman's correlation coefficients for each protein as a percentage of the total protein concentration between the two visits, also showed good repeatability. The main indigenous proteins lysozyme, lipocalin and lactoferrin showed excellent repeatability with coefficients of 0.93, 0.93 and 0.98 respectively.

A limitation of tear analysis with the Bioanalyzer is its inability to detect smaller or less abundant proteins, such as the many cytokines and chemokines which are altered during inflammation in DED (ng/ml to pg/ml concentrations).[25] This method is also unable to directly identify a protein from electropherogram peak without prior knowledge of theoretical molecular weight, which requires further developments. In addition, proteins with adjacent molecular weights could be challenging to differentiate and may have an overlapping range. The current study was not powered to detect differences between dry eye and participants with 'healthy' eyes, but rather recruited a representative sample of the general population. Future studies will assess tear film biomarkers between participants with and without dry eye. A retrospective analysis of sample size calculation based on lysozyme, lactoferrin and lipocalin concentration found in this pilot study was conducted, which suggest a total of 77, 46, and 52 participants will be required to differentiate DED with non-DED participants. This calculation was performed with G-Power (version 3.1.9.7) using the difference between two dependent means (matched pairs) t test, with a two-tailed α-level of 5%, an 80% power level and a calculated effect size of 0.62.

In conclusion, the results obtained from this pilot study demonstrates that the inter-visit tear protein profiles measured by the Agilent 2100 Bioanalyzer are repeatable, thus emphasising it as a reliable

method for investigating a panel of tear proteins. This method is quick, affordable, requires only 4µL of tear and is relatively easy to perform which can provide proportionate quantification of several proteins. This microfluidic approach will need incorporation in large clinical studies characterising variety of patients groups, which may help improve the diagnosis and monitoring of various ocular surface diseases.

Figure 1. Tear collection using a 10 µl glass microcapillary tube

Figure 2. Protein 230 LabChip (Agilent technologies)

Figure 3 (A) Total protein concentrations at each visit for each participant. (B) Box plot showing the distribution of total protein concentrations during visit 1 and 2. Total protein concentration found for participant-1 visit-2 was higher indicated as an outlier.

Figure 4: Gel images from the bioanalyzer electropherograms with protein bands found for each participant. To assess repeatability results of visit 1 and 2 for each participant are reported alongside each other.

Figure 5. Protein concentrations displayed as the percentages of total protein concentration for each participant at visits 1 and 2. ZAG indicates Zinc-α2- glycoprotein, ~100kDa indicates an unidentified protein at 100kDa.

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