SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILISOPHY (PhD)

# New opportunities in the imaging and proteomic studies of tear

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UNIVERSITY OF DEBRECEN

DOCTORAL SCHOOL OF CLINICAL MEDICINE

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The PhD Defense will be held on 16th May, 2022. at 1 p.m.

Live online access will be provided. If you wish to join the discussion, please send and an e-mail to the toth.noemi@pte.hu address until 12 a.m. at latest on the previous day of the defense (13th May, 2022.). For technical reasons, after the deadline, it will not be possible to join the defense.

# 1. Introduction and literature review

A stable preocular tear film is a feature of a healthy eye, forming the primary refractive surface for light entering the visual system in addition to protecting and hydrating the cornea. Three layers (two phases) of the tear film are distinguished: the mucin layer, that covers the ocular surface, reduces the presumed hydrophobicity of the epithelial cells, the aqueous layer lubricates the ocular surface, provides it with nutrients and has antibacterial properties, and the lipid layer prevents evaporation of the tear film.

Various methods to reveal the structure of the tear film are found in literature, ranging from conventional tests such as fluorescein tear break-up time (TBUT) and Schirmer test to the rotator Scheimpflug imaging study of the fluorescein-stained ocular surface (Pentacam, Oculus, Germany) and the simultaneous video imaging of the fluorescein-stained ocular surface, which can be combined with imaging of the lipid layer of the tear film.

The novel device, LacryDiag® (Quantel Medical, France), can diagnose dry eye syndrome (DED) using a range of non-contact tests, such as measurement of lower tear meniscus height (LTMH), upper (MeibS) and lower (MeibI) eyelid meibography, interferometry (INT) and non-invasive tear break-up time (NIBUT).

In addition to oxygen, metabolites and electrolytes, tear film contains antimicrobial peptides, proteins and soluble immunoglobulins that protect the ocular surface from infections. As a body fluid, tear is widely tested during the diagnostic work-up of ophthalmic diseases. The total protein concentration of tear is about 10 mg/ml, which is approximately seven times less than the serum protein level. Proteins, small organic molecules, metabolites,

electrolytes and lipids in tear may be potential biomarkers for ophthalmic and systemic diseases.

The sensitivity of modern proteomic techniques has enabled the identification of more than 1500 proteins. Qualitative proteomics, which can identify the presence of a protein in a sample, has been replaced by quantitative proteomics with the development of instruments used to measure post-translational modifications on proteomic platforms. However, multiplex methods are needed to analyze multiple proteins in the same sample. Approaches using combined methods include a combination of antibody-based detection and quantitative polymerase chain reaction (qPCR). By combining two separate high-throughput analytical techniques, proximity extension assay (PEA) allows the relative quantification of multiple proteins in very small sample volumes (1 µl), providing an efficient tool for the analysis of small volumes of body fluids.

## 2. Objectives

- The aim of our study was to investigate the reliability and clinical applicability of a modern tear film imaging device by comparing inter- and intraobserver differences.

- We examined the difference between the traditional reference method and the modern diagnostic tool by comparing TBUT to NIBUT measured with LacryDiag®.

- We applied the PEA method for qualitative and quantitative tear analysis in non-invasively collected tear samples.

- In order to make a better understanding of post-trabeculectomy wound healing processes, we analyzed the molecular abnormalities after surgery with focusing on flap complications.

# 3. Patients and methods

## 3.1. Patient groups

### 3.1.1. Patient groups in the imaging study

Our study was carried out at the Department of Ophthalmology of the Clinical Center of the University of Pécs. The 50 healthy volunteers (20 males and 30 females) included in the study did not have any prior general or ophthalmological diseases, did not wear contact lenses, and did not undergo intraocular or any type of refractive surgery during or prior to the study. In accordance with the principles of the Declaration of Helsinki, all patients were informed in detail and their written informed consent was obtained prior to their inclusion. The research was approved by the local ethics committee.

### 3.1.2. Patient groups in the proteomics study

Tear samples were collected from glaucoma patients who underwent trabeculectomy at the Department of Ophthalmology of the University of Debrecen. The sampling was performed in accordance with the Declaration of Helsinki and was approved by the local ethics

committee. The 8 patients (2 males and 6 females) included in the study gave written informed consent for tear sampling before undergoing trabeculectomy (day 0), as well as on the first and fourth postoperative day, and in the third postoperative month. In one case, the sample was collected after 10 months instead of 3 months. Patients were retrospectively divided into two groups: “complications” and “no complications”, depending on whether they required the use of eye drops to reduce intraocular pressure in the first postoperative year. In three patients, surgical intervention failed to achieve the targeted IOP and therefore medicinal therapy was necessary. No early postoperative complications were reported in the patients studied. The following events were considered early postoperative complications: trabecular bleb leak, low intraocular pressure (hypotony), hyphema, choroidal effusion, hemorrhage or inflammation (blebitis, endophthalmitis). Exclusion criteria included concomitant diseases like autoimmune pathologies, systemic inflammatory conditions and non-glaucomatous diseases of the ocular surface.

## 3.2. Clinical trials

### 3.2.1. Imaging study

Comprehensive ophthalmological examination was performed: visual acuity measurement was followed by anterior segment slit-lamp examination, LacryDiag® (Quantel Medical, France) and TBUT measurements. TBUT was performed at least 5 minutes after the LacryDiag ®test. LTMH, MeibS, MeibI, INT and NIBUT parameters measured by the device meet the criteria of the TFOS DEWS 2 report; therefore, these were the non-contact tests evaluated in our study.

The LacryDiag® test and TBUT measurement were performed by the same experienced physician for each patient. Two independent experienced investigators selected and analyzed

LTMH, MeibS, MeibI and INT. The second investigator re-analyzed the data one month later. The test took about 10 minutes and none of the patients complained of pain, discomfort or visual disturbance.

With LacryDiag®, LTMH is measured semi-automatically. During the test, the height of the tear accumulated in the lower meniscus is measured by the examiner using a caliper. The average of five measurements was used for our analysis.

During Meib, the LacryDiag® uses infrared imaging and image analysis of the Meibomian glands, with automatic border detection and manual corrections if necessary. The technique is based on white light transillumination of the everted eyelid. In the semi-automatic method, the instrument determines the percentage of gland loss after the examiner manually selects the area to be examined.

INT is a qualitative and quantitative analysis of the lipid layer. This method allows the detection of a thin layer of oily phase on the surface of the aqueous phase. The kinetics and reflectance pattern of the oily phase allow the thickness of the lipid layer to be assessed. This layer of the tear film is mostly produced by the Meibomian glands. The instrument allows the comparison of video recordings of the patient's ocular surface with reference videos (7 pre-recorded videos).

NIBUT determines the stability of the tear film based on the rate of evaporation. It is based on the observation of the reflection of a pattern projected to the surface of the eye. The NIBUT software automatically detects blinking, records the interval between two blinks and calculates the NIBUT value. The investigator's task is limited to starting and stopping the recording and does not involve any classification task. According to the manual, if the interval between blinks reaches 12 seconds, the recording should be stopped. For statistical analysis,

an ordinal scale was used to reduce statistical bias. In comparing the two methods, the cut-off value for the ordinal scale of NIBUT and TBUT was set at 10 seconds based on the literature, but a cut-off value of 12 seconds for NIBUT and 8 seconds for TBUT showed better diagnostic ability, thus statistical calculations were performed with both cut-off values.

#### 3.2.2. Proteomic analysis

##### 3.2.2.1. Tear sampling

Non-invasive tear collection was performed using sterile glass capillary tubes (VWR Ltd. Radnor, PA, USA) for 2 minutes from the lateral lower meniscus without the use of local anesthesia or stimulation. After centrifugation (at 4°C with 2.4 xg for 10 minutes), supernatants were divided into five µl aliquots, deep-frozen and stored at -70°C until analysis. After tear sampling, ophthalmic examination was performed (visual acuity, intraocular pressure measurement, slit-lamp examination of the anterior and posterior segments).

##### 3.2.2.2. Relative quantification of proteins by proximity extension assay

PEA studies were performed by Olink Proteomics (Uppsala, Sweden). Using the inflammatory and cardiovascular disease II (CVD II) panels, relative quantification of a total of 184 proteins was performed. We selected the proteins to be tested based on our previous research. These proteins were found in the inflammation panel and the CVD II panel, and therefore these two panels were chosen. The values for each protein were obtained in NPX units, the relative protein abundance unit introduced by Olink Proteomics.

##### 3.2.2.3. Functional analysis

Hierarchical clustering of the proteins detected in more than 30% of the samples was performed using Gene Cluster 3.0 (http://cluster2.software.informer.com/) and heatmap

analysis was performed using Java TreeView v1.1.6r4. No filtering or adjustment was performed on the data prior to clustering, distance/similarity measures were based on Pearson's correlation, and clustering was performed using full correlation analysis. The network of selected proteins was plotted using String 10.5 with default settings and medium stringency, and gene ontology (GO) features with higher representation (i.e., enriched) compared to the random distribution were highlighted. The String database is an easy-to-use tool for analyzing protein-protein interaction networks and provides information on protein structure and function. For proteins with statistically significant changes, pathway analysis was performed using the Wikipathways search function. (https://www.wikipathways.org/index.php/WikiPathways). Pathways were assessed manually.

## 3.3. Statistical methods used

### 3.3.1. Imaging study

Statistical analysis was performed using Intercooled Stata for Windows (version 13.0). In all cases, examination data from the right eye were used. For descriptive statistics, mean, standard deviation (SD) and 95% confidence interval (95% MT) of the mean were reported. For LTMH, MeibS and MeibI, intraclass correlation coefficients (ICC) were calculated to estimate interobserver and intraobserver reliability. An ICC below 0.4 indicates poor reliability, an ICC between 0.4 and 0.59 indicates satisfactory reliability, a result between 0.6 and 0.74 indicates good reliability, and a result between 0.75 and 1.0 indicates excellent reliability. Weighted Cohen's kappa statistic was used to evaluate the agreement index between INT and NIBUT and TBUT due to their ordinal category. Cohen's kappa coefficient

values indicate low (below 0.2), satisfactory (0.21–0.40), moderate (0.4–0.60), substantial (0.61–0.80) or almost perfect (0.81–1.0) agreement. Bland-Altman plots were used to visualize the differences between the evaluations and to determine the limits of agreement (LoA; mean difference ± 1.96 × the standard deviation of the difference). In these plots, the middle line indicates the mean difference between instruments and the top and bottom lines indicate the 95% LoA values. A value of $p < 0.05$ was defined as statistically significant.

#### 3.3.2. Proteomic analysis

A non-parametric Mann–Whitney U-test was used to compare the mean NPX values for the two groups. Statistical analysis was performed using the SPSS 25.0 program (IBM Inc., USA). To examine the effect of surgical outcome and/or time on relative protein levels, linear mixed model and analysis of variance (ANOVA) were applied using the ImerTest function of the R program. It was performed by Olink (Sweden). Tear samples from eight different patients, taken at five different time points, were included in the analyses. Proteins with less than 30% detectability were removed from the analysis, resulting in the analysis of 138 proteins out of 184.

Time and complications were considered as fixed effects, subject ID as a random effect. Due to the small number of patients included in the study, the effect of gender was not examined. Fixed effect p-values were estimated using Satterthwaite's approximation of degrees of freedom and corrected using the Benjamini–Hochberg method. For all significant examinations, post hoc tests were performed to estimate the population mean and pairwise differences between groups. Post hoc p-values were calculated using the Tukey method.

# 4. Results

## 4.1. Imaging study

Of the fifty healthy volunteers tested, 15.6% of the TBUT measurements were under 8 seconds and 28.12% were under 10 seconds. When measuring NIBUT, 28.12% of subjects were under 10 seconds and 75% were under 12 seconds.

For LTMH, both inter- and intraobserver variability were excellent (interobserver ICC = 0.805; intraobserver ICC = 0.868). Between two examiners, MeibI ICC was poor (MeibI ICC = 0.464), but MeibS ICC was good (MeibS ICC = 0.666). The intraobserver variability of MeibI and MeibS was excellent (MeibI ICC = 0.760; MeibS ICC = 0.771) The Bland–Altman plots of LTMH, MeibI and MeibS show a high degree of variability in LoA values between groups.

Between the two examiners and between two examinations of the same examiner, the agreement index for INT evaluation was fair and moderate (INT interobserver value = 0.301; $p = 0.0002$; INT intraobserver value = 0.566; $p < 0.001$), but the agreement index was higher for the intraobserver comparison. Bland–Altman plots for INT show a high degree of variability in LoA between groups for all parameters.

When comparing the NIBUT and TBUT methods, there was little agreement for different cut-off values (NIBUT cut-off: 12 sec and TBUT: 8 sec: kappa coefficient = 0.075; $p = 0.099$; NIBUT and TBUT cut-off: 10 sec: kappa coefficient = 0.054; $p = 0.376$).

## 4.2. Proteomic analysis

Tear sample analysis with proximity extension assay

Tear samples were analysed by Olink Proteomics (Uppsala, Sweden) with PEA using CVD II and inflammatory panels. Each panel contained 92 proteins and four internal controls, providing information on a total of 184 proteins. A technical problem was encountered with the analysis of brain-derived neurotrophic factor, so analysis of this protein was not feasible. Internal controls were added to each sample to control the analysis and the quality of each sample. Quality control (QC) was performed in two steps: i) each sample plate was evaluated based on the SD of the internal controls and only those sample plates that passed the QC (SD <0.2 NPX) were included, ii) the quality of each sample was evaluated by the deviation of the median value of the controls. Samples that deviated from the median by less than 0.3 NPX passed the quality control. It occurred for 55 out of 60 proteins for CVD II and 57 out of 60 for the inflammatory panels. Most proteins could be measured with a coefficient of variation (CV) of <5%, with intraassay CV typically being 4%. Based on the above, the reliability of the PEA method was found to be adequate and applicable to the analysis of tear samples.

Examination of protein level changes after trabeculectomy

Proteins with at least a 20% intergroup difference (between “complications” and “no complications” group) were introduced into the String network analysis program. The network of proteins less likely to occur in the samples of patients in the “no complications” group contained 17 proteins. von Willebrand factor–cleaving protease, alpha-1-microglobulin/bikunin precursor, chemokine (C-C motif) ligand 3, CD40 ligand, CD84 molecule, carbonic anhydrase 5A, 2,4-dienoyl-CoA reductase 1, low affinity receptor for IgG Fc fragment, fibroblast growth factor

23, gastric intrinsic factor, leptin, collagen macrophage receptor, atrial natriuretic factor, renin, thrombopoietin, tumor necrosis factor superfamily member 14 and UDP-glucose:glycoprotein glucosyltransferase 1 are less likely to be found in samples from patients with complications. Six proteins, however, were more likely to be present in the “complications” group: interleukin 17C, interleukin 10 receptor subunit alpha, interleukin 20 receptor subunit alpha, fibroblast growth factor 19, artemin and tumor necrosis factor superfamily member 11.

For a more accurate assessment, qualitative analysis was followed by a quantitative analysis. Out of 184 proteins, 46 proteins were detected in less than 30% of the samples and were excluded from further statistical analysis. The changes in the relative abundance of proteins were visualized on a heat map. Hierarchical clustering of data showed that these protein patterns did not clearly distinguish the “complications” group from the “no complications” group. However, for 14 proteins, higher abundances were observed in samples of the “complications” group: caspase 8, S100A12 protein, TNF superfamily member 14, monocyte chemotactic protein 3, chemokine (C-C motif) ligand 23, nuclear factor kappa-B essential modulator, carcinoembryonic antigen-related cell adhesion molecule 8, spondin 2, superoxide dismutase, glyoxalase 1, osteoclast-associated immunoglobulin-like receptor, poly-ADP ribose polymerase-1, signal transducing adaptor molecule and eukaryotic initiation factor.

Nine proteins showed statistically significant differences between the two groups according to the non-parametric Mann–Whitney U-test: TNFRSF10A $p=0.033$; TNFRSF11A $p=0.018$; IL-18 $p=0.014$; polymer immunoglobulin receptor $p=0.033$; prolargin $p=0.023$; MMP12 $p=0.016$; IL8 $p=0.03$; MCP-1 $p=0.023$; CCL3 $p=0.019$. The amount of all proteins except PIGR were increased in the samples from the “complications” group. The analysis showed a

network with few connections and the enriched pathways were associated with the immune response.

In order to obtain more information about the events that regulate wound healing and to track the effects of time and/or complications on relative protein levels, we performed a rigorous, in-depth statistical analysis using a linear mixed model. Regarding the effect of time, IL-6 and MMP-1 levels changed in a statistically significant manner. Both protein levels increased in a statistically significant manner on the first postoperative day, remained high on the second and fourth day, and returned to the original level three months after surgery. The changes were statistically significant for comparison between sampling days. Changes between the "complications" and "no complications" groups were not statistically significant.

With technological advances, both imaging tools and biochemical methods are expected to provide more accurate and precise information about structural and functional changes in tear, but more case-control studies are needed to develop guidelines for the widespread clinical use of these methods.

## 5. Main results and conclusions

Lacrydiag®

- The repeatability of the imaging technique was reliable, in addition the LTMH has shown excellent interobserver accuracy as well. However better results were achieved in the case of the other parameters, if the examination was performed by the same investigator. These results suggest that follow-up examinations of patients should be performed by the same ophthalmologist.

- The slight disagreement of the NIBUT and TBUT comparisons suggests that follow-up should be performed with the same method.
- The LacryDiag® instrument provides a non-invasive, standard way to help monitor the patient by video and image recording of test results.

Proximity extension assay

- Our results showed that in the samples of the “complications” group, proteins involved in different phases of wound healing showed an increase in both qualitative and quantitative aspects compared to the “no complications” group. In addition, we observed an increase in the levels of several proteins that can be measured at low levels in case of prolonged wound healing (e.g., GLO1, SOD2). However, proteins that regulate the normal progression of wound healing were less abundant in the samples of the “no complications” group. In conclusion, the analysis of tear proteins suggests the existence of wound healing imbalances that may lead to late surgical complications.
- Proteins involved in immune response and wound healing are detected in different amounts and/or frequency after surgery between the two groups of patients.
- We were the first to test tear samples using PEA, and our data suggest that PEA can be used to quantify the relative abundance of hundreds of proteins in individual tear samples.

# 6. Authorized list of references and other publications on which the thesis is based

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Registry number: DEENK/438/2021.PL
Subject: PhD Publication List

Candidate: Noémi Tóth
Doctoral School: Doctoral School of Clinical Medicine

**List of publications related to the dissertation**

1. **Tóth, N.**, Szalai, E., Rák, T., Lillik, V., Nagy, A. C., Csutak, A.: Reliability and clinical applicability of a novel tear film imaging tool.
   *Graefes Arch. Clin. Exp. Ophthalmol. 259* (7), 1935-1943, 2021.
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2. Csősz, É., **Tóth, N.**, Deák, E., Csutak, A., Tőzsér, J.: Wound-Healing Markers Revealed by Proximity Extension Assay in Tears of Patients following Glaucoma Surgery.
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   DOI: https://doi.org/10.3390/ijms19124096
   IF: 4.183

**List of other publications**

3. **Tóth, N.**, Silver, D. M., Balla, S., Káplár, M., Csutak, A.: In vivo corneal confocal microscopy and optical coherence tomography on eyes of participants with type 2 diabetes mellitus and obese participants without diabetes.
   *Graefes Arch. Clin. Exp. Ophthalmol. [Epub ahead of print]*, 2021.
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4. Szalai, E., **Tóth, N.**, Kolkedi, Z., Varga, C., Csutak, A.: Comparison of various intraocular lens formulas using a new high-resolution swept-source optical coherence tomographer.
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   In: A száraz szem kezelése. Szerk.: Berta András, Csutak Adrenne, Kolozsvári Bence, Módis László, Bauch & Lomb, [s.l.], 4-11, 2017.

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