UNIVERSITY of DEBRECEN

FACULTY OF MEDICINE
Department of Biochemistry and Molecular Biology
H-4002 Debrecen, Egyetem tér 1. P.O.B. 400.
Phone: +3652/416-432, email: bmbi@med.unideb.hu

Final report

in relation to OTKA proposal

"Proteomic alterations related to impaired wound healing leading to glaucoma surgery complications" (PD116817)

Principal investigator: Dr. Éva Csősz

Ethical committee (DE KK RKEB/IKEB) approval number: 423-2014

Glaucoma is a multifactorial neurodegenerative disease affecting the optic nerve, leading to impaired vision, and in advanced cases to blindness [1]. The neuropathy of the optic nerve and the loss of retinal ganglion cells can be observed, resulting in atrophy of the optic nerve and impairment of visual functions, leading to blindness [2,3].

Using various proteomic methods; such as LC-MS-based analyses and immunological methods, hundreds of proteins were found to be related to glaucoma, most of which were obtained by highly invasive techniques from the retina and the trabecular meshwork [4–9]. Biological fluids are often used instead of tissue samples for biomarker studies [10], among the ocular biological fluids used in the examination of glaucoma, the aqueous humor (AH) was frequently analyzed [9]. The AH is the product of the ciliary body, and is drained from the eye through the trabecular meshwork and the uveoscleral pathway. Proteomic analyses of AH samples from patients with glaucoma and controls revealed important proteins involved in metabolism, inflammatory response and antioxidant defense [9,11]. Extensive studies were conducted by different research groups to analyze the amount of inflammatory cytokines revealing higher levels of pro-inflammatory cytokines in AH originating from patients with glaucoma as compared to controls [12–16]. Despite the usefulness of AH as a source of biomarkers of trabecular meshwork and retinal ganglion cell damage, the invasive collection of this biological fluid hinders its application for the purpose of screening or follow-ups. While tear; given their availability and ease of collection, may contain glaucoma-related proteins traced directly to the AH, thereby providing a better alternative. Tear is a valuable biological fluid, widely studied in eye diseases for the purpose of identifying potential biomarkers for eye-related and systemic diseases [10].

During the study the possibility of utilization of tear as an accessible biological material was examined. For this reason the scientific literature was searched for studies which utilize tear as a source for potential biomarkers for ocular and systemic diseases. In the same time the possible quantitative proteomics methods were examined in order to find the most suitable method for tear analysis.

The data obtained from the literature searches regarding the utilization of body fluids and quantitative proteomics were summarized in a publication:

Éva Csősz, Gergő Kalló, Bernadett Márkus, Eszter Deák, Adrienne Csutak, József Tőzsér (2017) Quantitative body fluid proteomics in medicine - A focus on minimal invasiveness. J Proteomics, 153:30–43.

Beside the literature review focusing on the application of body fluids for biomarker research and the available quantitative proteomics methods, the literature related to ocular wound healing was also examined. The search results regarding the ocular wound healing were presented as part of a review publication about another eye-related neurodegenearative disease, the diabetic retinopathy:

Éva Csősz, Eszter Deák, Gergő Kalló, Adrienne Csutak, József Tőzsér (2017) Diabetic retinopathy: Proteomic approaches to help the differential diagnosis and to understand the underlying molecular mechanisms. J Proteomics, 150:351-358.

In glaucoma therapy the only controllable factor is the intraocular pressure, making the reduction of the increased intraocular pressure (IOP) the most important form of glaucoma treatment. This treatment can delay the disease process and prevent visual field loss [3]. Most of the patients are initially treated with eye drops but when the desired IOP cannot be reached surgery becomes necessary. Some minimal invasive microsurgery procedures are available, but the most widely used gold standard surgical intervention in open angle glaucoma is, still, filtration surgery. The latter involves trabeculectomy, which is an invasive procedure associated with relatively high complication and failure rate [17,18]. During trabeculectomy, a part of the trabecular meshwork is removed and a channel is created between the anterior chamber and the

FACULTY OF MEDICINE
Department of Biochemistry and Molecular Biology
H-4002 Debrecen, Egyetem tér 1. P.O.B. 400.
Phone: +3652/416-432, email: bmbi@med.unideb.hu

subconjunctival space leading to controlled leaking of the aqueous humor and, thus, lowering of the IOP. One of the key features of the success of trabeculectomy is the wound healing which might be impaired making the postoperative IOP control impossible [17].

Wound healing is a well-organized cascade of events in skin starting with a coagulative and inflammatory phase, followed by the proliferative and repair phase, and ending with the remodeling phase [19]. Ocular wound healing differs from that observed in case of skin [20] mainly in the timing of the phases and because of the three distinct tissue types involved: ocular epithelium, stroma and endothelium.

Following injury in the first few hours in the lag or latency phase, the released cytokines (mainly IL-1, IL-6, TNF-α and IL-8) orchestrate the early events of epithelial wound healing. The damaged cells are dying mainly by apoptosis, and the recruited immune cells help the debridement and the clearance of apoptotic cells [21,22]. MMPs are activated by IL-1 and other factors, and an extensive extracellular matrix rearrangement starts [23,24]. The epidermal (EGF), hepatocyte (HGF), keratocyte (KGF), platelet-derived (PDGF) and nerve (NGF) growth factors released upon injury or by the action of cytokines help the wound healing process. During this phase, some of the existing cellular junctions are removed, and there is a fibronectin polymerization to help the cell migration, and focal contacts are formed at the wound margin, preparing the conditions for cell migration. In the migration phase, cells migrate to the site of wound to cover the wound bed. The migration starts approx. 5 h after the injury and is directed by IL-6, KGF, HGF, PDGF, which is followed by cell proliferation stimulated by the strong mitogenic effect of growth factors. At the same time, extensive synthetic processes are taking place and the formation of basement membrane and restoration of the barrier functions happen [22,23,25].

In the stroma, injury is followed by the keratocyte apoptosis and recruitment of the immune cells. IL-1 and TGFβ released in the epithelial cell layer diffuse to the stroma due to the defects of the basement membrane and regulate the early events of stromal wound healing. The immune cells and keratocytes transform to fibroblasts and myofibroblast mainly upon the action of TGFβ, and migrate to the site of injury to fill up the wound [26–28]. Meanwhile the keratocytes and fibroblasts secrete growth factors that help the cell proliferation both in the stroma and in

the epithelial cell layer. The stromal and the epithelial wound healing ends with a slow and long remodeling phase that lasts for up to a year. During this phase in the epithelial cell layer, the stratification of the cells happens and the firm adherence of the cells to the underlying structures is reestablished. In the stroma, there is an extensive collagen remodeling, and the myofibroblasts disappear. Regarding the endothelial injury, cell migration is the most important process; the endothelial cells migrate to the site of injury and fill up the gap and secrete new basement membrane to restore the barrier functions, if necessary [23,24].

Under normal conditions the ocular wound-healing process ends up with scarless healing that is necessary for proper vision [23,24,29]. Each step of the process can be affected, leading to complications and ineffective surgical intervention. Reepithelization typically occurs mainly three to five days after surgery [24], but complications appear later highlighting the importance of the early events of wound healing [30,31].

Complications following trabeculectomy such as (flap related complications, chorioidal ablation, bleb failure) usually appear 6 months or 1 year after the surgical intervention and can lead to the ineffectiveness of the treatment. These patients may require additional topical treatment or another trabeculectomy.

Markers able to predict the appearance of complications have high importance giving possibility for ophthalmologists for the establishment of proper treatment protocols.

One possibility to address this question would be the examination of AH collected during surgery. One group have identified the elevated thrombospondin-1 level in AH as a risk factor for trabeculectomy failure at 1 year in patients with PACG [32]. However a more accessible body fluid, in this case the tear, would further help the prognostic process. If the risk group for trabeculectomy failure could be determined before the surgery that would give the possibility for the ophthalmologists to choose more appropriate therapeutical methodologies. In order to be able to state the utility of tear, first we need to answer the question if the tear can be used instead of AH.

During this study my aim was to collect as much information as possible on the differences between the protein profiles of patients who show complications following

trabeculectomy and of those without complications in order to be able to find potential protein markers for the prediction of trabeculectomy complications.

Patients and samples

The sample collection was done in accordance with the Declaration of Helsinki, and was approved by the Ethical Committee of the University of Debrecen (approval number: 4234-2014). Altogether 225 patients have been recruited into the study. Recruited subjects were patients of the Department of Ophthalmology, Faculty of Medicine, University of Debrecen, and gave written informed consent for sample collection. All of the patients underwent trabeculectomy surgery to reduce intraocular pressure at the Department of Ophthalmology, Faculty of Medicine, University of Debrecen. Exclusion criteria were the presence of autoimmune disease and/or any ocular surface disease other than glaucoma. The non-invasive tear collection was carried out before the trabeculectomy (day 0), on 1st, 2nd, and 4th day after trabeculectomy (day 1, day 2, day 4) and 3, 6 and 12 months following the surgical intervention. An attempt was made to collect tear also at day 10 and 1 months following trabeculectomy, but as a result of low patient compliance many of the patients did not come back on the indicated day, in this way the sample collection could not be done exactly in the indicated time points. The 7 day interval for sample collection in these time points were considered two long so the samples collected in these time points were omitted from further examinations. Samples were collected 2 and 4 hours after the trabeculectomy, but all of these samples were contaminated with blood, so we could not use them for the analysis of tear proteins. This fact led us to stop sample collection at these very early time points.

The non-stimulated tear sample collection was carried out with sterile glass capillary tubes (VWR Ltd., Hungary) for two minutes from the lateral inferior meniscus without local anesthesia or stimulation [33]. Tear samples were centrifuged at 4°C at 2.4xg for 10 minutes in a benchtop Eppendorf centrifuge; then, the supernatants were aliquoted to five-µl aliquots and deep frozen and stored at –70°C until analysis.

The AH samples were collected during trabeculectomy surgery through a limbal paracentesis by the same operator, using sterile glass capillary, care was taken to prevent blood

and intraocular tissue contamination. The samples were expelled from the capillaries to 0.2 mL PCR tubes and processed in an identical way as the tears. Protein concentration of tear and AH samples was determined using the Bradford method [34].

Ophthalmological examination

At the time of tear collection, an ophthalmological examination was carried out. The best corrected visual acuity was determined followed by applanation tonometry (Goldmann), slit-lamp examination, and in most of the cases anterior segment photo documentation. The presence of early and late complications such as infection, blebitis, endophthalmitis, visual acuity reduction, cataract, early and late flap related complications, bleb failure, postoperative hypotony and chorioidal ablation were also examined.

Comparison of tear and AH cytokine levels

In order to be able to answer the more elaborate questions regarding the time course of cytokine changes during the wound healing first the comparison of AH and tear had to be done.

As far as according to the scientific literature the inflammation has a very profound role in wound healing I have chosen the examination of cytokines and chemokines.

For the comparison of the tear and aqueous humor, 20 patients (11 male and 9 female; mean age: 58.8 ± 14.8) with glaucoma (8 patients with primary angle closure glaucoma (PACG) and 12 patients with POAG) were recruited and the preoperative tear and aqueous humour samples were collected. The concentration of interleukins IL-1β, IL-1Ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, eotaxin, basic fibroblast growth factor (bFGF), granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), interferon (IFN) γ, interferon gamma-induced protein 10 (IP-10), monocyte chemoattractant protein 1 (MCP1), macrophage inflammatory proteins MIP1α and MIP1β, platelet-derived growth factor (PDGF-BB), regulated on activation, normal T cell expressed and secreted (RANTES), tumor necrosis factor α (TNFα) and vascular endothelial growth factor (VEGF) was analyzed in tear and AH samples using a multiplex immunobead

FACULTY OF MEDICINE
Department of Biochemistry and Molecular Biology
H-4002 Debrecen, Egyetem tér 1. P.O.B. 400.
Phone: +3652/416-432, email: bmbi@med.unideb.hu

system based on xMAP technology (Luminex, USA). The 27-plex Bio-Plex kit (Bio-Rad Laboratories, USA) was utilized strictly adhering to the manufacturer's instructions.

In our samples all the 27 cytokines were present and could be examined. Considering that we did not include healthy control because our aim was to compare the two type of samples originating from the same patient, first we compared our results to data available in the literature. It was found that our results are in accordance with data presented in the literature, however, some differences could be detected. The higher level of some cytokines observed in our study compared to the values from the literature might indicate a more pronounced proinflammatory condition, which might be due to the fact that the patients recruited into our study had a more advanced phase of glaucoma, finally succumbing to trabeculectomy.

It should be noted that it is hard to compare results among the different studies, because the stage and type of the glaucoma, the tear sample collection method and the applied analytical methods all affect the level of cytokines.

When the effect of gender and type of glaucoma on the cytokine levels, tear production rate and tear protein concentration was examined, no statistically significant difference could be found between any of the studied groups.

The IOP of the patients and the presence of complications following trabeculectomy was monitored in case of the recruited patients. We could collect ophthalmological data in case of 19 out of 20 recruited patients and we examined the effect of the presence of complications on the level of tear cytokines, tear total protein concentration and tear production rate. In case of three proteins statistically significant differences could be observed; the concentration of IFN-γ was almost two times higher in the group of patients without complication, and the same tendency was observed in case of GM-CSF and IL-5 as well (Figure 1).

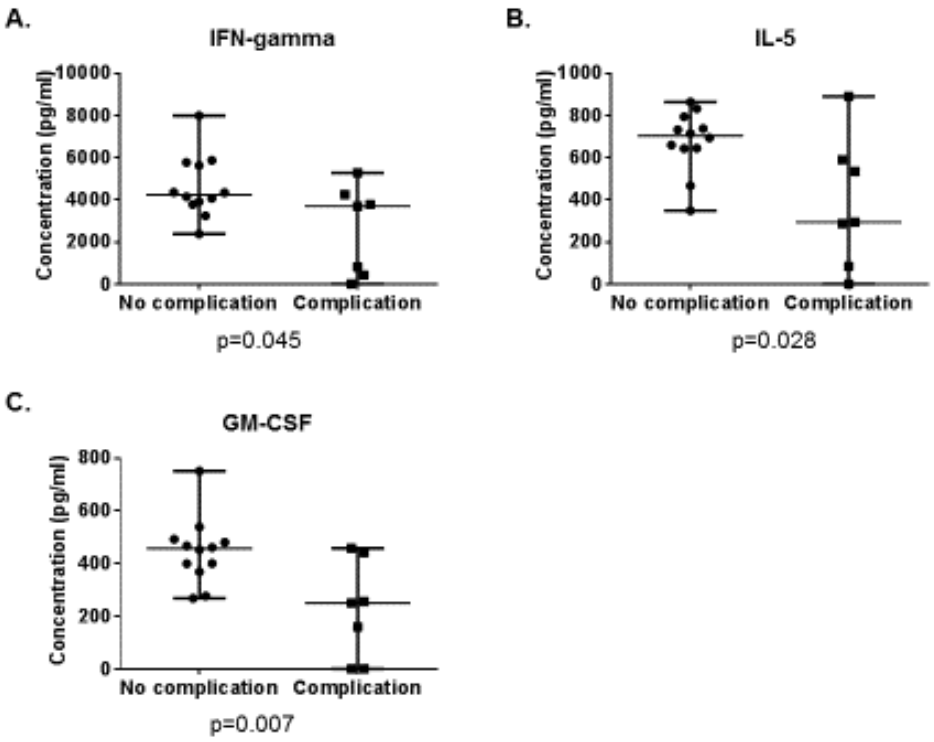

Figure 1. Concentration of cytokines with statistically significant difference between the groups with complications and no complication. The concentration (pg/ml) of IFN-gamma (A), IL-5 (B) and GM-CSF (C) in the two groups is plotted. Below each plot the p value is indicated.

According to our data the group with complications shows reduced levels in case of all of these cytokines, however according to the scientific literature faster wound healing, which might be one reason for bleb failure would require increased levels of these compounds. It should be noted that none of the studies regarding IL-5, IFN-γ and GM-CSF mentioned above were carried out on tear samples. The phenomena leading to bleb failure are not known in details and most probably multiple, more complex mechanisms take part in the regulation of the process. Despite the fact that at this point our results observed in human tear contradict to the results observed by others in skin or in rodent ocular wound healing models the IL-5, IFN-γ and GM-CSF can serve as a good starting point for further biomarker studies and verifications.

When the same type of analysis was carried out in case of AH as well, no statistically significant difference could be found between any of the studied groups.

The comparison of the concentration of cytokines found in AH and tear indicated a higher level of cytokines in tear compared to AH and this increase was statistically significant in all cases but IL-2 (Fig 2).

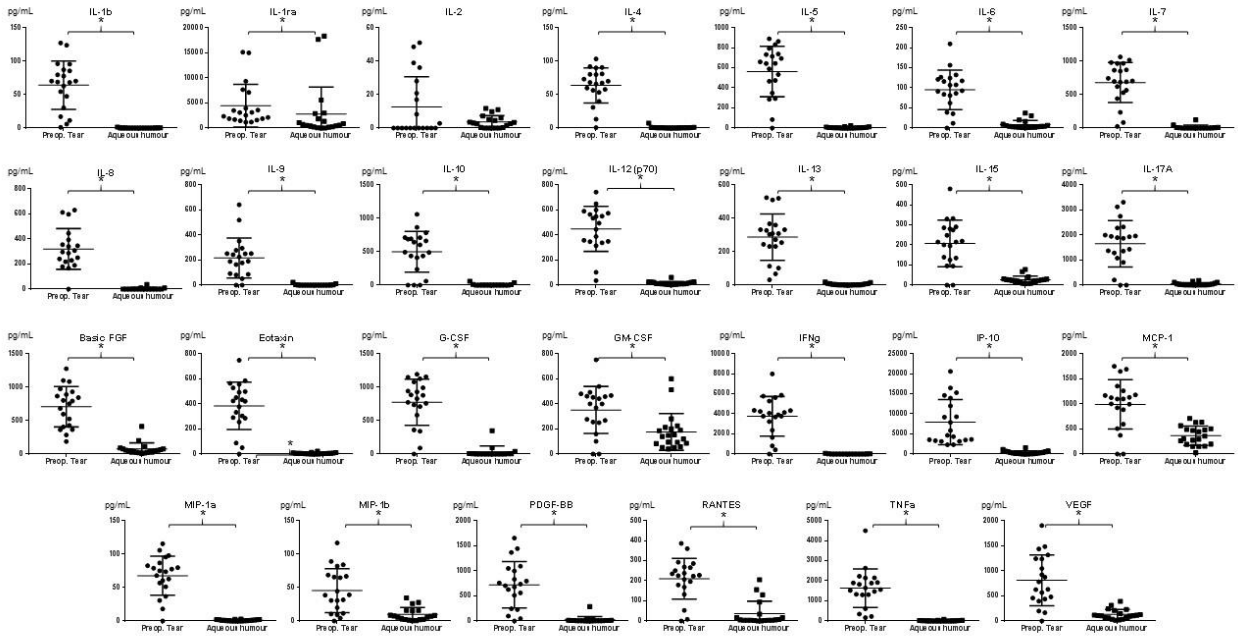

Figure 2. Concentration of 27 cytokines and chemokines in preoperative tear and aqueous humour samples from patients with glaucoma. The "x" axis shows the sample type, while "y" axis shows the concentration of the analyte in pg/ml. * indicate statistically significant ($p<0.05$) difference.

These data indicate that tear and AH samples are not identical from inflammatory point of view and tear cannot replace AH. Most probably the level of cytokines in the two sample types are controlled by different mechanisms. The higher cytokine levels in tear compared to AH might reflect not only the glaucoma-related, but the eye-drop- or other factors-induced proinflammatory conditions as well.

The altered concentration of three tear proteins, the tear IFN-γ, GM-CSF and IL-5 was associated with the presence of complications however, more patients have to be recruited to be able to identify/verify these proteins as potential risk factors for the occurrence of complications.

Our data highlight the promising potential of this continuously available, easy-to-collect body fluid for dynamic testing makes it worth exploring as an alternative to samples collectable by invasive methods. These data were summarized in a manuscript:

Éva Csősz, Eszter Deák, Noémi Tóth, Carlo Enrico Traverso, Adrienne Csutak, József Tőzsér: Comparative analysis of tear and aqueous humour cytokine profiles in patients with glaucoma - identification of potential predictive tear biomarkers for trabeculectomy complications and submitted to International Journal of Molecular Sciences.

As far as according to our data tear is a valuable biological fluid for the examination of glaucoma and in the same time it can be collected any time, a follow-up study examining the level of cytokines and chemokines on different postoperative days was carried out. Initially we intended to use the mass spectrometry and our aim was that with the help of a cytokine mix containing stabile isotope labeled reference peptides for more than 300 cytokines and chemokines to monitor the concentration of selected 25 molecules in the samples. Unfortunately the cytokine mix did not work and despite the extensive optimization and consultation with the vendor, the endogenous cytokine peptides could not be detected.

In order to get information on the changes in the cytokine profile after trabeculectomy tear samples were collected at day 0, day 1, day 2 and day 4 following trabeculectomy. For the follow-up study the same BioPlex system (27plex Luminex multiplex immunobead-based technique in duplicates) was used as that administrated for the comparison of tear and AH cytokine content. The data were examined in a similar way as before.

During the follow-up, on day 1 an increase was observed in the level of most of the cytokines but none of the differences were statistically significant. When the possible effect of other factors (gender, type of glaucoma, application of express shunt or mitomycin C during surgery, presence of complication) on the level of cytokines was examined statistically significant

differences in cytokine levels could be observed only in case of express shunt implantation and mitomycin C (MMC) administration.

In many cases during the surgery express shunt is utilized to help the AH drainage or antimitotic agents (5-fluorouracil or MMC) are administrated to prevent excessive scar formation and to improve the surgical success. The administration of the express shunt led to decreased IL-8 levels on day 1 ($p=0.041$). The utilization of 0.2 mg/ml MMC for 2 minutes during the surgery influenced the level of cytokines observed on all postoperative days (Figure 3).

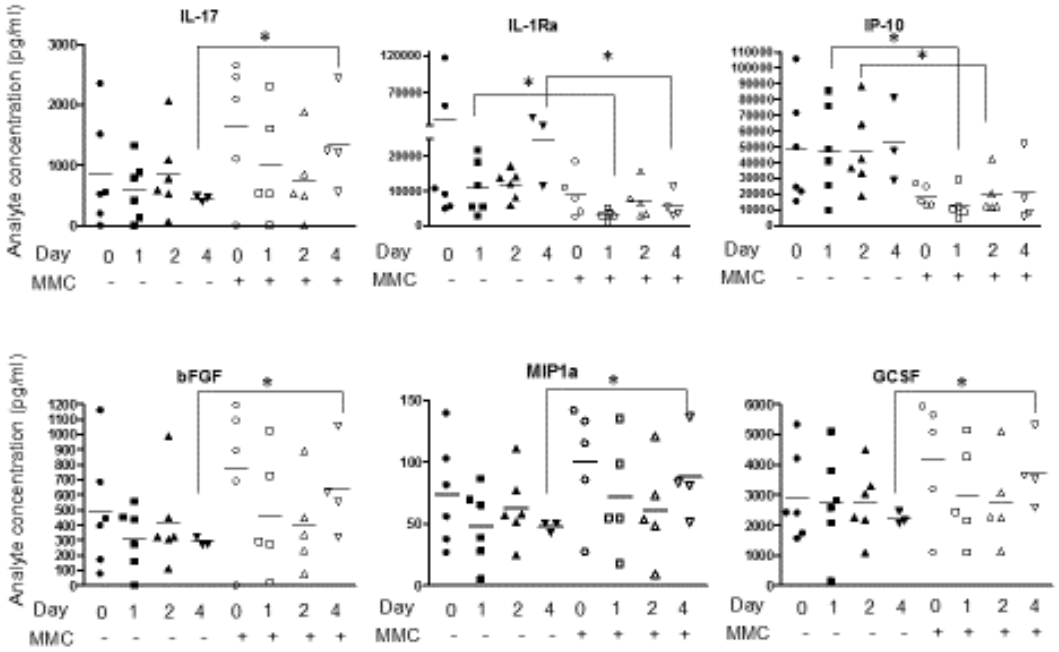

Figure 3. Concentration of cytokines showing statistically significant difference between the MMC treated vs. non-treated groups. The "x" axis shows the days and the administration of MMC, while "y" axis shows the concentration of the analyte in pg/ml. * indicate statistically significant ($p<0.05$) differences.

As it was expected, on day 0 no statistically significant difference was detected, while on day 1 the level of IL-1Ra and IP-10 decreased in the tears of patients treated with MMC. On day 2, the same phenomenon was observed in case of IP-10, and on day 4, a statistically significant

decrease in the level of IL-1Ra and increase in the level of IL-17, bFGF, G-CSF and MIP1α could be detected.

Examination of wound healing markers

By the examination of cytokine levels we could get information on the early inflammatory events taking place after trabeculectomy. One of my aims was to enlarge our knowledge regarding the proteins which play a role in the wound healing process and to analyze proteins other than cytokines. I intended to examine samples from three selected patients with wound healing complication and three selected ones without any complication after trabeculectomy with shotgun proteomic analyses. The iTRAQ method to be used in this stage of the research was tested and on standards worked well. However, unfortunately the amount of the tear and the sensitivity of the mass spectrometer available did not permit the analysis of tear samples in duplicates, so the application of the mass spectrometry for tear analysis was stalled. As far as pooling is not accepted in tear proteomics, I have decided to explore multiplex protein analysis assays with superior sensitivity to the available mass spectrometry system. I have decided to use the benefits of the proximity extension assay (PEA) which makes possible the sensitive and scalable analysis of multiple proteins in a single run from 1 µl sample by combining antibody-based detection with the well-defined methods used during quantitative PCR. This method makes possible the relative quantification of multiple proteins in very low sample amounts providing and effective tool in the analysis of body fluids available in low amounts (https://www.olink.com/).

The proteins which I intended to examine by mass spectrometry, some cytokines, the HGF, KGF, EGF, NGF and PDGF along with other proteins which, according to the scientific literature were shown to be important in wound healing, especially ocular wound healing, such as TGF, FGFs, matrix metalloproteinases etc. were included into our analyses. Previously I have designed SRM-based experiments for the targeted examination of growth factors in tears, but the sensitivity of the system was not sufficient for the proper detection and quantification of HGF, KGF, EGF and IGF-1 in tear of patients undergoing trabeculectomy.

With the approval of the NKFI Office (Dr. Gyula Szigeti, research and innovation vice-chairman) two panels the Inflammation (https://www.olink.com/products/inflammation/) and Cardiovascular II (https://www.olink.com/products/cvd-ii-panel/) panels were chosen and the relative quantities of 184 proteins were examined in 60 tear samples originating from 3 patients with and 5 patients without complications.

The amount and the frequency of the 184 tested proteins was examined in the two groups, first doing a qualitative analysis, followed by quantitative analysis, heat map analysis, hierarchical clustering and statistical analysis. The proteins with altered frequency or amount between the groups with and without complications were subjected to functional analysis. First the network of proteins was created with the help of String (https://string-db.org/) and analyzed followed by the GO enrichment analysis. In this way I could identify proteins present more or less likely in the samples of patients presenting complications, and the functional analysis of proteins with altered amounts between the two groups revealed the importance of wound healing and immune response in the group with complications. For the examination of the effect of the time and complication a linear mixed model was applied and significantly higher levels of IL-6 and MMP1 in the early time points (day 1, 2 and 4) following trabeculectomy could be observed. The protein amounts went back to the level observed before the surgery few months after the intervention.

A pathway analysis was carried out using the Wikipathways search function. There were 7 pathways containing both IL-6 and MMP1, and all of them were manually evaluated for relevance. Two of the pathways were specific to liver, two were versions of androgen regulation and the final two were very general: IL-4 and IL13 signaling and insulin-like growth factor transport and binding. As far as the photodynamic therapy-induced NF-kappa B pathway might have relevance in wound healing this one was selected. According to this pathway the NF-kappa B signaling is activated which in turn activates among the others, the expression of interleukins such as IL-6 and of matrix metalloproteinases such as MMP-1. When this pathway is activated in tumor cells it leads to an inflammation, followed by leukocyte migration into the tumor tissue leading to the apoptosis of the tumor cells. During ocular wound healing the damaged cells die by apoptosis and necrosis and the immune cells are recruited to help the

tissue regeneration and the clearance of dead cells. The higher activity of this pathway in the first few days (day 1, 2 and 4) after surgery might be responsible for the inflammatory part of the wound healing process.

My research had two main readouts: first I could demonstrate the applicability of proximity extension assay for tear analysis and second, I could observe altered frequency and amount of proteins having role in immune response and wound healing in the tears of patients with complications following glaucoma surgery. These results highlight the importance of inflammation in wound healing complications and in the same time indicate the utility of PEA in tear analysis.

These data were summarized in a scientific publication:

Éva Csősz, Noémi Tóth, Eszter Deák, Adrienne Csutak and József Tőzsér (2018) Wound healing markers revealed by proximity extension assay in tears of patients following glaucoma surgery. International Journal of Molecular Sciences, 19, 4096.

29 proteins which showed differential expression in the PEA were subjected to further mass spectrometry experiments. The proteotypic peptides were identified by an *in silico* design procedure applied in our lab previously [35,36] and all the transitions were recorded on Orbitrap Fusion tribrid mass spectrometer (Thermos Scientific). In the parallel reaction monitoring (PRM) analyses some of the previously examined proteins were also included [36]. The mass spectrometry data were acquired, and 12 out of 39 proteins could be detected and quantified in tear collected on day 0, day1, day 2 and day 120 from three patients with and three patients without complications. The in-depth analysis is still in progress, but according to the preliminary results some proteins show differential expression between the two groups.

Additional experiments carried out in order to find new ways for tear examination in glaucoma

Beyond the examination of protein profile changes we wanted to acquire information on other proteins having role in wound healing process. It is well known that transglutaminases and especially factor XIII has a role in the extracellular matrix remodeling and wound healing

FACULTY OF MEDICINE
Department of Biochemistry and Molecular Biology
H-4002 Debrecen, Egyetem tér 1. P.O.B. 400.
Phone: +3652/416-432, email: bmbi@med.unideb.hu

[38] and the involvement of α2-plasmin inhibitor (α2-PI) in the process was also demonstrated [39]. As far as both of these proteins were detected in tears [40,41] and we had a collaboration with Dr. Eva Katona and Dr. Laszlo Muszbek we have made an attempt to examine the level of these two proteins in the tear of patients with or without complications following trabeculectomy.

The previously developed immunoassay was used for the examination of factor XIII [41] and a highly sensitive chemiluminescent sandwich ELISA was developed to measure α2-PI in tiny volumes of tear.

The factor XIII could not be detected in any of the samples, most probably due to technical problems, but the α2-PI was present in case of samples collected on day 0, day 1 and day 2 originating from 5 out of 6 patients. This was just a pilot study but the presence of this protein in tear seems to be promising for further research on larger sample size.

Considering the important role of inflammation in the wound healing, beside the cytokines and chemokines I wanted to get information also on the level of eicosanoids. Tear proteins were precipitated and a targeted mass spectrometry-based method was adapted for the examination of eicosanoid molecules in the supernatant. Different precipitating agents were tested and the precipitation with acetontirile showed the best results. The prostaglandin E2 and D2, thromboxane B2 and arahidonic acid were detected in tear, however their concentration was near the detection limit of the instrument and in this way they could not be quantified.

With the acquisition of a new mass spectrometry system the profile of our laboratory broadened with the examination of amino acids. After setting up the analytical methods we tried to examine the level of free amino acids in tear. We could find literature data on tear amino acid analysis [42] and elevated homocystein leves were observed in glaucoma [43]. Although we could not analyze the homocystein levels, we were curious on the level of the proteinogenic amino acids in tear. One main obstacle in our study was the low available tear amount, so in order to be able to do the derivatization reaction, a pooling was required. POAG and PACG pools were created; a pool of tears from 10 patients in each group was generated, derivatized using the AccQ-Tag derivatization protocol by the Waters Company and analyzed in duplicates on Waters Acquity H-class UPLC. The clear distinction between Asp and Asn could not be

achieved, instead of individual amino acids, data for both of them in form of Asx is given. Similarly, Glx contains the combined results for Glu and Gln. Statistically significant differences could be observed between control and POAG groups in case of Gly, Trp, Phe, His, Thr, Ala, Pro, Asx, Leu, Tyr, Ser, Lys, Ile and Val, between the control and PACG groups in case of Gly, Trp, Phe, His, Thr, Ala, Pro, Asx, Leu, Tyr, Arg and Glx. The PACG and POAG groups could be discriminated from each other, significantly higher levels of Gly and Leu were observed in PACG compared to POAG. As far as measuring the amino acid concentration in tear is a relatively simple analytical method, the differences in the amino acid levels can serve as potential biomarkers helping the diagnosis of glaucoma. In the next step we will need to modify the method to be usable for the analysis of individual tear samples of low volumes.
These new examination possibilities not presented previously in the Workplan can lead us to new discoveries helping to better understand the wound healing mechanism in physiological and in pathological conditions.

Conclusion

A complex proteomics strategy involving antibody- and mass spectrometry- based methods, statistical analysis, protein network and pathway analyses was applied to study the proteomic profile changes in the tear and aqueous humour samples collected from patients who underwent trabeculectomy. In the same time we have done the first comparison of the tear and aqueous humour and we could observe that the tear and AH are not identical biological fluids from inflammatory point of view. Despite of the differences observed, tear can be used instead of AH for the examination of complication-related proteins. We could observe an increase in the level of the examined tear proteins in the early postoperative days, and with these data we could recapitulate on human tear samples a previous observation made on a rodent experimental trabeculectomy model [37]. The statistically significant time-dependent changes observed in case of IL-6 and MMP-1 in our study show an elevation in their concentration in the early postoperative days and go back to their preoperative level 3 months after the surgery. We could also show that proteins having role in wound healing and inflammation show altered abundance and amount in the group with complications compared to samples originating from patients

without complications. By using the Olink panels, we have demonstrated for the first time the utility of tear for PEA analysis.

It is very important to be able to group patients into low or high risk groups for the appearance of late complications after trabeculectomy, thus the identification of potential predictive biomarkers are crucial. In preoperative tear samples we could identify three proteins (IL-5, IFNg and GM-CSF) with lower level in the group with complications highlighting their potential as predictive biomarkers for the appearance of late complications after trabeculectomy.

According to our data, the most important events in the appearance of late complications are most probably related to the early phases of ocular wound healing. In these phases the proinflammatory processes dominate and I could demonstrate altered levels of proteins having role inflammation and wound healing in the samples originating from patients with complications. Most probably a shifted proinflammatory - antiinflammatory balance along with the change in the amount and abundance of proteins having role in wound healing leads to an altered wound healing starting in the first few postoperative days finalizing with the appearance of complications in the late remodeling phase of wound healing.

The metabolomic studies carried out highlighted the importance of amino acid analysis in the diagnosis of glaucoma and opened up new ways of tear examination.

My future plan is to combine the proteomic and metabolomic data with the ophthalmological data using a system biology approach and to make all the data collected during the study publicly available.

References

1. Kingman S. Glaucoma is second leading cause of blindness globally. Bull World Health Organ. 2004;82: 887–888. doi:/S0042-96862004001100019
2. Križaj D, Ryskamp DA, Tian N, Tezel G, Mitchell CH, Slepak VZ, et al. From mechanosensitivity to inflammatory responses: new players in the pathology of glaucoma. Curr Eye Res. 2014;39: 105–19. doi:10.3109/02713683.2013.836541
3. Chang EE, Goldberg JL. Glaucoma 2.0: Neuroprotection, neuroregeneration,

neuroenhancement. Ophthalmology. 2012;119: 979–86. doi:10.1016/j.ophtha.2011.11.003

4. Hagan S, Martin E, Enríquez-de-Salamanca A, Aass C, Norheim I, Eriksen E, et al. Tear fluid biomarkers in ocular and systemic disease: potential use for predictive, preventive and personalised medicine. EPMA J. 2016;7: 15. doi:10.1186/s13167-016-0065-3
5. Pieragostino D, Agnifili L, Fasanella V, D'Aguanno S, Mastropasqua R, Di Ilio C, et al. Shotgun proteomics reveals specific modulated protein patterns in tears of patients with primary open angle glaucoma naïve to therapy. Mol Biosyst. 2013;9: 1108–16. doi:10.1039/c3mb25463a
6. Yang X, Hondur G, Li M, Cai J, Klein JB, Kuehn MH, et al. Proteomics analysis of molecular risk factors in the ocular hypertensive human retina. Investig Ophthalmol Vis Sci. 2015;56: 5816–5830. doi:10.1167/iovs.15-17294
7. Höhn R, Mirshahi A, Nickels S, Schulz A, Wild PS, Blettner M, et al. Cardiovascular medication and intraocular pressure: results from the Gutenberg Health Study. Br J Ophthalmol. 2017; 1–5. doi:10.1136/bjophthalmol-2016-309993
8. Tezel G. A proteomics view of the molecular mechanisms and biomarkers of glaucomatous neurodegeneration. Prog Retin Eye Res. Elsevier Ltd; 2013;35: 18–43. doi:10.1016/j.preteyeres.2013.01.004
9. Funke S, Perumal N, Bell K, Pfeiffer N, Grus FH. The potential impact of recent insights into proteomic changes associated with glaucoma. Expert Rev Proteomics. 2017;14: 311–334. doi:10.1080/14789450.2017.1298448
10. Csosz É, Kalló G, Jakob BM, Deák E, Csutak A, Tozsér J. Quantitative body fluid proteomics in medicine - A focus on minimal invasiveness. J Proteomics. 2016;153: 30–43. doi:10.1016/j.jprot.2016.08.009
11. Kaeslin MA, Killer HE, Fuhrer CA, Zeleny N, Huber AR, Neutzner A. Changes to the aqueous humor proteome during glaucoma. PLoS One. 2016;11: 1–15. doi:10.1371/journal.pone.0165314
12. Takai Y, Tanito M, Ohira A. Multiplex cytokine analysis of aqueous humor in eyes

with primary open-angle glaucoma, exfoliation glaucoma, and cataract. Investig Ophthalmol Vis Sci. 2012;53: 241–247. doi:10.1167/iovs.11-8434

13. Khalef N, Labib H, Helmy H, Abd M, Hamid E, Moemen L, et al. Levels of cytokine in the aqueous humor of eyes with primary open angle glaucoma, pseudoexfoliation glaucoma and cataract. Electron Physician. 2017;9: 3833–3837. doi:http://dx.doi.org/10.19082/3833
14. Ohira S, Inoue T, Iwao K, Takahashi E, Tanihara H. Factors influencing aqueous proinflammatory cytokines and growth factors in uveitic glaucoma. PLoS One. 2016;11: 1–13. doi:10.1371/journal.pone.0147080
15. Du, Shaolin et al. Multiplex cytokine levels of aqueous humor in acute primary angle-closure patients: fellow eye comparison. BMC Ophthalmol. BMC Ophthalmology; 2016;16: 1–8. doi:10.1186/s12886-016-0182-8
16. Gu R, Zhou M, Jiang C, Yu J, Xu G. Elevated concentration of cytokines in aqueous in post-vitrectomy eyes. Clin Exp Ophthalmol. 2016;44: 128–134. doi:10.1111/ceo.12638
17. Van Bergen T, Van de Velde S, Vandewalle E, Moons L, Stalmans I. Improving patient outcomes following glaucoma surgery: state of the art and future perspectives. Clin Ophthalmol. 2014;8: 857–67. doi:10.2147/OPTH.S48745
18. Bar-David L, Blumenthal EZ. Evolution of Glaucoma Surgery in the Last 25 Years. Rambam Maimonides Med J. Israel; 2018;9. doi:10.5041/RMMJ.10345
19. Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M. Growth factors and cytokines in wound healing. Wound Repair Regen. United States; 2008;16: 585–601. doi:10.1111/j.1524-475X.2008.00410.x
20. Bukowiecki A, Hos D, Cursiefen C, Eming SA. Wound-healing studies in cornea and skin: Parallels, differences and opportunities. Int J Mol Sci. 2017;18: 1–24. doi:10.3390/ijms18061257
21. Eslani M, Movahedan A, Afsharkhamseh N, Sroussi H, Djalilian AR. The Role of Toll-Like Receptor 4 in Corneal Epithelial Wound Healing. Investig Opthalmology Vis Sci. 2014;55: 6108. doi:10.1167/iovs.14-14736
22. Ljubimov A, Saghizadeh M. Progress in corneal wound healing. Prog Retin Eye Res.

2016;49: 17–45. doi:10.1016/j.preteyeres.2015.07.002.Progress

23. Ashby BD, Garrett Q, Willcox MD. Corneal Injuries and Wound Healing – Review of Processes and Therapies. Austin J Clin Ophthalmol. 2014;1: 1–25. doi:10.1073/pnas.1019055108
24. Maycock NJR, Marshall J. Genomics of corneal wound healing: a review of the literature. Acta Ophthalmol. 2014;92: e170–e184. doi:10.1111/aos.12227
25. Yu FSX, Yin J, Xu K, Huang J. Growth factors and corneal epithelial wound healing. Brain Res Bull. 2010;81: 229–235. doi:10.1016/j.brainresbull.2009.08.024
26. West-Mays JA, Dwivedi DJ. The keratocyte: Corneal stromal cell with variable repair phenotypes. Int J Biochem Cell Biol. 2006;38: 1625–1631. doi:10.1016/j.biocel.2006.03.010
27. Barbosa FL, Chaurasia SS, Cutler A, Asosingh K, Kaur H, de Medeiros FW, et al. Corneal myofibroblast generation from bone marrow-derived cells. Exp Eye Res. Elsevier Ltd; 2010;91: 92–96. doi:10.1016/j.exer.2010.04.007
28. Wilson SE, Liu JJ, Mohan RR. Stromal-epithelial interactions in the cornea. Prog Retin Eye Res. 1999;18: 293–309. doi:10.1016/S1350-9462(98)00017-2
29. Spadea L, Giammaria D, Trabucco P. Corneal wound healing after laser vision correction. Br J Ophthalmol. 2016;100: 28–33. doi:10.1136/bjophthalmol-2015-306770
30. Olayanju JA, Hassan MB, Hodge DO, Khanna CL. Trabeculectomy-Related Complications in Olmsted County, Minnesota, 1985 Through 2010. JAAMA Ophthalmol. 2015;133: 574–580. doi:10.1016/j.chemosphere.2012.12.037.Reactivity
31. Csutak A, Steiber Z, Tozser J, Jakab A, Berta A, Silver DM. Plasminogen activator activity in tears of pregnant women. PLoS One. United States; 2017;12: e0177003. doi:10.1371/journal.pone.0177003
32. Zhu J, Xie L, Li Z, Lu X. The prognosis of trabeculectomy in primary angle- closure glaucoma patients. 2019;1. doi:10.18240/ijo.2019.01.10
33. Berta A. Collection of tear samples with or without stimulation. Am J Ophthalmol. 1983;96: 115–6.
34. Bradford MM. A rapid and sensitive methode for the quantitation of microgram

quantites of proiteins utilizing the principle of protein-dye binding. Anal Biochem. 1976;72: 248–254.

35. Kallo G, Chatterjee A, Toth M, Rajnavolgyi E, Csutak A, Tozser J, et al. Relative quantification of human beta-defensins by a proteomics approach based on selected reaction monitoring. Rapid Commun Mass Spectrom. England; 2015;29: 1623–1631. doi:10.1002/rcm.7259
36. Kalló G, Emri M, Varga Z, Ujhelyi B, Tozsér J, Csutak A, et al. Changes in the chemical barrier composition of tears in Alzheimer's disease reveal potential tear diagnostic biomarkers. PLoS One. 2016;11. doi:10.1371/journal.pone.0158000
37. Seet L-F, Finger SN, Chu SWL, Toh LZ, Wong TT. Novel insight into the inflammatory and cellular responses following experimental glaucoma surgery: a roadmap for inhibiting fibrosis. Curr Mol Med. Netherlands; 2013;13: 911–928.
38. Griffin M, Casadio R, Bergamini CM. Transglutaminases: nature's biological glues. Biochem J. 2002;368: 377–396. doi:10.1042/BJ20021234
39. Kanno Y, Hirade K, Ishisaki A, Nakajima K, Suga H, Into T, et al. Lack of α2-antiplasmin improves cutaneous wound healing via over-released vascular endothelial growth factor-induced angiogenesis in wound lesions. J Thromb Haemost. John Wiley & Sons, Ltd (10.1111); 2006;4: 1602–1610. doi:10.1111/j.1538-7836.2006.01978.x
40. Lembach M, Linenberg C, Sathe S, Beaton A, Ucakhan O, Asbell P, et al. Effect of external ocular surgery and mode of post-operative care on plasminogen, plasmin, angiostatins and alpha(2)-macroglobulin in tears. Curr Eye Res. England; 2001;22: 286–294.
41. Orosz ZZ, Katona E, Facsko A, Berta A, Muszbek L. A highly sensitive chemiluminescence immunoassay for the measurement of coagulation factor XIII subunits and their complex in tears. J Immunol Methods. Netherlands; 2010;353: 87–92. doi:10.1016/j.jim.2010.01.001
42. Dammeier S, Martus P, Klose F, Seid M, Bosch D, D'Alvise J, et al. Combined Targeted Analysis of Metabolites and Proteins in Tear Fluid With Regard to Clinical Applications. Transl Vis Sci Technol. United States; 2018;7: 22.

doi:10.1167/tvst.7.6.22

43. Roedl JB, Bleich S, Schlotzer-Schrehardt U, von Ahsen N, Kornhuber J, Naumann GOH, et al. Increased homocysteine levels in tear fluid of patients with primary open-angle glaucoma. Ophthalmic Res. Switzerland; 2008;40: 249–256. doi:10.1159/000127832

Conference Posters

1. **Éva Csősz,** Eszter Deák, Gergő Kalló, Adrienne Csutak, József Tőzsér: Tear protein analysis in the different phases of wound healing following glaucoma surgery, 16th Human Proteome Organisation World Congress, HUPO, Dublin, Ireland, 2017.
2. **Éva Csősz,** Eszter Deák, Adrienne Csutak, József Tőzsér: Tear analysis of cytokine level changes in the different phases of wound healing following glaucoma surgery, Hungarian Molecular Life Sciences 2017, Eger, Hungary, 2017.
3. **Éva Csősz,** Eszter Deák, Gergő Kalló, Adrienne Csutak, József Tőzsér: Identification of tear as a source for possible biomarkers for wound healing complications following glaucoma surgery, Central and Eastern European Proteomics Conference, Budapest, Hungary, 2016.

Conference Lectures

1. **Éva Csősz**, Eszter Deák, Adrienne Csutak, József Tőzsér: Tears as a good candidate for follow-up studies in case of patients having glaucoma surgery, 10th Molecular Cell and Immune Biology Winter Symposium, Debrecen, 2017.
2. **Eva Csosz**: The Beauty and the Beast or the pros and cons of tear proteomics, Liquid Biopsy Precongress Workshop, 16th Human Proteome Organisation World Congress, HUPO, Dublin, Ireland, 2017 – invited speaker.

Accepted Publications

1. Éva Csősz, Noémi Tóth, Eszter Deák, Adrienne Csutak and József Tőzsér (2018) Wound healing markers revealed by proximity extension assay in tears of patients following glaucoma surgery. International Journal of Molecular Sciences, 19, 4096.

UNIVERSITY of DEBRECEN

FACULTY OF MEDICINE
Department of Biochemistry and Molecular Biology
H-4002 Debrecen, Egyetem tér 1. P.O.B. 400.
Phone: +3652/416-432, email: bmbi@med.unideb.hu

2. Éva Csősz, Gergő Kalló, Bernadett Márkus, Eszter Deák, Adrienne Csutak, József Tőzsér (2017) Quantitative body fluid proteomics in medicine - A focus on minimal invasiveness. J Proteomics, 153:30–43.
3. Éva Csősz, Eszter Deák, Gergő Kalló, Adrienne Csutak, József Tőzsér (2017) Diabetic retinopathy: Proteomic approaches to help the differential diagnosis and to understand the underlying molecular mechanisms. J Proteomics, 150:351-358.

Manuscripts under review

1. Éva Csősz, Eszter Deák, Noémi Tóth, Carlo Enrico Traverso, Adrienne Csutak, József Tőzsér: Comparative analysis of tear and aqueous humour cytokine profiles in patients with glaucoma - identification of potential predictive tear biomarkers for trabeculectomy complications and submitted to International Journal of Molecular Sciences.

Debrecen, 2019. 01. 30.

Dr. Éva Csősz
principal investigator