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Final report

in relation to OTKA proposal

"Complex proteomics analysis of tears and aqueous humor for the examination of post-operative wound healing complications following glaucoma surgery" (PD121075)

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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 (Kingman, 2004). 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 (Chang & Goldberg, 2012; Križaj et al., 2014). 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 (Chang & Goldberg, 2012). Most of the patients are initially treated with eye drops but when the desired IOP cannot be reached surgery becomes necessary. The most widely used gold standard surgical intervention in open angle glaucoma is trabeculectomy, which is an invasive procedure associated with relatively high complication and failure rate (Bar-David & Blumenthal, 2018; Van Bergen, Van de Velde, Vandewalle, Moons, & Stalmans, 2014). During trabeculectomy a channel is created in the trabecular meshwork 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 (Van Bergen et al., 2014).

Wound healing is a well-organized cascade of events. Ocular wound healing differs from that observed in case of skin (Bukowiecki, Hos, Cursiefen, & Eming, 2017) 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 (Eslani, Movahedan, Afsharkhamseh, Sroussi, & Djalilian, 2014; Ljubimov & Saghizadeh, 2016). MMPs are activated by IL-1 and other factors, and an extensive extracellular matrix rearrangement starts (Ashby, Garrett, & Willcox, 2014; Maycock & Marshall, 2014). 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 (Ashby et al., 2014; Ljubimov & Saghizadeh, 2016; Yu, Yin, Xu, & Huang, 2010).

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 (Barbosa et al., 2010; West-Mays & Dwivedi, 2006; Wilson, Liu, & Mohan, 1999). 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 (Ashby et al., 2014; Maycock & Marshall, 2014).

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Under normal conditions the ocular wound-healing process ends up with scarless healing required for proper vision, but each step of the process can be affected, leading to complications (Ashby et al., 2014; Maycock & Marshall, 2014; Spadea, Giammaria, & Trabucco, 2016). Flap related complications, chorioidal ablation, bleb failure etc. usually appear 6 months or 1 year after trabeculectomy and can lead to the ineffectiveness of the treatment. These patients may require additional topical treatment or another trabeculectomy.

A better understanding of the molecular events behind the ocular wound healing can help to design better treatment strategies. At the same time, markers able to predict the appearance of late complications early, would have high importance giving possibility for ophthalmologists for the establishment of proper treatment protocols.

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 (Berta, 1983). 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 aqueous humour 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 (Bradford, 1976).

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.

Finding the optimal biological sample for biomarker studies and identification of potential early biomarkers for late complications of trabeculectomy

Biological fluids are often utilized in biomarker studies. In case of glaucoma, the most frequently examined body fluid was the aqueous humor (AH) (Funke, Perumal, Bell, Pfeiffer, & Grus, 2017). Proteomic analyses of AH samples from patients with glaucoma and controls

revealed important proteins involved in metabolism, inflammatory response and antioxidant defense (Funke et al., 2017; Kaeslin et al., 2016). 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 (Du, Shaolin, 2016; Gu, Zhou, Jiang, Yu, & Xu, 2016; Khalef et al., 2017; Ohira, Inoue, Iwao, Takahashi, & Tanihara, 2016; Takai, Tanito, & Ohira, 2012). 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. Contrary to AH tear is an easily accessible, continuously available body fluid with high potential in follow-up studies, which were designed in the current proposal. In order to find the best suitable biological sample for our study both tear and AH was collected. 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 primary open angle glaucoma (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 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. 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.

When we have analyzed the level of examined cytokines in tear and AH, no correlation between their levels in the two sample types could be observed indicating 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.

In order to see if our results are acceptable 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. This 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.

As a next step, groups were formed according to the available data and the level of cytokines was analyzed between the groups. Grouping features were the gender, type of glaucoma, tear production rate, tear protein concentration and presence of complications. Gender and the type of glaucoma were not associated with a statistically significant difference in the cytokine levels, tear production rate and tear protein concentration in any of the studied groups. The group of patients using three or more different eye-drops was compared to the group using less than three eye-drops, but we could not find any statistically significant difference. We could detect statistically significant differences only between groups with complication and no complication. 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.

According to our data ocular complications are associated with reduced levels of these three observed cytokines. However, according to the scientific literature faster wound healing, which might be one reason for bleb failure, would require increased levels of these factors. It

should be noted that none of the studies involving IL-5, IFN-γ and GM-CSF were carried out on tear samples. The phenomena leading to bleb failure are not known in detail and most probably multiple, more complex mechanisms take part in the regulation of the process. In spite of the fact that our results observed in human tear contradict the results observed by others in skin or in rodent ocular wound healing models, we consider IL-5, IFN-γ and GM-CSF as a good starting point for further biomarker studies and verifications.

When the same cytokine levels measured in AH were examined in the same groups as those mentioned in case of tear, no statistically significant difference could be found between any of the studied groups.

The tear IFN-γ, GM-CSF and IL-5 were identified as good biomarker candidates; their reduced levels 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. If validated, these proteins could predict the occurrence of late complications before the surgical intervention itself, making possible the adjustment of therapeutic strategies applied during trabeculectomy. At the same time, our data highlight the promising potential of this continuously available, easy-to-collect body fluid for dynamic testing.

These data were summarized in a scientific paper:

Éva Csősz, Eszter Deák, Noémi Tóth, Carlo Enrico Traverso, Adrienne Csutak, József Tőzsér: Comparative analysis of cytokine profiles of glaucomatous tears and aqueous humour reveals potential biomarkers for trabeculectomy complications.
FEBS Open Bio, 2019, 9:1020-1028.

Examination of wound healing markers

Wound healing in the eye is a complicated row of events orchestrated by cytokines and growth factors. With the examination of cytokine levels we could get information on the early inflammatory events taking place after trabeculectomy. As far as the Luminex assay contained no growth factor which might be relevant in our case, an SRM-based assay was designed for

insulin-like growth factor 1 (IGF-1), epidermal growth factor (EGF), hepatocyte growth factor (HGF) and keratocyte growth factor (KGF). The test was positive, the standards gave good signal, but no endogenous peptide in tears could be detected so far. Based on the mass spectrometry results it seems that the detection limit of the mass spectrometry system is insufficient to analyze growth factors in tear.

In order to get more information regarding the proteins which play a role in the wound healing process 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, 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. 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. The level of IL-6 in all postoperative states was shown to be higher in the group with complications however the difference was not statistically significant.

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.

Targeted proteomic method development for the examination of the wound healing markers

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 (Kallo et al., 2015; Kalló et al., 2016) and all the transitions were recorded on Orbitrap Fusion tribrid mass spectrometer (Thermos Scientific). In the Workplan we have planned SRM-based analyses, but in the study we have utilized parallel reaction monitoring (PRM) analyses. At the time when the proposal was submitted we had access only to a 4000 QTRAP mass spectrometer allowing the utilization of SRM-based targeted proteomics analyses. During the study period we acquired a more performant, Orbitrap Fusion tribrid mass spectrometer which allowed the utilization of PRM-type targeted proteomics experiments for the relative quantification of the proteins. The basis of the two different methods is very similar in that sense, that in both cases the proteotypic peptides characteristic for the examined proteins should be determined first. While in case of SRM only the previously determined and set transitions will be recorded, in case of PRM all the transitions will be recorded and then, from the result files the best transitions will be selected. In this sense, the PRM gives a higher flexibility and in our case it showed as a superior technique to SRM making possible the examination of more transitions from the same samples. 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 180 from three patients with and three patients without complications. More optimization of the developed method is needed in order to be

able to improve protein detection. A statistical analysis was carried out and in case of peptides corresponding to three proteins statistically significant difference could be detected (Figure 1).

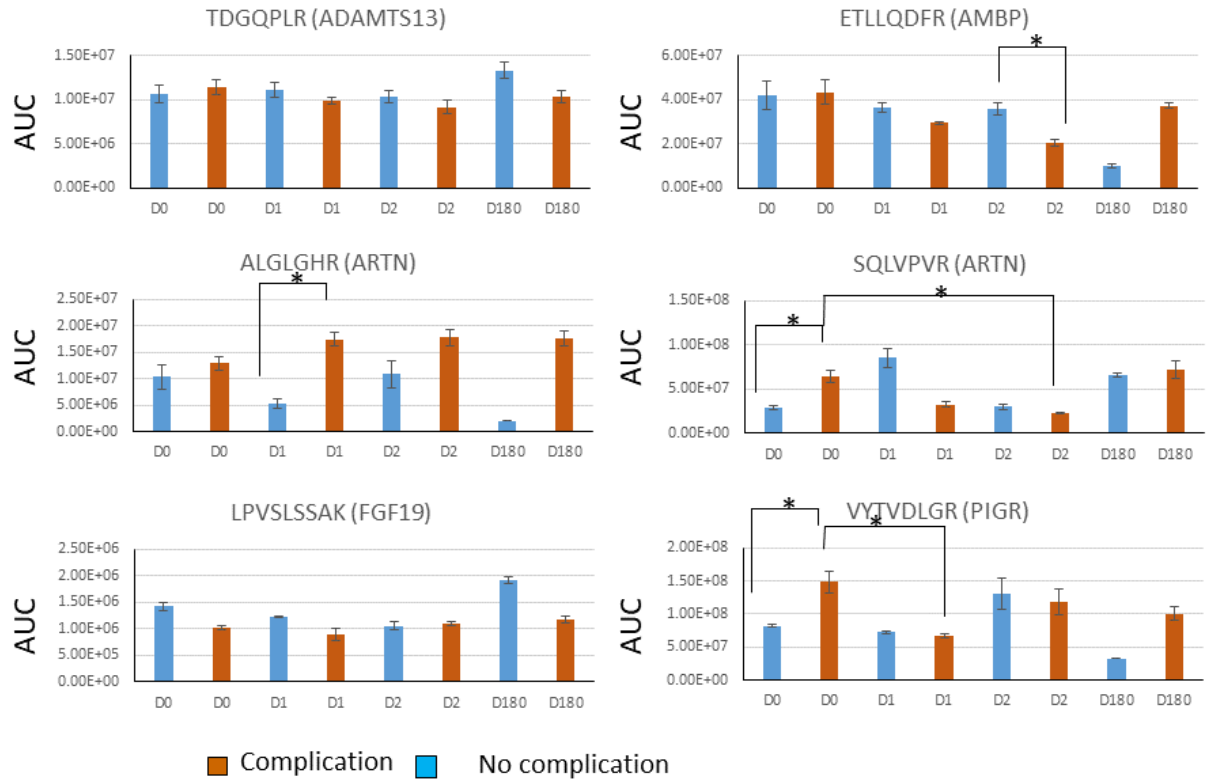

Figure 1. Relative quantification of the selected peptides by PRM.

The x axis shows the days of sample collection, the y axis shows the AUC values measured with mass spectrometer and calculated by the Skyline software.

According to the previous data obtained by PEA the level of AMBP, polymeric immunoglobulin receptor and artemin proteins changed in a statistically significant manner between the group with and without complications.

Based on our results, more optimization of the method is required, but the developed targeted proteomics method is suitable for the examination of tear samples.

Examination of cytokines in a follow-up study following glaucoma surgery

As far as according to our data tear is a valuable biological fluid for the examination of glaucoma and at 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. 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 2).

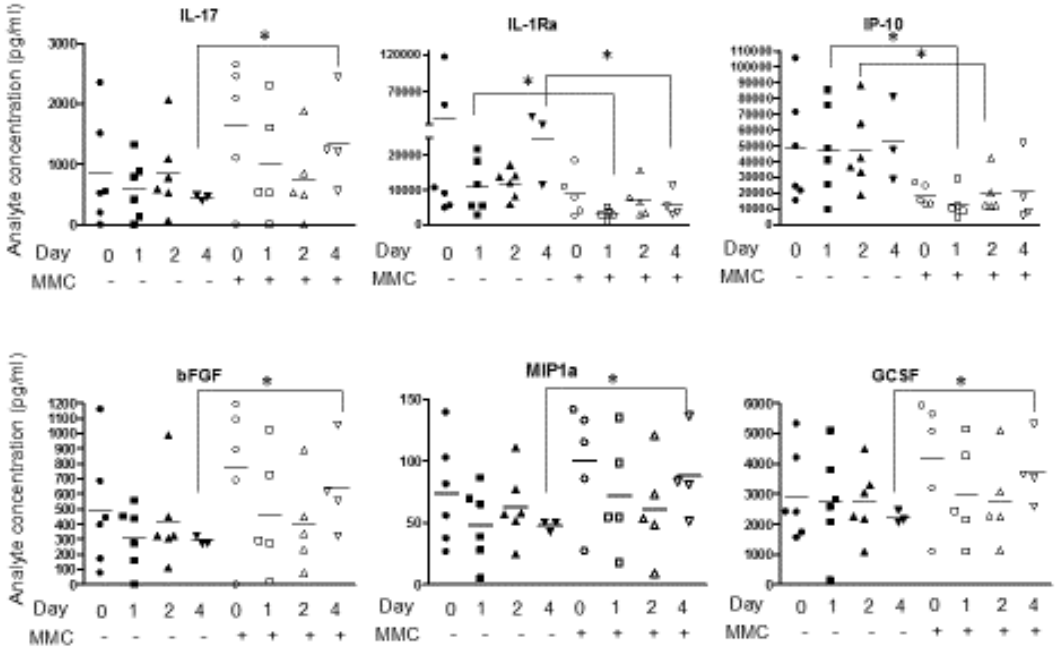

Figure 2. Concentration of cytokines showing statistically significant difference between the MMC treated vs. non-treated groups. The "x" axis shows the days and the administration of MMC, while "y" axis shows the concentration of the analyte in pg/ml. * indicate statistically significant ($p<0.05$) 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.

Analysis of factor XIII and α2-plasmin inhibitor in tear

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 (Griffin, Casadio, & Bergamini, 2002) and the involvement of α2-plasmin inhibitor (α2-PI) in the process was also demonstrated (Kanno et al., 2006). As far as both of these proteins were detected in tears (Lembach et al., 2001; Orosz, Katona, Facsko, Berta, & Muszbek, 2010) 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 (Orosz et al., 2010) 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.

Metabolomic examination of tear samples originating from patients with different forms of glaucoma

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 (Dammeier et al., 2018) and elevated homocystein leves were observed in glaucoma (Roedl et al., 2008). 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, PACG and POAG with diabetes (POAGD) 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 POAG and PACG groups in case of Arg, Ala and Gly (Figure 3). No statistically significant difference could be detected between POAGD and any of non-diabetic glaucoma groups.

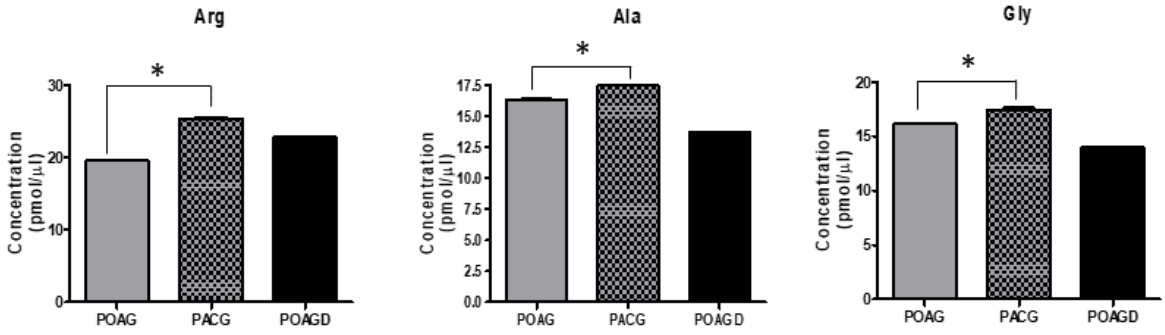

Figure 3. Concentration of amino acids showing statistically significant difference between POAG, PACG and POAGD groups. The "x" axis shows the group, while "y" axis shows the concentration of the amino acid in pg/ml. * indicate statistically significant ($p<0.05$) differences.

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. As a next step we have modified the protocol in order to be able to use it for the analysis of individual tear samples. According to the current protocol, 3 µl of tear sample is enough for the examination of the amino acids. We have measured the amino acid content in case of more than 20 samples originating from patients with different types of glaucoma and the evaluation of these results is in progress.

These new examination possibilities not presented previously in the Workplan can lead us to new discoveries helping to get more information related to glaucoma and on the wound healing mechanism in physiological and in pathological conditions.

Conclusion

A complex proteomics and metabolomics 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. At the same time, we have carried out the first comparative analysis of the tear and aqueous humour samples 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 (Seet, Finger, Chu, Toh, & Wong, 2013). 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 is 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 we have optimized an analytical method for tear amino acid analysis opening up new ways of tear examination.

As far as we were able to update our method for the analysis of individual tear samples, I will be able to perform a complex, system biology-based examination of the different data types. All the so far collected data are published in open access journals and the proteomic data are deposited in publicly accessible databases.

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Publications related to the scientific topic elaborated during the study period

Conference Posters

1. **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, Eger, Hungary, March 31- April 2, 2017.
2. **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 Organization World Congress, Dublin, Ireland, 17 - 21 September 2017, http://hupo2017.ie/
3. Orsolya Bába, Gergő Kalló, Tímea Székely, Renáta Kovács, Adrienne Csutak, **Éva Csősz**: Quantitative analysis of amino acids in tear samples from patients with glaucoma and diabetic retinopathy. 12th Molecular Cell and Immune Biology Winter Symposium, Debrecen, 10-11 January, 2019.
4. Nikolett Bertók, Gergő Kalló, Adrienne Csutak, **Éva Csősz**: Quantitative analysis of tear proteins from patients with diabetic retinopathy with different mass spectrometry-based methods. 12th Molecular Cell and Immune Biology Winter Symposium, Debrecen, 10-11 January, 2019.

5. **E. Csosz**, N. Tóth, E. Deák, G. Kalló, A. Csutak, J. Tőzsér: Multiapproach tear proteomics analyses in glaucoma with emphasis on wound healing following trabeculectomy. EUPA 2019, Potsdam, Germany, 24-28 March, 2019.

Conference Lectures

1. **Éva Csősz:** State-of-the art proteomics to dig deeper into the proteome, 10th Molecular Cell and Immune Biology Winter Symposium, Debrecen, 6-7 January, 2017.
2. **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, 6-7 January, 2017.
3. **Eva Csosz**: The Beauty and the Beast, or the pros and cons of tear analysis, HUPO Precongress Workshop, Liquid Biopsy, Dublin, Ireland, 16 September 2017 - invited speaker
4. **Csősz Éva**: MRM alapú módszerek alkalmazása testfolyadékokból történő biomarker vizsgálatokhoz. Sciex szeminárium, Budapest, Október 25, 2017.
5. **Csősz Éva**, Kalló Gergő, Márkus Bernadett, Tőzsér József: Fehérje biomarker kutatások, avagy hol állunk a shotgun analízis - célzott proteomika - ELISA tengelyen? Tömegspektometriai Szakmai Nap, Budapest, December 6, 2017.
6. **Csősz Éva**: Complex proteomics and metabolomics examination of wound healing markers in glaucoma patients following trabeculectomy. Hungarian Molecular Life Sciences Conference, Eger, March 29-31, 2019.
7. **Csősz Éva**: Proteomikai technikák a biomarker kutatásban és a diagnosztikai laboratóriumban. MOLSZE XVI. Nagygyűlése, Budapest, Augusztus 30-31, 2019.
8. **Csősz Éva**: Biomarkerek - mi az amit hihetünk és mi az amit nem? Hatvani István Szakkolégium, Debrecen, május 15, 2019.

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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. IF: 4,183
2. Éva Csősz, Eszter Deák, Noémi Tóth, Carlo Enrico Traverso, Adrienne Csutak, József Tőzsér (2019) Comparative analysis of cytokine profiles of glaucomatous tears and aqueous humour reveals potential biomarkers for trabeculectomy complications. FEBS Open Bio, 9:1020-1028. IF: 1,959

Debrecen, 2019. 10. 25.

Dr. Éva Csősz
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I agree with the present report

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