

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

MEDIATORS IN THE TEARS OF KERATOCONIC PATIENTS

by

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The Examination takes place at 12 a.m., February 07, 2014.

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The PhD Defense takes place at The Lecture Hall of Bldg. A, Department of Internal Medicine, University of Debrecen at 2 p.m. February 07, 2014.

Abbreviations

AveK	- average K value
CBA	- cytometric bead array
CCL5/RANTES	- Chemokine(C-Cmotif)ligand 5/Regulated on Activation, Normal T Expressed and Secreted
CKI	- Central Keratoconus Index
CXCL8/IL-8	- C-X-C chemokine ligand 8/interleukin-8
CXL	- corneal collagen cross-linking
EGF	- epidermal growth factor
IHA	- index of height asymmetry
IL	- interleukin
INF	- interferon
ISV	- index of surface variance
K1	- keratometry value in the flat meridian
K2	- keratometry value in the steep meridian
KI	- Keratoconus Index
MMP	- matrix metalloprotease
NGF	- nerve growth factor
NK	- natural killer cells
OSI	- Opposite Sector Index
PAI	- plasminogen activator inhibitor
RGP	- rigid gas permeable
Rmin	- minimum of radius
SD	- standard deviation
SDP	- standard deviation of power (corneal refractive)
TH	- T-helper cell
t-PA	- tissue-type plasminogen activator
ThCT	- thinnest corneal thickness
VEGF	- vascular endothelial growth factor

1. Introduction and literature review

Keratoconus (KC), historically viewed as a non-inflammatory disease, is an ectatic corneal disorder associated with progressive thinning of the corneal stroma inducing irregular astigmatism leading to impairment in the quality of vision. It affects young adults and has an incidence of about 1:2000 in the general population. The cause and the factors governing the progression and stabilization of the disease are unknown, but eye rubbing, atopic disorders, genetic inheritance, and contact lens (CL) wear are thought to be associated with KC.

Changes in the quality and quantity of the tears are important parameters of the various anterior segment eye diseases including KC.

Recently, cytokine imbalance in KC disrupting the corneal homeostasis has been established, and inflammatory and other mediators have been reported to be altered in the tears of KC patients: increase of interleukin (IL)-6, epidermal growth factor (EGF) and tissue inhibitor of metalloproteinases-1 (TIMP-1), decrease of IL-12, interferon (IFN)- γ , IL-4, IL-13, chemokine (C-C motif) ligand 5/Regulated on Activation, Normal T Expressed and Secreted (CCL5/RANTES), vascular endothelial growth factor (VEGF) and alteration in the levels of tumor necrosis factor (TNF)- α and nerve growth factor (NGF). IL-13 decrease was associated with the severity of the disease. Moreover, increased binding of IL-1 α by keratoconic corneal fibroblasts suggests a role of inflammation in the onset or progression of KC as well. Elevated matrix metalloproteinase (MMP)-9 levels in the tear fluid of KC patients indicates a tissue degenerative process - the hallmark of the disease - contributing to the thinning and weakening of the corneal connective tissue. KC is a disease with multivariable origin, in which corneal ectasia caused by the degradation of stromal collagen is accompanied by expression of pro-inflammatory cytokines, cell adhesion molecules, and matrix metalloproteinases playing important role in the pathogenesis.

Rigid gas-permeable (RGP) CLs are routinely used to neutralize the otherwise uncorrectable irregular astigmatism in KC. Similar to eye rubbing, CLs are relevant factors causing mechanical irritation of the corneal surface, possibly contributing to the development and progression of KC by activating mediators

and growth factors. As CLs are routinely used nonsurgical option for patients with KC, therefore the analysis of human tears following CL wear may provide important information about the effect CLs have and expand our knowledge of the pathogenesis of KC.

Corneal collagen crosslinking (CXL) is a procedure that mitigates the progression of KC. Many clinical studies have provided data supporting the efficacy of the treatment: improvement in corneal shape, including a decrease in corneal higher-order aberrations, keratometry (K) values and several quantitative indices of corneal topography. The mediators determining the progression or stabilization of KC have not been well characterized and there is limited understanding on the effect of CXL on these factors. Although biomechanical and architectural improvements of the cornea after CXL have already been well documented in the literature, changes in the tear film biomarkers have not yet been explored. It is important to analyze the predicted influential role of these components on the effect of CXL.

2. Aims

- I. To determine associations between the different types of mediators of the tear fluid and topographic indices characterizing the severity of KC. This work helps to broaden our body of knowledge on tear mediators in KC.
- II. To evaluate the concentration of mediators in tear samples of patients with KC versus control subjects at time points prior to and during continuous wear of CLs. The tested multifunctional mediators were chosen as representative molecules that are associated with corneal degradation in KC (MMP-9, MMP-13, TIMP-1, tissue-type plasminogen activator (t-PA), plasminogen activator inhibitor-1 (PAI-1)), or with CL-related processes (IL-6, IL-13, CXCL8/IL-8, CCL5/RANTES) as well as regulatory and healing mechanisms (NGF, EGF).

- III. To further assess the pathomechanism of KC after CXL, we evaluated the short and the long-term effect of CXL on the concentration of mediators (IL-6, -13, -17A, IFN γ , CXCL8, CCL5, MMP-9, -13, TIMP-1, t-PA, PAI-1, EGF and NGF) in tears of patients with KC. In addition, the changes in concentration of mediators were correlated with the changes in outcomes (K values, Thinnest corneal thickness (ThCT), Radii Minimum (Rmin), Keratoconus-Index (KI), Center Keratoconus Index (CKI), Index of Height Asymmetry (IHA) and Index of Surface Variance (ISV)) measured by Pentacam over 12 months.

3. Patients and methods

Prospective studies were performed at the Department of Ophthalmology, Medical and Health Science Center, University of Debrecen. None of the subjects were taking any medication that could interfere with tear production, and none suffered from any disease of known immunological origin. Following the tenets of the Helsinki Declaration, informed written consent was signed by all participants. Studies were approved by the Institutional Ethics Committee of the University of Debrecen.

The criteria for diagnosing KC were defined as one, or as the combination of the following clinical signs: central or paracentral stromal thinning of the cornea, conical protrusion, Fleischer's ring, Vogt's striae by slit-lamp examination as well as topographic changes. The stage of KC was graded as mild when the steepest keratometric reading (K2) was <45 diopters (D), moderate if K2 was between 45 and 52 D, and severe with K2 >52 D. K2 has been considered to be a reliable quantitative clinical variable to assess the severity of KC.

Exclusion criteria included active inflammatory or infectious systemic or ocular disease, current treatment with systemic or local drugs, use of eye drops and patients with eye rubbing. Eyes with previous ocular surgery and trauma were also excluded from the study. Both eyes of the patients were used if they met the above mentioned criteria.

3.1. Patients groups and clinical examinations

3.1.1. To determine the associations between mediators and topographic indices in KC

Tear samples were collected from 14 eyes of 11 KC patients (mean age: 29.3 years, range 19-50 years, standard deviation (SD): 8.8). Patients were recruited from the Contact Lens Unit, between February, 2010 and April, 2010. All patients were before their first use of contact lenses. Both eyes of each patient had a complete ophthalmologic evaluation, including keratometry, best-corrected visual acuity measurements, slit-lamp biomicroscopy, and corneal topography (Topographic Modeling System corneal topographer, software version 4, Tomey Corp.). The following indices were measured: maximum K value (K2), average K value (Ave K), Klyce/Maeda Keratoconus Index (KCI), Smolek/Klyce Keratoconus Severity Index (KSI), Opposite Sector Index (OSI), Center/Surround Index (CSI), Keratoconus Prediction Index (KPI), Standard Deviation of Corneal Power (SDP).

3.1.2. To evaluate the mediators prior to and during CL wear in KC

Tear samples were collected from 15 eyes of 12 beginner keratoconic RGP contact lens wearers (Group I) (mean age: 26.5 years, range 19-50 years, SD: 28.3) over a 6 weeks period following CL fitting and in case of 5 eyes over one year.

Two control groups were formed. Group II consisted of 19 eyes of 10 myopic beginner soft contact lens wearers (mean age: 21.2 years, range 15-38 years, SD: 7.2). Sample collection was performed over 6 weeks after CL wearing and in case of 13 eyes over one year. Group III consisted of 8 eyes of 5 myopic or hyperopic beginner RGP contact lens wearers (mean age: 22.6 years, range 15-30 years, SD: 5.0). Tear samples were collected over 6 weeks after contact lens wear.

Patients with keratoconus and normal subjects were recruited from the Contact Lens Unit, from February, 2010 through April, 2011. All of the subjects had never worn CLs before. After study enrollment, all subjects wore the CL for more than 8 hrs/day. Both eyes of each patient had a complete ophthalmologic evaluation, including keratometry, best-corrected visual acuity measurements, slit-

lamp biomicroscopy, and corneal topography (Topographic Modeling System Corneal Topographer (software version 4, Tomey Corp.). All of the keratoconic patients were scar-free and had a best spectacle corrected visual acuity under 20/20, all being correctable to 20/20 with RGP contact lenses.

3.1.3. To evaluate the effect of CXL on mediators in KC

Twenty-six eyes of 23 patients (mean age: 28.2 years, range: 16-60 years, standard deviation (SD): 10) with progressive KC were enrolled and treated in this prospective study. All eyes underwent comprehensive ophthalmological examination and tear sample collection before and after CXL during the 1-year follow-up period at regular intervals: preoperatively and at day 4, day 10 visits, and 1, 3, 6 and 12 months after CXL. Twelve eyes of 12 healthy controls (mean age: 27.8 years, range: 16-67 years, standard deviation (SD): 15.3) were also enrolled in this study.

The inclusion criteria included 16 years of age or older and axial topography consistent with KC. Progressive KC was defined as 1 or more of the following changes over 24 months: an increase of 1.0 diopter (D) or more in the steepest K value, an increase of 1.0 D or more in manifest cylinder, or an increase of 0.5 D or more in manifest refraction spherical equivalent. Exclusion criteria were: previous ocular surgery, abnormality in lens or retina on biomicroscopic examination, chemical injury or delayed epithelial healing, corneal pachymetry less than 300 μm , and pregnancy or lactation during the course of the study.

Crosslinking Treatment (CXL) was performed using InPro CCL-Lix device (Norderstadt, Germany). Topical anesthesia was administered and the corneal epithelium removed by mechanical debridement over the central 8.0 mm. A 0.1% riboflavin solution (with 20% dextran) was then administered topically every 2 minutes for 30 minutes. After riboflavin administration, its absorption throughout the corneal stroma and anterior chamber was confirmed by a slitlamp examination. Pachymetry (obtained with Pentacam) was performed and if the cornea was less than 400 μm (4 out of 26 eyes), hypotonic riboflavin (0.1% in sterile water; Medio Cross sine, Medio-Haus Medizinprodukte GmbH) was

administered, 1 drop every 10 seconds for 2 minute sessions, after which pachymetry was performed to confirm that the stroma had swollen to 400 μm or more. This was repeated until adequate corneal thickness was obtained. The cornea was exposed to ultraviolet-A (UV-A) 365 nm light for 30 minutes at an irradiance of 3 mW/cm^2 . The postoperative treatment was antibiotic eye drops for 7 days (tobramycin), steroid eye drops (fluorometholone) and artificial tear drops for at least 3 months. No contact lenses were used postoperatively.

All eyes had a complete ophthalmological evaluation, including keratometry, best-corrected visual acuity measurements, slit-lamp biomicroscopy (under low illumination to avoid reflex tearing), and Rotating Scheimpflug topography (Pentacam HR, Oculus Optikgeräte GmbH, Wetzlar, Germany) before CXL and during the 1-year follow-up, at each ophthalmological visit. The following data were exported to Microsoft Excel (Microsoft Corp, Redmond, Washington): Holladay equivalent keratometry values in the flat (K1) and steep (K2) meridian, ThCT, Rmin, KI, CKI, IHA, ISV. For height data measurements, the toric ellipsoid reference surface was used.

3.2. Tear collection and mediators' analysis

Non-traumatic tear collection was carried out in the morning with capillary tubes from the inferior meniscus, without topical anesthesia for 2 min; the total volume of each collected tear samples were registered. The samples were immediately transferred to Eppendorf tubes tubes (disposable 0.2 mL PCR tubes, No: 732-0548; VWR International, West Chester, PA, USA) and frozen at $-80\text{ }^{\circ}\text{C}$ without centrifugation within 15 min from collection. To avoid pipetting and dilution errors, collected tear samples of $< 4\text{ }\mu\text{l}$ were excluded. Occasionally, tear collection could not be carried out due to dry eyes.

The microparticle-based flow cytometric bead array (CBA) technology allowing quantification of multiple proteins in small individual tear samples were used in these studies. Combined FlowCytomix™ Simplex Kits were used with the appropriate FlowCytomix Basic Kit with minor modifications of the manufacturer's instructions (eBioscience, Bender MedSystems GmbH, Vienna, Austria).

Multiparametric data acquisition was performed on a FACS Array cytometer (BD Biosciences Immunocytometry Systems, San Jose, CA). Data were analyzed with the FlowCytomix Pro 2.3 software. Additional serial dilutions of the standard were applied to obtain better sensitivity and therefore, modified standard curves were generated in the analysis. The detection limits were for IL-6: 1.2 pg/ml, IL-13: 4.5 pg/ml, CXCL8/IL-8: 0.5 pg/ml, CCL5/RANTES: 25 pg/ml, MMP-9: 95 pg/ml, MMP-13: 50 pg/ml, TIMP-1: 28 pg/ml, NGF: 126.8 pg/ml, EGF: 22.7 pg/ml, t-PA: 4.8 pg/ml, PAI-1: 13.5 pg/ml, IL-17A: 2.5 pg/m and INF γ : 1.6 pg/ml.

3.2.1. The concentration of IL-6, IL-13, CXCL8, CCL5 (RANTES), MMP-9, MMP-13, TIMP-1, NGF and EGF were measured by the CBA method.

3.2.2. Tear collections were made before and after CL wear at regular intervals: 10 and 30 min after CL fitting, and 2 and 6 weeks after continuous CL wear (more than 8 hrs/day). One year after the CL fitting, tear samples could also be collected from some patients. During collection, the CLs were not removed. The concentration of IL-6, IL-13, CXCL8, CCL5 (RANTES), MMP-9, MMP-13, TIMP-1, NGF, EGF, t-PA and PAI-1 were measured by CBA.

3.2.3. Tear samples were collected from all 26 eyes of 23 keratoconic patients before CXL and during the 1-year follow-up at each ophthalmological visit and once from the 12 control eyes. In keratoconic patients, the concentrations of IL-6, CXCL8/IL-8, CCL5/RANTES, MMP-9, MMP-13, TIMP-1, NGF, t-PA and PAI were measured in all 157 tear samples, while the volume of 105 tear samples allowed a second measurement for IL-13, IL-17A, INF γ and EGF.

3.3. *Statistical analysis*

The statistical package applied was Stata version 11. The significance criterion was set at $\alpha=0.05$.

3.3.1. Variables were described in terms of means and SDs. The rate of release of the mediators into the tears was calculated from the concentration (pg/ μ l) and the volume of tears (μ l) collected in 2 minutes. Values for the mediators in the tear

samples were transformed to improve normality using the method with the best effect thereto. Multiple linear regression was used to analyze the association between the release of mediators (explanatory variable) and keratometric readings (outcome). Models were fitted separately for each possible pairing between mediators and keratometric variables, with age used in continuous form either as an adjustment covariate or in interaction with the mediator. In case of a significant interaction, mediator effects were evaluated at the sample 10th, 50th, and 90th percentiles of age.

3.3.2. The quantities of mediators released into tears were calculated as products of concentrations (pg/ μ l) and tear volumes (μ l) collected over 2 minutes. Continuous variables were described in each patient group and in the overall study sample using standard statistics.

Intergroup differences at baseline and at 1 year were tested using ANOVA or a non-parametric equivalent subject to distributional assumptions being satisfied. Trends of release intensity over time were assessed using linear regression with an interaction term between measurement day and patient group. Because some patients were observed on both eyes, standard errors were adjusted for patient-level clustering (non-independence) of observations by using the robust calculation formula. Trend linearity was visually inspected by comparing with the empirical trend obtained using kernel-weighted local polynomial smoothing.

Intergroup differences of release levels over time (up until week 6) were assessed using multilevel mixed-effects linear regression with adjustment for baseline and age, taking into account the clustering of observations within eyes and eyes within patients. Measurement occasions were allowed to be categorical (indicator variables for 1st, 2nd, etc.) or continuous (number of days since baseline); randomness was allowed to appear at the slope or intercept level; interaction was allowed between patient groups and measurement occasion if significant. The combination of these options delivering the best fitting model was sought out for each mediator. Models were compared using likelihood ratio tests. Variables were transformed to improve normality unless this failed to produce substantially better fit assessed by normality of standardized residuals.

3.3.3. Variables were described in terms of means and SD on their native scales. For all analytical procedures, keratometric variables and mediators in the tear samples were transformed to improve normality using zero-skewness log transformation.

Paired 2-tailed Student's t tests were used to analyze post-CXL changes from baseline for all 13 mediators and the 8 variables obtained from Pentacam.

Multilevel mixed-effects linear regression was used to analyze the association between the concentration of the 13 mediators and keratometric readings obtained from Scheimpflug Camera (ThCT, K1, K2, Rmin, KI, CKI, IHA, ISV). Models were fitted separately for each possible pairing between mediators and keratometric variables, with adjustment for contact lens wear and tear volume, and interaction terms between measurement occasion and mediator concentration.

4. Results

4.1. Determination of the associations between mediators and topographic indices in KC

The average topographic data were as follows: K2 : 51.7 D, SD: 4.7; Ave K: 48.2 D, SD: 3.4; KCI: 80.7%, SD: 26.5%; KSI: 61.4%, SD: 25.5%; OSI: 9.7, SD: 4.8; CSI: 2.4, SD: 1.5; KPI: 0.4, SD: 0.1; SDP: 4.9, SD: 1.6. The K2 for 10 eyes were between 45 and 52 D, and for 4 eyes were over 52 D.

The average concentrations measurements in the tear fluid were as follows: IL-6: 58.8 pg/ml, SD: 162.4; IL-13: 426 pg/ml, SD: 602; CXCL8/IL-8: 699 pg/ml, SD:1632; CCL5/RANTES: 398 pg/ml, SD:413; MMP-9: 39.6 ng/ml, SD:63.7; MMP-13: 1.1 ng/ml, SD: 0.94; TIMP-1: 38.0 ng/ml, SD: 42.0; NGF: 286 pg/ml, SD: 315; and EGF: 7972 pg/ml, SD:7577.

The average release amounts in the collected tear fluid were as follows: IL-6: 0.64 pg, SD: 1.0; IL-13: 19.7 pg, SD: 33.9; CXCL8/IL-8: 9.5 pg, SD:10.2; CCL5/RANTES: 17.4 pg, SD:20.8; MMP-9: 674 pg, SD:1089; MMP-13: 38.1 pg, SD:

48.0; TIMP-1: 1225 pg, SD: 1110; NGF: 10.7 pg, SD: 15.1; and EGF: 126 pg, SD:55.

Significant positive associations were found between CCL5 and CSI ($p=0.04$); between MMP-13 and K2 ($p=0.002$), Ave K ($p=0.002$) and SDP ($p=0.01$); and between NGF and K2 ($p=0.001$), Ave K ($p=0.001$), KSI ($p=0.02$), OSI ($p=0.04$), CSI ($p=0.01$), KPI ($p=0.03$) and SDP ($p=0.001$). Significant negative correlations were found between IL-6 and KCI ($p=0.02$). Age-dependent associations were observed between IL-13 and KCI ($p=0.02$), KSI ($p<0.001$), OSI ($p=0.02$), KPI ($p=0.02$); between CXCL8 and K2 ($p=0.045$), Ave K ($p=0.03$), CSI ($p=0.001$), SDP ($p=0.04$); between CCL5 and KSI ($p=0.04$); between MMP-13 and CSI ($p=0.002$).

4.2. Evaluation of mediators prior to and during CL wear in KC

11 eyes had moderate KC (K2 between 45 and 52 D), and 4 eyes had severe keratoconus (K2 >52 D). The mean K2 was 51.5 (SD: 4.7). CL related side effects or adverse events did not happen.

Intergroup differences at baseline: Keratoconus (Group I) vs. Controls (Group II and Group III): No age-related statistical differences were detected between patients with KC and both control groups. No significant differences were found in the collected tear fluid volume between the three groups. Overall, we found that baseline MMP-9 and IL-6 release was 2 fold, CCL5 - 3 fold, IL-13 - 4 fold and PAI-1 - 7 fold elevated, while t-PA decreased to 25% (based on mean values) in KC compared to all control samples (taken together the two control groups), but did not reach statistical significance due to the large variation (standard deviation) in levels between the subjects and the small sample size. Prior to CL wear, the EGF release was significantly lower in the KC group compared to controls ($p=0.03$).

Differences between the linear trend over six weeks and during CL wear between the groups: In KC, an increasing linear trend over time was found for MMP-9 ($p=0.07$), EGF ($p=0.04$) and CXCL8 ($p=0.01$). The same increasing trend was seen in the case of MMP-9 ($p=0.04$), MMP-13 ($p=0.002$), IL-6 ($p=0.04$) and CXCL8 ($p=0.001$) in Group III (patients wearing RGP contact lenses). Soft CL wearers (Group II) had no significant effect on any of these mediators. Significant

differences in linear trend over time between patients with KC wearing RGP lenses (Group I) and ametropic patients wearing RGP lenses (Group III) were observed for MMP-13 (both groups having an increasing trend, being more pronounced in Group III; $p=0.01$) and TIMP-1 (decrease in Group I and increase in Group III; $p=0.02$). In addition, significant differences between patients with KC wearing RGP lenses (Group I) and ametropic patients wearing soft CLs (Group II) were observed for MMP-9 (decrease in Group II and increase in Group I; $p=0.03$) and CXCL8 (decrease in Group II and increase in Group I; $p=0.02$). The same significant differences were found between ametropic patients wearing RGP lenses (Group III) and ametropic patients wearing soft CLs (Group II) in case of MMP-9 ($p=0.02$), CXCL8 ($p=0.002$) and MMP-13 ($p=0.002$) indicating the influence of RGP lens itself on the release levels of these mediators and not the effect of the KC disease.

The releases of MMP-9 at week six ($p=0.0001$) and NGF at 10 minutes were higher ($p=0.01$), but NGF at week two was lower ($p=0.04$) in the KC group as compared to the soft CL wearers group (Group II). In KC, the release levels of MMP-13 and NGF at week two ($p=0.001$ in both) and six ($p<0.0001$, $p=0.02$), respectively, were lower than in the RGP group, similar to IL-6 and CXCL8 ($p<0.0001$ in both) at the second week and PAI-1 ($p=0.02$) at all time points. Inversely, the release levels of TIMP-1 were higher ($p=0.02$) than in the RGP group, but only at 10 and 30 minutes.

In this study, we did not find differences in the level of mediators after one year of contact lens wear between the patients with KC (RGP CL wearers) and ametropia (soft CL wearers). Only 3 out of 5 tear samples from KC (depending on the type of mediators) and 10 out of 13 tear samples from controls were available after one year of the study. Based on these limited data, there was a decreasing trend of TIMP-1 (almost to half of the baseline level) and of PAI-1 (reaching the detection sensitivity levels in tears of patients with KC after one year of CL wear), and an increasing trend of IL-6 release (4 fold) in KC compared to control.

4.3. Evaluation of the effect of CXL on mediators in KC

The collected tear volume ($p < 0.0001$) and the thinnest corneal thickness ($p = 0.0005$) increased significantly 4 days after the treatment. Despite the excessive tearing at day 4, there were statistically significant increases in the concentrations of IL-6 and CXCL8 ($p < 0.0001$) when compared to the pre-operative (pre-CXL) baseline levels. At the same time, the concentration of IL-13 ($p = 0.01$), IL-17A ($p = 0.001$), IFN γ ($p = 0.02$), CCL5 ($p = 0.001$), MMP-13 ($p = 0.02$), EGF ($p < 0.0001$), NGF ($p = 0.01$) and PAI-1 ($p = 0.001$) decreased significantly, and there were no significant changes in the concentrations of MMP-9, TIMP-1 and t-PA. The changes in the concentration of all the mediators 38 days after CXL compared with the baseline failed to reach statistical significance, and the volume of the collected tears returned back to the pre-CXL levels. The thinnest corneal thickness were significantly decreased ($p < 0.0001$) after CXL compared to the baseline data at day 38.

At 6 months, there was a significant decrease in ThCT and ISV ($p = 0.03$ and $p = 0.0005$) and increase in Rmin ($p = 0.007$). At 12 months, the change in K1, ISV and KI were statistically decreased compared to the baseline ($p = 0.0045$, $p = 0.002$, $p = 0.0005$; respectively), while Rmin was increased ($p = 0.0004$). At 6 months, there was a significant increase in t-PA ($p = 0.023$), while at 12 months, there was a significant decrease in the concentration of IL-6 and CXCL8 ($p = 0.005$ and $p = 0.047$).

We examined the linear association between the different mediators and the tomography data. After 3 months, KI was associated negatively with IL-17A ($p = 0.002$) and MMP-13 ($p = 0.01$); similarly IHA was negatively associated with IL-17A ($p = 0.016$) and EGF ($p = 0.032$). After 6 months, CKI and ISV showed significant associations with IL-17A ($p = 0.001$ and $p = 0.016$, respectively), similar to CKI with IL-13 ($p = 0.002$) and ThCT with IL-13 ($p = 0.01$). After 12 months, there were reverse associations between ThCT and IL-6 ($p = 0.005$), IL-13 ($p = 0.017$), IL-17A ($p = 0.049$), INF γ ($p = 0.017$), CCL5 ($p = 0.02$) and PAI-1 ($p = 0.011$).

5. Discussion

5.1 The etiology of KC still remains unclear, just like the factors predicting disease progression. Besides the typical clinical signs detected by slit-lamp biomicroscopy, corneal topography and tomography are the standard tools in the detection and classification of KC.

MMPs and TIMPs play important role in the degradation of extracellular matrix proteins and are secreted in response to cytokines and growth factors. We found a strong positive correlation between MMP-13 level and the severity of the disease. In contrast, we could not detect any correlation in case of MMP-9 and TIMP-1, which suggests, that other MMPs or other enzymes might play a more crucial role in the underlying molecular mechanisms of KC; moreover, the level of enzyme activity at the time of measurement may in itself influence their final effect.

The impairment of corneal innervation has been suggested to have a role in the pathogenesis of KC and - to our best knowledge – the NGF levels in the tear fluid were measured for the first time in our study at patients with KC. NGF level showed a significant positive correlation with the topographic indices studied (7 positive out of 8) in all age groups. This finding underscores the importance of NGF in the pathophysiology of KC and raises the attention to carry out further studies to determine its possible role in the disease progression.

Our study showed a negative correlation between IL-6 level and the KCI, although no correlation could be found for K2. Our results predict that there is an active interplay between MMPs and cytokines as IL-6 can stimulate the production of several MMPs. IL-13 showed a negative correlation with the severity of KC, especially above age 30. The chemokine CXCL8 showed negative correlation with the progression of the topographic indices. The chemokine CCL5 showed positive correlation with CSI and negative correlation with KSI at ages 30 and older.

Age-dependent associations were observed in case of IL-13, CXCL8, CCL5 and MMP-13. These findings underscore the importance of age and stage of the disease. Our data suggest that IL-13, CXCL8, CCL5 and MMP-13 have different effect on the disease depending on the age of the patient and the severity of the disease.

Our study has limitations, such as population size and the fact that the enrolled patients had mainly moderate KC and not all stages were equally presented. This study does not exclude the possibility of other inflammatory mediators being involved in the pathophysiology of KC nor does it identify the source and the activity of the measured mediators in the tears. However, several new correlations can be established from these results that can serve as the basis for further studies in KC.

5.2. Despite extensive clinical and basic studies on KC, the precise role of CL wear in the pathophysiology of the disease still remains unknown and ambiguous. RGP CLs are routinely used for the adequate correction of the visual impairment in KC and improving the quality of life. Our studies verify the hypothesis that the level of several mediators in the tear fluid gets altered due to CL wear depending on the material of the lenses and also on the corneal pathology. Changes in the level of specific mediators in connection with contact lens wear suggest that the lens itself may play a role in KC. According to our results, the elevation of MMP-9 and CXCL8 level in KC after CL wear and the concomitant decrease of NGF, TIMP-1 and PAI-1, may play a possible role in matrix degradation that is the hallmark of keratoconic corneas. Furthermore, the time course and levels of mediators support the concept that there is an imbalance between cytokines, enzymes and their inhibitors, and this imbalance can play important role in the development of the disease.

We have shown that novice commencement of RGP CL wear associates with increased MMP-9 and MMP-13 levels in patients with- and especially without KC; the same result was found with soft CL wearers, indicating the influence of RGP lens itself to increase MMP level and not the effect of the keratoconic disease. This is not a primary finding in KC, but a secondary effect of the RGP CL wear. We found that the levels of TIMP-1 were even higher in patients with KC than without in the early time points after fitting the RGP CLs, but the linear trend over the six weeks became inversed: it decreased in KC and increased in the controls. These changes that were resulted from the wear of RGP CLs in KC have a great importance, because the increased level of the degrading enzymes in connection

with concomitant decrease of a regulatory/inhibitory mediator suggests an imbalance and therefore, might influence the progression of the disease. In KC the release of PAI decreases more than in ametropes reaching undetectable levels after one year. This finding may be important as PAI can cause MMP-9 activation and thus contribute to more excessive extracellular matrix degradation. So far, PAI has not been studied in connection with CL wear, and has neither been investigated in the tears of patients with KC.

The physical presence of a CL, especially RGP CL, causes mechanical irritation and consequently higher release of inflammatory mediators. We have confirmed a significant increase of IL-6 level after wearing RGP CLs. In contrast, we have demonstrated that starting to wear soft CLs causes no alteration in any of the examined inflammatory cytokines. Whether the RGP CL-related increase of IL-6 level is sufficient to facilitate the long-term progression rate of KC is not yet known. We have observed an increasing linear trend over time for CXCL8 not only in KC RGP lens wearers, but also in controls wearing RGP lenses.

We found, that the use of RGP CLs did not alter the release of NGF in the tear fluid of patients with KC. At weeks 2 and 6, the NGF release was significantly lower in KC when compared to the ametropic group wearing RGP CLs. The level of EGF was significantly lower in the tear fluid of patients with KC prior to CL wear. We present a significantly increasing linear trend over 6 weeks for EGF in case of beginner RGP CL-wearers with KC in contrast to the controls. RGP lens wear in KC induces elevated release of EGF, possibly due to mechanical stress on the corneal epithelium.

The main limitations of our study are the limited population size and the fact we could not collect tear samples from all the patients after one year (due to loss, corneal cross-linking or discontinued CL wear).

5.3. To the best of our knowledge, there are no studies evaluating the effect of the corneal cross-linking procedure on different tear biomarkers. The morphological regression effect of CXL has been reported earlier, but this is the first high-throughput study to demonstrate the effect of CXL on tear mediators. In our study, the postoperative changes in 8 Pentacam topography indices were

evaluated and the associations between the different protein concentrations were followed up to 12 months providing a comprehensive analysis of the potential effect of CXL on keratoconic corneas. Cytokines, chemokines, enzymes, and growth factors are important in wound healing and tissue redistribution response of corneal tissue, regulating the wound healing, apoptosis, cell cycling and migration processes under physiological or pathological conditions. It is assumed that biomarkers from this complex network may be responsible for clinical outcomes after CXL treatment.

The improvements of K1, KI, ISV, ThCT and Rmin detected in our study are consistent with previous findings supporting the fact that the cornea is assuming a more regular shape after CXL and that collagen CXL is an effective procedure.

The CXL treatment of de-epithelialized corneas causes a prompt, excessive release of several mediators independent of the long-term effect of the procedure, and due to the mechanical (epithelial removal), chemical (riboflavin soaking) and physical (UVA irradiation) stress on the cornea. Even though the excessive tearing after CXL, the concentration of IL-6 and CXCL8 significantly increased in our study 4 days after CXL ($p < 0.0001$), suggesting the prompt involvement of inflammatory cells in the process. By day 4, the ThCT increased significantly ($p = 0.0005$) and this swelling dramatically decreased at day 38 ($p < 0.0001$). This edematous process in the corneal stroma in the early post-CXL phases has already been described earlier. The concentration of several mediators decreased significantly because of the diluting effect of the tearing. The CXL treatment retards the progression of KC by cross-linking of collagen molecules. Although the complex pathophysiology of CXL is unclear, the early clinical worsening and the transitional alteration of the mediators coincide with the epithelial debridement, re-epithelization process and post-operative keratocyte apoptosis and repopulation as well as new collagen synthesis. After UVA exposure the keratocytes in the outer layers of the treated part of the cornea undergo cell death. By definition, in order to crosslink tissue, keratocytes must be killed. When cells die through necrosis, a strong inflammatory response is initiated and a release of various mediators including chemokines occurs. In the early-phase after CXL, the extremely elevated release of IL-6 and CXCL8, detected in our study, significantly contributes to the

corneal epithelial wound healing process and the recruitment of inflammatory cells into the corneal stroma, resulting the prevention of tissue damage from excessive inflammation by clearance of damaged cells. After CXL, all corneal layers regenerate rapidly, even the epithelial regrowth is complete after four days. The re-epithelisation of the cornea is followed by remodelling and reorganization, new keratocytes migrate into the central area for several months following CXL. In the early stages of the recovery from CXL and at 38 days after CXL, the concentration of all the mediators and even the volume of the collected tears during 2-min returned to the pre-CXL levels. Gradual repopulation of the corneal stroma, starting between the second and third month after the intervention, is usually completed within six months.

The decrease in the concentration of IL-6 ($p=0.005$) 12 months after CXL is in line with previous studies. In addition, we have shown a strong negative correlation between IL-6 and ThCT after 1 year following CXL. We could also detect significant decrease of CXCL8 after 1 year following CXL, which also supports the assumption that cytokines and chemokines play an important role in the pathomechanism of KC and that CXL treatment might alter the inflammatory response. The decrease in the level of IL-6 and CXCL8 after CXL treatment could be considered as positive for corneal health because the level of these cytokines of healthy controls in our study were significantly lower compared to patients with KC. The decreased level of cytokines could be considered as a contributing factor in the stabilization of this corneal disease. The analysis of the long-term associations after CXL have revealed reverse association between ThCT and IFN γ . IL-13 plays crucial role in the amplification of the T_H2 response, and the decreased levels suggest that T_H2 responses may be suppressed in KC. Dermal fibroblasts stimulated with IL-13 upregulate the production of collagens type I and type III. IL-17 is produced primarily by the T_H17 subset T lymphocytes, and it can also mediate induction of fibroblasts as well as production of tissue degrading proteases and cytokines. IL-13 and IL-17 in our study showed significant long-term association with the topography indices (ISV, CKI, ThCT). Our baseline findings that KI negatively correlates with CCL5 and reversely a significant association exists between ThCT and CCL5 1 year after CXL, underline the importance of CCL5 in the pathomechanism of KC.

MMPs are secreted in response to cytokines and growth factors and elevated level of MMPs in the tear fluid of KC patients indicates a tissue degenerative process contributing to the thinning of the cornea. MMPs and cytokines interact with each other forming a complex network, including the stimulation of MMP-9 and MMP-13 by IL-6. The active form of t-PA converts plasminogen to plasmin, which can also degrade several components of the extracellular matrix and trigger activation of the MMP pathway. MMPs and PAs in turn are partially regulated by TIMPs and PAIs, inhibiting this cascade system and therefore influencing KC progression. The PAI-1 gene can be induced by several growth factors and cytokines, and it can inhibit the activity of t-PA enzymes. A significant increase in t-PA was detected in our study 6 months after the CXL treatment and 1 year after the CXL a reverse association was observed between ThCT and PAI-1. In our study, a negative correlation could be observed between the concentration of MMP-13 and KI at baseline and 3 months post-CXL. TIMPs are natural inhibitors of the different MMPs, and there have been various studies with conflicting reports on the expression of TIMP-1 in KC corneas. In our study, we could not detect any correlation or alteration in the level of MMP-9 and TIMP-1, which suggests, that other MMPs, as well as other enzymes might play a more crucial role in the underlying molecular mechanism following CXL, and probably, the actual enzyme activities influence the final effect. TIMP-1 has been observed to prevent TIMP-3 induced apoptosis of keratocytes, therefore the lack of the alteration of TIMP-1 concentration after CXL may be beneficial to KC patients.

The nerve regeneration after CXL is very rapid, and is almost complete six months after the treatment and in our study, during the early postoperative period, when the re-epithelization process is dominant, a slight increase in the concentration of NGF could be observed, but no association or long-term changes in the concentration of NGF could be detected after CXL. It is important to mention that the use of steroid eye drops and duration of treatment after CXL highly depend on the ophthalmologists' practice. We have used fluorometholone drops for minimum 3 months and found no increase in the mean intraocular pressure postoperatively. Corneal thickness and posterior elevation at minimum pachymetry proved to be highly reliable diagnostic parameters of KC and to monitor the efficacy of treatment after CXL. Currently, the long-term effects of CXL treatment are not well known and based on our findings, the steroid treatment may also

influence the post-CXL changes. Our finding that the ThCT did not decrease 1 year after CXL should be studied further.

A limitation of this study is that we could compare the post-CXL findings with the baseline data of the patients and also with a healthy control group, but ideally, because of possible progression of the disease that can be accompanied with alteration in the mediator levels, the same number of fellow eyes with the same severity of KC without any treatment would have been desired to be the control group, but when progression is detected, treatment is usually indicated and the long term results would be limited to reach. Although it has been demonstrated that mediators are associated with the severity of KC, it is unclear what kind of proteins can be used to distinguish the non-progressive form of KC from progressive form at those cases when the early CXL treatment would be very important. It would be of great benefit to understand which biomarkers may promote the effect of CXL treatment. Tear samples are an essential tool to understand the molecular mechanism behind CXL and the multiplex platform is ideally suited for the detection of biomarkers from tear samples.

6. Summary of new results

I. Several correlations were observed between the mediators (IL-6, IL-13, CXCL8, CCL5 (RANTES), MMP-13, NGF) and the topographic indices (K2, AveK, KCI, KSI, OSI, CSI, KPI, SDP) suggesting their role in the pathophysiology of KC. Our data indicate that some mediators have different effects on the severity of KC in an age-dependent manner.

II. We have confirmed that several secreted mediators (IL-6, CXCL8, MMP-9, MMP-13, TIMP-1, NGF, EGF, PAI-1) are altered in the tears of KC patients wearing RGP CLs, and a complex imbalance of various mediators might have an impact on the development of the disease.

III. We have reveals that many mediators including cytokines (IL-6, CXCL8/IL-8, CCL5/RANTES, IL-13, IL-17A, INF γ), enzymes (MMP-13, t-PA, PAI) and growth factors (EGF, NGF) being altered in the tears of KC patients after CXL with

concomitant changes in the shape of KC corneas. These alterations detected in tear samples may have an impact on the effect of this treatment.

7. Usefulness of the scientific results

Keratoconus affects young patients and it is one of the leading causes of corneal transplantation. Ocular surface changes are predicted to have an impact on the composition of tear fluid and these changes in return are likely to have an effect on the ocular surface, emphasizing the importance of investigations regarding the role of tear fluid components in the the pathophysiology of keratoconus.

We have revealed alterations in the levels of mediators in the tears of patients with KC providing a useful tool for monitoring the severity of the disease and possibly giving a better insight into the progression of the disease. Our results highlight the fact that many mediators including cytokines, growth factors and enzymes play in the complex underlying molecular mechanism of KC.

The precise role of mediators needs to be defined and it is important to confirm the observed changes in a larger study to gain further insight into the pathomechanism of KC and molecular alterations after contact lens wearing and the corneal CXL treatment. Our findings suggest the importance of these data for directing future clinical and basic research, including investigations of the tolerability of the different types of CLs.

Further studies determining the pre-operative predictors of patients in whom outcomes significantly improve or worsen after CXL treatment are also needed. Additionally, studies are required to further elucidate the effect of the change of mediators after CXL with the different stages of KC. This might then serve as a platform for local inhibition of pathologic corneal thinning or individualized treatment.

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List of publications related to the dissertation

*1. Fodor, M., **Kolozsvári, B.L.**, Petrovski, G., Kettesy, B., Gogolák, P., Rajnavölgyi, É., Ujhelyi, B., Módis, L., Petrovski, B., Szima, G.Z., Berta, A., Facsó, A.: Effect of contact lens wear on the release of tear mediators in keratoconus.

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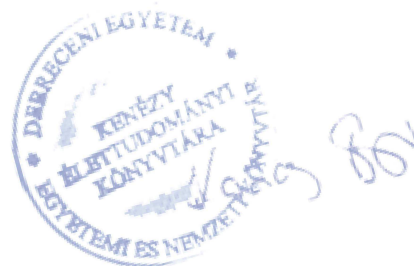
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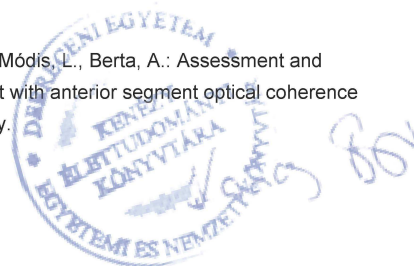


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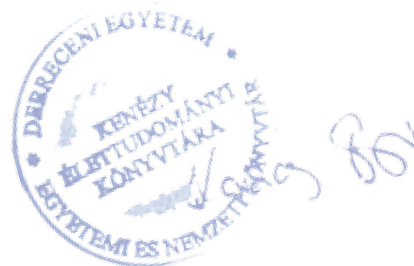
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Fodor M., **Kolozsvári B.**, Kettesy B., Ujhelyi B., Berta A., Rajnavölgyi É., Gogolák P., Szima G., Facskó A.: Kontaktlencse által kiváltott könnyben lévő mediátorok vizsgálata, különös tekintettel keratoconus esetén. 2011.03.18. Magyar Műlencse Implantációs és Refraktív Sebészeti Társaság Kongresszusa (SHIOL); Keszthely.

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