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

Effects of aging on corneal parameters measured with Pentacam in healthy subjects and investigation of tear mediators in progressive and non-progressive keratoconus

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We provide publicity in an online form. If interested, please email us (vitalygdr@gmail.com) for the link until 17.00 on the day before the defense (15.03.2021)

ABBREVIATIONS

AstigF: corneal astigmatism on the frontal surface

AstigB: corneal astigmatism on the back surface

AxisF: flat axis of astigmatism on the frontal corneal surface

AxisB: flat axis of astigmatism on the back corneal surface

AUC: areas under the receiver operating characteristic curves (ROC)

CCL5/RANTES: C-Cmotif ligand 5/Regulated on Activation, Normal T Cell Expressed and Secreted

CCT: central corneal thickness

CKI: central keratoconus index

CXCL8/IL-8: C-X-C chemokine ligand 8/interleukin-8

CXL: corneal cross-linking therapy

C Vol D 10 mm: corneal volume measured in 10mm diameter from frontal corneal apex

D: diopter

D-index: Belin-Ambrósio deviation index

ECM: extracellular matrix

IFN: interferon

IHA: index of height asymmetry

IHD: index of height decentration

IL: interleukin

ILC: innate limfoid cell

ISV: index of surface variance

IVA: index of vertical asymmetry

J45 vector: 45° degree component of Jackson's crosscilinder force vector

K1F: diopter along the flat axis on the frontal surface

K1B: diopter along the flat axis on the back surface

K2F: diopter along the vertical axis on the frontal surface

K2B: diopter along the flat axis on the back surface

KC: keratoconus

KI: keratoconus index

Kmax Front: maximal diopter, maximal keratometrics on the frontal surface

MMP: matrix metalloproteinase

NGF: nerve growth factor

NLR: neutrophil-limfocyte ratio

NPV: negative predicted value

Pachy Min: thinnest point of the cornea (smallest pachymetric value)

PAI: plasminogen activator inhibitor

PPV: positive predictive value

ROC: receiver operating characteristic curves

ROS: reactive oxygen species

SD: standard deviation

TIMP-1: tissue inhibitor of metalloproteinase 1

t-PA: tissue plasminogen activator

1. INTRODUCTION

Aging is a physiological process and occasionally it is hard to differentiate between time dependent biological changes and damages from environmental insults. Age-related changes occur in all structures of the eye with various consequences. Corneal aging generates structural and functional changes including steepening of keratometry indices and a rotation of the axis of astigmatism resulting in a shift from with-the-rule to against-the-rule astigmatism. Alterations of higher-order aberrations of the cornea are also well known. Corneal thickness decreases throughout infancy and around 3 years of age it reaches adult thickness and appears to be stable over time. However age-related change in corneal thickness appears to be rather controversial in other studies. A complete and precise evaluation of the cornea must take keratometric, astigmatic vectorial as well as pachymetric characteristics into account. There are clinical situations where it is important to distinguish between pathological and normal age-related changes. The assessment of the progression of ectatic corneal disorders in young patients determines the optimal treatment.

2. LITERATURE REVIEW

Keratoconus (KC) is a progressive ectatic corneal disorder with heterogeneous clinical severity and varying progression. Its prevalence is around 1:375 in the general population highlighting its public health importance. The pathogenesis is related to a combination of genetic, biomechanical, biochemical and environmental risk factors including inflammation, oxidative stress, and allergy. Corneal collagen crosslinking (CXL) proved to be effective in halting the progression of KC and the best results can be obtained in early stage. This possible treatment underscores the challenge of identifying the appropriate patients as early as possible.

Altered levels of various cytokines, enzymes, regulatory and growth factors, and diagnostic markers of inflammation and tissue injury have been found in the tears or in the cornea of patients with KC, pointing to the crucial role of the immune system in the pathogenesis of keratoconus. These include proinflammatory cytokines (tumor necrosis factor (TNF), interleukin (IL)-1β, IL-6), inflammatory chemokines (C-X-C chemokine ligand 8 (CXCL8)=IL-8, C-C motif ligand 5 (CCL5)= Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES)), inflammatory mediators (IL-12, interferon (IFN)-γ, IL-17), the anti-inflammatory cytokine IL-10, cytokines associated with allergy development (IL-4, IL-13), enzymes and their co-factors associated with tissue remodeling (matrix metalloproteinases (MMPs), tissue inhibitor of metalloproteinase-1 (TIMP-1), cathepsin B). Various growth factors, other enzymes, enzyme inhibitors, cellular proteins which can serve as diagnostic markers in the context of cellular and tissue injury or inflammation were also described, including epidermal growth factor, vascular endothelial growth factor; insulin-growth factors, nerve growth factor, lipocalins, lipophilins, phospholipase A2, cystatins, albumin, type I and

type II keratins, lactoferrin, Prolactin-Induced Protein, α-fibrinogen, α1-antitrypsin; apolipoprotein A1, lysozyme C, zinc-α2-glycoprotein, metabolic enzymes (e.g. glyceraldehyde-3-phosphate dehydrogenase), different immunoglobulins (IgA, IgG1, and the κ-isotype of the Ig light chains) and the polymeric immunoglobulin transport receptor. Disease specific changes in these molecular markers can be of diagnostic value in KC.

To the best of our knowledge, there are no studies examining these mediators in the tear fluid to predict the progression of the disease. However, early detection of progression in keratoconus is of high importance because early identification of the progressive nature of the disease allows early treatment to reduce the risk of visual impairment. If tear biomarker profiling could predict the progression of keratoconus earlier than the parameters used nowadays, it would be ideal for clinical application.

The MMP-9, MMP-13 are collagenases produced by various cell types during tissue remodeling, involving injuries and tissue repair. Their functions are regulated directly or indirectly by inhibitors (e.g. TIMP-1) or activator proteases and their respective activators and inhibitors (e.g. tissue plasminogen activator (t-PA), plasminogen activator inhibitor (PAI-1)). The other investigated mediators are associated with different type of inflammatory processes involving the Th1 or type 1 innate lymphoid cell (ILC1) produced mediator: IFN-γ which take part in the classical inflammatory macrophage activation. Pro-inflammatory cytokines and chemokines produced by various innate inflammatory cell types: IL-6, CXCL8/IL-8, CCL5/RANTES. Cytokines mediating the resolution of the inflammation: IL-13, IL-10 produced by Th2 cells or ILC2 cells. IL-17α is an inflammatory cytokine produced by Th17 cells or ILC3 cells normally mediating neutrophil granulocyte rich inflammation and other antimicrobial defence processes in the epithelial tissues. NGF takes part in regulatory and healing mechanisms.

3. AIMS

I. To determine age-related changes of corneal parameters using a Pentacam device in a healthy cohort aged between 14 and 67 years at baseline.

II. To determine the concentrations of 13 different immune mediators (IFN-γ, IL-6, IL-10, IL-13, IL-17A, CXCL8/IL-8, CCL5/RANTES, MMP-9, MMP-13, NGF, TIMP-1, t-PA és PAI-1) in tear samples of patients with KC. The tested multifunctional mediators were chosen as representative molecules that are associated with corneal degradation in KC. Their exact role is not exactly defined yet in the process.

III. To correlate the changes of these mediators (IFN-γ, IL-6, IL-10, IL-13, IL-17A, CXCL8/IL-8, CCL5/RANTES, MMP-9, MMP-13, NGF, TIMP-1, t-PA and PAI-1) to the changes of Pentacam parameters which are used to detect progression.

IV. To find combinations of mediators which can predict better the progression of the disease than single mediators.

4. PATIENTS AND METHODS

4.1. Patient selection and clinical examination

The first part of our examination comprised 35 healthy participants of European descent with 20/20 Snellen equivalent distance visual acuity with low refraction error (lower than 1.5 diopters [D]) but without other ophthalmological disorders. Following the tenets of the Declaration of Helsinki, written informed consent was signed by all participants and/or their parent and/or legal guardian for study participation prior to enrollment. The study protocol was approved by the Regional and Institutional Research Ethics Committee of the University of Debrecen (DEOEC-RKEB/IKEB 3313-2011). Exclusion criteria were: refraction error more than 1.5 D, active inflammatory or infective systemic or ocular disease, current treatment with systemic or local drugs, use of eyedrops, contact lens wear, previous ocular surgery, abnormality in the lens or retina on biomicroscopic examination, precedent chemical injury or delayed epithelial healing, age less than 14 years, and pregnancy or lactation. Prior to a standard ophthalmological investigation, three images were captured of one randomly selected eye of each patient with high-resolution Pentacam (Pentacam HR, Oculus Optikgeräte GmbH, Wetzlar, Germany, software version 1.17r139) using a 12-mm wide Scheimpflug imaging technique. All participants underwent repeated ophthalmological examination during a follow-up period of 3.6 years on average. At the baseline and follow-up visit the following parameters were recorded for each eye: examination date, Snellen visual acuity, spherical equivalent. The following data were exported from Pentacam to Microsoft Excel (Microsoft Corp, Redmond, Washington): Holladay equivalent keratometry values in the flat (K1) and steep (K2) meridian of the front and the back surface, maximal keratometry values of the front surface (Kmax), corneal astigmatism of the front and the back surface (AstigF and AstigB, respectively), flat axis of corneal astigmatism (AxisF, AxisB), corneal thickness at the thinnest point of the cornea (Pachy Min), the volume of the cornea in a diameter of 10 mm centered on the anterior corneal apex (C Vol D 10 mm), index of surface variation (ISV), index of vertical asymmetry (IVA), index of height asymmetry (IHA) and index of height decentration (IHD). The change in Pentacam parameters were analyzed always in comparison with the baseline values. All examinations in this study were performed between 8 and 10 am to correct for daily corneal thickness changes.

4.2. Keratoconus patient selection, Pentacam measurements and tear collection/analysis

In the second part of our study we performed an observational cohort study involving well-characterized keratoconus patients recruited from the Department of Ophthalmology, Faculty of Medicine, University of Debrecen, Hungary.

Keratoconus was diagnosed upon the presence of one or more 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, and topographic changes. Exclusion criteria included the existence of active inflammatory or infectious systemic or ocular disease (including atopic dermatitis), history of chronic, abnormal eye rubbing, and current treatment with systemic or local anti-inflammatory drugs. Patients who were pregnant or lactating during the course of the study and eyes with a history of ocular surgery (including corneal cross-linking) or trauma were also excluded.

Altogether, 42 keratoconic patients (mean (standard deviation=SD) age 36.4 (12.3), range 15-68 years) were enrolled in the study. Both eyes of each participant underwent repeated ophthalmological evaluation, including clinical history, automated kerato-refractometry (KR-8900; Topcon Co, Tokyo, Japan), uncorrected and corrected distance visual acuity determinations, slit-lamp biomicroscopy (under low illumination to avoid reflex tearing) and Rotating Scheimpflug tomography (Pentacam HR, Oculus Optikgeräte GmbH, Wetzlar, Germany). Patients were examined with a Pentacam HR at baseline and at the end of the one-year follow-up to determine the progressive nature of the disease. The following parameters were exported to Microsoft Excel (Microsoft Corp, Redmond, Washington): K1, K2; Kmax Front; AstigF; Pachy Min; Keratoconus Index (KI); Central Keratoconus Index (CKI); and Belin-Ambrósio deviation index (D-index). KC progression was defined as an increase in K2 and/or Kmax and/or in astigmatism of 1.00 diopter (D) or more in the prior 12 months. Grade of KC was defined as mild if the steepest keratometric reading K2 was <45 diopters, moderate if K2 was between 45 and 52D, and severe if K2 was >52D. We involved only one eye of each patient at baseline that met all inclusion but no exclusion criteria, except for patients with one progressive and one non-progressive keratoconic eye, in which case one eye was enrolled into the non-progressive, and the other eye into the progressive group.

Non-traumatic, non-stimulated tear collection using micro-capillary tubes from the inferior meniscus were carried out at the same time of the day, between 8.00 and 9.30 a.m. at the baseline visit and at the end of the one-year follow-up. We calculated tear volumes from the length of the tear column in the tube on a micrometer scale. The tear samples were analyzed for IL-6, IL-10, IL-13, IL-17A, CXCL8/IL-8, CCL5/RANTES, IFN-gamma, MMP-9, MMP-13, TIMP-1, NGF, t-PA, and PAI-1 concentrations using the Cytometric Bead Array method. Combined FlowCytomix Simplex Kits were used with the appropriate FlowCytomix™ Basic Kit, with minor modifications to the manufacturer's instructions (eBioscience, Bender Med Systems GmbH, Vienna, Austria). The subsequent detection limits were as follows: IL-6: 1.2 pg/ml; IL-10: 1.9 pg/ml; IL-13: 4.5 pg/ml; IL-17A: 2.5 pg/ml; IFNγ: 1.6 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; t-PA: 4.8 pg/ml; and PAI-1: 13.5 pg/ml.

4.3. Statistical analysis

4.3.1 Age related alterations

Outcome parameters reported by the Pentacam system were mostly treated as untransformed continuous variables on their natural scales and units. Exceptions included corneal axis angles, which were consolidated into the range 0° to 90° to derive a laterality-independent measure of location within the range running from horizontal to vertical; and posterior corneal astigmatism values, which were sign reversed. Jackson's cross cylinder power vector components (J_0 and J_{45}) were calculated for the anterior corneal surface (i.e., the simulated keratometric data) with the method described by Thibos et al.. Unadjusted comparisons of follow-up vs baseline readings were based on paired t-tests if normality assumptions were satisfied or Wilcoxon's matched-pairs signed-ranks tests otherwise. Male subjects were compared to females in terms of age using Wilcoxon's rank-sum test. Multilevel mixed-effects linear regression was used for adjusted estimation of changes in outcome parameters through follow-up time. Adjustment variables included baseline readings of the outcome parameter, baseline age, and interaction terms between age and follow-up time, and between baseline reading and follow-up time (sex proved unnecessary to adjust for). Findings were expressed as estimated annual changes with 95% confidence intervals either as a function of baseline variable(s) (in presence of a significant interaction) or as a single overall estimate (in absence of significant interactions), and visualized using scatter plots of baseline reading of outcome parameter vs. age at baseline, with symbology indicating direction, significance, and magnitude of change over time. The statistical package applied was Stata version 11. The significance criterion was set at $\alpha<0.05$.

4.3.2 Keratoconus progression

If both eyes of the patient were in the same group, i.e. non-progressive or progressive, then we only included one randomly selected eye. Eye selection was based on generating random values using Microsoft Excel set to produce numeric indicators with equal probabilities for either eye. We included automatically the fellow, keratoconic eye of the patient if the other eye had a history of an invasive procedure (CXL, transplantation) and was therefore subject to exclusion from the study. We included both eyes if one was progressive and the other was non-progressive in nature. Mediator concentration and Pentacam parameter variables were inspected for distribution shape and transformed to improve normality if necessary. The quantities of mediators released into tears were calculated using concentrations (pg/µl) and tear volumes (µl) collected over 2 minutes. First we analyzed the Pentacam data at baseline and end of follow-up to determine the progression of the disease, and to be able to classify the eyes of the 42 patients into two disease groups, namely the non-progressive and the progressive groups. Then we determined statistical correlations between

the changes in the levels of tear mediators and changes in Pentacam parameters during follow-up using linear regression. As a third step, the predictive performance of tear fluid mediators was evaluated using logistic regression. All possible pairs of mediator concentration variables, and similarly of release level variables, were formed and used as continuous explanatory variables complete with an interaction term between them, in models with observed progression as the binary outcome. After each model fit, observations were sorted on predicted probability and dichotomized for model diagnostic purposes such that the number of predicted cases of progression above the cut should equal the number of observed cases. Observations with predicted probability of at least 50% were used as predicted positives in evaluating the system for sensitivity, specificity, and positive and negative predicted values (PPV and NPV, respectively). For reference, the procedure was repeated using Pentacam parameter pairs as explanatory variables. Comparison was based on a non-parametric approach evaluating the areas (AUC) under the receiver operating characteristic curves (ROC) of each Pentacam parameter model against those of each of the best two mediator release models. For these models, cutoffs were not set on the measurement scales of the predictor variables. Instead, model-predicted probabilities greater than or equal to 50% were regarded as positive predictions (progressive disease).

5. RESULTS

5.1. Age related alterations

Our study included thirty-five randomly selected eyes of 35 healthy volunteers of European descent (22 women and 13 men; 19 right and 16 left eyes). Participants were predominantly female (63%). There was no statistically significant difference regarding age between genders. The mean age at the first visit was 31.8 years (SD: 12.4, range: 14.2-66.9 years). The average time interval between visits was 3.6 years (SD: 0.92, range: 2.1-5.7 years). The mean age at the control visit was 35.4 years (SD: 12.3, range: 16.4 to 69.2 years).

Both keratometric values (K1 and K2) on the anterior corneal surface decreased significantly during observation ($p = 0.002$ and 0.048, respectively), with no accompanying changes to K max. No significant changes in K1 and K2 were observed with unadjusted analysis on the posterior surface. The astigmatism parameter only changed significantly with aging on the posterior surface of the cornea ($p=0.022$). The mean deviation from horizontal of the flat axis angle on the posterior surface (K1 B) was 8.5° (SD=7.3°, range: 0.9-35.4°) at baseline and 10.2° (SD=7.3°, range: 1.6-33.3°) at the end of follow-up. The difference was significant ($p=0.027$). There was no significant difference between baseline and follow-up for the flat axis angle on the anterior corneal surface (K1 F). Regarding power vectors of the anterior surface, Jackson's cross cylinder power vector component at 45° demonstrated weak changes with advancing age ($p = 0.047$).

The examination of pachymetric features of the cornea revealed that Pachy min decreased significantly with age ($p < 0.001$). In line with this, corneal volume in the central 10 mm also decreased significantly with age ($p < 0.001$). During follow-up, the Pentacam indices IHA and IHD showed significant increase ($p \leq 0.001$), while ISV and IVA did not change. Taking baseline age into account, the front keratometric values (K1 and K2) decreased more substantially in younger than in older individuals. Although unadjusted analysis shows no significant changes in K1 and K2 on the posterior surface, adjusted statistical modeling revealed that the keratometric reading on the posterior corneal surface in flat axis (K1B) changes in a heterogeneous fashion across baseline value and, to some extent, age. Low-range keratometric values, which are observed in younger subjects, increase significantly with time, while those in the high range do the opposite. Neither the keratometric astigmatism parameter on the anterior surface of the cornea nor the J0 vector changed significantly with time. Adjusted analysis also shows that these two readings are stable across follow-up throughout the entire range of baseline readings and ages. The borderline significant change detected in Jackson's cross cylinder power vector component at 45° (unadjusted $p = 0.047$) is explained by a significant increase in older subjects with low to mid-range initial J45 values; values in younger subjects and in those with mid to high-range baseline readings did not change with advancing age. Investigation into age-related progression of posterior corneal surface astigmatism revealed baseline dependent tendencies: if the baseline value of the astigmatism parameter was low, it tended to significantly increase, and if it was high, to significantly decrease during follow-up; this was true across the whole range of baseline age in our sample. Adjusted analysis shows that both the volume of the central 10 mm diameter of the cornea and the Pachy min parameter decrease significantly, especially from higher baseline measured values, with some suggestion of dependence on baseline age. Readings that are low at baseline, and also those registered in older subjects, seem to have a limited capacity to change.

Based on our analysis, the estimated annual change of Pentacam parameters of a normal, healthy eye are: on the anterior surface of the cornea, K1 and K2 decrease significantly (although maintaining a stable Kmax); on the posterior surface, however, only high-range values of the flat keratometry parameter (K1) decrease significantly over time ($p = 0.019$). Front astigmatism seems to remain stable with aging, while higher initial absolute values of posterior astigmatism tend to reduce. Anterior axes of astigmatism do not seem to vary over time; initially horizontal posterior axes shift significantly towards the vertical, and those in the vertical or oblique angle at baseline do so towards the horizontal position. While J0 seems to be stable, J45 significantly increases from a low baseline ($p = 0.012$). The volume of the central 10 mm diameter of the cornea and the Pachy min parameters decrease stronger in thicker corneas.

5.2. Keratoconus progression

A total 45 eyes of 42 patients (mean (SD) age 36.4 (12.3), range 15-68 years) were enrolled in the study. At the end of follow-up, eyes were classified into a non-progressive KC group (29 eyes) and a progressive KC group (16 eyes) determined by Pentacam parameters. Generally, the study only involved one eye of each patient, except in three patients where one eye was in the non-progressive, and the other in the progressive group. At baseline, KC grade in the non-progressive vs progressive group was mild in 4 vs 0, moderate in 18 vs 8, and severe in 7 vs 8 eyes. After one year, these counts were 5 vs 0, 17 vs 8, and 7 vs 8, respectively. At baseline, there were significant differences in K2 ($p=0.035$), Kmax ($p=0.014$), D-index ($p=0.016$) but not in any other Pentacam values including astigmatism. At the end of follow-up, there were significant differences in K2 ($p=0.007$), Kmax ($p=0.0008$), D-index ($p=0.0396$) and also in corneal astigmatism of the front surface ($p=0.027$).

At baseline, there were no significant differences between the two groups in the release or concentration of mediators. At the end of follow-up, there were significant differences in the release of IFNγ, IL-13, IL-17A, CCL5, MMP-13, and PAI-1 between the two groups however, no significant differences in mediator concentrations between the two groups were observed.

Significant differences were observed between the progressive and the non-progressive group in the way changes in the levels of the different tear mediators were correlated with changes in Pentacam parameters. Changes in five out of eight analyzed Pentacam parameters correlated positively with changes in IFNγ, IL-13, IL-17A, CXCL8, CCL5, TIMP-1, and t-PA.

Of all possible baseline mediator pairings, those showing reasonable predictive power for keratoconus progression included IFNγ with NGF and IL-13 with NGF released quantities. High released levels of NGF, and moderately high levels of NGF coupled with low levels of IFNγ or IL-13, were associated with increased odds of progression. The plane defined by these variable pairs was possible to separate, to a certain degree, into non-progressive and a progressive area. The model based on released quantities of IFNγ with NGF had a reasonable specificity and NPV but a moderate sensitivity and PPV estimate (ROC AUC = 0.9385), while the one based on released IL-13 and NGF (AUC = 0.9692) had very high specificity and PPV with high NPV and reasonable sensitivity.

The predictive performance indicators of Pentacam parameter pairs were similar to or poorer than (especially in relation to IL-13 and NGF) those estimated for mediator release pairs. AUC values ranged from 0.3692 to 0.7385 and were found significantly smaller in 40 out of 56 comparative relations with the two models based on mediator release.

6. DISCUSSION

6.1. Age related alterations

A wide range of changes occur in the aging cornea some of which are clinically relevant for planning surgical procedures and also for the management of corneal ectatic disorders. Reports on age-related changes of corneal thickness in healthy eyes are somewhat contradictory. Central corneal thickness seemed to be stable (mean change of CCT was -1.9 ± 14 µm) over 1.5 years in children, while other studies reported no significant association between corneal thickness and age. On the other hand, Rieth et al. detected significantly increased corneal thickness in the elderly in a cross sectional study. In line with our observations, vast majority of previous studies report a statistically significant inverse relationship between age and corneal thickness. In the present work we detected a 14-µm average decrease at the thinnest point of the cornea representing the strongest decrease reported in longitudinal studies so far (5 to 14 µm across 8.2 years, 3.5 µm across 3.8 years, 2.6 µm across 5 years reported by Weizer, Brandt and Hashemi et al., respectively). The mean ages of these longitudinal study samples were 50 to 60 years at the baseline visit, which may explain why our younger patient population (mean age 31.8 years) showed more pronounced corneal thinning. This observation indicates that corneal tissue degradation is more pronounced in younger ages. The only study which analyzed the change of the thinnest point thickness with Pentacam contained individuals between 41 and 64 years old (average 49.9 years) with mean corneal thickness of 524.9 µm (SD 32). This was 13 µm thinner than in our younger study group of 14 to 67-year-old subjects (average 31.8 years). The difference in corneal thickness between these two different age groups confirms our observation on the age dependent corneal thinning.

Little attention was paid to corneal volume in earlier studies although it may sensitively follow topographical and pachymetric changes of the cornea and therefore is a sensitive indicator of corneal health. Recently, the change in this parameter was assessed during accommodation, in connection with phacoemulsification, refractive surgery and keratoconus. In the present study we found an age-dependent decrease in corneal volume of the central 10 mm. Interestingly the change was more pronounced in case of higher baseline values and younger individuals. This change is very similar to that observed with corneal thickness however their reason is still largely unknown. The thinner cornea in line with the higher degree of rigidity in older aged patients might be a hint of physiologically cross linking process in elderly individuals. Investigation of the age-related, long-term changes of corneal rigidity including corneal hysteresis and corneal resistance factor together with the changes of Pentacam parameters could extend our knowledge on corneal physiology and could help to understand the underlying processes. Nevertheless, some authors failed to find a significant change in CCT over a decade. Physiological age-dependent decrease of these parameters should be considered when evaluating the progression of corneal ectatic disorders. We must aim to reliably predict progression and monitor the effectiveness of corneal cross-linking treatment in corneal ectatic disorders taking normal age-related cornea changes into account.

We have found that both keratometric values (K1 and K2) on the anterior corneal surface decreased significantly during follow-up, while K max remained stable. Adjusted statistical analysis of our data revealed that anterior surface keratometric values decrease stronger in younger than in older subjects. On the other hand, higher K values proved to be more stable over time independent of age. In a middle-aged to older population sample in a longitudinal study, K max increased 0.38 Diopters ($p<0.001$) in 5 years. In contrast with these results Orucoglu et al. reported a significant positive correlation between anterior K1 and age, without any correlation between K2 and age. In our study, unadjusted analysis shows no significant changes in K1 and K2 values of the posterior surface. However, adjusted statistical modeling revealed that the rate of change of keratometric values of the posterior corneal surface in the flat axis (K1B) may vary with baseline value and age. Low-range keratometric values increase significantly with time, while those in the high range do the opposite, suggesting a tendency for values to progress away from distributional extremes.

It is important to investigate corneal astigmatism with highly repeatable and reproducible methods such as Scheimpflug imaging to provide reliable data on age-related changes of astigmatism in order to improve long-term outcomes after intraocular lens implantation and refractive surgery. Previous reports on age-related changes in astigmatism were all cross-sectional studies and/or were performed with other devices. Total refractive astigmatism has been shown to change from with-the-rule to against-the-rule with age. According to Ho et al. and Nemeth et al. who both used Pentacam there is an age-related shift towards against-the-rule and with-the-rule astigmatism for the anterior and posterior corneal surfaces, respectively. To the best of our knowledge, the current study is the first longitudinal study in the literature examining age-related changes of keratometric astigmatism at both the anterior and posterior surfaces of the cornea. Moreover, we examined keratometry values in a population covering the widest age range reported so far. In a cross-sectional and a longitudinal study alike, the prevalence of astigmatism increased with age, however in other cross-sectional studies it was demonstrated that keratometric astigmatism decreases significantly at both the anterior and posterior surfaces of the cornea with age. Our findings are similar to these, although we only found evidence for keratometric astigmatism at the posterior surface to decrease significantly ($p=0.02$), while astigmatism remained stable at the anterior surface. Moreover, posterior astigmatism showed a baseline-dependent significant increase from lower and a significant decrease from higher initial values, with no evidence of heterogeneity across baseline age. Our findings are in contrast with the conclusions of Németh et al. that the posterior surface of the cornea is much more stable with advancing age than the anterior surface, even though there are no differences between the two studies in ethnic composition or in methods used. Over time, the mean flat axis angle of the posterior keratometry shifted towards horizontal from a vertical ($p=0.004$) or oblique ($p=0.004$) baseline, which is in line with earlier studies proving that anterior corneal topographic astigmatism drifted from with-the-rule to against-the-rule astigmatism in older subjects.

Vector analysis allows for a complete description of astigmatism characteristics. In the assessment of anterior surface power vectors, Jackson's cross cylinder power vector

component at 45° showed significant changes with advancing age (unadjusted p = 0.047), which were explained by a substantial increase in older subjects with low to mid-range initial J45 values; values in younger subjects and in those with mid to high-range baseline readings did not change with advancing age. Our findings are consistent with previous results that in addition to the frontal astigmatism, the J0 vector also remains stable with age. Based on our study with almost four years of mean follow-up, the axis of anterior astigmatism did not change significantly.

Limitations of this study include a modest sample size precluding robust conclusions and the fact that only European descent of low refractive error were included. We do not know at this time, whether the changes observed in our study are linear with age. Moreover, because patients with high refractive error were excluded, we cannot estimate the effect of refractive surgery on the outcomes over decades. Despite these limitations, it is important to emphasize that our results highlight the fact that K1F, K2F, K1B, AstigB, PachyMin, and CV decreased significantly, IHA, IHD, and J45 vector increased significantly, and the mean AxisB shifted significantly with age.

The strength of our longitudinal study is that it gives reliable, reproducible data obtained with a Pentacam HR device. Moreover, the study group consisted of 14 to 67-year-old individuals providing a wide age range to estimate age-dependent changes associating to different baseline ages. We explored for the first time that age and baseline value of corneal parameters have an effect on age-dependent corneal changes. To the best of our knowledge, this is the first prospective longitudinal study with a long follow-up period that reveals the clinically relevant consequences of aging on multiple parameters of the healthy cornea measured with Pentacam. In summary, based on our observations age-related changes of the anterior and posterior corneal surfaces should be considered when planning a surgical procedure for astigmatism correction.

6.2. Keratoconus progression

Several studies have revealed that inflammatory factors play a key role in the pathomechanism of KC and several associations were revealed between the levels of inflammatory mediators and the severity of the disease. Tear mediator profile as a noninvasive biomarker of keratoconus can act as a prognostic biomarker and may aid in the timely treatment of this heterogeneous disease. To the best of our knowledge, there is no longitudinal study evaluating mediators in order to predict the true progression of keratoconus.

The real clinical importance and relevance of our study lies in the ability to tell from current mediator levels whether KC is likely to progress in the future. This dictates an approach where baseline levels, strictly without their follow-up counterparts, are the explanatory variables and KC progression is a binary outcome. Another interesting aspect is the possibility of interaction between various mediators: it might be that no single mediator is strongly predictive on its own, but a combination where high levels of one are accompanied by low

levels of another is. Our findings suggest that certain mediators could predict the progression of KC and have outstanding roles in the pathomechanism of KC. Future directions may include targeting these inflammatory factors in the management of KC to restore the dysregulated inflammation in KC pathogenesis. This opens the potential to explore anti-inflammatory strategies to either halt or delay the progression of KC.

In our study, IFNγ with NGF and IL-13 with NGF released quantities showed reasonable predictive power for keratoconus progression: high levels of NGF, and moderately high levels of NGF coupled with low IFNγ or IL-13 were associated with keratoconus progression. Based on the models, prediction based on released IL-13 and NGF seems to be more useful because it has 100 % specificity and PPV with 93 % NPV and reasonable (80 %) sensitivity. Martínez-Abad et al. designed a predictive model for keratoconus progression based on refractive, topographic and aberrometric changes. Our findings suggest that compared to Pentacam parameters, mediator release levels might have at least similar, but potentially greater, predictive power for keratoconus progression. This is to be fully clarified by future research specifically targeted at the question. Karaca et al. used the serum neutrophil-to-lymphocyte ratio (NLR) to predict keratoconus progression, based on its predictive property of systemic inflammation in several diseases. Although NLR is simple and inexpensive, it was found to predict the presence of KC progression with only 79% sensitivity and 81% specificity.

We think that tear collection is a non-traumatic and non-invasive procedure and tears can truly reflect the local pathological disorders such as keratoconus. Based on the findings of our study, IFNγ, IL-13, IL-17A, CXCL8, CCL5, MMP-13, TIMP-1 t-PA, PAI-1 and NGF seem to have a crucial function in the progression of keratoconus; as to prediction, IL-13 and NGF have the most important role. We found a significant positive association in tears of patients with KC between CCL5, MMP-13 and NGF levels and several topographic data and showed that IL-13, CXCL8, CCL5 and MMP-13 have different effects on the severity of disease depending on age. Age influences the immune response and also the progressive nature of keratoconus. We revealed significant differences in the release of IL-13, CCL5, MMP-13 and also IFNγ, IL-17A and PAI-1 between the two groups showing their pivotal role not only in the pathomechanism of KC but in the course of progression. Produced by Th1 cells, IFNγ is associated with autoinflammatory and autoimmune diseases. We measured increased IL-13 and IFNγ concentrations in the tears of KC patients. Jun et al. showed significantly increased tear levels of IL-17 and decreased IL-13 and CCL5 in keratoconus compared to normal controls. IL-17 is the principal proinflammatory cytokine produced by T helper 17 cells and is associated with many chronic inflammatory conditions. Based on our study IFNγ with NGF and IL-13 with NGF are predictive indicators of keratoconus progression. IFNγ has a broad range of biological functions and IL-13 is produced by Th2 cells, as IL-10. IL-13 plays crucial roles in amplification of the Th2 response which is dampened in keratoconus. Balasubramanian et al. detected increased expression of IL-10 and IL-6 and classified keratoconus as an inflammatory disease. Interestingly, we could not reveal any differences in the tear levels of IL-6 or IL-10 between the progressive and non-progressive groups, although Lema et al. observed increased levels

of IL-6, while Sorkhabi et al. measured decreased levels of IL-10 in keratoconic tears. IL-6 increases chemokine activation, including CXCL8 (which was higher in our study in the progressive group), and IL-10 inhibits IL-6. These results suggest that because of cytokine interaction, many of them have a role in the pathomechanism but are less significantly involved in the progression and only some mediators can be used as progression predictors. The goal of our study is to identify some of these biomarkers that contribute to KC progression in order to recognize the progression of this visually debilitating disease earlier.

Higher levels of NGF existing in tears of patients with progressive keratoconus is in line with the well-known neural sensitizing role of this neuromediator. Kolozsvári et al. revealed not only disease specific mediators in the tear fluid of patients, but confirmed several associations between the levels of mediators and the severity of keratoconus, including NGF. Lacrimal glands are known to produce, release, and be responsive to NGF, and NGF is a normal constituent of the tear film. Correlation between NGF tear levels and the severity of corneal damage in the tear film of patients with dry eye disease was established; moreover, NGF promotes corneal healing in physiologic and pathologic conditions, and it has been shown that corneal injuries induce an increase in local NGF and NGF receptors capable of stimulating epithelial healing. These observations suggest that NGF may be involved in local tissue damage. The inadequate balance between pro-inflammatory cytokines, proteolytic enzymes, protease inhibitors, inflammatory modulators and antioxidants may lead to increased activity of metalloproteinases. Several studies have investigated the role of proteolytic enzymes such as MMPs in KC. MMPs are involved in the degradation of extracellular matrix (ECM) or activation of cellular apoptosis. In keratoconus, the cornea expresses elevated levels of MMP-13 and the levels of MMP-1, -3, -7 and MMP-13 are also increased in the tear fluid. The enzymatic activity of MMPs depends on TIMP, which is a potent inhibitor of MMPs. Pouliquen et al. suggested that cytokines, including CXCL8 might regulate the protease cascade including the plasmin system, involving t-PA and PAI and also MMPs which would lead to ECM changes in keratoconus.

Since keratoconus progression results in severe irreversible loss of visual acuity, it indicates a relevant need to find biomarkers which promptly and sensitively indicate disease progression in the future in order to select the progressive forms in time for CXL treatment. The limitations of this study are that it cannot exclude the possibility of other mediators being involved in the progression of the keratoconic cornea and that the identification of the source and activity of the mediators has not been investigated. Tear samples are crucial in understanding the molecular mechanism of the progression process and the multiplex platform is perfectly suited for the detection of biomarkers from tear samples. Ideally, because the progression of the disease will stop at a point, which can be accompanied by an alteration in mediator levels including a decrease of IL-13/NGF, it would have been useful to monitor the tears of eyes with progressive keratoconus without any treatment for a longer time to find out about the time of progression arrest; however, in this study the vast majority of the eyes with progressive keratoconus needed and received CXL treatment, making such

long-term observations largely unavailable. Despite these limitations, it is important to indicate that our results underline the fact that many mediators are involved in the complex mechanisms of keratoconus progression. It remains to be determined in further studies which of these mediators or any others are principal in predicting keratoconus progression.

To conclude, our study confirms that different mediators in the tear fluid could predict the progression of keratoconus and may underscore the important roles of NGF and IL-13, which together seem to be useful in the prediction of the progression with 100% specificity and PPV, 93% NPV, and 80% sensitivity. Locally released mediators serve as additional proof for considering corneal cross-linking treatment in an attempt to stop KC progression. As next steps, the critical levels of these mediators and the precise roles of the identified predictive biomarkers need to be defined on a larger cohort to reveal their potential use not only as diagnostic markers but as therapeutic targets as well. The potential roles of biomarkers include being components in a KC progression predictive system combined with other input variables such as Pentacam parameters, age, or other data available in a real-life clinical setting.

7. SUMMARY OF NEW RESULTS

I. We prospectively analyzed the age-related changes of corneal Scheimpflug parameters in healthy subjects and we found several associations between age and changes of corneal structure. We explored for the first time that age and baseline value of corneal parameters have an effect on age-dependent corneal changes.

II. We determined the concentrations of 13 different immune mediators (IFN-γ, IL-6, IL-10, IL-13, IL-17A, CXCL8/IL-8, CCL5/RANTES, MMP-9, MMP-13, NGF, TIMP-1, t-PA és PAI-1) in tear samples of patients with KC and at the end of follow-up, there were significant differences in the release of IFNγ, IL-13, IL-17A, CCL5, MMP-13, and PAI-1 between KC w/wo progression.

III. In our study, changes in five Pentacam parameters correlated positively with changes in tear IFNγ, IL-13, IL-17A, CXCL8, CCL5, TIMP-1 and t-PA.

IV. In this study IFNγ with NGF and IL-13 with NGF released quantities showed reasonable predictive power for keratoconus progression: high levels of NGF, and moderately high levels of NGF coupled with low IFNγ or IL-13 were associated with keratoconus progression. Based on the models, prediction based on released IL-13 and NGF seems to be useful with 100 % specificity and PPV with 93 % NPV and reasonable (80 %) sensitivity.

8. SUMMARY

Aging is a physiological process and occasionally it is hard to differentiate between time dependent biological changes and damages from environmental insults. Age-related changes occur in all structures of the eye with various consequences. Several studies examined normal age-related changes of corneal parameters, most of what were cross sectional and not longitudinal in nature.

KC is a progressive ectatic corneal disorder with heterogeneous clinical severity and varying progression. Recent studies suggest that the pathogenesis is related to a combination of genetic, biomechanical, biochemical and environmental risk factors including inflammation, oxidative stress and allergy.

Our purposes were to prospectively analyze the age-related changes of corneal Scheimpflug parameters in healthy subjects and to find immunomediator combinations which could sensitively indicate KC progression.

In the first part of our study thirty-five eyes of 35 volunteers (age 14–67 years) were investigated with an average interval of 3.6 years. Changes of corneal parameters and indices were analyzed. K1F and K2F decreased significantly during observation and showed stronger decrease in younger than in older individuals. Higher values proved to be more stable. K1B decreased significantly and the degree of decrease was dependent on its baseline value and age: in young subjects low values increased, high values decreased. AstigB decreased significantly and showed a baseline-dependent significant increase from lower and a significant decrease from higher initial values. Over time, the mean AxisB shifted significantly. PachyMin and CV decreased significantly with age, especially from higher baseline values in younger subjects. IHA and IHD increased significantly.

In the second part of our study tear samples of 42 patients with KC were collected at baseline and at the end of the one-year follow-up. The concentrations of 13 mediators were measured by CBA (cytometric bead array). Based on Pentacam HR examination eyes were divided into a non-progressive and a progressive group. At the end of the follow-up significant differences were observed in the release of IFNγ, IL-13, IL-17A, CCL5, MMP-13 and PAI-1 between the two groups. Changes in five Pentacam parameters correlated positively with changes in IFNγ, IL-13, IL-17A, CXCL8, CCL5, TIMP-1 and t-PA. We found that tear level of IL-13 in combination with NGF can predict the progression of KC with 100% specificity and 80% sensitivity.

The results of our first longitudinal study suggest that both corneal surfaces change significantly with age. We demonstrate for the first time that age and baseline values influence the age-related changes of corneal parameters. The findings of our second longitudinal study may underscore the importance of NGF and IL-13 tear levels in the prediction of keratoconus progression.

To create the future's progress new approaches are needed to complete the complex mechanism of normal aging of the cornea and also it's pathological conditions. One of the fundamental theories of aging is the free-radical theory. Based on this, free radicals — reactive oxygen species (ROS) — are the main origin of aging by causing oxidative cellular injuries. On

the other hand, oxidative stress induced cell damages play a crucial role in the pathomechanism of keratoconus. According to these observations further studies based on tear examinations are needed to understand the role of free radicals in the aging process and also in the progression of corneal ectatic disorders.

9.APPENDIX

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List of publications related to the dissertation

1. Fodor, M., **Vitályos, G.**, Losonczy, G., Hassan, Z., Pásztor, D., Gogolák, P., Kolozsvári, B. L.: Tear Mediators NGF along with IL-13 Predict Keratoconus Progression.
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   Sci Rep. 9 (1), 3419, 2019.
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List of other publications

3. Pintér, Z., Rill, L., **Vitályos, G.**, Borbásné Farkas, K., Kolarovszki, B., Frank, D.: A Moyers-féle vegyes fogazati analízis alkalmazhatóságának vizsgálata a magyarországi lakosság körében.
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10. KEY WORDS

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