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

TEAR MEDIATORS IN CORNEAL ECTATIC DISORDERS

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The Examination takes place at the Department of Gynecology, Faculty of Medicine, University of Debrecen; 11 a.m. January 24, 2018

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ABBREVIATIONS

ART Max: maximum Ambrósio's relational thickness

Astig: corneal astigmatism

BAD-D: Belin-Ambrósio deviation index

B.Ele.Th.: back elevation at the thinnest location

CCL5/RANTES: kemokin(C-Cmotif) ligand 5/regulated on activation, normal T cell expressed and secreted

CKI: central keratoconus index

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

CXCL10/IP-10: C-X-C kemokin ligand 10/ interferon-gamma-inducible protein 10

D: diopter

ECM: extracellular matrix

F.Ele.Th.: front elevation at the thinnest location

FFKC: forme fruste keratoconus (an incomplete or atypical form of the disease)

IFN: interferon

IHA: index of height asymmetry

IHD: index of height decentration

IL: interleukin

ISV: index of surface variance

IVA: index of vertical asymmetry

K1: Holladay equivalent keratometry value in the flat meridian

K2: Holladay equivalent keratometry value in the steep meridian

KC: keratoconus

KCS: keratoconjunctivitis sicca

KI: keratoconus index

Kmax: maximal keratometry

MMP: matrix metalloproteinase

NGF: nerve growth factor

Pachy Apex: corneal thickness at the apex of the cornea

Pachy Min: corneal thickness at the thinnest point of the cornea

Pachy Pupil: corneal thickness at the pupil's center

PA: plasminogen activator

PAI: plasminogen activator inhibitor

PMD: pellucid marginal degeneration

PPI Ave: average pachymetric progression index

PPI Max: maximal pachymetric progression index

PPI Min: minimal pachymetric progression index

Rmin: minimal radius of curvature

SD: standard devation

TIMP-1: tissue inhibitor of metalloproteinase-1

TNF: tumor necrosis factor

t-PA: tissue plasminogen activator

1. INTRODUCTION AND LITERATURE REVIEW

Corneal ectatic disorders have considerable importance in public health. They are characterized by progressive deformation of the corneal architecture—including corneal thinning at different locations—depending on the type of the ectasia. **Keratoconus (KC)** is the most common primary corneal ectatic disease that gives rise to a cone-shaped cornea, while **pellucid marginal degeneration (PMD)** is a very rare peripheral thinning disorder of the inferior (in atypical cases of the superior) cornea. It is not known whether KC and PMD are distinct diseases or whether they represent different clinical presentations of the same underlying disease process. In early cases of PMD, the cornea may look relatively normal and, in severe cases, PMD may be difficult to differentiate from KC. PMD is usually discovered in the later decades of life (between the second and the fifth) compared to KC. It is important to note that PMD is not associated with vascularisation and lipid or iron deposition. The clinical significance of distinct PMD compared to KC is that the clinical continuum and treatment modalities—including the surgical interventions for these two entities—are different.

In the past few decades, slit-imaging technologies provided further improvement in corneal imaging. Nowadays, we can measure not only the front but also the back surface of the cornea with pachymetric mapping and can typify corneal architecture in three dimensions. Furthermore, Ambrósio et al. established numerous indices to improve the screening of the ectasia.

Corneal ectatic disorders are generally believed to be non-inflammatory diseases with a multivariable origin. However, a recent review by Galvis and colleagues has suggested that ectatic disorders are partly inflammatory conditions. Biomarkers in the tear film have been studied in more depth in patients with KC but no studies have been reported for PMD. Elevated levels of interleukin- (IL-) 1b, -4, -5, -6, -8, and -17; tumor necrosis factor (TNF)-α, and-β; interferon-(IFN-) γ; matrix metalloproteinase (MMP-) 1, -3, -7, -9, and -13; cathepsin B; and lipocalin-1 have been found in the tears of patients with KC. Decreased levels of IL-4, -10, -12, and -13; TNF-α; IFN-γ; chemokine (C-C motif) ligand 5 (CCL5/RANTES, regulated on activation, normal T cell expressed and secreted); lipophilin C and A; lactoferrin; α-fibrinogen; zinc-α2-glycoprotein; immunoglobulin A; immunoglobulin κ-chain; polymeric immunoglobuline receptor ; phospholipase A2; cystatin S,

SN and SA have also been found in the tear fluid. Kenney et al. have found a decreased level of tissue inhibitor of metalloproteinase-1 (TIMP-1) in KC corneas, compared to normal corneas.

In addition, an association between KC and bronchial asthma has been identified almost 50 years earlier.

There are only a few preliminary studies examining the association between mediators (mainly cytokines) in the tear fluid and the severity of KC. Lema and Durán analyzed 28 eyes, Jun et al. checked 18 patients, and Kolozsvári et al. studied only 14 keratoconic eyes. In a recent study, Shetty et al. examined the association between MMP-9, IL-6, TNF-α, and different stages of KC. The crucial limitations of these studies are the small number of patients, or the few examined mediators, or the lack of subclinical cases.

2. AIMS

I. To compare the concentrations of 11 mediators [IL-6, -10, chemokine (C-X-C motif) ligand 8 (CXCL8)/IL-8, chemokine (C-X-C motif) ligand 10 (CXCL10)/interferon-gamma-inducible protein 10 (IP-10), CCL5/RANTES, MMP-9 and-13, TIMP-1, nerve growth factor (NGF), tissue plasminogen activator (t-PA) and plasminogen activator inhibitor (PAI)-1] in the tear of PMD patients and keratoconic patients who were similar to the PMD patients not only in age but also in severity, and in normal eyes.

II. To determine associations between the different types of mediators in tear fluid—IL-6, -10, CXCL8/IL-8, CCL5/RANTES, MMP-9 and -13, TIMP-1, t-PA, and PAI-1—in the whole spectrum of keratoconic eyes (suspect, subclinical, and manifest cases of KC) and normal eyes.

III. To explore associations between these mediators and the Scheimpflug parameters which characterize the severity of KC.

IV. To examine the relationship between the Scheimpflug imaging parameters and bronchial asthma in KC.

3. PATIENTS AND METHODS

In these studies, patients with primary corneal ectasia and normal subjects were recruited from the Outpatient Unit, Department of Ophthalmology, Faculty of Medicine, University of Debrecen. Following the tenets of the Declaration of Helsinki, written informed consent was signed by all the participants prior to enrolment. These studies were approved by the Institutional Ethics Committee of the University of Debrecen.

PMD patients with classic slit-lamp signs of PMD (an inferior, narrow band of corneal thinning separated from the limbus by a relatively uninvolved area 1–2 mm in width, and a protruding cornea above the area of thinning, associated with a flat vertical meridian and a sharp change in curvature or shape at or immediately above the band of thinning) were included.

The criteria for diagnosing KC were defined as one or a 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. We used Pentacam (Pentacam HR, Oculus Optikgeräte GmbH, Wetzlar, Germany) for the precise diagnosis of corneal ectasias.

Both eyes of each participant underwent ophthalmological evaluation, including clinical history (especially bronchial asthma and contact lens usage), automated keratorefractometry (KR-8900; Topcon Co, Tokyo, Japan), uncorrected and corrected distance visual acuity determinations, slit-lamp biomicroscopy, Rotating Scheimpflug tomography (Pentacam HR), and non-stimulated tear sample collection with glass capillaries.

The exclusion criteria were: active inflammatory or infective systemic or ocular disease, current treatment with systemic or local drugs, use of eye drops, previous ocular surgery, moderate and severe KCS (keratoconjunctivitis sicca), abnormality in the lens or retina on biomicroscopic examination, chemical injury or delayed epithelial healing and pregnancy or lactation. Patients with allergic symptoms and patients with ocular allergic signs on slit-lamp biomicroscopy were excluded from the study. We took a detailed case history of every patient, and asymptomatic asthmatic disease and asymptomatic allergic dermatic disorders were recorded. Contact lens wearing was discontinued at least two weeks before the measurements and tear sample collection.

3.1. Patients groups and clinical examinations

3.1.1. To compare the concentrations of mediators in the tear of pellucid marginal degeneration patients and keratoconic patients who were similar to the pellucid marginal degeneration patients in severity, and in normal eyes

Non-stimulated tear samples were collected from nine eyes of seven PMD patients (mean (SD) age: 46.4 (9.9) years, male/female ratio: 3/4, range 36–59 years) and 55 eyes of 55 KC patients (mean (SD) age: 44.2 (8.3) years, male/female ratio: 31/24, range 29–61 years). We do not include the fellow eye of the PMD patients without a definitive diagnosis of corneal ectasia. Because of the rarity of PMD, in the instance of this ectasia, both eyes of the patients were used if they met the aforementioned inclusion criteria. Twenty-four randomly selected right or left eyes of 24 healthy controls were also enrolled on this study (mean (SD) age: 44.5 (10.6) years, range: 29–67 years). KC patients were included if they met at least one of three criteria on the similarity of severity parameters. These were: the subject's Holladay equivalent keratometry values in the steep meridian (K2) to be within the PMD group's mean K2 ±0.2 D; maximal keratometry (Kmax), within PMD mean Kmax ±0.8 D; and minimal radius of curvature (Rmin), within PMD mean Rmin ±0.1 mm. The recruitment procedure was run exhaustively on all of the potential controls and the KC patients who were available for us to contact.

The following data were exported to Microsoft Excel (Microsoft Corp, Redmond,Washington): Holladay equivalent keratometry values in the flat (K1) and steep meridian (K2), Kmax, corneal astigmatism (Astig), Rmin, corneal thickness at the apex (Pachy Apex) and at the thinnest point of the cornea (Pachy Min), index of surface variation (ISV), index of vertical asymmetry (IVA), keratoconus index (KI), central keratoconus index (CKI), index of height asymmetry (IHA) and index of height decentration (IHD).

3.1.2. To determine associations between the mediators in tear fluid of keratoconic eyes and normal eyes, to explore associations between these mediators and the Scheimpflug parameters, to examine the relationship between the Scheimpflug imaging parameters and bronchial asthma

We examined patients with KC at all stages (severe, moderate, and mild KC, subclinical KC or forme fruste keratoconus) and normal, control patients. We have categorized the participants based on the clinical stage of KC, but group allocation was not a factor in the analysis. The stages of KC were divided between mild, if the K2 was <45 diopters (D); moderate, when K2 was between 45 and 52 D; and severe if K2 was >52 D. At present, there are no specific or universally accepted criteria categorizing an eye as having subclinical KC or forme fruste KC (FFKC).

The criteria for diagnosing subclinical KC or FFKC were defined as one or a combination of the following clinical signs: if it was the fellow eye of a patient with KC and had a normal cornea on slit-lamp examination (in several cases, keratoplasty or corneal cross-linking was performed on the keratoconic eye), or using a Pentacam, the BAD-D (Belin-Ambrósio deviation index), ART Max (Maximum Ambrósio's Relational Thickness), and PPI Ave (Average Pachymetric Progression Index); or where the back elevation values at the thinnest location (B.Ele.Th.) were not in the normal range of the Pentacam; or where the criteria of KC did not apply but the maximum keratometric reading (Kmax) was more than 47 D. We included a randomly selected eye from each participant, although both eyes were examined. Altogether, 69 patients (mean (SD) age 30.7 (10.3), range 13–68 years) with the following diagnoses—severe KC: 25, moderate KC: 21, mild KC: 5 and subclinical KC: 18—and 19 normal controls (mean (SD) age 31.7 (11.5), range 18–67 years) were enrolled in the study.

The following data were exported to Microsoft Excel: K1, K2, Kmax, Astig, Rmin, corneal thickness at the pupil's center (Pachy Pupil), Pachy Apex and Min, ISV, IVA, KI, CKI, IHA, IHD, front elevation at the thinnest location (F.Ele.Th.), B.Ele.Th., minimal and maximal pachymetric progression indices (PPI Min and Max), PPI Ave, ART Max and BAD-D.

3.2. Tear collection and mediators' analysis

Non-traumatic tear collection was carried out using sterile thin glass microcapillary tubes from the inferior meniscus, without anesthetic drops or stimulation. The tear volume was calculated and registered accordingly. All the collected tear volumes were over 4 µl. Tears were collected for two minutes and then promptly transferred to Eppendorf tubes and frozen at −80°C without centrifugation, within 15 minutes of collection. A microparticle- based flow Cytometric Bead Array (CBA) technology allowing the quantification of multiple proteins in small individual tear samples was used in the study. Combined FlowCytomix Simplex Kits were used with the suitable FlowCytomix Basic Kit, with minor modifications to the manufacturer's orders (eBioscience, Bender Med Systems GmbH, Vienna, Austria). Multiparametric data acquisition was executed using a FACS Array cytometer (BD Biosciences Immunocytometry Systems, San Jose, CA). The data were analyzed with the FlowCytomix Pro 2.3 software (eBioscience). Additional serial dilutions of the standard were applied to achieve better sensitivity and modified standard curves were thus generated in the analysis. The detection limits were, for IL-6: 1.2 pg/ml, IL-10: 1.9 pg/ml, CCL5/RANTES: 25 pg/ml, CXCL8/IL-8: 0.5 pg/ml,CXCL10/IP-10: 6.0 pg/ml, MMP-9: 95 pg/ml, MMP-13: 50 pg/ml, TIMP-1: 28 pg/ml, t-PA: 4.8 pg/ml, PAI-1: 13.5 pg/ml and NGF: 126.8 pg/ml.

3.3. Statistical analysis

3.3.1. Only one eye per patient in the KC and normal groups was analyzed to avoid bias due to inter-eye correlations. Conversely, both eyes of the PMD patients were analyzed due to the rarity of this corneal ectasia. Statistical methods appropriate for the presence of eyes nested within subjects were used. The variables were described in terms of means and SD on their native scales.

Multilevel mixed-effects linear regression was used to compare the patient groups in terms of mediator concentrations and keratometric readings obtained from a Scheimpflug camera (K1, K2, Kmax, Astig, Pachy Apex and Min, Rmin, KI, CKI, IHA, ISV, IVA, IHD). Models were fitted separately for each outcome. The statistical package applied was Stata version 11. The significance criterion was set at α=0.05.

3.3.2. We categorized the participants for descriptive purposes based on clinical severity (group allocation was not used as a variable in the analysis). Mediator concentration variables were inspected for the distribution shape and natural log transformed (for t-PA and PAI-1, square root transformed) to improve normality. Similarly, Pentacam parameters were subjected to one of these transformations if that improves distributional symmetry.

Pentacam parameters were unified in a composite index referred to as the Standardized Pentacam Score. This was calculated by centering, standardizing, and direction correcting the source variables (so that the higher values invariably represent more severe pathologies), running a principal component analysis and deriving the first principal component.

Associations between all possible pairs of mediator levels, as well as those between mediators in the tear fluid and Scheimpflug parameters, were evaluated using simple linear regression, including a quadratic term by a curvature in the relationship, if required. Associations between pairs of mediators and the Standardized Pentacam Score were assessed using multiple linear regressions adjusted for age, the presence of asthma, and contact lens usage. Mediator variables were used in linear and quadratic forms, and interactions between those terms were also included in order to accommodate the model to curvatures in the outcome space. Relationships were expressed as the overall significance of the mediator pair effect and also as additive differences in the Standardized Pentacam Score at sample-covered locations, defined by mediator pair concentrations versus an arbitrary reference point.

4. RESULTS

4.1. The concentrations of mediators in the tear of pellucid marginal degeneration patients and keratoconic patients who were similar to the pellucid marginal degeneration patients in severity, and in normal eyes

The average values (SD) of the parameters measured with Pentacam in patients with PMD, KC and in healthy controls [PMD (SD)/ KC (SD)/ Control (SD)] were as follows: K1 (D): 42.4 (5.6)/ 45.6 (4.0)/ 43.4 (1.4); K2 (D): 49.1 (4.0)/ 49.3 (4.1)/ 44.3 (1.6); Kmax (D): 55.2 (7.2)/ 54.4 (5.4)/ 44.8 (1.6); Astig (D): 6.7 (2.9)/ 3.7 (1.9)/ 0.9 (0.6); Rmin (mm): 6.2 (0.8)/ 6.3 (0.6)/ 7.6 (0.3); Pachy

Apex (µm): 502 (47)/ 486 (49)/ 551 (32); Pachy Min (µm): 489 (45)/ 468 (51)/ 545 (33); ISV: 97.9 (45.6)/ 80.8 (31.3)/ 15.1 (4.4); IVA: 1.04 (0.51)/ 0.86 (0.42)/ 0.11 (0.04); KI: 1.24 (0.23)/ 1.21 (0.12)/ 1.02 (0.02); CKI: 1.01 (0.34)/ 1.04 (0.04)/ 1.00 (0.01); IHA: 17.8 (11.5)/ 23.3 (19.7)/ 3.8 (2.4); IHD: 0.12 (0.08)/ 0.08 (0.05)/ 0.01 (0.00).

Differences between the parameters measured with Pentacam among the patient groups were as follows: **PMD v. Control:** K1: p=0.312, Astig: p<0.0001, K2, Kmax, Rmin, Pachy Apex, Pachy Min, ISV, IVA, KI: p<0.0048, CKI: p=0.834, IHD: p<0.0001, IHA: p=0.0465. **KC v. Control:** K1: p=0.01, Astig: p<0.0001, K2, Kmax, Rmin, Pachy Apex, Pachy Min, ISV, IVA, KI: p<0.0001, CKI: p<0.0001, IHD: p<0.0001, IHA: p<0.0001. **PMD v. KC:** K1: p=0.006, Astig: p<0.0001, K2, Kmax, Rmin, Pachy Apex, Pachy Min, ISV, IVA, KI: p≥0.0814, CKI: p=0.004, IHD: p=0.038, IHA: p=0.224.

We enrolled the eyes of keratoconic patients who were similar to the PMD patients not only in age but also in severity (K2, Kmax and Rmin), which explains the absence of significant differences not only in K2, Kmax and Rmin but also in Pachy Apex, Pachy Min, KI, IHA, ISV and IVA.

The average concentrations (SD) of the mediators in the tear film of patients and healthy controls [PMD (SD)/ KC (SD)/ Control (SD)] were as follows: IL-6 (pg/ml): 213 (251.2)/ 160.2 (265.7)/ 217.1 (172.2); IL-10 (pg/ml): 43.06 (129.2)/ 280.3 (832.8)/ 625.1 (708.2); CCL5/RANTES (pg/ml): 636.5 (731.2)/ 412.5 (542.1)/ 218.8 (194.1); CXCL8/IL-8 (pg/ml): 1719 (905.8)/ 2231 (2857)/ 4026 (2681); CXCL10/IP-10 (pg/ml): 96.9 (72.4)/ 91.9 (87.3)/ 124.7 (123.3); MMP-9 (ng/ml): 170.8 (294.7)/ 51.3 (131.9)/ 36.7 (61.5); MMP-13 (ng/ml): 0.371 (1.1)/ 36.6 (88.0)/ 96.7 (77.1); TIMP-1 (ng/ml): 69.7 (174)/ 127.1 (251.7)/ 160.9 (164.6); t-PA (pg/ml): 960 (1023)/ 4066 (8545)/ 7304 (5737); PAI-1 (ng/ml): 2.21 (2.3)/ 2.08 (2.6)/ 2.16 (2.1); NGF (pg/ml): 4603 (2823)/ 4523 (4266)/ 3160 (2584).

Differences between the concentrations of the mediators in the tear film of patients among the patient groups were as follows: **PMD v. Control:** IL-8: p=0.031, MMP-9: p=0.004, MMP-13: p=0.004, t-PA: p=0.031; **KC v. Control:** IL-8: p=0.011, MMP-9: p=0.655, MMP-13: p=0.004, t-PA: p=0.094; **PMD v. KC:** IL-8: p=0.479, MMP-9: p=0.005, MMP-13: p=0.198, t-PA: p=0.226.

MMP-9 was the only mediator that significantly differed between the two ectatic patient groups (p=0.005).

Although we could not establish relevant differences because of the high standard deviations, the anti-inflammatory cytokine IL-10 was 14-times lower in PMD than in the controls, and seven-times lower than in KC. In addition, CCL5, which is chemotactic for T cells, eosinophils and basophils, and which plays an active role in recruiting leukocytes into inflammatory sites, was the highest in PMD compared to the two other groups. In line with this, TIMP-1, one of the most important MMP-inhibitors, was the lowest in PMD as compared to KC and the controls. The ratios of MMP-9 and TIMP-1 were 2.45, 0.40 and 0.23 in PMD, KC and the controls, respectively. In contrast with these findings, MMP-13, IL-8 and t-PA were the lowest in the PMD group.

4.2. Associations between the mediators in tear fluid in the whole spectrum of keratoconic eyes (suspect, subclinical, and manifest cases of KC) and normal eyes

A number of significant positive associations were observed between pairs of mediator concentrations in tear fluid collected from KC patients and subjects with normal eyes: IL-6 and IL-10, CXCL8, CCL5, MMP-13, TIMP-1, t-PA, PAI-1: p<0.0001, IL-6 and MMP-9: p=0.0203; IL-10 and CXCL8, CCL5, MMP-13, t-PA, PAI-1: p<0.0001; CXCL8 and CCL5, MMP-9, MMP-13, TIMP-1, t-PA, PAI-1: p<0.0001; CCL5 and MMP-13, t-PA, PAI-1: p<0.0001; MMP-9 and TIMP-1, t-PA: p<0.0001; MMP-13 and TIMP-1, t-PA, PAI-1: p≤0.0001; TIMP-1 and t-PA, PAI-1: p<0.0001; t-PA and PAI-1: p<0.0001.

4.3. Associations between mediators in the tear fluid and Scheimpflug parameters

Significant positive associations were found between BAD-D and CXCL8 (p=0.020), BAD-D and MMP-9 (p=0.005), and K2 and MMP-9 (p=0.031). Significant negative associations were found between Pachy Min and CXCL8 (p=0.027) and Pachy Min and t-PA (p=0.023).

4.4. Associations between pairs of mediators and the Standardized Pentacam Score (a composite parameter calculated from pentacam readings)

A significant association was found between the TIMP-1 concentration, the MMP-9 concentration, and the Standardized Pentacam Score: the combination of high TIMP-1 and low MMP-13 levels was characterized by a low score, while high levels of both mediators—as well as low TIMP-1 concentrations coupled with moderate MMP-9 levels—were associated with an elevated score. Significant associations were also found between the concentrations of a number

of other pairs of mediators and the Standardized Pentacam Score: between CXCL8 and IL-6: p=0.014, CXCL8 and CCL5: p=0.028, CXCL8 and t-PA: p=0.024; CCL5 and t-PA: p=0.026; MMP-9 and CCL5: p=0.04, MMP-9 and TIMP-1: p=0.001; MMP-13 and TIMP-1: p=0.043; t-PA and TIMP-1: p=0.014; PAI-1 and t-PA: p=0.02.

4.5. The Effect of Bronchial Asthma on Standardized Pentacam Score

There was a history of asthma in five patients (7.25 %) and one of contact lens usage in 15 patients (21.74 %). A strong, significant positive association between asthma and the Standardized Pentacam Score was found by linear regression adjusted for age and contact lens usage. Asthmatic patients' scores were, on average, an estimated 5.7 units (95% CI: 2.0 to 9.4, p=0.003)—or 1.32 standard deviations—higher than those of subjects without the condition.

5. DISCUSSION

5.1. To compare the concentrations of mediators in the tear of pellucid marginal degeneration patients and keratoconic patients who were similar to the pellucid marginal degeneration patients in severity, and in normal eyes

To the best of our knowledge, this is the first study that has aimed to reveal the biochemical differences between PMD and KC. Most of the ectatic corneal disorder cases are KC, and although PMD is far rarer it is no less important. PMD and KC differ relevantly in terms of prognosis and management, and distinguishing between these two conditions is of potential clinical importance. If biomarkers in the tear film can help in differentiating between the two entities, this non-invasive procedure could be advocated in the diagnostic tree. There are reports suggesting that PMD, KC and keratoglobus may belong on a spectrum of the same pathophysiology rather than separate disease processes, but based on our preliminary results it seems that PMD and KC are distinct diseases and might not be the phenotypic variations of the same disorder.

In this study we evaluated the concentration of different mediators in tear samples. MMPs participate in degrading and remodeling the extracellular matrix (ECM), and elevated levels of MMP-9 in the tear fluid of PMD and KC patients indicate a tissue-degenerative process

contributing to the thinning of the cornea. MMP-9 was the only mediator which significantly differed between PMD and KC ($p=0.005$). The more elevated MMP-9 in the tear fluid of PMD patients compared to KC patients might suggest a basic difference in the underlying pathological tissue-degradative processes.

Elevated levels of MMP-1, -3, -7, -9 and -13 have been found in the tears of patients with KC. The ocular surface inflammation is activated by proteases, including MMP-9, and based on our study it may help to differentiate between KC and PMD. MMPs and cytokines interact with each other by forming a complex network, including the stimulation of MMP-9 and MMP-13 by IL-6. Strongly expressed MMP-13 was reported in KC suggesting a role in intra- and extracellular pathological collagen destruction. In contrast with this finding, we found a significantly lower MMP-13 concentration ($p=0.004$) in the tears of patients with both disorders. Although the difference was not statistically significant, patients with PMD had the lowest MMP-13 concentration, which should be further investigated because of the complexity of the cascade of the degenerative processes.

The active form of t-PA converts plasminogen to plasmin, which can also degrade several components of the extracellular matrix and trigger the activation of the MMP pathway. MMPs and plasminogen activators (PAs) in turn are partially regulated by TIMPs and PAIs, inhibiting this cascade system and therefore influencing the progression of ectatic corneal diseases. In earlier studies, non-specific, slightly elevated t-PAs were detected in KC, but until now there have been no studies examining this protein in PMD. The PAI-1 gene can inhibit the activity of t-PA enzymes. Interestingly, in our study, the t-PA concentration was significantly lower in PMD compared to the controls while the PAI-1 concentrations were the same in the three groups, which suggests that other enzymes might play a more important role in the underlying molecular mechanism, and probably that the actual enzyme activities influence the final-effect impact on tissue degradation in ectatic corneal disorders. TIMPs are natural inhibitors of the different MMPs, and there have been various studies presenting conflicting reports on the expression of TIMP-1 in keratoconic corneas. In our study, TIMP-1 (one of the most important MMP inhibitors) was the lowest in PMD as compared to KC and the controls, and this reduced activity might have an impact on tissue degradation. Evaluating the balance between MMP-9 and TIMP-1, the ratios of MMP-9

and TIMP-1 were 2.45, 0.40 and 0.23 in PMD, KC and the control groups, respectively, indicating a more pronounced tissue degradation in PMD than in KC. Future studies may be needed to confirm the active interplay between MMPs and TIMPs as well as ILs, especially IL-10.

KC is defined as a non-inflammatory disease of the cornea, but an increasing number of studies have shown the role of the over-expression of several cytokines; therefore, classifying the disease as 'non-inflammatory' may now be inappropriate. In contrast with these previous findings based on younger keratoconic patients, in our study IL-6 in the tears of ectatic patients and in the controls did not differ significantly. Additionally, elevated levels were detected in inflammatory ocular surface diseases as well as in KC, and CXCL8, which is the predominant chemoattractant in the tear fluid, was significantly lower both in PMD and KC than in the controls in our study. We could detect that CCL5, which is chemotactic for T cells, eosinophils and basophils, and which plays an active role in recruiting leukocytes into inflammatory sites, was highest in PMD as compared to the two other groups (but this result was not significant). Our finding that in both corneal ectasias the concentrations of CCL5 are higher than normal are in contrast with Jun et al. Additional studies are needed to further validate the role of IL-6, CXCL8 and CCL5 in the pathomechanism of corneal ectasias and in tissue damage in PMD and KC. The anti-inflammatory cytokine, IL-10, was 14-times lower in PMD than in the controls and seven-times lower than in KC (but this result was not significant). The lower concentration of the anti-inflammatory cytokine IL-10 in the tear fluid of PMD patients compared with keratoconic patients and with the controls in the current study supports the assumption that cytokines and chemokines play an important role in the pathomechanism of KC. IP-10/CXCL10 is a fibrotic and angiostatic chemokine produced by macrophages, endothelial cells and fibroblasts, and it did not show significant differences between our patient groups. Our observation supports the fact that the cornea in KC is avascular and that scarrring occurs only in the severe stage, which was presented in our cohort in just 11%. It has been suggested that the impairment of corneal innervation has a role in the pathogenesis of KC, and a significant reduction in NGF expression has been detected in KC corneas as part of an imbalance in the NGF signaling pathway. Our results are in contrast with the previous findings because we could not establish differences in the concentration of NGF between the patient groups; nor were we able to find any correlation between the severity of KC and NGF.

The limitations of our study are the small number of patients with PMD and the fact that only older patients (mean ages over 44 years) were included. However, because of the rarity of PMD, the nine eyes with PMD involved in this study are a proportional representation of our around four hundred ectatic patient population. The cause of the older patient age is that the manifestations of PMD are evident at a later age, and we wanted to involve true PMD cases in this study. From this point of view, we could have compared PMD patients with KC cases of an older age, when KC is less progressive. It also would have been desirable to involve the early stages of PMD among younger people in the investigation, because of the possible progression of the disease which can be accompanied with alterations in the mediator levels. Furthermore, a limitation of this study is that it cannot exclude the possibility of other mediators being involved in the pathomechanism of PMD nor the identification of the source and activity of the mediators (including MMP-9) and the expression of the different receptors. Although it has been demonstrated that mediators reveal the biochemical differences between PMD and KC, it is unclear which proteins can be used to distinguish the non-progressive form of corneal ectasia from the progressive type (where early treatment would be very important).

Tear samples are an essential tool to understand the molecular mechanism behind corneal ectasias, and the multiplex platform is ideally suited for the detection of biomarkers from the small volumes of tear samples. Further studies with a larger sample might be required to validate our preliminary findings and to demonstrate the importance of MMP-9.

In summary, this study reveals several mediators being altered in the tears of PMD and KC patients, and these alterations may have an impact on the differences between these ectatic diseases. Additionally, research is required to further elucidate the differences and the importance of mediators in the pathomechanism of PMD and KC.

5.2. To determine associations between the mediators in tear fluid of keratoconic eyes and normal eyes, to explore associations between these mediators and the Scheimpflug parameters, to examine the relationship between the Scheimpflug imaging parameters and bronchial asthma

To the best of our knowledge, this is the first study that aimed to reveal associations between pairs of mediators and the severity of KC, evaluated using a Pentacam. Despite the

intensive clinical and biochemical investigations, the pathogenesis of KC is not yet known in detail. Classically, KC was considered a non-inflammatory disease; however, recently published articles have suggested that inflammation is involved in the pathogenesis of KC. Only few studies investigated the associations between a range of cytokines in the tear fluid and the severity of KC. The limitations of these reports are the small number of patients, or the few examined mediators, or the lack of subclinical cases.

In the current study, we have determined the associations between mediators in the tear fluid of keratoconic patients covering the whole spectrum of the disease (from subclinical to manifest KC). In accordance with earlier studies, we proved that different mediators—including cytokines, chemokines, enzymes, and inhibitors—in the tear fluid cooperate and take part in a complex immunological network. Significant associations were explored between the concentrations of different mediators and the Standardized Pentacam Score, which is a composite index statistically unifying all Pentacam parameters. As far as we know, there are no studies evaluating the association between different tear mediators and Pachy Min and also BAD-D, which was designed to present comprehensive data based on anterior and posterior corneal elevation and a pachymetric evaluation. Our outcomes support the linkage between the complex network of the various mediators and the comprehensive Pentacam indices. Based on our results, inflammation not only seems to be involved in the pathogenesis of KC, but also plays a crucial role in the pathological corneal processes from the initial stage to the final one. We have found only a few significant associations between the single mediators and Pentacam indices, highlighting the fact that mediators, including cytokines, take part in a complex cascade. The examined mediators overlap, neutralize and enhance the effects of one another. It is in line with evidence showing that various multitargeted mediators in the serum collaborate in different diseases, suggesting that examination of only one or a few mediators is not enough to explore complex immunopathological processes.

TIMP-1 is the inhibitor of MMPs, which prevents pro-MMP activation and, furthermore, presents antiapoptotic properties. MMP-13 was categorically reported in KC. Additionally, proMMP-13 activation is partially inhibited by TIMP-1.In line with these studies, we have found significant associations between TIMP-1 concentration, MMP-13 concentration, and the

Standardized Pentacam Score. The combination of high TIMP-1 and low MMP-13 levels was characterized by a low score, while high levels of both mediators—as well as low TIMP-1 concentrations coupled with moderate MMP-13 levels—were associated with elevated scores. Additionally, significant positive associations were found between MMP-9 and BAD-D, and also with K2.

Based on our study and others, the collagenolytic milieu of the human cornea seems to be more complex than expected. Further studies are required to understand the exact mechanisms of collagenases and inhibitors. Numerous interactions have been observed between the fibrinolytic and MMP systems taking part in proteolytic activation. PAs are partially regulated by PAIs, inhibiting this cascade system and, therefore, influencing KC progression. Different growth factors and cytokines induce the PAI-1 gene and inhibit the activity of the t-PA enzyme. In addition, PAs could affect the proteolytic inactivation of growth factors. Significant associations between the severity of keratoconus and pairs of t-PA/TIMP-1 and t-PA/PAI-1 were detected in our study. In addition to this observation, significant negative associations were found between the corneal thickness at the thinnest point of the cornea and t-PA. Apart from the elements of the proteolytic and fibrinolytic systems, we have examined different cytokines, such as the proinflammatory IL-6 and chemokine CXCL8/IL-8 and the anti-inflammatory IL-10; we found that all of these cytokines cooperate with each other and play a crucial role in the pathogenesis of KC. The significant positive association between BAD-D and CXCL8 and the significant negative correlation between Pachy Min and CXCL8 highlight the roles of the chemokines.

Bronchial asthma is a chronic airway inflammatory disease. The infiltration of eosinophils, mastocytes, and T lymphocytes and the release of several inflammatory mediators play an important role in asthma's pathogenesis. Different proteins are altered in the serum of these patients, such as IgE; IL-1, IL-4, IL-6, IL-8, and IL-13; CCL5; TNF-α; MMP-9; TIMP-1; t-PA; and serum Angiopoietin-1. Due to an increase in the number of known mediators, additional anti-inflammatory options are becoming available in the therapy of asthma. The connection between asthma and other atopic diseases with KC was first published almost 50 years ago and has been confirmed several times since then. We found a strong significant positive association between asthma and the severity of KC, meaning that asthmatic patients have 5.7 higher score than

nonasthmatic subjects. Based on our study, bronchial asthma has an impact on the severity of KC. This result albeit was a secondary outcome of our study, confirms the previous hypothesis related to the connection between KC and asthma. Further prospective studies are required to examine the effect of the systemic treatment of asthma on the pathomechanism of KC.

The strengths of our study are the large number of participants (88 subjects) and the consideration of a wide range of tear mediators that could be associated with Pentacam parameters. Our study has limitations, such as the lack of examination of enzyme activities, as well as the fact that we did not examine the progression of KC and the effect of asthma medication. In addition, this study does not exclude the role of other inflammatory molecules in the pathophysiology of KC. It would be interesting to measure more and different types of mediators, but this remains to be determined in subsequent studies. However, several new correlations can be revealed from our results that can be the basis for further study of this topic. As a next step, the precise role of these mediators needs to be defined, aswell as examination of the progression of KC and exploration of other mediators' functions. These studies might then serve as a platform for finding targets for local inhibition of pathological corneal thinning, or eventual treatment.

6. SUMMARY OF NEW RESULTS

I. Our study has aimed to reveal the biochemical differences between PMD and KC. Our results show significant differences in the levels of MMP-9 in tear samples between these two ectatic corneal disorders.

II. A number of significant positive associations were observed between pairs of mediator concentrations in tear fluid collected from KC patients and subjects with normal eyes.

III. We aimed to reveal associations between pairs of mediators and the severity of keratoconus, evaluated using a Pentacam. Significant positive associations were found between BAD-D and CXCL8, BAD-D and MMP-9, and K2 and MMP-9. Significant

negative associations were found between Pachy Min and CXCL8 and Pachy Min and t-PA.

IV. Significant associations were also found between the concentrations of a number of other pairs of mediators and the Standardized Pentacam Score.

V. A strong, significant positive association between asthma and the Standardized Pentacam Score was found by linear regression adjusted for age and contact lens usage. Our study confirms the effect of bronchial asthma on keratoconus.

7. SUMMARY

Corneal ectatic disorders have importance in public health. They are characterized by progressive deformation of the corneal architecture. Pellucid marginal degeneration (PMD) is a very rare peripheral thinning disorder of the inferior cornea, while keratoconus (KC) is the most common primary corneal ectatic disease that gives rise to a cone-shaped cornea. It is not known whether PMD and KC are distinct diseases or whether they represent different clinical presentations of the same underlying disease process. Corneal ectatic disorders are generally believed to be non-inflammatory diseases, however, recent studies have suggested that ectatic disorders are inflammatory conditions. Biomarkers in the tear film have been studied in patients with KC but no studies have been reported for PMD.

Our aim was to determine and to compare the concentrations of various mediators (IL-6, IL-10, CCL5/RANTES, CXCL8/IL-8, CXCL10/IP-10, MMP-9, MMP-13, TIMP-1, t-PA, PAI-1 and NGF) in the tear film of patients with PMD and KC in order to reveal any possible biochemical differences between these two entities. MMP-9 presented relevant variances between the two patient groups. The ratios of MMP-9 and TIMP-1 were 2.45, 0.40 and 0.23 in PMD, KC and the controls, respectively.

A few preliminary studies examining the association between biomarkers in the tear fluid and the severity of KC, but the limitations of these reports are the small number of patients, or the few examined mediators, or the lack of subclinical cases. We aimed to determine associations between biomarkers in tear fluid (IL-6, IL-10, CXCL8/IL-8, CCL5/RANTES, MMP-9, MMP-13, TIMP-

1, tPA, and PAI-1) in the whole spectrum of keratoconic eyes and normal eyes. An additional goal was to explore associations between these mediators and the Scheimpflug parameters which characterize the severity of KC. Our aim was also to examine the relationship between the Scheimpflug imaging parameters and bronchial asthma in KC.

This study reveals the cooperation of the different mediators in tears all taking part in the complex pathomechanism of KC. Our study reveals associations between tear biomarkers and Scheimpflug parameters, confirms that inflammation is involved in the pathogenesis of KC. Determination of the precise role of these mediators, as well as examination of the progression of KC serve then as a platform for local inhibition of pathological corneal thinning, or eventual treatment.

8. APPENDIX

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Subject: PhD Publikációs Lista

Candidate: Dorottya Pásztor
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Doctoral School: Doctoral School of Clinical Medicine

List of publications related to the dissertation

1. **Pásztor, D.**, Kolozsvári, B. L., Csutak, A., Berta, A., Hassan, Z., Kettesy, B., Gogolák, P., Fodor, M.: Scheimpflug Imaging Parameters Associated with Tear Mediators and Bronchial Asthma in Keratoconus.
   J. Ophthalmol. 2016 (9392640), 1-7, 2016.
   IF: 1.463 (2015)
2. **Pásztor, D.**, Kolozsvári, B. L., Csutak, A., Berta, A., Hassan, Z., Ujhelyi, B., Gogolák, P., Fodor, M.: Tear Mediators in Corneal Ectatic Disorders.
   PLoS One. 11 (4), e0153186, 2016.
   DOI: http://dx.doi.org/10.1371/journal.pone.0153186
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List of other publications

3. Kolozsvári, B. L., Losonczy, G., **Pásztor, D.**, Fodor, M.: Correction of irregular and induced regular corneal astigmatism with toric IOL after posterior segment surgery: a case series.
   BMC Ophthalmol. 17 (3), 1-6, 2017.
   DOI: http://dx.doi.org/10.1186/s12886-016-0397-8
   IF: 1.238 (2015)

4. **Pásztor, D.**, Kolozsvári, B. L., Losonczy, G., Fodor, M.: Femtosecond laser assisted keratoplasty combined with cataract extraction in a patient with keratoconus and oculocutaneous albinism.
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   J. Immunol. Res. 2014, 1-8, 2014.
   DOI: http://dx.doi.org/10.1155/2014/570685
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9. KEY WORDS

keratoconus

pellucid marginal degeneration

tears

mediators

Scheimpflug parameters

Pentacam

topographic and tomographic indices

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