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

KERATOCONUS: ROLE OF HEREDITY IN THE PATHOGENESIS AND DIAGNOSIS OF SUBCLINICAL CASES

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The Examination takes place at the Department of Ophthalmology, Faculty of Medicine, University of Debrecen; 11:30 a.m. June 24, 2019

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen;
at 13:00 p.m., June 24, 2019.

INTRODUCTION

On the front surface of the eye there is the smooth and transparent cornea which has a watch-glass shape. Keratoconus is the most common, primary and progressive, bilateral ectatic disorder of the cornea, in which a circumscribed lower or middle part assumes a conical shape due to stromal thinning and protrusion. The refractive power of the cornea progressively increases in the center. Myopia, myopic astigmatism and irregular astigmatism become more pronounced, and higher order optical abberrations appear. The more advanced the disease process is, the more frequent the typical clinical signs are which can be observed by slit-lamp examination. Regarding our research, the most important signs are: central stromal thinning, Fleischer ring (a subepithelial iron deposition which partially or completely surrounds the cone) and prominent corneal nerves. Keratoconus (KC) occurs in all races, and in all parts of the world. The estimated prevalence of the disease lies between 50 and 230 per 100 000 in the general population, and is still considered to be one of the major causes for corneal transplantation in developed countries. The disease process often starts at the end of the teenage years or at the beginning of the twenties on one of the corneas, but ultimately both eyes become affected, usually in different degrees. The eye in which the typical clinical signs have not yet appeared, called subclinical keratoconus in the literature. Previously the term keratoconus suspect was rather used. Concerning the etiology, the disease is assuredly complex, multifactorial; in which genetic and environmental factors, their complicated interactions, and epigenetic mechanisms might play a role. Several different pathologic processes presumably lead to the development of keratoconus, which is considered as a final common pathway. In subjects having genetic predisposing factors, the appropriate environmental effect may trigger the development of the disease.

Genetic influence

There is important evidence of genetic influence in KC: though it is considered to be an isolated condition, many associated genetic disorders (Down syndrome, osteogenesis imperfecta, Ehlers-Danlos syndrome, mitral valve prolapse) as well as ocular diseases (Leber`s congenital amaurosis, diffuse tapetoretinal degeneration), and several corneal dystrophies (Avellino, Fuch`s endothelial, granular, posterior polimorf corneal dystrophy) have been reported. Most of these inherited systemic diseases are related to

abnormalities of the connective tissues, moreover, isolated keratoconus can presumably be caused by these, as well. Observations in twin and pedigree studies support the role of genetic factors, too. Concordance rate is higher in monozygotic than in dizygotic twins, similarly to consanguineous offspring. Familial occurrence of myopia, of different forms of astigmatism and of keratoconus has been demonstrated in several studies, where the frequency of familial KC cases was estimated to be 6–23%. A recent study, which confirmed familial aggregation, examined a large cohort of sporadic keratoconus families, where carrier status could be identified based on videokeratographic indices. These quantitative topographical indices served as subclinical phenotypes, and based on these, complex segregation analysis was performed.

In relatives of KC patients, Marc Amsler demonstrated similar but less pronounced corneal topographical changes by Placido disc, and called this quite frequent phenomenon forme fruste KC. He proved that classic and forme fruste KC constituted the same entity and that must be considered in genetic studies. Though the nomenclature is not unified, nowadays the term subclinical KC is more frequently used even instead of forme fruste. Topographic examination of relatives of KC patients revealed aggregation of asymmetric corneal patterns and increased videokeratoscopy indices. Relatives of patients with KC have a high prevalence of undiagnosed KC and KC traits, therefore, keratorefractive surgery should be considered cautiously in these individuals. Topographic features similar to subclinical KC were observed among some refractive surgery candidates, especially among the ones suffering from progressive keratectasia after the intervention. Thus, identifying subclinical KC is necessary for screening potential high risk refractive surgery candidates, too. However, diagnosing subclinical KC is still challenging nowadays. Many studies examine KC family members to define subtle characteristics associated with subclinical KC.

Fleischer ring

One of the most important pathognomic signs is the Fleischer ring, which can frequently be observed by slit-lamp examination, and is caused by iron deposition in the cytoplasm of basal epithelial cells and in intercellular spaces. Though its origin is controversial, many studies imply an association between locations of iron deposition and changes in the corneal surface epithelium. Changes in the iron metabolism in corneal epithelium possibly result in iron

deposition, which may be a part of a cascade of events, ultimately leading to keratoconus. Moreover, it can play a role in post-LASIK keratectasia, as well.

Prominent corneal nerves

Prominent corneal nerves are not only signs of KC, but they are part of the pathophysiological processes in several ways; moreover, they play a role in the progression of the disease, too.

Significant microstructural changes have been observed in all layers of the keratoconic cornea. In addition, up- and down-regulation of several proteins, enzymes, cytokines and altered levels of important extracellular matrix proteins and proteoglycans involved in cell proliferation and migration, have been reported. All these factors imply that keratoconus is a complex, multifactorial disease.

AIMS

Our first aim was to study whether genetic factors can play a role in the development of keratoconus. For this purpose, we carried out a segregation analysis of topography parameters from keratoconus probands and family members. Keratoconus patients treated and cared for in our department were called together with their family members for clinical and corneal topographic examination. Age and gender matched controls were assigned to them, and pachymetric central corneal thickness examination was also performed on a subpopulation. We wanted to know the extent of familial aggregation in our population, and the most probable mode of inheritance based on videokeratography parameters. Complex segregation analysis, a powerful statistical approach was used to determine the mode of inheritance of particular topography indices from family data, i.e. to evaluate the transmission of a trait within pedigrees. In the complex segregation analysis of our own population, we tested not only mendelian but non-mendelian inheritance models, too. We analysed videokeratography indices as continuous traits; whereas clinical associations of KC such as corneal Fleischer ring and gender were included as covariates in the analysis.

As a second aim, we examined characteristic clinical signs of KC - the occurrence of Fleischer ring, prominent nerves and corneal thinning - in patients, in their relatives and in healthy controls. We investigated the correlation of corneal Fleischer ring and prominent nerves with videokeratographic signs of KC, both among unaffected family members and healthy control individuals to find out if these signs can be used to better identify subclinical KC.

PATIENTS AND METHODS

The design of our study followed the tenets of the Helsinki Declaration, informed consent of patients and controls was obtained before examination. After detailed information, all subjects voluntarily contributed to their involvement. Families of keratoconus patients treated and cared for in our department were examined. These pedigrees were considered sporadic, and none of the family members had any of the known genetic associations of KC. Healthy controls were recruited from students, personnel and patients of the department with healthy corneas. Normal individuals were defined as having refractive errors less than ± 6.0 D spherical or ± 3.0 D cylindrical, without any former corneal disease or eye operation and without history of keratoconus, corneal disease or transplantation in the family. All of the enrolled subjects were Hungarian patients with Caucasian ancestry. Data of eyes that had previous corneal injury or surgery, including perforating keratoplasty, were not used in the analyses.

Examined subjects: keratoconus pedigrees and controls

Genetic examination, complex segregation analysis

For complex segregation analysis, sixty unrelated, sporadic keratoconus families ascertained by probands, were involved. Out of all living family members (274), 212 subjects could participate in our examinations, and age and gender matched controls were assigned to each of them. Since the occurrence of KC is very rare in the first decade of life and the age of onset of our youngest proband was 9 years, children under that age were excluded from the study group.

Clinical correlation analysis

For clinical correlation analysis, 47 pedigrees were involved, and 142 controls having healthy corneas were assigned to them. Among family members of probands, we found two KC patients with manifest disease, healthy individuals and some subclinical KC cases. We separated the non-affected KC family members from KC patients. The group of non-affected family members comprised relatives who had ≤ 3 D astigmatism or ≤ 6 D spherical refractive error, and whose *KISA* or *KSI* indices, which are widespread in clinical practice, did not imply the presence of manifest disease (*KISA*<100%, *KSI*<30%).

Examination methods

For each KC proband, diagnosis was based on clinical examination, including measurement of visual acuity, slit-lamp biomicroscopy, direct ophthalmoscopy, retinoscopy and videokeratography. All KC family members and control individuals consented to slit-lamp clinical examination and corneal topography. For clinical correlation analysis, central corneal pachymetry examination was also performed on a subpopulation. 15 KC patients, 45 family members and 26 controls consented to ultrasonic pachymetry (27 eyes, 89 eyes and 52 eyes, respectively). The reason for the missing data in this group is unilateral keratoplasty of 3 probands, and previous corneal injury of a family member. By slit-lamp biomicroscopy, the presence of Fleischer ring in the epithelium, and prominent corneal nerves were thoroughly searched for. Fleischer ring was considered as present when at least 2 mm curved paracentral subepithelial iron deposit was detected.

Corneal topographic (videokeratographic) examination

The corneal topograph is an instrument which is used to examine the anterior corneal surface, curvature and refractive power. Its principle is similar to the earlier used hand-held keratoscopes: from a distance of a few centimeters, it projects 25 mire rings onto the cornea from apex to limbus, which is reflected from its surface. The instrument detects this image on 256 points per each ring, then with the aid of the controlling computer software, based on the degree of distortion of the mire rings, it generates a color-coded corneal surface map. Topographic examination was performed on both eyes of each study participant, using a TMS-4 videokeratoscope (Tomey Corporation, Nagoya, Japan). At least four images per eye were taken. Data obtained from the best image of each eye, selected upon the quality of the keratoscopy mires by visual inspection, were used.

Topographic (videokeratographic) indices

Videokeratographic indices given by the TMS-4 software and those calculated from them were selected for further analysis.

Indices used in complex segregation analysis

1.) *KISA*: K*eratoconus Percentage Index*, describes the typical topographic features of KC with the combination of 4 different indices.

$$\mathrm{KISA} = \frac{\mathrm{K} \cdot (\mathrm{I} - \mathrm{S}) \cdot \mathrm{AST} \cdot \mathrm{SRAX}}{300} \times 100$$

In the formula, *K*: central corneal power, *I-S*: Inferior-Superior Dioptric Asymmetry Index, *AST*: an index, which shows the degree of regular astigmatism, *SRAX*: skewed radial axis, characterizing irregular astigmatism. We calculated *KISA* index based on this formula. For familial aggregation statistics, values were used as was defined in the original study: (*KISA*: <60% healthy eyes, 60-100% subclinical KC cases, >100% KC). The natural logarithm transformation of the *KISA* index was used to obtain normal distribution for the statistical analysis (*ln(KISA)*). In complex segregation analysis, the further linear transform, *ln(KISA)+3* was used to provide input for analyzing *KISA* as a continuous trait.

2.) *KSI*: *Keratoconus Severity Index*. *KSI* uses 10 different videokeratographic indices as input parameters for a classification neural network, which was developed for KC screening. *KSI* expresses the severity of the disease in percentage, i.e.: >30% implies manifest KC, while >15% is characteristic of subclinical KC eyes. The software calculated the values of *KSI*.

3.) *6 mm Fourier Asymmety Index*: Fourier series harmonic analysis is a mathematical method by which the raw data of corneal refractive power from topographic images can be deconstructed into a series of trigonometric functions. This analysis decomposes the matrix of corneal refractive powers into 4 indices, which are calculated within the area of mire rings 3 mm and 6 mm from the center by the software of the topograph. In case of KC, the most relevant is the Asymmetry Index, thus we used that for our analyses. *6 mm Fourier Asymmetry Index* is the decentration (first harmonic) component in the Fourier series, on mire rings 1–20 which approximately represent the central 6 mm of the cornea. The x10 multiple of the *6 mm Fourier Asymmetry Index* was used as the input for analyzing it as a continuous trait. Out of the pairs of indices relating to the right and left eyes, the one with a higher value was assigned to each person. Topographic indices of operated eyes were ignored.

Indices used in clinical correlation analysis

1.) *KISA*: *Keratoconus Percentage Index*

2.) *KSI*: *Keratoconus Severity Index*

3.) *3 mm and 6 mm Fourier Asymmety Indices:* decentration (first harmonic) component in the Fourier series, on mire rings 1-9, and rings 1-20 which approximately represent the central 3 or 6 mm of the cornea.
4.) *I-S: Inferior-Superior Dioptric Asymmetry Index*. Its value can be positive or negative, depending on which corneal hemisphere is steeper or has higher refractive power, thus we used the absolute value of the *I-S* for each eye.

Statistical analysis of the data

Complex segregation analysis

The program SIGMASTAT (version 3.5) was used to verify the age and gender matching of probands, family members and controls at α=0.05 level of significance. The ability of *KSI, KISA* and *6 mm Fourier Asymmetry* to distinguish KC and non-KC family members, and healthy eyes was tested by ANOVA. Analysis of the 60 keratoconus pedigrees was performed using the program package S.A.G.E. 6.1.0 (2010): Statistical Analysis for Genetic Epidemiology, http://darwin.cwru.edu/sage/. For general analysis of the families, including descriptive statistics on pedigree structure, the program PEDINFO was used. Complex segregation analysis is the main statistical methodology used to analyse the mode of inheritance of a pathological or non-pathological trait within pedigrees. Complex segregation analysis of the 60 keratoconus families was performed using the program SEGREG as follows: videokeratographic indices *ln(KISA)+3*, *KSI* and *6 mm Fourier Asymmetry Index x10* were used as input parameters for analyzing them as continuous traits.
For analysis of quantitative traits, Bonney's class *A* regressive model was used, which assumes that an individual`s phenotype depends on the phenotypes of earlier relatives only through his own parents` phenotypes and genotypes. Siblings are dependent only because of common parentage. The segregation of a possible major locus is allowed for by letting one or more parameters depend on an unobserved latent qualitative factor *u = AA, AB or BB*, which is called an individual's type. If segregation is mendelian, type represents the putative genotype that underlies the observed phenotype, and segregation is realized through a single autosomal locus with two alleles, *A* and *B*. *A* is the hypothesized disease allele. The frequencies of allele *A* and *B* are denoted *qA* and (*1- qA*), respectively. The distribution of types in the

population is assumed to be in Hardy-Weinberg equilibrium, therefore the type frequencies in the population can be defined by *qA*. Individuals of types *AA, AB* and *BB* are assumed to transmit component *A* (allele *A*, if the type is a genotype) to their offspring with transmission probabilities τAA, τAB, and τBB, respectively. Regarding this, type is best defined in terms of the expected distribution of an individual's offspring, so we use the type as a general term to allow for several kinds of discrete transmission, whether mendelian or not. The means associated with each type regarding the analyzed trait were μAA, μAB, μBB, respectively, and one common variance was estimated. To allow for a multifactorial component, residual familial correlations were defined between pairs of relatives as follows: ρFM denotes familial correlations between spouses (father and mother), ρPO for parent-offspring, (ρFO for father-offspring, ρMO for mother-offspring), and ρSS for sib-sib pairs, respectively.

Eight different models were applied regarding topographic indices to explore the source of variation among family members.

1.) The analysis was started with an unrestricted, *general* model, where all parameters were allowed to be estimated by the program for the best fit of the data. It is a full or multifactorial model, which comprises effects of a major gene, of further genes (polygenic inheritance) and of the environment. This general model which fit our data best was compared with the other 7 models, in which various restrictions about the mode of transmission were incorporated; thus, appropriate parameters were fixed to specific values in these models. The *general* model was considered the alternative hypothesis to be compared to each of the 7 restricted models as null hypotheses. By these comparisons we tried to explore whether any mode of inheritance or regularity could be applied to our data, with which they could be explained. The specific models were the following:

2.) *sporadic* model,

3.) *environmental* model,

4.) m*endelian major gene* model,

5.) *polygenic* model,

6.) *non-mendelian major gene* (MG) model,

7.) *mixed* model, a *non-mendelian major gene with a polygenic* factor model,

8.) *non-mendelian major gene with an environmental* factor model (MG+environmental).

In covariate analysis, presence of Fleischer ring (1: present, 0: not present)

and gender (1: male, 0: female) was included as a binary covariate. Polygenic variance was assessed with the FPMM program, which is part of S.A.G.E. 6.1.0 (2010) program package.
Parameters were estimated by the maximum likelihood method.

Two criteria were used for comparison of the models:
1.) Hypothesis tests were based on the likelihood ratio criterion, comparing each restricted model to the *general*, unrestricted model. The *likelihood ratio test* (*LRT*) was calculated as twice the difference in the natural logarithm of the likelihoods of the restricted (null hypothesis) and the unrestricted (*general*) models

$LRT = -2\Delta ln(L) = -2ln(L)_{\text{restricted model}} - [-2ln(L)_{\text{unrestricted model}}]$,

where *L* is the maximum likelihood of the individual models. Its distribution is asymptotically equivalent to the χ^2 (chi-square) statistic, its number of degrees of freedom is equal to the difference in the number of independent parameters estimated in the compared models. Thus, we calculated the values of our likelihood variables by subtracting the *–2ln(L)* values of the *general* model from each *–2ln(L)* value of the specific models, then we read the associated *p* values in the appropriate χ^2 statistical table. Significance level here was considered α=0.05, too. We wanted to know whether there is one or more specific, restricted model out of the seven which also fits our data, according to the *general*, unrestriced, all-including, complete model fitting our data best. Therefore, in the case of $p>0.05$, meaning it is "not significantly different" from the *general* model, could we declare that we also accept that certain, specific model, because it also fits our data well. $p>0.1$ implied the best fit. If we got $p<0.05$ for a model based on the *likelihood ratio test*, then we did not accept that model because this *p* value means that this certain, specific model is significantly different from the *general* model best fitting our data. Thus, this model is not susceptible to provide an explanation of the mode of inheritance or regularity which is probable, based on our data.
2.) In addition, the *Akaike's Information Criterion, AIC* was used to select the most parsimonious model among equally likely models for the data ($p>0.05$). Since $AIC = -2ln(L)+2n$, where *n* is the number of estimated parameters, the model with the lowest *AIC* value was considered to be the most parsimonious. The *–2ln(L)* and *AIC* values were given by the SEGREG program with the results of the analysis. Since families were recruited through the probands,

single ascertainment was performed, that is, the likelihood of each pedigree was conditioned on the affection status of the proband only.

Clinical correlation analysis

For calculating statistics, data of both the right and left eyes of each individual were included, except for those of operated or previously injured eyes. In KC family members, 3 series of correlation statistics were tested: the presence of Fleischer ring, central corneal pachymetry data and the presence of prominent corneal nerves were correlated with one another and with topographic (videokeratographic) indices. Correlation statistics were also performed on the data of control individuals; however, these results were not reliable because of the low number of cases with Fleischer ring or prominent corneal nerves. The presence of Fleischer ring and prominent nerves in the cornea were tested as binary variables. Central corneal pachymetry data and all topographic indices were continuous variables in the analysis. Correlation between variables was calculated using Pearson correlation statistic, *p* value and the correlation coefficient (*r*) were determined. Parameter values between groups were compared with *t*-test or *Mann-Whitney rank sum* test. The program Sigma Stat 3.5 version was used for calculating statistics and α=0.05 was the level of significance.

RESULTS

Genetic examination, complex segregation analysis

Descriptive statistical analysis of pedigrees

Sixty sporadic, i.e. non-familial keratoconus pedigrees were enrolled in the study, who were not related. Mean size of families and its standard deviation was 4.57 (±1.55) members per pedigree. The range of family size and sibship size was 3-11, and 1-4, respectively. The majority of the pedigrees, 44 (73.33%) were nuclear families, including parents and their offspring, while 16 of them (26.67%) were three generational. Out of all living family members (274), 212 relatives could participate in the detailed clinical and corneal topographic examination, 100 males (47.17%) and 112 females (52.83%). Based on the *KISA* index, previously undiagnosed KC were found in 11 first degree relatives (seven parents and four sibs). In our population, the estimated prevalence of manifest keratoconus was 7.6% (11/145, 95% confidence interval, CI: 3.3–11.9), 33–152 times higher compared to the prevalence of keratoconus in the general population (0.05–0.23%). These data show familial aggregation of KC, and support the existence of genetic factors in its development. Fleischer ring also showed accumulation in KC families: it was observed in 65.9% of KC patients and in 25.3% of their family members. The proportion concerning non-KC relatives is remarkable, for this reason we considered its more profound, further examination important.

Distribution of KC indices, and their ability to separate KC and healthy eyes

All indices were significantly ($p<0.001$) different across the KC, non-KC family member, and healthy control groups when analysed with *ANOVA*. *KISA* and *6 mm Fourier Asymmetry* differentiated all groups from each other ($p<0.05$), while *KSI* only differentiated KC from healthy controls and from non-KC family members, but not the non-KC family members from healthy controls. The distribution of selected videokeratographic indices in the study population was as follows:

1.) Distribution of *ln(KISA)+3* is apparently bimodal. Cutoff values of *KISA* suggested for subclinical and manifest KC are: 60 and 100, while the same values for the transformed *ln(KISA)+3* are: 7.1 and 7.6, respectively. These are very near owed to the logarithmic transformation, and both appear to separate normal from diseased. The normal population is log-normally

distributed, as evidenced by the quasi Gaussian pattern after log transformation.
2.) A similar bimodality is visible in the case of *6 mm Fourier Asymmetry*. The cutoff value presumed from our data separates KC patients and non-KC relatives. The first minimum of the distribution histogram is here at this value, then the relative frequency again increases. This first part of the distribution histogram before the possible cutoff represents the healthy population. Its mean value estimated from the histogram well correlates with values fitted for the healthy population in segregation analysis, and they are in accordance with the values published in the original study. The distribution of both *KISA* and *6 mm Fourier Asymmetry* shows a continuum between low and high diseased values.
3.) Conversely, *KSI* separates three groups, normal, subclinical KC and KC, as defined in the original paper of Smolek and Klyce. We indicated the suggested cutoff values (15 and 30) based on that.

Complex segregation analysis
Since all three parameters (*KSI, KISA* and *6 mm Fourier Asymmetry Index*) present a continuum from healthy through subclinical KC to diseased, we have analysed the indices as continuous traits. In complex segregation analysis, we allowed the program to estimate the mean for diseased and non-diseased corneas in order to reach the best fit in each case, regardless of the previously reported cut-off levels.
In the case of *KISA*, the most parsimonious model was the *non-mendelian major gene (MG)+environmental* effect, but this model did not differ significantly from other accepted models involving a MG (*MG only* or *MG with polygenic component; p*>0.1). Analysis of *KSI* and *6 mm Fourier Asymmetry* resulted in the acceptance of a *non-mendelian major gene effect (MG)* as the most parsimonious model. At the same time, *sporadic*, *polygenic* and *mendelian major gene* models were consistently rejected for all indices ($p<0.001$), whereas in the case of *KISA* and *6 mm Fourier Asymmetry*, the *environmental* model was also accepted. In spite of recruiting families with no known relatives having KC, we identified 4 two-generational families where affected individuals were found in every generation and mendelian dominant mode of inheritance could be suspected. To exclude the confounding effect of these pedigrees, segregation analysis was repeated by exclusion of the above 4 families. In the remaining 56 families, the *non-mendelian major gene* model

remained the most parsimonious model for all indices unitedly, although with lower *AIC* levels, indicating decreased heterogeneity among the families. The above mentioned 4 pedigrees were separately examined, and running of the complex segregation analysis was attempted for their 16 members in all. Interpretable results could not be gained because of the limited data, therefore the possibility of truly dominant inheritance in these families could not be confirmed. When only family members above the age of 20 years were included in the analysis – assuming that the disease becomes fully manifest by that age – the accepted model remained the *non-mendelian major gene* effect. For *KSI* and *KISA*, *MG alone* was the best model, and its likelihood increased in comparison to the fit where younger family members were included. It was coherent with the expected better manifestation of symptoms at an older age. For *6 mm Fourier Asymmetry*, *MG+polygenes* remained the best fitting restricted model, however, the identity of the likelihood of this model to that of the general model became less probable ($p<0.05$). The presence of Fleischer ring as covariate greatly improved the fit of the most parsimonious *non-mendelian major gene* model for all indices, in complex segregation analysis. Gender as covariate with *KSI* minimally decreased the fit of the *MG model*. Conversely, a slightly improved fit for *KISA* and *6 mm Fourier Asymmetry* was found with gender as covariate. The presence of Fleischer ring had a powerful effect in these analyses also, which confirmed our assumption that its possible importance would warrant a more profound examination.

For the most parsimonious *non-mendelian major gene* models, the estimated allele frequency was relatively high and penetrance was low. On the other hand, phenotypes apparently not carrying the disease allele can also have the probability of passing on the diseased phenotype. A major gene effect with polygenic influence (*mixed* model) was also accepted for all indices, although the estimated heritability due to polygenes was low. In accordance with this, variance due to polygenes was estimated as negligible. Concerning the *MG models*, restricting parameters to strictly dominant or recessive models did not yield acceptable means, thus, information about the dominant or recessive nature of the underlying major gene effect could not be gained.

Clinical correlation analysis

Characteristics of the examined population

Controls were not significantly different from the members of KC families, concerning age (p=0.11) and gender distribution (p=0.153). In the cohort used for clinical correlation analysis, in KC patients, Fleischer ring was detected in 71.7%, and prominent nerves of the cornea in 15.2%. In KC family members, corneal Fleischer ring was observed in 30.8% and prominent nerves were detected in 14.5%. Among control individuals, interestingly, these clinical signs were present to a small extent: Fleischer ring and prominent nerves were observed in 2.1% and 2.8%, respectively. Corneal shapes suspectable to KC were observed in some KC family members, which were associated with the presence of Fleischer ring; however, in these cases the *KSI* index did not reach its cutoff value concerning subclinical KC. Certain control individuals had Fleischer ring in their corneas with prominent nerves or else, iron deposition not reaching the criteria of Fleischer ring with prominent corneal nerves. The corneal shapes of these individuals were asymmetric and, though to a small extent, resembled the patterns specific for keratoconus.

Correlation statistics in KC relatives

Among KC family members, there was a significant negative correlation between central corneal pachymetry data and the presence of Fleischer ring (r = -0.234). Each topographic index showed a significant positive correlation with Fleischer ring. These correlation results imply that Fleischer ring occurred in thinner and more asymmetric corneas of KC relatives. There was a significant positive correlation with prominent corneal nerves for all topographic indices, except for *I-S*. Significant negative correlation between prominent nerves and central corneal pachymetry data indicated that prominent nerves were more likely to appear in thinner corneas (r = -0.235). Central corneal pachymetry values negatively correlated with all examined topographic indices (r < -0.3 for all indices, correlation was the highest for *Fourier Asymmetry Indices 3* and *6 mm:* r = -0.425, r = -0.427, respectively). There was no significant correlation between Fleischer ring and prominent nerves in these corneas, indicating that prominent nerves and Fleischer ring did not frequently occur in the same individuals.

Comparison of family member groups with t-test

In accordance with correlation statistics, siginificant differences were found between relatives having or not having clinical signs of KC, by *t*-test. Relatives with Fleischer ring had significantly thinner corneas and higher values of each videokeratographic index compared to those without Fleischer ring. Relatives with prominent corneal nerves displayed higher *KSI*, *3* and *6 mm Fourier Asymmetry Indices* than those having no prominent nerves. Since Fleischer ring and prominent corneal nerves were not frequently concomitant, *t*-test comparison of corneas with prominent nerves to all other family member corneas including corneas with Fleischer ring – which were thinner and more asymmetric – yielded significant differences only for *KSI* and *Fourier Asymmetry Indices*. Accordingly, when relatives with prominent nerves were compared to those who had neither prominent corneal nerves nor Fleischer ring in their corneas, significant differences were found for all parameters except for *KISA*. Out of 142 control individuals, 1 had only Fleischer ring, 2 had both Fleischer ring and prominent nerves, 2 had only prominent nerves. Correation statistics in this dataset was not reliable because of low number of cases. These 5 control individuals with Fleischer ring and prominent corneal nerves were separated from the other 137 controls, and *t*-test (*Mann-Whitney Rank Sum test*) was performed for the two groups. This showed that controls with Fleischer ring and prominent nerves had significantly higher values of *KSI* (p=0.048) and *KISA* (p=0.012) indices, but were not significanly thinner than controls without these signs in their corneas (p>0.1).

DISCUSSION

In genetic, complex segregation analysis we showed that pathologically high *KISA* values are aggregated in keratoconus families. The complex segregation analysis demonstrated that the inheritance of *KISA, KSI* and *6 mm Fourier Asymmetry* indices are mainly influenced by a non-mendelian major gene (MG) effect. Our results clearly underline the genetic background of keratoconus, and suggest the role of a major gene effect. Although the non-mendelian MG model was the most parsimonious in our analysis, results slightly varied depending on the examined indices. Distribution histograms in our analysis showed that the more complex an index is, the more effectively it separates KC and non-KC groups. *KSI* also discriminates an intermediate peak, likely corresponding to subclinical KC cases. In line with this, *KSI* yielded the most consistent segregation results, indicating the presence of a non-mendelian major gene effect in all analyses. In the case of *KISA* and *6 mm Fourier Asymmetry*, the environmental model was also accepted besides the MG models, but exhibited less parsimony. However, sporadic, polygenic and mendelian major gene models were consistently rejected for all indices. Our results also emphasize the importance of the examination method used in the diagnosis of KC when genetic studies are planned. Modern corneal tomographic methods, which allow for measurement of corneal thickness and anterior-posterior elevation of the cornea, can also help determine the inheritance of KC. Covariate analysis with Fleischer ring and gender indicate in our view that factors influencing topographic features of KC and the development of Fleischer ring are driven by the same genetic determinants. On the other hand, gender may influence corneal asymmetry, as it improved the fit for the indices (*6 mm Fourier Asymmetry* and *KISA*) that put emphasis on corneal asymmetry. Nevertheless, in KC development, significant population based differences may exist in gender influence as well, since some studies clearly describe male or female preponderance in KC. The low polygenic variance estimated in FPMM analysis indicates that variability in KC penetrance is rather due to environmental than polygenic effects. In our analysis, dominant or recessive models could not be fitted to the MG effect, indicating also the importance of environmental or epigenetic factors rather than genetic interactions in disease development. Altogether, our data indicate that the complex non-mendelian major gene effect underlying KC would be transmitted by a relatively frequent allelic variant, whose penetrance is low and is highly influenced by environmental factors and only weakly by the effect

of other genes. At the same time, our results do not exclude genetic heterogeneity. Similarly to many complex traits, it can be assumed that damage to different genes may ultimately evolve a similar disease phenotype. Our data indicating that KC is a complex, non-mendelian disease are in accordance with the widely accepted view of many researchers and clinicians. However, our study is the first to provide objective evidence of the complex, non-mendelian inheritance of KC. Though, it has to be emphasized, that this study is based on the analysis of multiple sporadic families and therefore is different from analyzing large multigenerational families, which is an established practice of inherited, familial KC. Familial KC may have another genetic origin and its suggested mode of inheritance is mostly autosomal dominant. A number of genetic mechanisms were reported in the background of complex, non-mendelian inheritance. One of them is variable expressivity, namely, the phenomenon that KC gene effect may cause other curvature or refractive changes of the cornea (i.e. astigmatism, myopia). Digenic inheritance has also been suggested in KC families earlier, namely, that the damage of two different genes are simultaneously required for the disease development. In addition, complexity may arise from epigenetic influences besides genetic mechanisms, such as effects of regulatory RNAs and DNA methylation status. Cross-talk between proteins along the same metabolic pathway may be one of those protein interactions that affect penetrance. Complex inheritance results in a relatively low genotype-phenotype correlation, making evaluation of linkage studies in sporadic KC families difficult; therefore, other approaches may be more straightforward for studying the background of KC in sporadic families. Notwithstanding, in rare mendelian pedigrees, linkage analysis could result in successful identification of genes involved in KC development, although the low genotype-phenotype correlation could make analysis difficult even in such cases. Because of the complexity of the disease, whole genome sequencing, carefully planned genom-wide association studies, new generational sequencing techniques – such as WES: whole exome seqencing, WGS: whole genome sequencing, targeted sequencing –, epigenetic studies and transcriptome, proteome or pathway analyses may be more adequate to reveal common pathways leading to KC development.

By the examination of clinical signs characteristic of keratoconus, occurrence of Fleischer ring and prominent corneal nerves was observed even in corneas of unaffected relatives. The most remarkable one was the corneal Fleischer

ring which was present in almost every third family member, similarly to its occurrence detected in mild KC. In manifest KC, we found Fleischer ring in 71.7 % of the patients, which is consistent with the results of previous studies (57%-87%). Even in the healthy control population, prominent nerves (2.8%), Fleischer ring (2.1%) and corneal asymmetry still occurred rarely. In the corneas of KC family members and controls, the possible presence of Fleischer ring was thoroughly searched for, and though it occurred in many subjects, it was mostly faint. To the best of the authors' knowledge, this is the first study of an examination of Fleischer ring and prominent corneal nerves among unaffected relatives of KC patients. Based on our correlation and *t*-test results, the presence of Fleischer ring and prominent nerves was associated with features of KC; thus, family members who exhibited these signs had thinner and more asymmetric corneas than those without Fleischer ring and prominent nerves. In control subjects, corneas displaying a Fleischer ring and prominent nerves were more similar to KC corneas (shown by significantly higher *KSI* and *KISA* values), but were not thinner than other normal corneas. Interestingly, prominent nerves and Fleischer ring did not always occur together in the same cornea. It is well known that these signs occur with unequal frequencies in KC. It is believed that KC is a complex disease, both genetic and environmental factors playing a role in its pathogenesis. Fleischer ring is supposed to develop as a consequence of altered response of KC epithelial cells to oxidative stress. In KC, prominent corneal nerves are part of the pathophysiological processes in several ways and they play a role in the disease progression, too. Recent corneal in vivo confocal microscopy studies consistently have shown that even in mild KC, the subbasal nerve morphology is grossly abnormal. This might be explained by altered regulation of nerve growth factor (NGF) receptor expression in the KC cornea. These different etiological factors might explain why Fleischer ring and prominent nerves do not always occur together in KC or subclinical KC corneas. It was unexpected to find Fleischer ring in corneas of normal controls. Nevertheless, supposing that subclinical KC is an incompletely developed KC, its occurrence in normal controls is possible. A recent study reported the presence of bilateral Fleischer ring in a subject who had only mild topographic asymmetry, average central corneal thickness, low myopia with stable refraction, normal best corrected visual acuity and normal retinoscopic reflexes. The example of this patient, who was considered as subclinical KC, indicated that corneal Fleischer ring can occur without a positive family history and characteristic topographic signs

of KC. In our family members, minimal topographic alterations were observed and associated with the presence of Fleischer ring and prominent corneal nerves. One of the examined parameters in our study was central corneal thickness measured by ultrasound pachymetry. Central corneal pachymetry alone is unreliable in the diagnosis of KC, nevertheless, decreased thickness can be a good indicator of similarity to KC, and together with the videokeratographic indices this parameter supported that Fleischer ring and prominent nerves tend to occur in corneas more similar to KC. However, the examination of the posterior corneal surface and pachymetry with modern diagnostic methods (Scheimpflug imaging or Orbscan) would be helpful in future studies, since the earliest signs of KC can be more accurately detected with these techniques, and correlations will be given further impact with the addition of posterior topographic information. In spite of sophisticated imaging techniques, detecting subclinical KC is still challenging in many cases. Identifying subclinical cases, however, is important both for genetic studies and selecting high risk refractive surgery candidates. Based on our results, it can be suggested that a thorough search for Fleischer ring and prominent nerves in the cornea can help to decide whether or not to diagnose subclinical KC in borderline cases.

SUMMARY

Keratoconus (KC) is the most common, primary and progressive, bilateral, ectatic disease of the cornea, in which the lower and middle part of the cornea assumes a conical shape because of thinning and protrusion. Along with environmental effects, genetic factors may also play a role in its development. Complex segregation analysis was performed to reveal the presumed mode of inheritance, using clinical and videokeratographic data of sporadic KC families. Based on the data of 212 family members ascertained through 60 probands, and 212 age and gender matched healthy controls, familial aggregation and distribution of videokeratographic parameters were examined. Segregation of *KSI*, *KISA* and *6 mm Fourier Asymmetry* alone or in covariate analysis with gender or the presence of Fleischer ring, exploring mendelian and non-mendelian models of inheritance was tested using complex segregation analysis with the S.A.G.E. statistical program package. Based on *KISA* index, our population showed strong familial aggregation. All examined videokeratographic indices were able to differentiate between KC and non-KC family members as well as normal controls. Hypothesis accepted as most parsimonius model of inheritance for all indices indicated the presence of a non-mendelian major gene effect. Inclusion of Fleischer ring as covariate improved the fit of non-mendelian major gene models. Mendelian, sporadic and polygenic models were consistently rejected. Of the commonly known clinical signs of keratoconus, we examined the occurrence of Fleischer ring, prominent corneal nerves and thinning, in 117 unaffected family members of KC patients and in 142 healthy control individuals. With the use of Pearson correlation and *t*-test statistics, Fleischer ring, prominent corneal nerves and central pachymetry data were tested with each other and with videokeratographic indices (*KSI*, *KISA*, *3* and *6 mm Fourier Asymmetry*, and *I-S*). Unaffected KC family members who exhibited Fleischer ring or prominent nerves had thinner and more asymmetric corneas than those without Fleischer ring or prominent corneal nerves. We frequently observed Fleischer ring and prominent nerves on the corneas of unaffected family members. Though rarely, Fleischer ring and prominent corneal nerves occurred among normal controls, indicating the existence of subclinical KC cases in the normal population. Control subjects, who had corneal Fleischer ring or prominent nerves had corneas more similar to KC than other controls, based on *t*-test. The results of complex segregation analysis indicate a strong genetic contribution to the transmission of keratoconus. Inheritance is most probably due to a non-mendelian major gene effect. Low genotype-phenotype correlation in sporadic KC families can make linkage studies difficult, thus, genome wide association studies, new generational sequencing techniques, epigenetic and pathway analyses may provide more information on disease pathogenesis in non-familial keratoconus. Searching for the possible presence of Fleischer ring or prominent nerves on the cornea may help in the decision whether or not to diagnose subclinical KC in a borderline case.

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