Corneal and Conjunctival Alteration of Innate Immune Expression in First-Degree Relatives of Keratoconus Patients.

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Key messages:

- Corneal and conjunctival TLR2/TLR4 are overexpressed in eyes with KC.
- Corneal and conjunctival TLR2/TLR4 are overexpressed in first-degree relatives of KC patients.
- TLR2/TLR4 overexpression in KC relatives is not related to sex, age, and the presence or absence of allergic disease, eye itching and eye rubbing.
- TLR2/TLR4 expression in KC relatives is not related to abnormal clinical, topographic, aberrometric and tomographic parameters.

ABSTRACT

Purpose: The innate immune Toll-like receptors 2 (TLR2) and 4 (TLR4) may play a key role in the physiopathology of keratoconus (KC). Therefore, the aim of this study was to compare TLR2/TLR4 expression in corneal and conjunctival epithelial cells between healthy first-degree relatives of patients with KC and healthy controls as well as KC patients.

Methods: Case-control study in 72 healthy eyes of 36 control subjects, 53 eyes of 27 first-degree relatives and 109 eyes with KC (60 patients). All participants were subjected to a clinical, topographic, aberrometric and tomographic examination with extraction of corneal and conjunctival epithelial cells through scraping. TLR2/TLR4 expression was measured by flow cytometry, and was compared among controls, first-degree relatives and KC patients. The relationship between TLRs expression and epidemiological-clinical variables or topographic-aberrometric-tomographic parameters was also analyzed.

Results: Mean TLR2/TLR4 expression showed a significant gradual increase among groups: controls< first-degree relatives < KC patients. Mean expression of TLR2 in corneal epithelial cells and both TLR2/TLR4 in conjunctival epithelial cells were significantly higher in relatives than in controls ($p=0.026$, $p<0.001$ and $p=0.031$, respectively). Sex, age, allergic disease, eye itching, rubbing and topographic-aberrometric-tomographic parameters were not associated to TLR2/TLR4 expression in relatives. TLR2 conjunctival expression was independently associated to relatives (OR,1.001;CI95%,1.000-1.002,p=0.043) after adjustment by sex, age and rubbing.

Conclusion: TLR2 and TLR4 are overexpressed in corneal and conjunctival epithelial cells of KC relatives compared to controls. Both biomarkers may monitor early ocular changes in first-degree relatives who not show any abnormal clinical-topographic-aberrometric-tomographic parameters.

Keywords: Corneal and conjunctival epithelial cells, Innate immunity, Keratoconus, Relatives, Toll-like receptor 2 and 4.

DECLARATIONS

Funding: This project has been awarded grants from the Spanish Ministry of Economy and Competitiveness (Instituto de Salud Carlos III: PI14/00247, PI18/00159); Xunta de Galicia (Consellería de Economía e Industria: IN607A2018/3), and the European Union program ERFD. T. Sobrino (CPII17/00027) is recipient of a research contract from the Miguel Servet Program (National Institute of Health Carlos III). Similarly, U. Regueiro was awarded a predoctoral Fellowship from the Xunta de Galicia (ED481A-2015/001). The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Authors' contributions: Conception and design of the study (T.S., I.L.). Data acquisition and analysis (U.R., M.L-L., P.H., T.S.). Clinical data acquisition and analysis (U.R., M.L-L., I.L.). Handled funding and supervision (T.S., I.L.). Statistical analysis (U.R., P.H.). Manuscript drafting (U.R., I.L.). Critical revision for important intellectual content (M.L-L., P.H., T.S.). Supervision (T.S., I.L.). All authors reviewed and approved the manuscript.

List of abbreviations.

Arbitrary fluorescence units (AFUs), confidence interval (CI), corneal astigmatism (dK), diopters (D), dioptric central power (DCP), heat shock proteins (HSP), inferior-superior dioptric asymmetry (I-S asymmetry), interleukin 1beta (IL-1β), interleukin 4 (IL-4), keratoconus (KC), maximum dioptric power (maxPD), micrometers (μm), minimum thickness point (MTP), nuclear factor-kappaB (NF-κB), odd ratio (OR), percentage (%), posterior elevation (PE), standard deviation (SD), steeper corneal meridian (K2), toll–like receptors (TLRs), toll-like receptor 2 (TLR2), toll-like receptor 4 (TLR4), tumor necrosis factor alpha (TNF-α), vertical coma ($Z_3^{\pm 1}$).

Introduction

The term “keratoconus” (KC) reflects a degenerative-chronic process characterized by the pathological weakening, thinning and protrusion of the corneal tissue [1]. Usually, it is a bilateral and asymmetric condition in which the distortion of the corneal curvature results in the appearance of irregular astigmatism and impaired vision [2], involving a significant public health problem [3-4]. KC has an early onset age, affecting young people in their second decade of life, and their evolution is often associated with itching and eye rubbing. Regarding the etiology, it has not been fully elucidated yet; nevertheless, KC is recognized as a disease associated with some environmental, biomechanical, genetic and biochemical mechanisms [5].

Several studies have confirmed the overexpression of inflammatory mediators measured in tears of KC patients, being this overexpression strongly correlated with the progression of the disease [6–8]. In line with the above, we have confirmed the overexpression of Toll-like Receptor 2 (TLR2) and 4 (TLR4) in corneal and conjunctival epithelial cells as well as in blood monocytes and neutrophils of KC patients [9-10]. TLR2 and TLR4 are both type I immune innate transmembrane proteins which detect the presence of exogenous and/or endogenous agents associated to cell damage [11]. Upon recognizing ligands, both TLRs trigger an immune response which contributes to the translocation of NF-kB factor to the nucleus and the subsequent expression of inflammatory genes [12]. It has been shown that the systemic innate immune overexpression observed in KC patients was correlated with the increase of inflammatory mediators and NF-kB factor in serum [10]. In addition, it was also confirmed a relationship between corneal and conjunctival expression of TLRs and the KC severity [9]. Consequently, we postulate that the alteration in the innate immune homeostasis acts at tissue level promoting an inflammatory response involving the molecular drivers that finally cause the tissue degradation in KC.

Moreover, genetic predisposition could also exacerbate the KC onset process. In this regard, the existence of families with several members affected of KC has described [13-14]. Regarding first-degree relatives, an incidence rate of KC up to 14% [15] and 19% [16] has been reported. In many cases, these first-degree relatives showed topographic corneal alterations [14-17]. For all these reasons, healthy first-degree relatives of patients with KC are considered a group of subjects with high probabilities to develop the disease.

In this study, we hypothesize that healthy first-degree relatives may show alterations in the expression of TLR2 and/or TLR4. Therefore, the main aim of this study was to measure the TLR2/TLR4 expression in corneal and conjunctival epithelial cells from healthy first-degree relatives of KC patients in comparison with control subjects and KC patients. Moreover, we evaluated the relationship between TLR2/TLR4 expression and epidemiologic, clinic, topographic, aberrometric and tomographic parameters. To the best of our knowledge, this is the first study that evaluates the implication of innate immunity receptors in first-degree relatives of KC patients.

Material and methods

The present study was performed following the principles of the Declaration of Helsinki of the World Medical Association (as revised in Brazil 2013). All participants were subjected to a common clinical, topographic, aberrometric, tomographic protocol with extraction of biological sample which was approved by the Ethics Committee for Clinical Research of Galicia (Code 2015/436). All examinations were conducted by the same researchers. Written informed consent was obtained from each participant prior to their inclusion in the study and after full explanation of the procedures. The ophthalmological examination included:

(a) Filiation data and full anamnesis: sex, age, patient's eye history, family history of corneal ectasia and medical history (allergy, eye itching, eye rubbing…). Atopic conditions such as rhinitis, asthma and atopic dermatitis were included as allergic diseases as well as food allergies, drug allergies, allergies to insect bites and animals. The presence or absence of eye itching and eye rubbing was noted according to the users report.

(b) Topographic and aberrometric parameters: dioptric central power (DCP), steper meridian (K2), corneal astigmatism (dK), maximum dioptric power (maxDP), inferior-superior dioptric asymmetry (I-S asymmetry) and high order aberrations in the anterior corneal surface [vertical coma ($Z_3^{\pm 1}$) and coma-like, for 6 mm of diameter]; collected using the TOPCON CA-100 System corneal topographer (Topcon Medical Systems, Inc., USA).

(c) Tomographic parameters: posterior elevation (PE) and minimum thickness point (MTP); collected using Orbscan II corneal tomographer (Orbtek, USA).

At the end of the ophthalmological examination both corneal and conjunctival epithelial cell samples was collected to determine the expression of TLR2 and TLR4:

(a) Sample collection: corneal and conjunctival epithelial cells was collected through superficial scraping with ophthalmic surgical lancets of PVA (NETCELL, Network Medical Products Ltd, UK), previously instilling an eye topic anesthetic (COLIRCUSÍ Double Anesthetics with tetracaine at 0.1% and oxybuprocaine at 0.4%, ALCON CUSÍ SA, Spain). The corneal sample was extracted from the lower third of the cornea and the conjunctival sample was conducted in the lower bulbar conjunctive without touching the palpebral edge. Each ophthalmic lancet was put into a sterile and nuclease-free cytometry tube with 1 mL of PBS. Superficial scraping performed for sample collection was a non-injurious invasive technique which only induced mild conjunctival hyperemia and/or mild superficial corneal staining during 1 or 2 hours after scraping.

(b) Sample analysis: TLR2/TLR4 expression was determined by Flow cytometry (Flow cytometer FACSAria iiu, BD Biosciences, USA; Software FACS Diva 6.02, BD Biosciences, USA), following the same protocol described by Malfeito et al [9]. Epithelial cells were marked with 5µL of fluorescein isothicyanate anti-TLR2-conjugated monoclonal antibodies and with 5µL of phycoerythrin anti-TLR4-conjugated monoclonal antibodies (INMUNOSTEP, Spain). To homogenize the cytometry analysis in order to avoid that the density of cells had impact on fluorescence intensity, a total of 1000 corneal events and 2000 conjunctival events were analyzed following the method described by Malfeito et al [9]. Results were expressed in Arbitrary Fluorescence Units (AFUs). Values have been adjusted to a negative control without antibodies.

Study subjects

This study included 123 subjects classified into three groups: 36 control subjects, 27 first-degree relatives of KC patients and 60 patients with KC. All of them were recruited in the Instituto Galego de Oftalmoloxía (INGO), Santiago de Compostela, Spain. The common inclusion criteria for all groups included: 1) signing of informed consent form; 2) conjunctival hyperemia <2 (Nathan Efron scale) [18]; 3) Schirmer >15mm in 5 minutes; and 4) at least 5 days with no contact lenses and no instillation of artificial tears or eye drops. Specific inclusion criteria for controls and relatives included: 1) normal biomicroscopic examination and

2) normal topography, aberrometry and tomography parameters, with no irregular astigmatism or abnormalities suggestive of corneal ectasia. Finally, the specific inclusion criterion for the KC group was the positive diagnosis of KC: evaluated with slit lamp examination, corneal topography, aberrometry and tomography [19–21].

On the other hand, common exclusion criteria for all groups included: 1) sickness or infection that interferes with the molecular markers of innate immunity; 2) dry eye disease, 3) active ocular or systemic inflammation (uveitis…); 4) local or systemic anti-inflammatory treatments; 5) recent eye surgery; 6) traumatism or corneal-conjunctival disease; and/or 7) pregnancy. In addition, control subjects with family history of KC were excluded.

Statistical analysis

Statistical analysis was made using SPSS 20.0 for Windows (IBM, USA). Results were expressed as percentages for categorical variables and as mean (± standard deviation (SD)) for continuous quantitative variables with normal distribution. The normality of a quantitative variable was verified by the Kolmogorov-Smirnov test. The bivariate comparison of groups was made with chi-square test (categorical variables) and with t-Student contrasts (normal continuous variables). The graphic representation of the comparisons between normal continuous variables was made using error bars. The one-way ANOVA test was used to make comparisons among more than two study groups (control, relatives and KC) following a Bonferroni post-hoc test. Bivariate correlations were analyzed using Pearson's coefficient (for normal distribution). In addition, a multivariable analysis strategy by logistic regression was carried out to correct potential confounding factors (sex, age and eye rubbing) in controls and relatives. A value of $p \leq 0.05$ was considered statistically significant in all tests.

The statistical EPIDAT 3.1 software was used for calculating sample size. This determination was based in preliminary non-published studies of TLR2 conjunctival levels. Accepting a confidence level of 95% ($\alpha = 0.05$) and 75% power ($\beta = 0.25$), at least 34 control eyes, 49 eyes of first-order relatives and 66 eyes with KC would be necessary.

Results

Sample features

We studied 72 healthy eyes from 36 control subjects (39% male; mean age, 31.3 ± 10.4 years), 53 eyes of 27 first-degree relatives of KC patients (58% male; mean age, 25.4 ± 10.2 years) and 109 eyes of 60 patients with KC (58% male; mean age, 33.1 ± 8.7 years). The lower mean age in relatives compared to the KC group is due to the fact that relatives were siblings or offspring of KC patients.

Table 1 shows the epidemiological-clinical characteristics and the topographic-aberrometric-tomographic parameters for each study group. Importantly, no statistically relevant differences were found in the percentage of allergic diseases. KC patients showed greater incidence of eye itching (85%) and consequently a greater rate of eye rubbing (80%) than the rest of the study subjects (controls and relatives). There were statistical differences between the pathological topographic, aberrometric and tomographic parameters of KC patients and the normal parameters of control and relatives groups. Therefore, our main study group (relatives) show totally normal epidemiological-clinical characteristics and topographic-aberrometric-tomographic parameters.

TLR2 & TLR4 expression

The mean (±SD) expression of TLR2/TLR4 in corneal and conjunctival epithelial cells for each study group is showed in **Table 1** and represented in **Figure 1**. Mean TLR2/TLR4 expression showed a gradual increase among groups: control subjects < relatives of KC patients < KC patients. Regarding the cornea, TLR2 expression was significant higher in relatives than in controls (1054±772 vs 752±629 ($p=0.026$), respectively). The same trend was observed for the conjunctiva, where relatives showed a significant increase in TLR2 and TLR4 compared with the control group (1117±595 vs 806±412 ($p\leq0.001$), and 3259±1305 vs 2669±1441 ($p=0.031$); respectively). However, only TLR2 expression in conjunctival epithelial cells was independently associated to relatives´ group (OR, 1.001; CI95%, 1.000 to 1.002, $p=0.043$) after adjustment for sex, age and rubbing **[Table 2].**

KC patients showed significantly higher expression values in corneal and conjunctival TLR2/TLR4 than controls. Likewise, KC patients showed significantly higher TLR2/TLR4 expression than relatives; with the exception of conjunctival TLR2, which did not show statistical difference between KC group and relatives **[Table 1]**.

First-degree relatives: Relationship between TLR2/TLR4 expression and epidemiological-clinical variables.

Once we have demonstrated the overexpression of several TLRs in relatives compared to controls, the next step was to analyze how epidemiological and clinical factors may influence the innate immune overexpression in relatives. We carried out a bivariate study for each TLR in relatives according to sex, age and the presence or absence of allergic disease, eye itching and eye rubbing **[Table 3]**. As a result, the corneal and/or conjunctival TLR2/TLR4 expression did not show statistically significant differences with these epidemiological-clinical variables. Therefore, in relatives, we did not observe an association between TLRs expression and the sex, the age and the presence or absence of allergic disease, eye itching and eye rubbing.

First-degree relatives: Correlation between TLR2/TLR4 expression and topographic, aberrometric and tomographic parameters.

Finally, we carried out a correlation study in order to evaluate the association between the topographic-aberrometric-tomographic parameters and the TLR2/TLR4 expression in relatives **[Table 4]**. As result, topographic (DCP, K2, dK, maxDP, I-S), aberrometric ($Z_3^{\pm 1}$, coma-like) and tomographic (PE, MTP) parameters did not show statistical correlation with the expression of TLR2/TLR4 in corneal and/or conjunctival epithelial cells in the relatives group.

Discussion

In this study we have demonstrated that first-degree KC relatives, with healthy epidemiological-clinical characteristics and normal topographic-aberrometric-tomographic parameters, show overexpression of corneal TLR2 and conjunctival TLR2/TLR4 compared with control subjects. However, only conjunctival TLR2 was associated with relatives after adjustment by sex, age and eye rubbing. To the best of our knowledge, this is the first study which evaluates the expression of innate immunity receptors in corneal and conjunctival epithelial cells from first-degree relatives of KC patients. In line with our results, Malfeito

et al [9] also observed the corneal and conjunctival TLR2/TLR4 overexpression in KC and subclinical KC compared with controls; confirming that corneal TLR2 expression may predict with high sensitivity and specificity the probability of KC.

TLR2 and TLR4 are immune innate transmembrane proteins that detect the presence of exogenous and/or endogenous agents associated with cell damage [11]. Therefore, TLR2 and TLR4 can be activated by infectious exogenous agents such as bacterial lipoproteins or lipopolysaccharides, and by endogenous ligands such as cellular fibronectin, heat shock proteins (HSP), lactoferrin or proteins with hormonal influence [22]. The TLR2/TLR4 activation by these ligands induces the expression of genes involved in the inflammatory response. Consequently, the TLR2/TLR4 overexpression in the ocular surface of first-degree KC relatives represents an ocular environment more predisposed to trigger a proinflammatory state, which could lead to the KC onset. Due to the fact that conjunctival TLR2 is the highest overexpressed receptor in KC relatives, but the corneal TLR2 is the main overexpressed receptor in patients with manifest KC [9]; we may hypothesize that the disease predisposition begins at conjunctival level and could lead to the disease pathological progression by the influence of multiple factors.

We have demonstrated that TLRs expression in relatives is not related to sex, age and the presence or absence of allergic disease, eye itching and eye rubbing. In addition, the topographic (DCP, K2, dK, maxDP, I-S asymmetry), aberrometric ($Z_3^{\pm 1}$, coma-like) and tomographic (PE, MTP) parameters neither showed statistical correlation with TLRs expression in relatives. In contrast, a strong association between TLR2 and TLR4 expression with the severity of KC quantified by topographic, aberrometric and tomographic parameters (steep meridian [K2], coma, minimum thickness point [MTP]) was observed in KC patients [9]; demonstrating that the more advanced the disease is, the greater the expression of toll-like receptors will be. The lack of relationship between the TLR2/TLR4 expression and the clinical-epidemiological values or the topographic-aberrometric-tomographic parameters in healthy KC relatives, confirms an exacerbated early innate immune predisposition at corneal and conjunctival level which may contribute to the KC onset process. In this regard, similarly, Ionescu et al [23] found significantly increased levels of interleukin 1 beta (IL-1β) and interleukin 4 (IL-4) in the tears of KC patients and their first-degree family members compared to normal subjects. IL-1β and IL4 has been widely recognized as cytokines associated to immune and proinflammatory processes and to the KC pathogenesis [24–26]. These results

are consistent with a previous study in which the systemic TLR2 and TLR4 immune overexpression observed in KC patients was correlated with the increase of inflammatory mediators and NF-kB factor in serum [10].

In the same way, the studies conducted by Kara et al [27] and by Ionescu et al [28] stated that the corneal biomechanical properties are also altered in KC relatives even before that the topographic indices. They propose that biomechanical parameter could be an early premonitory sign of a subclinical keratoconus [27-28]. Although the primary keratoconus origin remains unknown, the corneal structural disturbance is attributed to alterations in the entire ocular surface homeostasis [5]. Dogru et al [29] found several changes in the ocular surface of KC patients, including squamous conjunctival metaplasia, low goblet cell density and disorder of tear quality. In the same way, Ziangirova et al [30] reported the presence of immune inflammation in the conjunctival tissue of KC patients. Therefore, taking all these results into account we may hypothesize that the overexpression of the innate immune mechanisms in the corneal and the conjunctival epithelial cells, as well as the tear proinflammatory process, could trigger the corneal biomechanical alteration in first-degree relatives of KC patients.

Considering that relatives of KC patients have the highest risk of developing the disease [16-31]; the overexpression of innate immune receptors in the corneal and conjunctival epithelial surface, the expression of proinflammatory molecules in tears, as well as the evaluation of the corneal biomechanical capacities should acquire great importance as preoperative evaluations in these subjects, instead of the topographic-aberrometric-tomographic diagnostic methods currently used. It could avoid postoperative keratoectasia in relatives who wish to perform refractive surgery. Consequently, in order to bring this study closer to clinical practice, a device is currently being developed to test the TLR2/TLR4 expression in the ocular surface. This device will allow to carried out an easier and faster procedure different to the flow cytometry and the superficial epithelial scraping. Moreover, the findings of this study open the possibility to design new preventive or therapeutic approaches. Blocking or immunomodulating the TLR2 and/or TLR4 would at least decrease the typical proinflammatory state in the ocular surface of the KC patients. In addition, future studies could also focus on the importance of following these healthy first-degree relatives over time to identify who is progressing in KC development and determine if there is a correlation between their TLR2/4 levels at the time baseline and disease development.

Conclusion

TLR2 and TLR4 are overexpressed in corneal and conjunctival epithelial cells of first-degree KC relatives compared to control subjects. Therefore, these receptors (with in particular conjunctival TLR2 due to be the highest overexpressed) may be useful to monitor early ocular changes in first-degree relatives with healthy clinical values and with topographic, aberrometric and tomographic parameters inside the normal limits. In addition, they may be also useful to asses if a subject is in risk of developing KC. Finally, further studies are necessary in order to evaluate TLR2 and TLR4 as potential therapeutic targets for future preventive treatments in KC.

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FIGURES

Figure 1. Mean TLR2/TLR4 expression for each study group showing a significant gradual increase among groups: controls < first-degree relatives < KC patients.

Statistical differenced with regard to: † controls, § all groups. [P-values are showed in Table 1].

Sample size: Control= 72 eyes; Relatives= 53 eyes; KC= 109 eyes.

Abbreviations: KC, keratoconus; TLR2, toll-like receptor 2; TLR4, toll-like receptor 4; AFU, arbitrary fluorescence units.

TABLES

Table 1. Epidemiological-clinical characteristics, topographic-aberrometric-tomographic parameters and TLR2/TLR4 expression in corneal and conjunctival epithelial cells for each study group.

Table 2. Crude and adjusted OR of relative´s group for TLR expression.

Table 3. Mean expression (±SD) of TLR2/TLR4 in corneal and conjunctival epithelial cells of first-degree relatives according to their epidemiological (sex and age) and clinical (allergic disease, itching and rubbing) characteristics.

Table 4. Correlation (Pearson´s coefficient) between TLR2/TLR4 expression and quantitative topographic, aberrometric and tomographic parameters in first-degree relatives of patients with KC.

Figure 1

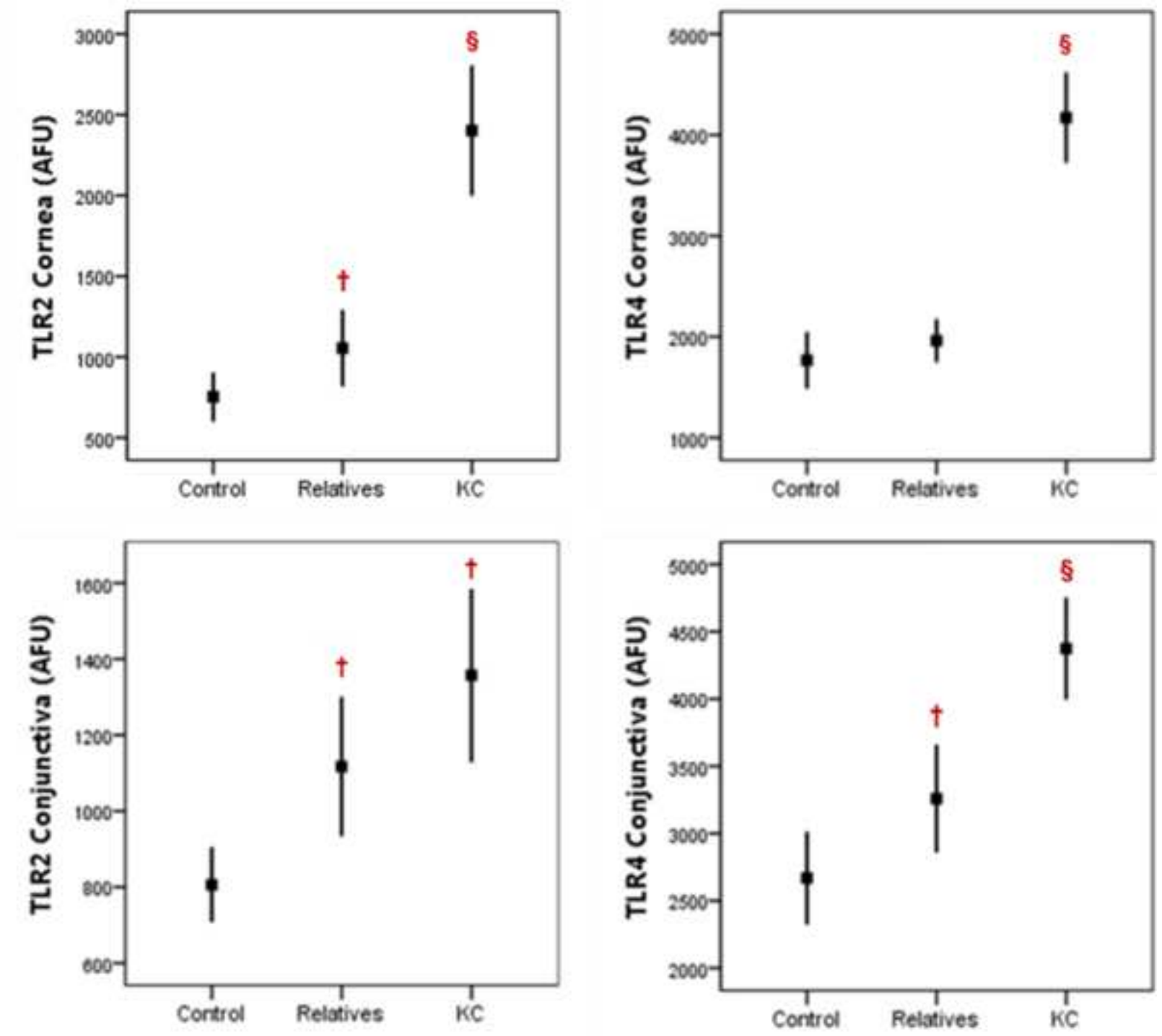

Table 1

TABLE.1. Epidemiological-clinical characteristics, topographic-aberrometric-tomographic parameters and TLR2/TLR4 expression in corneal and conjunctival epithelial cells for each study group.

	Control	Relatives	*P* †	KC	*P* †	*P* ‡
Sex, males (%)	38.9	57.7	0.138	58.3	0.066	0.957
Age, years	31.3 ± 10.4	25.4 ± 10.2	**0.032**	33.1 ± 8.7	0.353	**0.003**
Allergic disease (%)	47.2	42.3	0.707	60.0	0.228	0.133
Itching (%)	27.8	39.2	0.185	82.6	**<0.0001**	**<0.0001**
Rubbing (%)	33.3	35.3	0.823	71.6	**<0.0001**	**<0.0001**
DCP (D)	44.05 ± 1.65	44.30 ± 1.14	0.353	49.19 ± 5.67	**<0.0001**	**<0.0001**
K2 (D)	44.32 ± 1.75	44.54 ± 1.22	0.439	49.42 ± 4.28	**<0.0001**	**<0.0001**
dK (D)	0.84 ± 0.66	0.97 ± 0.74	0.298	3.75 ± 2.21	**<0.0001**	**<0.0001**
maxDP (D)	44.72 ± 1.80	45.21 ± 1.66	0.122	53.30 ± 5.33	**<0.0001**	**<0.0001**
I-S asymmetry (D)	-0.09 ± 0.51	-0.18 ± 0.57	0.400	5.69 ± 3.75	**<0.0001**	**<0.0001**
$Z_3^{\pm 1}$ (µm)	0.29 ± 0.15	0.26 ± 0.13	0.378	3.34 ± 9.92	**0.01**	**0.028**
Coma-like (µm)	0.35 ± 0.14	0.34 ± 0.14	0.774	4.33 ± 17.23	**0.049**	**0.040**
PE (µm)	28.0 ± 9.8	28.7 ± 12.3	0.730	94.7 ± 41.9	**<0.0001**	**<0.0001**
MTP (µm)	556 ± 37	537 ± 51	0.083	453 ± 62	**<0.0001**	**<0.0001**
TLR2 cornea (AFU)	752 ± 629	1054 ± 772	**0.026**	2402 ± 1819	**<0.0001**	**<0.0001**
TLR4 cornea (AFU)	1768 ± 1163	1962 ± 693	0.325	4172 ± 2018	**<0.0001**	**<0.0001**
TLR2 conjunctiva (AFU)	806 ± 412	1117 ± 595	**<0.001**	1357 ± 1035	**<0.0001**	0.164
TLR4 conjunctiva (AFU)	2669 ± 1441	3259 ± 1305	**0.031**	4374 ± 1723	**<0.0001**	**<0.0001**

Note: Significant *P*-values are given in bold.
Sample size: Control= 36 subjects, 72 eyes; Relatives= 27 subjects, 53 eyes; KC= 60 patients, 109 eyes.
Abbreviations: KC, keratoconus; DCP, dioptric central power; K2, steeper corneal meridian; dK, corneal astigmatism; maxDP, maximum dioptric power; I-S asymmetry, inferior-superior dioptric asymmetry; $Z_3^{\pm 1}$, vertical coma; PE, posterior elevation; MTP, minimum thickness point; TLR2, toll-like receptor 2; TLR4, toll-like receptor 4; AFU, arbitrary fluorescence units.
P † compared to control group.
P ‡ compared to relatives' group.

Table 2

TABLE.2. Crude and adjusted OR of relative´s group for TLR expression.

	OR (CI95%), p	OR (CI95%), adjusted p
TLR2 cornea	1.00 (1.00 to 1.00), **p=0.042**	1.00 (1.00 to 1.00), p=0.599
Sex		0.49 (0.15 to 1.57), p=0.231
Age		0.95 (0.89 to 1.01), p=0.111
Rubbing		1.19 (0.33 to 4.35), p=0.788
	OR (CI95%), p	**OR (CI95%), adjusted p**
TLR2 conjunctiva	1.00 (1.00 to 1.00), **p=0.004**	1.00 (1.00 to 1.00), **p=0.043**
Sex		0.46 (0.13 to 1.56), p=0.210
Age		0.95 (0.90 to 1.02), p=0.152
Rubbing		1.20 (0.33 to 4.30), p=0.780
	OR (CI95%), p	**OR (CI95%), adjusted p**
TLR4 conjunctiva	1.00 (1.00 to 1.00), **p=0.038**	1.00 (1.00 to 1.00), p=0.304
Sex		0.50 (0.15 to 1.61), p=0.245
Age		0.95 (0.89 to 1.01), p=0.113
Rubbing		1.43 (0.42 to 4.91), p=0.571

Crude and adjusted OR of relatives´ group for TLR expression.
Note: Significant P-values are given in bold. Dependent variable was the study group (control or relatives).
Sample size: Control= 36 subjects, 72 eyes; Relatives= 27 subjects, 53 eyes.
Abbreviations: CI, confidence interval; OR, odds ratio; TLR2, toll-like receptor 2; TLR4, toll-like receptor 4.

Table 3

TABLE.3. Mean expression (±SD) of TLR2/TLR4 in corneal and conjunctival epithelial cells of first-degree relatives according to their epidemiological (sex and age) and clinical (allergic disease, itching and rubbing) characteristics.

		TLR2 cornea	TLR4 cornea	TLR2 conjunctiva	TLR4 conjunctiva
Sex	**Male**	807 ± 440	1595 ± 565	1201 ± 685	3278 ± 1250
	Female	1268 ± 1381	2162 ± 775	1200 ± 764	2835 ± 1401
	P	0.288	0.067	0.998	0.455
Age	***≤24 years***	725 ± 309	1837 ± 870	1305 ± 696	3158 ± 1213
	≥25 years	1312 ± 1322	1839 ± 515	1086 ± 725	3011 ± 1455
	P	0.168	0.993	0.488	0.804
Allergic disease	**No**	1148 ± 1185	2015 ± 773	1385 ± 745	3123 ± 1094
	Yes	772 ± 364	1551 ± 499	901 ± 535	3031 ± 1668
	P	0.397	0.148	0.127	0.880
Itching	**No**	880 ± 434	2023 ± 661	962 ± 497	3329 ± 1565
	Yes	1213 ± 1059	1874 ± 773	1287 ± 693	3126 ± 992
	P	0.178	0.509	0.088	0.635
Rubbing	**No**	879 ± 426	1996 ± 644	989 ± 485	3284 ± 1507
	Yes	1256 ± 1114	1898 ± 815	1286 ± 737	3170 ± 1047
	P	0.134	0.668	0.126	0.793

Sample size: Relatives= 27 subjects, 53 eyes.
Abbreviations: TLR2, toll-like receptor 2; TLR4, toll-like receptor 4.

Table 4

TABLE 4. Correlation (Pearson´s coefficient) between TLR2/TLR4 expression and quantitative topographic, aberrometric and tomographic parameters in first-degree relatives of patients with KC.

	(Pearson correlation coefficient) *P*-value								
	DCP	**K2**	**dK**	**maxDP**	**I-S**	**$Z_3^{\pm 1}$**	**Coma-like**	**PE**	**MTP**
TLR2 cornea	(-0.160) 0.050	(-0.128) 0.094	(-0.158) 0.051	(-0.097) 0.160	(-0.147) 0.064	(0.086) 0.188	(0.064) 0.254	(0.135) 0.082	(-0.020) 0.417
TLR4 cornea	(-0.082) 0.200	(-0.061) 0.266	(-0.023) 0.408	(-0.027) 0.389	(-0.128) 0.093	(0.013) 0.447	(0.048) 0.312	(0.058) 0.274	(0.098) 0.157
TLR2 conjunctiva	(-0.135) 0.081	(-0.154) 0.055	(-0.060) 0.266	(-0.108) 0.133	(0.014) 0.443	(0.061) 0.264	(0.042) 0.331	(-0.020) 0.420	(-0.149) 0.062
TLR4 conjunctiva	(-0.105) 0.138	(-0.127) 0.095	(-0.131) 0.087	(-0.100) 0.151	(0.027) 0.388	(0.138) 0.076	(0.118) 0.110	(-0.131) 0.087	(-0.095) 0.164

Sample size: Relatives= 53 eyes.
Abbreviations: DCP, dioptric central power; K2, steeper corneal meridian; dK, corneal astigmatism; maxDP, maximum dioptric power; I-S asymmetry, inferior-superior dioptric asymmetry; $Z_3^{\pm 1}$, vertical coma; PE, posterior elevation; MTP, minimum thickness point; TLR2, toll-like receptor 2; TLR4, toll-like receptor 4.