Toll-Like Receptors as Diagnostic Targets in Pellucid Marginal Degeneration.

Uxía Regueiro [a,b], María Pérez-Mato [a], Pablo Hervella [a], Francisco Campos [a], Tomás Sobrino [a]*, Isabel Lema [a,b,c]*.

[a] Clinical Neurosciences Research Laboratory, Health Research Institute of Santiago de Compostela (IDIS), Santiago de Compostela, Spain
[b] Facultade de Óptica e Optometría, Universidade de Santiago de Compostela (USC), Santiago de Compostela, Spain
[c] Instituto Galego de Oftalmoloxía (INGO), Hospital Provincial de Conxo, Santiago de Compostela, Spain

*Address for correspondence:
Prof. Isabel Lema
Instituto Galego de Oftalmoloxía
Hospital Provincial de Conxo
15706 - Santiago de Compostela (Spain)
E-mail: mariaisabel.lema@usc.es
Telephone & Fax number: (+34) 981951098

*Address for correspondence:
Dr. Tomás Sobrino
Clinical Neurosciences Research Laboratory
Hospital Clínico Universitario
Travesía da Choupana S/N
15706 - Santiago de Compostela - Spain
E-mail: tomas.sobrino.moreiras@sergas.es
Phone: +34 981951098 / Fax: +34 981915098

KEYWORDS: Corneal ectasia; Innate immunity; Keratoconus; Pellucid marginal degeneration; Toll-like receptors 2; Toll-like receptors 4.

Funding: This project has been awarded grants from the Spanish Ministry of Economy and Competitiveness (Instituto de Salud Carlos III: PI14/00247); Xunta de Galicia (Consellería de Educación: GRC2014/027 and IN607A2018/3), and the European Union program ERFD. T. Sobrino (CPII17/00027) and F. Campos (CPII19/00020) re recipients 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.

Abbreviations and acronyms: anterior elevation (AE), arbitrary fluorescence units (AFUs), area under the curve (AUC), confidence interval (CI), diopters (D), dioptric central power (DCP), distance from the corneal center to the minimum thickness point (dMTP), inferior-superior asymmetry (I-S asymmetry), interleukin 1-beta (IL-1β), interleukin 6 (IL-6), keratoconus (KC), keratoconus prediction index (KPI), maximum dioptric power (PDmax), metalloproteinase-9 (MMP9), micrometers (μm), minimum thickness point (MTP), nuclear factor-kappaB (NF-κB), odd ratio (OR), pellucid marginal degeneration (PMD), percentage (%), posterior elevation (PE), receiver operating characteristic (ROC), standard deviation (SD), steeper corneal meridian (K2), tissue inhibitors of metalloproteinases-1 (TIMP-1), toll–Like receptors (TLRs), toll-like receptor 2 (TLR2), toll-like receptor 4 (TLR4), tumor necrosis factor alpha (TNF-α), vertical coma ($Z_3^{\pm 1}$).

ABSTRACT

The main purpose of this study is to evaluate the diagnostic role of Toll-like receptors 2 (TLR2) and 4 (TLR4) expression in corneal and conjunctival epithelial cells of eyes with pellucid marginal degeneration (PMD) compared to keratoconus patients (KC) and control subjects. A prospective case-control study in 29 PMD eyes, 109 KC eyes and 72 healthy eyes was done. All participants were subjected to a clinical, topographic, aberrometric and tomographic exam with extraction of corneal and conjunctival epithelial cells through scraping. The TLR2 and TLR4 expression was measured with flow cytometry. Receiver operating characteristic (ROC) curve analysis was used to determine the most appropriate cutoff point for predicting the risk of PMD and KC. Correlations between TLR2/TLR4 expression and the severity of PMD/KC were evaluated. A TLRs follow-up review was made 19±4 months after to the first review. As result, mean expression of TLR2 and TLR4 in both corneal and conjunctival epithelial cells was significantly higher in eyes with corneal ectasia (PMD and KC) than in control eyes (all $p<0.05$). Conjunctival TLR4 expression showed the highest capacity to diagnose the existence of PMD (odd ratio 42.84; 95% confidence interval:6.20-296.20; $p<0.0001$) after adjusting by eye rubbing and steeper corneal meridian. Moreover, we found an association between the TLR2/TLR4 overexpression with the severity of the PMD and KC measured by corneal topographic, aberrometric and tomographic quantitative parameters (all $p<0.05$). Differences on TLR2/TLR4 expression between study groups were maintained during the follow-up period. In conclusion, the TLR2/TLR4 overexpression in corneal and conjunctival epithelial cells of PMD and KC patients compared to healthy control subjects have demonstrated their role as diagnostic target in both corneal ectatic disorders.

KEYWORDS

Corneal ectasia; Innate immunity; Keratoconus; Pellucid marginal degeneration; Toll-like receptors 2; Toll-like receptors 4.

1. INTRODUCTION

Pellucid marginal degeneration (PMD) is a primary corneal ectasia characterized by the pathological weakening, protrusion and lower peripheral thinning of the corneal tissue, which show a typical topographic "crab claw" pattern (Belin et al. 2011; Fuchihata et al. 2014; Gomes et al. 2015). Like in keratoconus (KC), the distortion of the corneal curvature results in the appearance of irregular astigmatism and vision impairment which represents a significant public health problem (Kymes et al. 2008; Rebenitsch et al. 2011). PMD and KC reflect a degenerative, chronic process, usually bilateral, asymmetric and of unknown evolution which is often associated with itching and eye rubbing. Their pathogenesis has not yet been fully elucidated; nevertheless, PMD and KC are both recognized as diseases associated with ocular surface inflammation (Kolozsvári et al. 2014; Lema et al. 2010; Lema and Duran 2005; Pásztor et al. 2016). In fact, several studies have confirmed that the molecular drivers that cause tissue degradation in both ectatic conditions are dependent of inflammatory mechanisms (Balasubramanian et al. 2012; Chaerkady et al. 2013; Lema et al. 2009).

Recent studies conducted by our group showed that these inflammation seem also associated with alterations in the innate immune ocular homeostasis (Malfeito et al. 2018; Sobrino et al. 2017). Toll–Like receptors (TLRs) are type I transmembrane proteins which express not only in cells of the immune system, but also in epithelial and endothelial cells. There are at least 11 types of Toll-like innate immune receptors. Among them, TLR2 and TLR4 detect the presence of exogenous or endogenous agents associated to cell damage (Takeda and Akira 2005). Upon recognizing ligands associated to cell damage both TLRs trigger an immune response mainly using the gene MyD88-dependent signaling pathway, which contributes to the translocation of nuclear factor-kappaB (NF-kB factor) to the nucleus and the subsequent expression of inflammatory genes and eventually to the activation of adaptive immunity (Kawai and Akira 2007). In 2017, the first results showing the overexpression of TLR2 and TLR4 in blood monocytes and neutrophils in patients with KC were published (Sobrino et al. 2017). In addition, this systemic innate immune overexpression correlated with the serum increase of inflammatory mediators and NF-kB factor (Sobrino et al. 2017). Later studies confirmed the overexpression of TLR2 and TLR4 in corneal and conjunctival epithelium of patients with subclinical KC (Malfeito et al. 2018).

Nowadays, the involvement of both innate immunity receptors in PMD is unknown. Therefore, we hypothesize that TLR2 and TLR4 also could act as highly efficient diagnostic biomarkers for PMD. So, the main goal of this study was to measure the

expression levels of TLR2 and TLR4 in cells of corneal and conjunctival epithelium from PMD patients; these TLR expression was compared with the TLR2/TLR4 expression in corneal and conjunctival epithelium cells of KC patients and control subjects. The role of TLR2/TLR4 as diagnostic biomarkers of PMD and KC was evaluated by logistic regression analysis. In addition, in order to evaluate the innate immunity implication in the severity of corneal ectasias, we study the correlation between TLR2/TLR4 expression and some quantitative parameters involved in the degree of PMD and KC. Finally, we reevaluated the TLRs expression 19±4 months after the first review in order to test the hypothesis that the TLR expression could be higher over time if the disease progresses.

2. MATERIAL AND METHODS

2.1 Study Groups

This study comprised a total sample of 210 eyes divided into three groups: (1) Control group: 72 healthy eyes of 36 subjects who were not blood relatives of the rest of the groups and with no family history of corneal ectasia; (2) PMD group: 29 eyes of 15 patients with manifest PMD; (3) KC group: 109 eyes of 60 patients with manifest KC. All study groups were subjected to a common clinical, topographic, aberrometric and tomographic protocol with extraction of biological sample following the principles of the Declaration of Helsinki of the World Medical Association. This study was approved by the Ethics Committee for Clinical Research of Galicia (2015/436). All examinations were conducted by the same researchers.

Common inclusion criteria for all groups were: aged between 12 and 65 years, signing of informed consent form, conjunctival hyperemia <2 (Nathan Efron scale (Efron 1998)), Schirmer >15mm in 5 minutes, at least 5 days with no contact lenses and no instillation of artificial tear or eye drops. Specific inclusion criteria by groups were: (1) Control Group: normal topographic, aberrometric and tomographic parameters, with no irregular astigmatism or abnormalities suggestive of corneal ectasia; (2) PMD group: age of diagnosis ≥27 years, topographic crab claw pattern where the thinnest corneal area is located immediately below the area of greater corneal curvature, with topographic, aberrometric and tomographic parameters similar to KC taking into account a greater mean distance from the corneal center to the minimum thickness point (dMTP) (Lee et al. 2007); (3) KC group: presence of one or more characteristic biomicroscopic signs of the

disease (prominent corneal nerves, Vogt's striae, Fleischer ring…) (Krachmer, Feder, and Belin 1984) and meet the Rabinowitz (Rabinowitz 1995) topographic diagnostic criteria [dioptric central power (DCP) ≥48.7D and/or inferior-superior asymmetry (I-S)>1.9D], the Alió and Shabayek (Alió and Shabayek 2006) higher order aberrations [coma-like>0.65μm], the Orbscan II tomography criteria from (Rao et al. 2002) [minimum thickness point (MTP) <470μm and/or posterior elevation (PE) >40μm] and from (Fam and Lim 2006) [anterior elevation (AE) >16.5μm] and/or the KC Prediction Index (KPI) from (Maeda et al. 1994) >0.23%.

Common exclusion criteria for all groups: sickness, abnormality or infection that interferes with the molecular markers of innate immunity; dry eye disease; active ocular or systemic inflammation; local or systemic anti-inflammatory treatments; eye surgery; traumatism or corneal-conjunctival disease and/or pregnancy.

Moreover, we have taken special care to discriminate PMD from pellucid-like keratoconus. According with the Global Consensus on Keratoconus and Ectatic Diseases (Gomes et al. 2015), the best way to differentiate PMD from KC is by using a combination of approaches, which includes a full slit-lamp examination, corneal thickness map, anterior curvature map and anterior tomographic elevation map. Therefore, we pay special attention in the main distinctive clinical features between PMD and pellucid-like KC (Koc et al. 2018, Lee et al. 2017, Martínez-Abad et al. 2019): 1) PMD usually starts later in life and progresses slower than KC; 2) slit-lamp examination: Vogt striae, Fleischer ring, Munson sign are commonly present in moderate and advanced stages of KC but absent in PMD; 3) careful studying of corneal topographic and tomography with the main three maps to distinguish the "thinning location" and the "area of increased curvature below the thinning band" distinctive of PMD.

2.2 Clinical examination protocol

Filiation data and full anamnesis: age, sex, patient's eye history, family history of corneal ectasia and medical history (allergy, 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. Topographic and aberrometric parameters [collected using the TOPCON CA-100 System corneal topographer (Topcon Medical Systems, Inc., NJ, USA)]: DCP, maximum dioptric power (PDmax), I-S asymmetry, KPI index and high order aberrations in the anterior corneal surface (coma ($Z_3^{\pm 1}$) and coma-like) for 6 mm of diameter. Tomographic parameters [measured using the Orbscan IIz corneal topographer (Orbtek, UT, USA)]: MTP, dMTP,

PE and AE. Cells samples were collected from the corneal and conjunctival epithelium to determine the expression of TLR2 and TLR4. At 19±4 months after the initial examinations, a follow-up collection to the TLRs expression was made.

2.3 Sample Collection: Corneal and Conjunctival Epithelial Cells

The same sample collection method was used for all groups. The collection of corneal and conjunctival epithelial cells was made through scraping with ophthalmic surgical lancets of PVA (NETCELL, Network Medical Products Ltd, Kearsley, UK); following the same protocol as described by (Malfeito et al. 2018). Previously, a drop of eye topic anesthetic (COLIRCUSÍ Double Anesthetics with tetracaine at 0.1% and oxybuprocaine at 0.4%, ALCON CUSÍ SA, Barcelona, Spain) was instilled. The corneal sample was extracted in the lower third of the cornea. The conjunctival sample was conducted in the lower bulbar conjunctive, next to the limbus, without touching the palpebral edge. Every ophthalmic surgical lancet was placed in a 5 ml cytometry tube (sterile and nuclease-free) with 1 mL of PBS.

2.4 TLR2 & TLR4 expression analysis

All samples were analyzed using the same protocol. The expression of TLR2 and TLR4 in the cells of the corneal and conjunctival epithelium was determined using Flow cytometry (Flow cytometer FACSAria iiu, BD Biosciences, NJ, USA; Software FACS Diva 6.02, BD Biosciences, NJ, USA), following the same protocol as described by (Malfeito et al. 2018). The number of cells collected for each sample was quantified by manual cell counting with Neubauer Chamber. The number of cells collected through scraping with lancets was $1.31x10^5 \pm 0.3x10^5$cells/mL per sample. Briefly explained, the epithelial cells in each sample were marked with 5µL of fluorescein isothicyanate anti-TLR2-conjugated monoclonal antibodies and with 5µL of phycoerythrin anti-TLR4-conjugated monoclonal antibodies (from INMUNOSTEP, Salamanca, Spain) [dilutions 1:200]. A total of 1000 corneal events and 2000 conjunctival events were analyzed. The results were expressed as mean of Arbitrary Fluorescence Units (AFUs). Values have been adjusted to a negative control without fluorescent labeling.

2.5 Statistical Analysis

The statistical EPIDAT 3.1 software was used for calculating sample size. This determination was based in preliminary non-published studies of TLR4 conjunctival levels, in which PMD eyes showed 50% more TLR4 expression than control eyes.

Accepting a confidence level of 95% (α = 0.05) and 90% power (β = 0.9), at least 16 PMD eyes and 26 control eyes would be necessary.

The description of continuous quantitative variables was made using mean and standard deviation (SD) in the case of normal distribution. For non-normal distribution, the median and range of percentiles (25^{th} and 75^{th}) were used. Categorical variables were described as frequencies and percentages (%). The normality of a quantitative variable was verified by the Kolmogorov-Smirnov test.

The bivariate comparison of groups was made with t-Student contrasts (normal continuous variables), U-Mann-Whitney (non-normal continuous variables) and chi-square (categorical variables). The graphic representation of normal continuous variables was made using error bars. The ANOVA test was used to make comparisons among more than two study groups (control, PMD and KC).

Bivariate correlations were analyzed using Pearson's coefficient (for normal distribution) or Spearman's coefficient (for non-normal distribution). Logistic Regression analysis was used to determine the potential role of TLRs as biomarkers for corneal ectatic disorders diagnosis. OR analysis was made taking into account the ROC curve cutoff value of the TLRs studied. Cutoff values were selected looking for a good balance between sensitivity and specificity. To use these cutoff points as reference to another study, it´s necessary consider the same cytometer calibration.

A value of $p<0.05$ was considered statistically significant in all tests. The statistical analysis was made using SPSS 20.0 for Windows (IBM, New York, USA).

In addition, charts with dots and arrows were performed using Microsoft Office Excell to represent case by case the variations obtained between the initial and the follow-up TLRs expression.

3. RESULTS

3.1 Descriptive analysis of the sample

We studied 72 healthy eyes of 36 control subjects (39% male; mean age, 31.3 ± 10.4 years), 29 eyes of 15 patients with PMD (80% male; mean age, 45.2 ± 8.7 years) and 109 eyes of 60 patients with KC (58% male; mean age, 33.1 ± 8.7 years). Age difference among study groups is accounted for the different course of progression of each

condition. Mean age of presentation for PMD is higher than KC; this means that in order to be able to study both diseases in similar conditions of severity, the recruitment of patients must be done at different ages. **Table 1** shows the epidemiological and clinical characteristics of each study group. Control group showed less incidence of eye itching (85%) and consequently less rate of eye rubbing (80%) than patients with corneal ectasia. Importantly, no statistically relevant differences were found in the percentage of allergies. Moreover, topographic (K2, PDmax, KPI, I-S asymmetry), aberrometric ($Z_3^{\pm 1}$, coma-like) and tomographic (MTP, dMTP, PE, AE) values were significantly higher in PMD and KC than in control group. Some of these quantitative variables will be used as indicators of the degree of ectasia affectation in the correlation study between innate immune expression and severity of PMD/KC.

3.2 Expression of TLR2 and TLR4.

Levels of TLR2 and TLR4 expression in corneal and conjunctival epithelial cells were higher in PMD and KC than in the control group **[Table 1]**. In patients with PMD, corneal/conjunctival TLR4 demonstrate higher differences with control group ($p<0.0001$) than corneal/conjunctival TLR2. Regarding with KC patients, the main difference of TLR expression compared to the other groups was found for TLR2 and TLR4 in corneal cells ($p<0.0001$). **Figure 1** represents the mean expression (±SD) of TLR2/TLR4 in cells of the corneal and conjunctival epithelium for each study group. Age, sex and allergies are not correlated to the innate immunity receptors expression *(data not shown)*.

3.3 Role of TLR2 and TLR4 as predictive biomarkers of PMD and KC.

A Logistic Regression analysis was performed in order to explore the role of corneal and conjunctival TLR2/TLR4 expression as diagnostic biomarkers for PMD and KC. Firstly, according to the ROC curve analysis, TLR4 expression in conjunctival epithelial cells was the best TLR to diagnose PMD [area under the curve (AUC) of PMD vs Controls: 0.814 (confidence interval (CI) 95% 0.73-0.90) $p<0.0001$]. Likewise, corneal TLR4 expression is also useful for discriminate between PMD and controls [AUC: 0.789 (CI 95% 0.70-0.88) $p<0.0001$]. Furthermore, according to the ROC curve analysis, both TLR2 and TLR4 expression in corneal epithelial cells showed the best TLRs to diagnose KC [AUC of KC vs Controls to corneal TLR2: 0.882 (CI 95% 0.83-094) $p<0.0001$; AUC of KC vs Controls to corneal TLR4: 0.881 (CI 95% 0.82-0.94) $p<0.0001$] **[Figure 2**].

The TLRs cutoff values for PMD and KC were selected using the coordinates of each ROC curve [conjunctival TLR4 cutoff value for PMD: 3162 (86% sensitivity, 73% specificity); corneal TLR4 cutoff value for PMD: 1972 (70% sensitivity, 70% specificity);

corneal TLR2 cutoff value for KC: 1169 (80% sensitivity, 87% specificity); corneal TLR4 cutoff value for KC: 2516 (85% sensitivity, 81% specificity)]. These cutoff points were used in the logistic regression analysis. **Table 2** show the results of the logistic regression model performed for evaluating TLR2 and TLR4 expression as potential diagnostic biomarkers for ectatic disorders (PMD and KC).

Conjunctival TLR4 values ≥ 3162 UFA were independently associated to PMD diagnosis after adjusting by by rubbing and K2 [OR 42.84; 95%CI: 6.20-296.2; p<0.0001]; while a corneal TLR2 values ≥ 1169 UFA were independently associated to KC diagnosis [OR 27.98; 95%CI: 2.58-303.61; p=0.006] after adjusting by rubbing and K2. These cutoffs of TLR biomarkers represent the highest predictive values for the diagnosis of PMD and KC.

3.4 Relationship between TLRs expression and severity of corneal ectasia.

Table 3 shows the bivariate correlations between TLR2/TLR4 expression and several quantitative topographic, aberrometric and tomographic parameters involved in the severity of corneal ectasia (KPI, I-S asymmetry, $Z_3^{\pm 1}$, coma-like and PE for the PMD group; PDmax, KPI, I-S asymmetry, AE, PE and MTP for the KC group).

In patients with PMD, we observed a strong positive correlation between TLRs and KPI, I-S asymmetry and $Z_3^{\pm 1}$ values; whereas the coma-like and PE positively correlated with all the innate immune biomarkers except with conjunctival TLR2. In patients with KC, the expression of TLRs negatively correlated with MTP and positively correlated with PDmax, KPI, I-S asymmetry, AE and PE.

3.5 TLRs expression after a follow-up period of 19 months.

At 19±4 months after the initial examination, 56 participants (110 eyes) attended again for a follow-up review. Not all patients from the initial examination were included due to loss of follow-up. The sample used for this analysis is made up of 16 control subjects (32 healthy eyes), 11 PMD patients (21 eyes) and 29 KC patients (57 eyes). The statistically significant TLR2/TLR4 expression differences initially observed between groups were also maintained on the follow-up (*data not shown*). **Figure 3** represent case by case the variations obtained between the initial and the follow-up expression of TLR2 and TLR4. These graphs show a tendency to decrease in the corneal expression and a generalized tendency to increase in the conjunctival expression. This was the same pattern for all study groups. No morphological, topographic or tomographic changes were observed in the follow-up review to any study group (*data not shown*).

4. DISCUSSION

We have previously described the overexpression of TLR2 and TLR4 in corneal and conjunctival epithelial cells from KC patients compared to control subjects, but this expression remained unknown in other corneal ectatic diseases. The fact that PMD is a less common condition means that the study of its pathophysiology is more limited. To the best of our knowledge, this is the first study to evaluate the expression of TLR2 and TLR4 in corneal and conjunctival epithelial cells from PMD patients. Thanks to it, we have confirmed that PMD patients, similarly as KC patients, show a clear overexpression of TLR2 and TLR4 compared to control subjects. The innate immunity alteration observed in both types of corneal ectasia support the claim by the Global Consensus on Keratoconus and Ectatic Diseases (Gomes et al. 2015) that PMD and KC could be different clinical presentations of the same disease, representing a common end point of similar pathological processes.

In order to determine the molecular differences between PMD and KC, only (Pásztor et al. 2016) have studied the concentration of a variety of tear inflammatory mediators presents in both diseases. These researchers concluded that MMP9 was the only mediator which showed relevant differences between both groups of patients so that the ratio between this metalloprotease and its inhibitor (TIMP1) was greater in the case of PMD compared to both KC and control subjects (Pásztor et al. 2016). Therefore, it seems that similarities between PMD and KC abound at an inflammatory level. Regarding the innate immunity, in this study we have demonstrated that all TLRs studied are good diagnostic biomarkers to corneal ectatic diseases, although there are some differences between PMD and KC. Corneal and conjunctival TLR4 alteration play a greater role than the TLR2 expression in the PMD pathophysiology. In contrast, the results obtained in KC patients show that the corneal TLR2 and TLR4 alteration seems to be more relevant than the conjunctival one. In fact, conjunctival TLR4 value shows the highest capacity to diagnose the existence of PMD, while the bests KC biomarker is corneal TLR2. Perhaps, these results in the expression of innate immune biomarkers account for the morphological differences that exist between both types of corneal ectasia.

The correlation study with several parameters that act as indicators of corneal ectasia severity such as PDmax, KPI, I-S asymmetry, $Z_3^{\pm 1}$, coma-like, MTP, AE and PE (Choi and Kim 2012; Colak et al. 2016; Delgado et al. 2016; Gomes et al. 2015), demonstrate that the more advanced the disease is, the greater the expression of toll-like receptors will be. Thus, corneal and conjunctival TLR2 and TLR4 could act not only as diagnostic

biomarkers of corneal ectasia, but also as prognostic biomarkers that are supplementary to existing topographic and tomographic techniques.

According to our hypothesis, the expression of TLRs could be higher over time if the disease progresses. In the 19-month follow-up review, we have not observed any topographic-tomographic changes that show the progression of the disease; therefore, according to our hypothesis, the corneal TLR values should remain stable. However, our results showed a special decrease in TLR expression on corneal cells from KC patients 19 months after the initial examination. We did not find a plausible explanation about this result; however, we can speculate that the educational advice provided in the initial consultation allowed the reduction of the corneal immune-inflammatory environment. As educational advice, we have recommended that patients to avoid rubbing their eyes, to increase ocular lubrication (most of the artificial tears have anti-inflammatory components), and that contact lens wearers to not exceed the recommended hours of use. Regarding to the tendency to increase that the TLR2/TLR4 conjunctival expression profile showed at 19 months after the initial examination; knowing that our initial samples were analyzed predominantly during the warm months of the year, we could speculate that the conjunctival variations agree with the hypothesis of (Bind et al. 2016) who postulate that an temperature increase implies the hypomethylation of the gene responsible for transcribing TLR2 protein and, consequently, its underexpression. Nevertheless, (Khoo et al. 2011) show that vitamin D3, whose presence is directly proportional to sun exposure, can induce an TLR2/TLR4 increase. Both hypotheses are contradictory and it not clearly explains the different tendencies obtained in our study but, in any case, it is important to note that all the statistical differences observed in the first review between study groups (control, PMD and KC) were maintained in the follow-up, supporting that the variations of TLRs expression profile do not modify their high diagnostic capacity for predict corneal ectatic disease.

To the above said, we should add the possibility that any comorbidity of an inflammatory nature synergistically aggregates to the inflammation associated to both corneal ectasias thus exacerbating its pathogenic process (Balasubramanian, Pye, and Willcox 2013; Lema et al. 2008). As to itching and eye rubbing, our study also confirmed that they are risk factors associated to the development of corneal ectasia. Intense rubbing is known to increase the levels of proteases or inflammatory mediators on the ocular surface (Balasubramanian et al. 2013). Several studies confirm the high prevalence of eye rubbing in patients with ectasia (Lindsay, Bruce, and Gutteridge 2000; Naderan et al. 2015; Weed et al. 2008), suggesting that this rubbing could be associated to its pathogenesis and

progression. Moreover, it was described that the global impact of allergy is on the rise, with over 40% of the population currently suffering from some level of hypersensitivity; furthermore, recent studies predict that this incidence will continue to rise, with up to 50% of Europe projected to suffer from some form of allergy by 2025 (Aydin et al. 2019). Regarding the corneal ectasia, an allergy disease prevalence of 53% is estimated in KC patients, and PMD patients has also been associated with presence of allergic disease (Shimazaki et al. 2016; Zadnik et al 1998). Despite many studies confirm that allergy disease represent a factor associated to the corneal ectatic severity and to the triggering of a earliest disease presentation (Naderan et al. 2017; Shajari et al. 2016); recent studies suggest that allergy disease was only associated indirectly because the itch that it induced led to eye rubbing (Bawazeer et al. 2000; Ben-Eli et al. 2019). Our study showed that the presence of allergy was similar in all groups under study and not demonstrate any correlation with TLRs expression, supporting the hypothesis that eye rubbing may be a more relevant factor of KC than the presence of allergic condition.

The molecular changes associated to corneal ectasia are not only reflected on the ocular surface, but they can also be reflected on a systemic level. (Toprak et al. 2014) reported that in the pathogenesis of KC, oxidative stress is not confined to a local role but there are also blood abnormalities to the oxidative and antioxidant states. In line with these results, our working group demonstrated the overexpression of TLR2 and TLR4 in monocytes and neutrophils in patients with KC compared to control subjects, also finding a strong correlation with the serum levels of IL-1β, IL-6, TNFα, MMP9 and NF-κB (Sobrino et al. 2017). Therefore, we postulate that the inflammation found in corneal ectasia could be innate immune-dependent as is the case in other degenerative diseases. We could be addressing a progressive chronic process in which the alteration of the immunomodulation and repair lead to tissue destruction.

Our study has limitations. Specifically, the potential limitation of cell collection by scrapping technique in the ocular surface is that does not guarantee that all cells analyzed are pure populations of epithelial cells, and not resident immune cells such as dendritic cells or lymphocytes. Although the location of the scraping was performed in the cornea and in the conjunctiva where a high percentage of epithelial cells are localizated, we cannot exclude the existence of resident immune cells. Therefore, further studies are necessary to detect or not resident immune cells in the cellular scraping.

5. CONCLUSIONS

In conclusion, this study shows for first time that TLR2 and TLR4 are overexpressed in corneal and conjunctival epithelial cells of PMD patients, and confirms that they are also overexpressed in KC patients; demonstrating their key role as diagnostic target in both diseases. Furthermore, we found an association between TLR2 and TLR4 expression in both corneal and conjunctival epithelial cells with the severity of PMD and KC. Therefore, due to the importance of both TLRs have showed in PMD and KC patients, future studies should even focus their attention on these biomarkers as therapeutic target.

REFERENCES

Alió, J.L., Shabayek, M.H., 2006. Corneal Higher Order Aberrations: A Method to Grade Keratoconus. J. Refract. Surg. 22, 539–45.

Aydin, E., Gokhale, M., Azizoglu, S., Suphioglu, C., 2019. To See or Not to See: A Systematic Review of the Importance of Human Ocular Surface Cytokine Biosignatures in Ocular Allergy. Cells. 8, 620.

Balasubramanian, S.A., Pye, D.C., Willcox, M.D., 2013. Effects of Eye Rubbing on the Levels of Protease, Protease Activity and Cytokines in Tears: Relevance in Keratoconus. Clin. Exp. Optom. 96, 214–18.

Balasubramanian, S.A., Mohan, S., Pye, D.C., Willcox, M.D., 2012. Proteases, Proteolysis and Inflammatory Molecules in the Tears of People with Keratoconus. Acta Ophthalmol. 90, 303–9.

Bawazeer, A. M., Hodge, W. G., Lorimer, B., 2000. Atopy and keratoconus: a multivariate analysis. Br. J. Ophthalmol. 84, 834–836.

Ben-Eli, H., Erdinest, N., Solomon, A., 2019. Pathogenesis and complications of chronic eye rubbing in ocular allergy. Curr. Opin. Allergy. Clin. Immunol. 19, 526–534.

Belin, M.W., Asota, I.M., Ambrosio, R.Jr., Khachikian, S.S., 2011. What's in a Name: Keratoconus, Pellucid Marginal Degeneration, and Related Thinning Disorders. Am. J. Ophthalmol. 152, 157–62.

Bind, M.C., Coull, B.A., Baccarelli, A., et al., 2016. Distributional Changes in Gene-Specific Methylation Associated with Temperature. Environ. Res. 150, 38–46.

Chaerkady, R., Shao, H., Scott, S.G., Pandey, A., Jun, A.S., Chakravarti, S., 2013. The Keratoconus Corneal Proteome: Loss of Epithelial Integrity and Stromal Degeneration. J. Proteomics. 87, 122–31.

Choi, J.A., Kim, M.S., 2012. Progression of Keratoconus by Longitudinal Assessment with Corneal Topography. Invest. Opthalmol. Vis. Sci. 53, 927–35.

Colak, H.N., Kantarci, F.A., Yildirim, A., et al., 2016. Comparison of Corneal Topographic Measurements and High Order Aberrations in Keratoconus and Normal Eyes. Cont. Lens Anterior Eye. 39, 380–84.

Delgado, S., Velazco, J., Delgado Pelayo, R.M., Ruiz-Quintero, N., 2016. "Correlation of Higher Order Aberrations in the Anterior Corneal Surface and Degree of Keratoconus Measured with a Scheimpflug Camera. Arch. Soc. Esp. Oftalmol. 91, 316–19.

Efron, N., 1998. Grading Scales for Contact Lens Complications. Ophthalmic Physiol. Opt. 18, 182–86.

Fam, H.B., Lim, K.L., 2006. Corneal Elevation Indices in Normal and Keratoconic Eyes. J. Cataract Refrac. Surg. 32, 1281–87.

Fuchihata, M., Maeda, N., Toda, R., Koh, S., Fujikado, T., Nishida, K., 2014. Characteristics of Corneal Topographic and Pachymetric Patterns in Patients with Pellucid Marginal Corneal Degeneration. Jpn. J. Ophthalmol. 58, 131–38.

Gomes, J.A., Tan, D., Rapuano, C.J., et al., 2015. Global Consensus on Keratoconus and Ectatic Diseases. Cornea. 34, 359–69.

Kawai, T., Akira, S., 2007. TLR Signaling. Semin. Immunol. 19, 24–32.

Khoo, A.L., Chai, L.Y., Koenen, H.J., et al., 2011. Regulation of Cytokine Responses by Seasonality of Vitamin D Status in Healthy Individuals. Clin. Exp. Immunol. 164, 72–79.

Koc, M., Tekin, K., Inanc, M., Kosekahya, P., Yilmazbas, P., 2018. Crab claw pattern on corneal

topography: Pellucid marginal degeneration or inferior keratoconus?. Eye. 32, 11–18.

Kolozsvári, B.L., Petrovski, G., Gogolák, P., et al., 2014. Association between Mediators in the Tear Fluid and the Severity of Keratoconus. Ophthalmic Res. 51, 46–51.

Krachmer, J.H., Feder, R.S., Belin, M.W., 1984. Keratoconus and Related Noninflammatory Corneal Thinning Disorders. Surv. Ophthalmol. 28, 293–322.

Kymes, S.M., Walline, J.J., Zadnik, K., Sterling, J., Gordon, M.O., Collaborative Longitudinal Evaluation of Keratoconus Study Group, 2008. Changes in the Quality-of-Life of People with Keratoconus. Am. J. Ophthalmol. 145, 611–17.

Lee, B.W., Jurkunas, U.V., Harissi-Dagher, M., Poothullil, A.M., Tobaigy, F.M., Azar, D.T., 2007. Ectatic Disorders Associated with a Claw-Shaped Pattern on Corneal Topography. Am. J. Ophthalmol. 144, 154–56.

Lema, I., Duran, J.A., 2005. Inflammatory Molecules in the Tears of Patients with Keratoconus. Ophthalmology. 112, 654–59.

Lema, I., Duran, J.A., Ruiz, C., Díez-Feijoo, E., Acera, A., Merayo, J., 2008. Inflammatory Response to Contact Lenses in Patients with Keratoconus Compared with Myopic Subjects. Cornea. 27, 758–63.

Lema, I., Sobrino, T., Duran, J.A., Brea, D., Diez-Feijoo, E., 2009. Subclinical Keratoconus and Inflammatory Molecules from Tears. Br. J. Ophthalmol. 93, 820–24.

Lema, I., Brea, D., Rodríguez-González, R., Díez-Feijoo, E., Sobrino, T., 2010. Proteomic Analysis of the Tear Film in Patients with Keratoconus. Mol. Vis. 16, 2055–61.

Lindsay, R.G., Bruce, A.S., Gutteridge, I.F., 2000. Keratoconus Associated with Continual Eye Rubbing Due to Punctal Agenesis. Cornea. 19, 567–69.

Maeda, N., Klyce, S.D., Smolek, M.K., Thompson, H.W., 1994. Automated Keratoconus Screening with Corneal Topography Analysis. Invest. Ophthalmol. Vis. Sci. 35, 2749–57.

Malfeito, M., Regueiro, U., Pérez-Mato, M., Campos, F., Sobrino, T., Lema, I., 2019. Innate Immunity Biomarkers for Early Detection of Keratoconus. Ocul. Immunol. Inflamm. 27, 942-48.

Martínez-Abad, A., Piñero, D.P., 2019. Pellucid marginal degeneration: Detection, discrimination from other corneal ectatic disorders and progression. Cont. Lens. Anterior Eye. 42, 341–349.

Naderan, M., Rajabi, M.T., Zarrinbakhsh, P., Bakhshi, A., 2017. Effect of Allergic Diseases on Keratoconus Severity. Ocul. Immunol. Inflamm. 25, 418–23.

Naderan, M., Shoar, S., Rezagholizadeh, F., Zolfaghari, M., Naderan, M., 2015. Characteristics and Associations of Keratoconus Patients. Cont. Lens Anterior Eye. 38, 199–205.

Pásztor, D., Kolozsvári, B.L., Csutak, A., et al., 2016. Tear Mediators in Corneal Ectatic Disorders. PLoS One. 11, e0153186.

Rabinowitz, Y.S., 1995. Videokeratographic Indices to Aid in Screening for Keratoconus. J. Refract. Surg. 11, 371–79.

Rao, S.N., Raviv, T., Majmudar, P.A., Epstein, R.J., 2002. Role of Orbscan II in Screening Keratoconus Suspects before Refractive Corneal Surgery. Ophthalmology. 109, 1642–46.

Rebenitsch, R.L., Kymes, S.M., Walline, J.J., Gordon, M.O., 2011. The Lifetime Economic Burden of Keratoconus: A Decision Analysis Using a Markov Model. Am. J. Ophthalmol. 151, 768–73.

Shajari, M., Eberhardt, E., Müller, M., et al., 2016. Effects of Atopic Syndrome on Keratoconus. Cornea. 35, 1416–20.

Shimazaki, J., Maeda, N., Hieda, O., et al., 2016. National survey of pellucid marginal corneal

degeneration in Japan. Jpn. J. Ophthalmol. 60, 341–348.

Sobrino, T., Regueiro, U., Malfeito, M., et al., 2017. Higher Expression of Toll-like Receptors 2 and 4 in Blood Cells of Keratoconus Patiens. Sci. Rep. 7, 12975.

Takeda, K., Akira, S., 2005. Toll-like Receptors in Innate Immunity. Int. Immunol. 17, 1–14.

Toprak, I., Kucukatay, V., Yildirim, C., Kilic-Toprak, E., Kilic-Erkek, O., 2014. Increased Systemic Oxidative Stress in Patients with Keratoconus. Eye (London). 28, 285–89.

Weed, K.H., MacEwen, C.J., Giles, T., Low, J., McGhee, C.N., 2008. The Dundee University Scottish Keratoconus Study: Demographics, Corneal Signs, Associated Diseases and Eye Rubbing. Eye (London). 22, 534–41.

Zadnik, K., Barr, J.T., Edringtonet, T.B., et al., 1998. Baseline Findings in the Collaborative Longitudinal Evaluation of Keratoconus (CLEK) Study. Invest. Ophthalmol. Vis. Sci. 39, 2537–2546.

TABLES

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

	Control n= 72 eyes n= 36 subjects	**PMD** n= 29 eyes n= 15 patients	**p***	**KC** n= 109 eyes n= 60 patients	**p***	**p†**
Age of diagnosis (years)		32.93 ± 7.5		22.95 ± 5.82		**<0.0001**
Itching (%)	27.8	86.2	**<0.0001**	82.6	**<0.0001**	0.680
Rubbing (%)	33.3	86.2	**<0.0001**	71.6	**<0.0001**	0.125
Allergic disease (%)	47.2	40.0	0.639	60.0	0.228	0.169
DCP (D)	44.05 ± 1.65	45.36 ± 6.15	0.101	49.19 ± 5.67	**<0.0001**	**0.002**
K2 (D)	44.32 ± 1.75	47.72 ± 5.17	**<0.0001**	49.42 ± 4.28	**<0.0001**	0.076
PDmax (D)	44.72 ± 1.80	51.34 ± 6.30	**<0.0001**	53.30 ± 5.33	**<0.0001**	0.097
KPI Index (%)	0 [0, 0]	70 [55, 86]	**<0.0001**	100 [87, 100]	**<0.0001**	**<0.0001**
I-S Asymmetry (D)	-0.09 ± 0.51	7.19 ± 5.27	**<0.0001**	5.69 ± 3.75	**<0.0001**	0.096
$Z_3^{\pm 1}$ (µm)	0.29 ± 0.15	3.00 ± 3.31	**<0.0001**	3.34 ± 9.92	**0.01**	0.863
Coma-like (µm)	0.35 ± 0.14	4.24 ± 5.43	**<0.0001**	4.33 ± 17.23	**0.05**	0.980
MTP (µm)	556 ± 37	474 ± 99	**<0.0001**	453 ± 62	**<0.0001**	0.169
dMTP (mm)	0.85 ± 0.39	1.48 ± 0.72	**<0.0001**	1.01 ± 0.27	**0.003**	**<0.0001**
PE (µm)	28.0 ± 9.8	117.1 ± 71.7	**<0.0001**	94.7 ± 41.9	**<0.0001**	**0.042**
AE (µm)	9.8 ± 5.4	62.1 ± 40.4	**<0.0001**	41.2 ± 18.4	**<0.0001**	**<0.0001**
TLR2 cornea (AFU)	752 ± 629	1057 ± 510	**0.023**	2402 ± 1819	**<0.0001**	**<0.0001**
TLR4 cornea (AFU)	1768 ± 1163	2779 ± 1027	**<0.0001**	4172 ± 2018	**<0.0001**	**0.001**
TLR2 conjunctiva (AFU)	806 ± 412	1038 ± 609	**0.031**	1357 ± 1035	**<0.0001**	0.120
TLR4 conjunctiva (AFU)	2669 ± 1441	3969 ± 894	**<0.0001**	4374 ± 1723	**<0.0001**	0.230

Note: Significant P-values are given in bold. **p*=** compared to control group; **p†=** compared to PMD group.

Abbreviations: PMD = pellucid marginal degeneration; KC= keratoconus; DCP= dioptric central power; D=diopters; K2= steeper corneal meridian; PDmax= maximum dioptric power; KPI Index= KC prediction index; I-S asymmetry= inferior-superior asymmetry; $Z_3^{\pm 1}$= vertical coma; MTP= minimum thickness point; dMTP= distance from the corneal center to the minimum thickness point; PE = posterior elevation; AE= anterior elevation; TLR2= Toll-like Receptor 2; AFU= arbitrary fluorescence units; TLR4=Toll-like Receptor 4.

Table 2. Logistic Regression model of TLRs expression in corneal and conjunctival epithelial cells for PMD and KC.

	PMD		KC	
	OR (CI 95%) p value Unadjusted model	OR (CI 95%) p value *Adjusted model	OR (CI 95%) p value Unadjusted model	OR (CI 95%) p value *Adjusted model
TLR2 cornea	4.34 (1.68-11.21) = 0.002	2.91 (0.71-11.89) = 0.137	26.22 (10.65-64.57) <0.0001	27.98 (2.58-303.61) = 0.006
TLR4 cornea	5.44 (2.12-13.98) <0.0001	3.75 (0.97-14.48) = 0.055	24.67 (10.23-59.48) <0.0001	23.79 (1.97-287.40) = 0.013
TLR2 conjunctiva	1.89 (0.79-4.54) = 0.155	0.17 (0.026-1.11) = 0.064	9.53 (4.44-20.43) <0.0001	8.68 (2.24-33.66) = 0.002
TLR4 conjunctiva	16.78 (5.16-54.58) <0.0001	42.84 (6.20-296.2) <0.0001	8.99 (4.23-19.11) <0.0001	6.26 (1.69-23.24) = 0.006

Abbreviations: PMD= pellucid marginal degeneration; KC= keratoconus; OR= odd ratio; CI= confidence interval; TLR2= toll-like receptor 2; TLR4= toll-like receptor 4.

***** Adjusted by eye rubbing and steeper corneal meridian (K2); a common logistic regression model was performed for TLR2 and TLR4 due to have common metabolic pathways but it was made separately between cornea and conjunctiva because, although both are integrated into the ocular surface, there are marked differences in the regulatory mechanisms of each tissue clearly represented by the corneal avascularity.

Table 3. Bivariate correlations between TLR2/TLR4 expression and several quantitative topographic, aberrometric and tomographic parameters involved in the severity of corneal ectasia.

PMD	TLR2 cornea	TLR4 cornea	TLR2 conjunctiva	TLR4 conjunctiva
KPI	0.232 (p=0.013)	0.330 (p=0.001)	0.207 (p=0.023)	0.364 (p<0.0001)
I-S asymmetry	0.239 (p=0.011)	0.263 (p=0.006)	0.193 (p=0.031)	0.301 (p=0.002)
$Z_3^{\pm 1}$	0.336 (p=0.001)	0.217 (p=0.018)	0.199 (p=0.027)	0.280 (p=0.003)
Coma-like	0.351 (p<0.0001)	0.219 (p=0.017)	0.124 (p=0.116)	0.277 (p=0.003)
PE	0.214 (p=0.020)	0.286 (p=0.003)	0.144 (p=0.085)	0.304 (p=0.002)
KC	**TLR2 cornea**	**TLR4 cornea**	**TLR2 conjunctiva**	**TLR4 conjunctiva**
PDmax	0.396 (p<0.0001)	0.439 (p<0.0001)	0.253 (p=0.001)	0.289 (p<0.0001)
KPI	0.539 (p<0.0001)	0.575 (p<0.0001)	0.323 (p<0.0001)	0.455 (p<0.0001)
I-S asymmetry	0.448 (p<0.0001)	0.453 (p<0.0001)	0.280 (p<0.0001)	0.398 (p<0.0001)
AE	0.531 (p<0.0001)	0.498 (p<0.0001)	0.297 (p<0.0001)	0.334 (p<0.0001)
PE	0.471 (p<0.0001)	0.503 (p<0.0001)	0.250 (p=0.001)	0.338 (p<0.0001)
MTP	-0.435 (p<0.0001)	-0.468 (p<0.0001)	-0.277 (p<0.0001)	-0.385 (p<0.0001)

Abreviations: PMD= pellucid marginal degeneration; TLR2= toll-like receptor 2; TLR4= toll-like receptor 4; KPI= KC prediction index; I-S asymmetry= inferior-superior asymmetry; $Z_3^{\pm 1}$= vertical coma; PE= posterior elevation; KC= keratoconus; PDmax= maximum dioptric power; AE= anterior elevation; MTP= minimum thickness point.

FIGURE LEGENDS

Figure.1. TLR2/TLR4 expression in cells of the corneal and conjunctival epithelium for each study group from baseline visit. Statistical differences with regard to= * control, # all groups. PMD= pellucid marginal degeneration; KC= keratoconus; TLR2= Toll-like receptor 2; TLR4=Toll-like receptor 4; AFU= arbitrary fluorescence units. Data are shown as mean±SD.

Figure 2. Receiver operating characteristic (COR) curve analysis of TLR4 expression in conjunctival and corneal epithelial cells for predicting with the highest sensitivity and specificity the probability of existence of PMD compared to control subjects. Receiver operating characteristic (COR) curve analysis of TLR2 and TLR4 expression in corneal epithelial cells for predicting with the highest sensitivity and specificity the probability of existence of KC compared to control subjects. PMD= Pellucid Marginal Degeneration; KC= Keratoconus; TLR2= Toll-like Receptor 2; TLR4=Toll-like Receptor 4; AFU= arbitrary fluorescence units.

Figure.3. Changes in corneal/conjunctival TLR2/TLR4 for each study patient 19±4 months after the initial examination. Dots represent the initial TLR2/TLR4 values for each patient. Control group subjects are represented on the left side of the graph, PMD group on the central part and KC group on the right. For each dot, an arrow represents the increase (green) or decrease (red) in the expression of TLRs at follow-up review. PMD= Pellucid Marginal Degeneration; KC= Keratoconus; TLR2= Toll-like Receptor 2; TLR4=Toll-like Receptor 4; AFU= arbitrary fluorescence units. **[COLOUR FIGURE]**

FIGURE 1

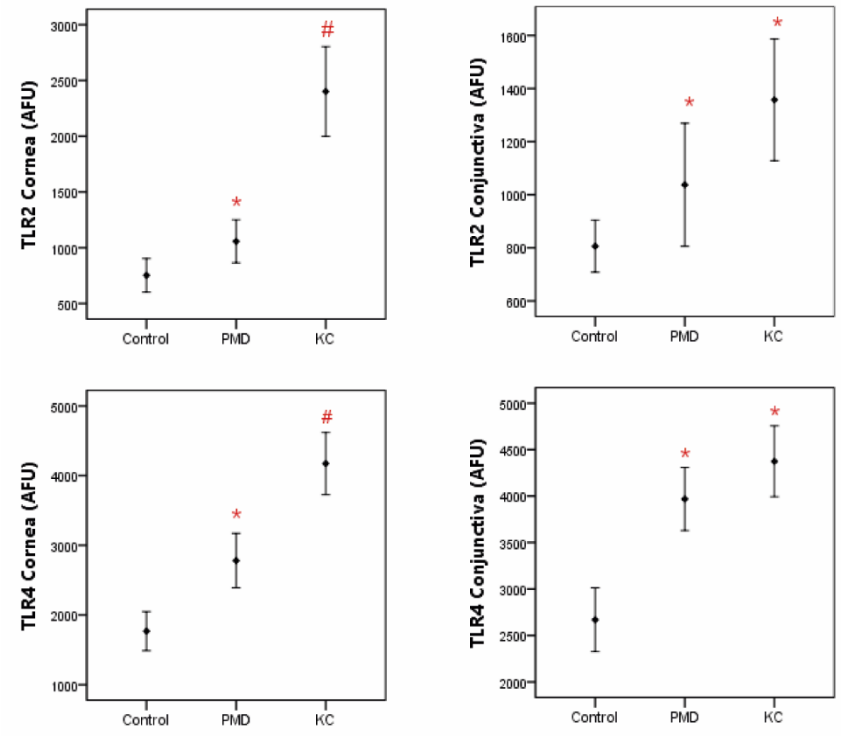
TLR2 Cornea (AFU)
3000
2500
2000
1500
1000
500
#
*
Control
PMD
KC
TLR2 Conjunctiva (AFU)
1600
1400
1200
1000
800
600
*
*
Control
PMD
KC
TLR4 Cornea (AFU)
5000
4000
3000
2000
1000
#
*
Control
PMD
KC
TLR4 Conjunctiva (AFU)
5000
4500
4000
3500
3000
2500
2000
*
*
Control
PMD
KC

FIGURE 2

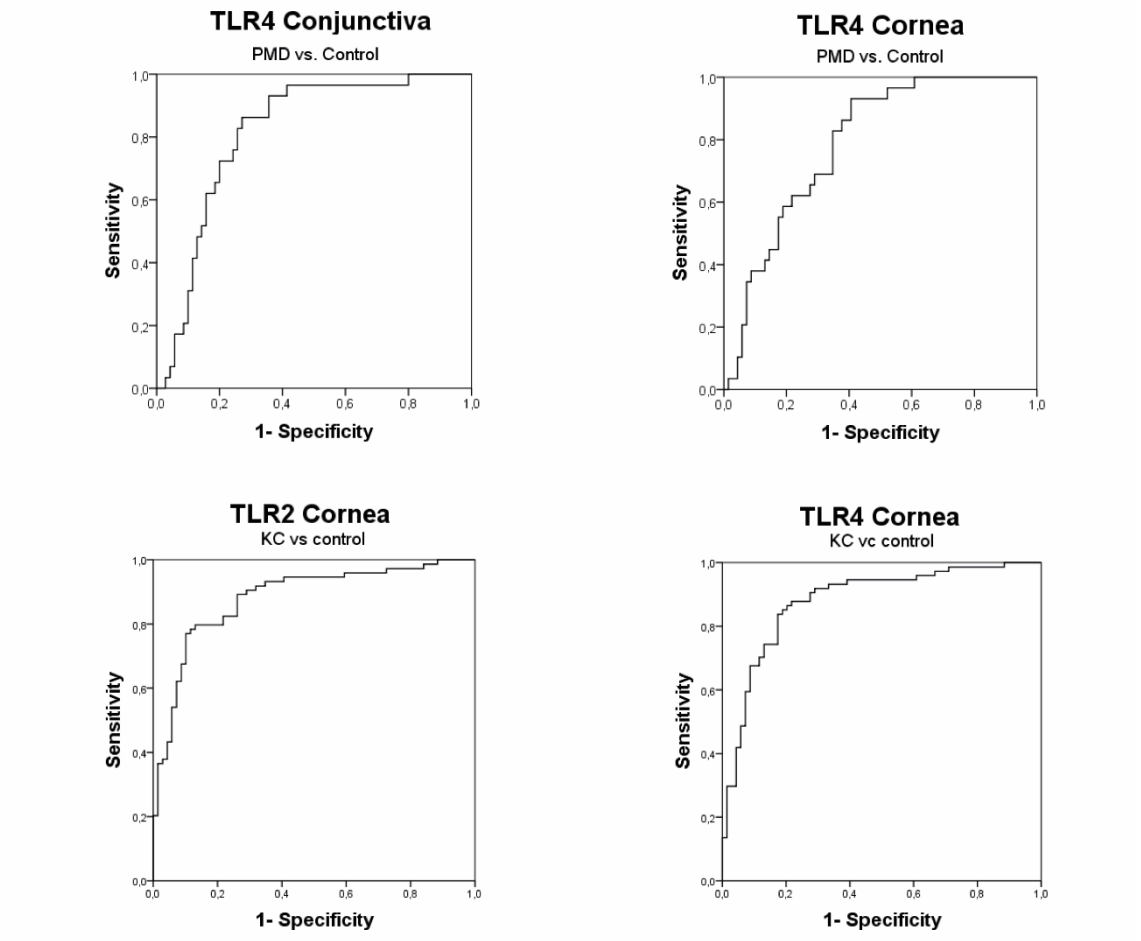
TLR4 Conjunctiva
PMD vs. Control
Sensitivity
1- Specificity
TLR4 Cornea
PMD vs. Control
Sensitivity
1- Specificity
TLR2 Cornea
KC vs control
Sensitivity
1- Specificity
TLR4 Cornea
KC vc control
Sensitivity
1- Specificity

FIGURE 3

(COLOUR FIGURE)