ORIGINAL ARTICLE

Innate Immunity Biomarkers for Early Detection of Keratoconus

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ABSTRACT

Purpose: To compare toll-like receptors 2 (TLR2) and 4 (TLR4) expression in cells of corneal and conjunctival epithelium between unilateral KC patients and control subjects.

Methods: Prospective cross-sectional case-control study, including 50 unilateral KC patients and 20 control subjects. TLR2 and TLR4 expression was measured with flow cytometry.

Results: Mean expression of TLR2 and TLR4, in corneal and conjunctival cells, was significantly higher in KC than in subclinical and control groups (all $p < 0.0001$). TLR2 expression in corneal epithelial cells can predict with the highest sensitivity and specificity the probability of KC existence compared to a control (area under the curve 0.995, 95% CI: 0.987–1.000; $p < 0.0001$). Corneal TLR2 expression also has a predictive capacity to discriminate between subclinical KC and controls (area under the curve 0.989, 95% CI: 0.975–1.000; $p < 0.0001$).

Conclusions: TLR2 and TLR4 expression in corneal and conjunctival epithelial cells may constitute predictive biomarkers for the early detection of KC.

Keywords: Biomarkers, Innate immunity, Toll-like receptor 2, Toll-like receptor 4, Unilateral keratoconus

Keratoconus (KC) is a degenerative condition, which is generally bilateral and progressive. It frequently results in visual loss with an onset in early adulthood.[1] In some cases, KC is unilateral, where KC affects only one eye and the fellow eye remains as forme fruste KC or subclinical KC.[2] Reported frequencies of unilateral KC in recent studies range from 0.5% to 4.5%.[3] The study of unilateral KC can provide insight into the pathogenesis of disease as the examination of the initially healthy eye may prove useful for the detection of changes of the disease.

The cornea is part of an integrated system, the ocular surface, which contains specific and non-specific immune molecules. Tissue degradation in thinning disorders like KC involves the expression of inflammatory mediators such as proinflammatory cytokines, cell adhesion molecules, and matrix metalloproteinases.[4,5] Previous studies by our group have demonstrated that interleukin (IL)-6 and tumor necrosis factor alpha (TNF-α) as well as matrix metalloproteinase-9 (MMP-9) are increased in the tears of patients with KC.[4] However, this overexpression of MMP-9 was not found in subclinical eyes, while increased levels of MMP-9 are associated with the corneal thinning.[6] Decreased levels of lactoferrin (Lf), zinc-α2-glycoprotein (ZAG), and immunoglobulin kappa chain (IGKC) have also been observed in the tears of patients with KC.[7] These molecules are involved in the immunity response. These results, therefore, suggest the involvement of immunological processes in the pathogenesis of

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ecstasies. In fact, the function of humoral and cellular components of the innate and adaptive response is modulated by Lf at the systemic and local level through toll-like receptors (TLRs).[8,9]

TLRs are a family of highly conserved innate immunity receptors that recognize damage-associated molecular patterns from endogenous molecules, which are released as a result of tissue damage.[10] These molecules, otherwise known as endogenous ligands, can activate TLR, thus promoting the recruitment of a number of adaptive proteins to activate nuclear factor-kB, which induces the expression of proinflammatory genes, inflammatory cytokines, and adhesion molecules[11]; these inflammatory markers are associated with KC and subclinical KC.[4,6] Besides, TLR4 is expressed in a variety of eye tissues and cells, including corneal epithelial cells and fibroblasts of the corneal stroma. TLR2 is also expressed in human conjunctival epithelial cells.[9] Importantly, treatment of corneal epithelial cells with neutralizing TLR2-antibodies induced a significant decrease of IL-6, IL-8, and TNF-α levels.[11] Consequently, it is tempting to postulate that the inflammatory response associated to KC might be a consequence of TLR activation. But no test has yet been conducted to ascertain the role of TLRs in patients with KC. The aim of this study was therefore to determine the expression levels of TLR2 and TLR4 in cells of corneal and conjunctival epithelium from patients with unilateral KC and control subjects for demonstrating their potential role as biomarkers for early detection of KC.

MATERIALS AND METHODS

Patients

We have designed a prospective, case-control study consisting of 50 patients with unilateral KC (100 eyes: 50 subclinical KC and 50 KC eyes) and 20 control subjects, with no KC clinical or topographic signs. Data collected included: age, sex, patient´s ocular history, medical history (atopy, eye rubbing), and family history of KC. All examinations were performed by the same researcher.

This study was conducted in accordance with the Declaration of Helsinki of the World Medical Association (2008) and had the approval of the Ethics Committee of the Servizo Galego de Saúde (Code: 2015/436). Both patients and control subjects gave their informed consent.

Inclusion criteria: (1) Subclinical KC group: Absence of clinical signs of QC; topographic values proposed by Rabinowitz and McDonnell[12]: central keratometry (<47.2 D), dioptric asymmetry of the lower-upper zone <1.4 D; we also considered the KSS index,[13] mean coma-like 0.35 μm, values of the point of minimum thickness >490 μm, and posterior elevation <0.045 mm,[14] also requiring a KPI <0.23%, a spontaneous AV or with spherocylindrical compensation <1.50 D of 1.0 (decimal Snellen).

2) Eyes with KC met the criteria of Rabinowitz-McDonnell,[12] and KSS index,[13] and presence of one or more typical biomicroscopic signs of KC.

3) Control subjects did not show biomicroscopic signs of KC, irregular astigmatism, or topographic values characteristic of KC or suspicion of KC.

Exclusion criteria: previous surgical intervention in the anterior segment, or childhood corneal trauma or corneal-conjunctival disease; existence of active systemic or ocular inflammation, or current treatment with systemic or local anti-inflammatory drugs. Hepatic, renal, hematologic, and immunologic diseases, disorders of thyroid function, uncontrolled diabetes, infections in the days preceding sample collection, and solid tumors. No contact lens wearing 10 days prior to clinical assessment and sampling.

The following quantitative topographic-tomographic parameters were analyzed: simulated central keratometry (Kc), flat meridian (K_1), steep meridian (K_2), maximum keratometry (Kmax), paracentral I-S dioptric difference, high-order aberrations of surface anterior (coma and coma-like), posterior elevation (PE), central corneal thickness (CCT), and minimum thickness point (MTP).

Ophthalmological Instruments

Specific examination instruments: TOPCON CA-100 System corneal topographer (Topcon Medical Systems, Inc., NJ, USA), and an Orbscan IIz corneal topographer (Orbtek, UT, USA).

Extraction of Corneal and Conjunctival Epithelial Cell Samples

Samples for the determination of TLR2 and TLR4 was extracted in the inferior bulbar conjunctiva, close to the limbus and the corneal sample in the lower third of the cornea; by using a surgical ophthalmic lancet (SOFT CELL®, OASIS®, CA, USA). Previously, anesthetic eye drops with 0.5% tetracaine and 0.05% naphazoline was installed.

TLR2 and TLR4 Expression Analysis

The cell samples, collected in cytometry tubes, were subjected to a vortexing process for 30 seconds. Subsequently, the tubes were allowed to stand 15 minutes for the cells to settle in the tube, and the microsponge was removed. Briefly, the expression of TLR2 and TLR4 was analyzed by direct flow cytometry according to the expression of specific surface antigens

(TLR2 and TLR4). Cells were labelled with fluorescein isothiocyanate (FITC) anti-TLR2-conjugated monoclonal antibodies and phycoerythrin (PE) anti-TLR4 conjugates (IMMUNOSTEP, Salamanca, Spain). After incubation, the samples were analyzed on a FACSAria iiu sorter-flow cytometer (BD Biosciences, NJ, USA). Expression of TLR2 and TLR4 in epithelial cells of the conjunctiva (2500 events) and cornea (1000 events) was analyzed using FACSDiva software 6.02 (BD Biosciences, NJ, USA). The results were expressed in arbitrary fluorescence units (AFUs).

The intra-assay coefficient of variation, obtained by a single investigator who performed the cellular study, and calculated by the analysis of two samples of the same patient, was 0.87. The analysis was carried out at the IDIS Clinical Neuroscience Research Laboratory by a blinded molecular biologist for both clinical and topographical data.

Statistical Analysis

The statistical EPIDAT 3.1 software was used for calculating sample size (http://www.sergas.es/ShowContidos_N3_T01.aspx? IdPaxina = 62714), accepting a confidence level of 95% ($\alpha = 0.05$) and 80% power ($\beta = 0.8$).

Results were expressed as percentages for categorical variables and mean (standard deviation) for continuous variables. Proportions were compared using the chi-square, whereas the continuous variables between groups were compared with Student's t. ANOVA test was used to compare the expression of TLR2 and TLR4 between study groups (KC, subclinical, and control).

ROC curve analysis was used to determine the most appropriate cutoff point for detecting control, subclinical and clinical KC from the expression values of TLR2 and TLR4 in corneal epithelial cells. Bivariate correlations were performed using Pearson coefficient. A value of $p < 0.05$ was considered to be statistically significant in all tests. The statistical analysis was conducted in SPSS 21.0 (IBM, Chicago, IL, USA) for Mac.

RESULTS

Clinical Features

No statistical differences were detected regarding sex or age: unilateral KC (64.0% males; mean age; 33.0 ± 9.2 years) and controls (45.9% males; mean age; 29.4 ± 6.7 years) ($p = 0.538$ and $p = 0.109$, respectively); 19 patients (38%) and 7 controls (35.0%) reported atopy ($p = 0.640$); 16 patients (32%) reported a family history of KC ($p < 0.0001$). The lapse of time from the first diagnosis of KC eye and the study ranged from 1 to 30 years (mean, 8.3 ± 6.2 years).

Table 1 shows topographical variables of the different study groups. All topographic values (Kc, K1, K2, Kmax, I-S, coma, and coma-like) were significantly higher in the KC than in subclinical and control groups ($p < 0.0001$). Moreover, subclinical group had I-S, coma, and coma-like values higher than the controls ($p < 0.0001$). Similarly, the PE was higher in the KC group ($p < 0.0001$). Likewise, the subclinical group showed a greater PE than the control ($p = 0.005$). CCT and MTP were lower in KC eyes ($p < 0.0001$); subclinical eyes also showed a lower CCT ($p = 0.002$) and MTP ($p = 0.002$) compared to the control group.

Expression of TLR2 and TLR4

Levels of TLR2 and TLR4 expression are shown in Table 2. TLR2 and TLR4 expression in corneal and conjunctival cells was higher in the KC group ($p < 0.0001$). Moreover, the expression of TLR2 and TLR4 was also higher in subclinical KC compared to the control group (control < subclinical < KC) (Table 2).However, the main difference of TLR expression from the control to subclinical and KC groups was found for TLR2 and TLR4 in corneal cells. Therefore,

TABLE 1. Topographical variables by study groups: control, subclinical, and keratoconus (KC).

Variables	Controls ($N = 40$)	Subclinical ($N = 50$)	p*	KC ($N = 50$)	p*	p**
Kc (D)	43.5 ± 1.8	43.5 ± 1.6	0.887	49.6 ± 5.9	<0.0001	<0.0001
K1 (D)	42.9 ± 1.9	43.0 ± 1.2	0.730	45.3 ± 3.2	<0.0001	<0.0001
K2 (D)	43.6 ± 1.8	43.9 ± 1.3	0.457	49.3 ± 4.7	<0.0001	<0.0001
Kmax (D)	44.1 ± 1.9	45.1 ± 1.3	0.170	53.4 ± 5.0	<0.0001	<0.0001
I–S (mm)	0.21 ± 0.15	0.77 ± 0.5	<0.001	5.03 ± 3.00	<0.0001	<0.0001
Coma (μm)	0.19 ± 0.12	0.29 ± 0.56	0.004	1.65 ± 0.74	<0.0001	<0.0001
Coma-like (μm)	0.24 ± 0.14	0.38 ± 0.56	<0.001	1.81 ± 0.77	<0.0001	<0.0001
Post. elevation (μm)	0.029 ± 0.01	0.036 ± 0.01	0.005	0.12 ± 0.14	<0.0001	<0.0001
CCT (μm)	552.0 ± 37.9	524.6 ± 41.8	0.002	476.4 ± 67	<0.0001	<0.0001
MTP (μm)	541.0 ± 38.7	513.5 ± 44.4	0.001	457.4 ± 69	<0.0001	<0.0001

p*: compared to control group; p**: compared to subclinical group. CCT = central corneal thickness; K2 = steep meridian; KC = keratoconus; MTP = minimum thickness point.

TABLE 2. Mean expression of toll-like receptor (TLR)2 and TLR4 in both corneal and conjunctival epithelial cells by study groups: control, subclinical and keratoconus.

Variables	Control (N = 40)	Subclinical (N = 50)	p*	KC (N = 50)	p*	p**
CORNEA						
TLR2 (AFU)	205.2 ± 111.7	1000.7 ± 443.3	<0.0001	2690.3 ± 1550.3	<0.0001	<0.0001
TLR4 (AFU)	1877.5 ± 891.2	2935.4 ± 1256.9	<0.0001	4319.5 ± 1450.2	<0.0001	<0.0001
CONJUNCTIVA						
TLR2 (AFU)	207.9 ± 130.6	321.8 ± 307.4	0.045	688.6 ± 657.4	<0.0001	0.003
TLR4 (AFU)	1759.3 ± 570.7	2173.8 ± 846.2	0.012	2509.0 ± 883.5	<0.0001	0.043

p*:compared to control group; p**:compared to subclinical group. AFU = arbitrary fluorescence unit; KC = keratoconus; TLR = Toll-like receptor.

predictive analyses were focused exclusively on the expression of TLR2 and TLR4 in corneal cells.

Predictive Value of TLR2 and TLR4 Expression in Corneal Cells for Detecting Early KC

According to the ROC curve analysis, TLR2 expression in corneal epithelial cells may predict with high sensitivity and specificity the probability of KC (both subclinical and KC) compared to controls (area under the curve: 0.995; $p < 0.0001$). Likewise, corneal TLR2 expression is also useful for predicting the probability of subclinical KC compared to the controls (area under the curve 0.989; $p < 0.0001$). Finally, corneal TLR2 expression also predict the probability of subclinical KC compared to the KC (area under the curve 0.893; $p < 0.0001$) (Figure 1a).

Corneal TLR4 expression may also predict with high sensitivity and specificity the probability of KC compared to the controls (area under the curve 0.846; $p < 0.0001$). Likewise, corneal TLR4 expression is also useful for predicting the probability of subclinical KC compared to the controls (area under the curve 0.756; $p < 0.0001$). Finally, corneal TLR4 expression also may predict the probability of subclinical KC compared to the KC (area under the curve 0.767; $p < 0.0001$) (Figure 1b).

Overall, TLR2 expression in corneal cells showed the highest predictive value for detecting the risk of onset of KC than the expression of TLR4.

Relationship between TLR2 and TLR4 Expression and KC Severity

Finally, we analyze the correlation of TLR2 and TLR4 expression with three topographic parameters associated with KC severity: K2, coma and MTP. We found a strong correlation between TLR2 and TLR4 expression with these topographic parameters (Table 3). It is noteworthy that the greatest association was found for the expression of TLR2 and TLR4 in corneal epithelial cells.

DISCUSSION

To the best of our knowledge, this is the first prospective study to evaluate the expression of TLR2 and TLR4 in corneal and conjunctival epithelial cells from KC patients compared to control subjects. Interestingly, TLR2 and TLR4 expression in both corneal and conjunctival epithelium was significantly higher in patients with unilateral KC with respect to controls. Moreover, a strong correlation was found between TLR2 and TLR4 expression in both corneal and conjunctival epithelial cells with topographic parameters of KC severity (K2, coma and MTP). Therefore, our results seem to demonstrate the potential role of innate immunity as therapeutic target for KC.

It is known that the onset of KC occurs in adolescence, and its progression is dependent on age. The unilateral KC patients included in this study showed a higher average age than reported in other studies,[3] and the elapsed time from the diagnosis of KC was 8.3 ± 6.2 years. For this reason, we think that most of our sample will not progress to bilateral KC. In our study, the subclinical and controls showed similar values for topographic variables such as Kc, K_1, K_2, and Kmax, as well as coma and coma-like, coinciding with other studies.[15] Overall, these facts mean strength in order to draw conclusions regarding subclinical KC. On the other hand, the frequency of atopy was similar in KC patients and controls, so this variable should not be influencing our results.

In spite of extensive basic and clinical studies of KC, the precise mechanisms underlying this pathology are not known. Pathophysiologic components of KC can be classified: alterations of the stroma composition, imbalance of proinflammatory and anti-inflammatory molecules, imbalance of the enzymes that cause extracellular matrix degradation and their corresponding inhibitors, oxidative stress, and cellular hypersensitivity. These events occur simultaneously. It is however yet unclear the order in which they occur and which of these events are necessary for the evolution of the disease.[16] In this respect, some clinical studies of KC defend that its pathogenesis involves immune and

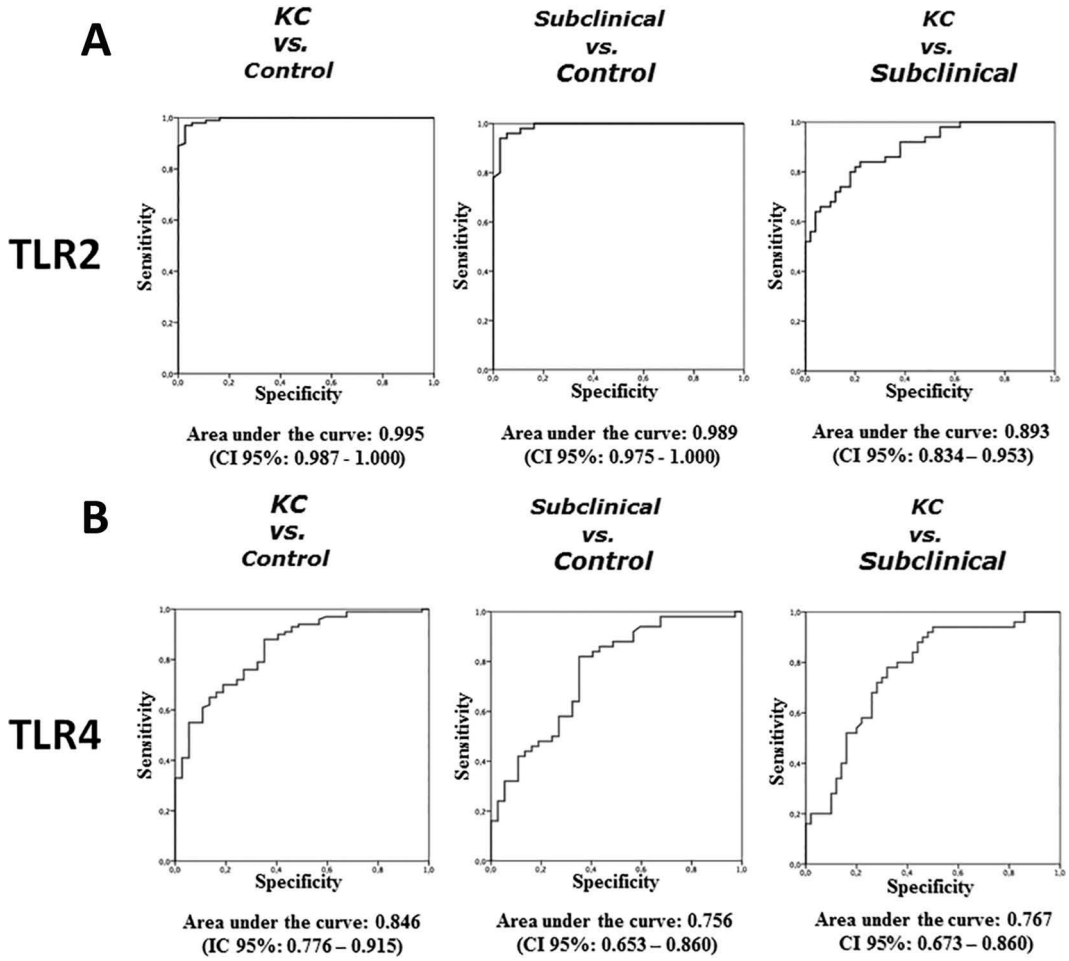

FIGURE 1. Receiver operating characteristic curve analysis of TLR2 (A) and TLR4 (B) expression in corneal epithelial cells for predicting with the highest sensitivity and specificity the probability of existence of KC (subclinical +KC) compared to control subjects, subclinical KC compared to control subjects and KC compared to subclinical. CI = confidence interval; KC = keratoconus; TLR = Toll-like receptor.

TABLE 3. Correlation between toll-like receptor (TLR)2 and TLR4 expression in both corneal and conjunctival epithelial cells with topographic parameters (steep meridian or K2, coma and minimum thickness point [MTP]) of keratoconus severity.

Markers/topography	Pearson coef.	*p*
TLR2 cornea/K2	0.542	<0.0001
TLR4 cornea/K2	0.446	<0.0001
TLR2 conjunctiva/K2	0.258	0.002
TLR4 conjunctiva/K2	0.270	0.001
TLR2 cornea/Coma	0.673	<0.0001
TLR4 cornea/Coma	0.452	<0.0001
TLR2 conjunctiva/Coma	0.308	0.003
TLR4 conjunctiva/Coma	0.229	0.009
TLR2 cornea/MTP	−0.475	<0.0001
TLR4 cornea/MTP	−0.337	<0.0001
TLR2 conjunctiva/MTP	−0.194	0.143
TLR4 conjunctiva/MTP	−0.251	0.003

K2 = steep meridian; MTP = minimum thickness point; TLR = toll-like receptor

inflammatory components.[4–6,17] In addition, more recent studies show that molecular changes associated to KC can be reflected systemically.[18,19] In this sense, previous studies by our group indicated that mean expression of TLR2 and TLR4 in both neutrophils and monocytes was significantly higher in patients with KC than in control subjects.[20] These findings are consistent with our results, which show that an innate immune response may occur in cells of corneal and conjunctival epithelium from patients with KC.

Furthermore, as we have reported elsewhere,[4,6] inflammatory markers (IL-6, TNF-α, and MMP-9) are increased in the tears of KC patients. We also noted decreased levels of Lf, IGKC and ZAG in the tears of patients with KC.[7] These results suggest an involvement of inflammatory and immunological processes in the pathogenesis of KC, even more so when these biomarkers were associated with the severity of the KC. There is evidence that the corneal microenvironment in KC is affected by inflammatory changes.[21] On the other hand, this inflammatory response, mainly mediated by proinflammatory cytokines,[17] could be activated by TLRs through endogenous ligands that promote the recruitment of a number of adaptive proteins to activate nuclear factor-kB, which finally induces the expression of proinflammatory

genes.[10,11] Consequently, it is tempting to postulate that inflammatory response in KC could be a consequence of TLR activation. In this regard, the results of the current study revealed that TLR2 and TLR4 expression in both corneal and conjunctival epithelial cells was significantly higher in KC eyes than in subclinical and control eyes; likewise, TLR2 and TLR4 expression was also higher in the subclinical group than in the control group. Additionally, several cell types, including keratocytes, express IL-6 in response to stimulation by IL-1β or TNF-α, and TLRs increase secretion of IL-6 and TNF-α in corneal fibroblasts.[22] Importantly, TLR2 in corneal epithelial cells was the biomarker more expressed in KC compared to the subclinical and control groups. This differential fact is important because previous studies have demonstrated that the treatment of corneal epithelial cells with neutralizing TLR2-antibodies induced a significant decrease in IL-6, IL-8, and TNF-α levels.[11]

Some studies suggest that the inflammatory response caused by the binding of TLRs to different ligands could be a key therapeutic measure for this immune response.[23,24] TLR-4 is known to be higher in the stroma of patients with vernal keratoconjunctivitis, but no difference in TLR-2 expression.[25]

Finally, we found a strong association between TLR2 and TLR4 expression with the severity of KC quantified by topographic parameters. This fact indicates that innate immunity may play a role in the pathophysiology of KC. Therefore, it is tempting to postulate that TLR2 and TLR4 may be considered potential biomarkers to identify asymptomatic (subclinical) eyes and clinical KC. Moreover, TLR2 and TLR4 can also be considered therapeutic targets in KC through their immunomodulation by blocking with neutralizing antibodies or preventing exposure to molecules stimulating TLRs. However, if a direct antagonist therapy on TLRs is a better option than preventing exposure to activating molecules needs to be elucidated in future studies.

In conclusion, this study demonstrated that TLR2 and TLR4 expression in corneal and conjunctival epithelial cells is higher in eyes with KC than in subclinical and control eyes. Furthermore, we found a strong association between TLR2 and TLR4 expression in both corneal and conjunctival epithelial cells with the severity of KC, quantified by topographic parameters such as K2, coma, and MTP. Therefore, TLR2 and TLR4 expression may constitute predictive biomarkers for the early detection of KC. Finally, these results also suggest that TLRs may play a relevant role as therapeutic target for KC.

DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

FUNDING

This work was supported by the Spanish Ministry of Economy and Competitiveness (Instituto de Salud Carlos III) [grant number PI14/00247]; Xunta de Galicia (Consellería de Economía e Industria) [grant number 10 PX113918156PR] and Consellería de Educación [grant number GRC2014/027]; and the European Union program ERFD. T. Sobrino (CPII17/00027) and F. Campos (CP14/00154) are researchers under a research contract with the Miguel Servet Program of Instituto de Salud Carlos III. Similarly, U. Regueiro was awarded a predoctoral Fellowship from the Xunta de Galicia [grant number ED481A-2015/001].

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