Contact Lens and Anterior Eye 42 (2019) 253–257

Contents lists available at ScienceDirect

Contact Lens and Anterior Eye

journal homepage: www.elsevier.com/locate/clae

Lactoferrin-loaded contact lenses counteract cytotoxicity caused *in vitro* by keratoconic tears

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ARTICLE INFO

Keywords:
Lactoferrin
Keratoconus
Tear cytotoxicity
Contact lenses

ABSTRACT

Lactoferrin (LF), an iron-binding protein with antioxidant activity, is significantly reduced in the lacrimal film of patients affected by keratoconus (KC) compared to healthy subjects, and this is supposed to be the cause for tear iron increase and consequent iron deposition and oxygen free radical accumulation in the cornea. We decided to study if LF-loaded contact lenses (LF-CLs) could exert antioxidant activity on epithelial cells incubated in tears collected from two patients affected by keratoconus (KC1 and KC2). Moreover through this model we indirectly estimated iron concentration in the tears of healthy and KC subjects.

Reflex tears were collected during the first 2 min of light or onion-induced lacrimation and stored at −20 °C. After incubation in tears for 18 h, mortality of epithelial cells was investigated by trypan blue exclusion test. Successively, LF-CLs were deposed on cells incubated in KC1 tears. For the indirect determination of iron content, cells were incubated with LF 1.2 mg/mL and different $FeSO_4$ concentrations, and for the estimation of iron from the patient's tears, cells were incubated with free serum medium and healthy tears (1:1) and different $FeSO_4$ concentrations.

Epithelial cells incubated with reflex tears of KC patients showed increased mortality (27.7 ± 3.9%, $p = 0.0003$, for KC1 and 17.6 ± 0.95%, $p = 0.014$, for KC2) compared to epithelial cells maintained in control healthy tears (8.6 ± 1.2%). This difference in mortality was correlated with tear iron concentration, which was estimated at 4.58 µg/mL for the healthy subjects, at 56.28 µg/mL for KC1, and at 8.7 µg/mL for KC2 patient. Application of LF-CLs counteracted KC tear cytotoxicity restoring viability obtained in the presence of control tears.

Therapeutic contact lenses obtained by LF loading can reduce oxidative stress induced by patients' tears and might represent an efficient device to arrest the progression of keratoconus.

1. Introduction

Many pathologies of the eye anterior segment are characterized by oxidative damage that affects in particular the cornea [1,2]. The production of high levels of free radicals causes lipid, protein and DNA peroxidation, and consequently serious alterations in the tissues that increase the disease severity. Oxidative damage from cytotoxic products has been extensively documented in keratoconus (KC), a corneal ectatic disease characterised by conical protrusion due to cornea thinning, which causes irregular astigmatism and myopia with consequent visual acuity reduction [2–5]. Although corneal stroma thickness decrease, induced by proteolytic activity and keratocyte apoptosis, appears to be the most severe modification the cornea undergoes, KC is generally marked by characteristic structural changes: elongation of the superficial epithelial cells at the apex of the cone, destruction of basal epithelium integrity, break in Bowmans' layer, and iron deposition superficially or within the epithelial layers, which constitute the Fleischer's ring [6–8].

Analysis of corneal buttons from patients undergoing primary keratoplasty or retransplantation showed significant decrease in the principal antioxidant agents of the cornea: thioredoxin reductase, peroxiredoxins, superoxide dismutase (SOD), the glutathione system, and aldehyde dehydrogenase [2,9–11]. Moreover, profiling of tear fluid demonstrated significant reduction in the level of small molecular antioxidants [12,13] and of the protein lactoferrin (LF) (0.7 ± 0.3 mg/mL in KC patients vs. 1.1 ± 0.3 mg/mL in healthy subjects [14]).

LF is a glycoprotein endowed with different activities, which in part depend on its ability of chelating ferric iron (Fe^{3+}) [15–22]. In its iron-

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https://doi.org/10.1016/j.clae.2018.12.004
Received 19 January 2018; Received in revised form 4 December 2018; Accepted 5 December 2018

unsaturated form it was able to suppress oxidative damage in rabbit corneal epithelial cells [23,24], suggesting that deregulation of its secretion by lacrimal glands may increase iron concentration in tears and consequently favour iron deposition and oxygen free radical accumulation within the cornea epithelium [8].

In a previous work we demonstrated that LF absorbed and released from contact lenses protected epithelial cells against cytotoxicity caused by hydrogen peroxide [25]. According to this observation, we decided to verify if the same LF-loaded contact lenses could exert antioxidant activity on epithelial cells incubated in tears collected from patients affected by keratoconus. For experimental reasons, we used reflex tears instead of tears from basal lacrimation. This was possible because literature demonstrates unvaried lactoferrin concentration between the two types of tears [26,27]. Moreover, since it is supposed that iron increases in tears of KC patients compared to healthy subjects, but control- and KC-iron levels are still not documented, in this paper we also estimated these values by using the *in vitro* model we have established.

2. Methods

2.1. Collection of tear samples

The study was approved by University of Milano - Bicocca Ethics Committee (protocol 247) in compliance with Helsinki Declaration and written informed consent was obtained from all participants. Reflex tears were collected within 2 min after onion or light stimulation in 1,5 mL microtubes placed near the inner corner of the eye and were conserved at −20 °C until use. Tears of four healthy subjects (28 ± 3 years old) without ocular pathologies were used as control. Two KC patients who acceded to the study succeeded in collecting a volume of tears sufficient for the experiments. The first subject (named KC1 through the manuscript) was aged 30 and showed a moderate level of pathology in the right eye, which had been diagnosed 4 years before this study. The second subject (KC2) was 41 years old and had a diagnosis of bilateral mild keratoconus two years before the collection of tears. At the moment of the study the disease was stable in both eyes. For confirmation of the altered composition of tears due to the pathology, the total protein content was measured by Bradford method which revealed a drastic reduction compared to the healthy subjects (4.6 ± 2.5 mg/mL in KC tears vs. 8.2 ± 1.4 mg/mL in healthy tears).

2.2. Apolactoferrin preparation and contact lens loading

Native LF (30 mg/mL) (kindly provided by Dr Stefano Farris, Department of Food, Environmental and Nutritional Sciences (DeFENS)—Packaging Lab, University of Milan) was dialyzed with a 15 kDa cut-off membrane (Sigma St. Louis, Mo, USA) against 0.1 M citric acid (pH 2) for 24 h to remove ferric ions. Then it was dialyzed against distilled water for 24 h, followed by dialysis against 25 mM KH_2PO_4 buffer (pH 6.6) containing 25 mM NaCl to prepare apolactoferrin (apoLF). The apoLF concentration was determined by absorbance at 280 nm (A_{280}), while its iron saturation was obtained by A_{280}/A_{465} ratio according to the method previously described [28]. Filcon V (silicone hydrogel) CLs were incubated in an apoLF solution (2 mg/mL) for 5 h at room temperature and the adsorbed apoLF was evaluated by A_{280} measurements of the protein remaining in the solution. As reported in Pastori et al. [25] LF released from each contact lens was about 30 µg. Considering tear film volume of about 10 µL, the concentration obtained corresponds to LF concentration in healthy tears: 3 mg/mL is the concentration of LF theoretically released in the tear film by contact lenses vs. 1–2 mg/mL which is the concentration reported in tears of healthy subjects [14]. LF-loaded CLs were deposed on cells incubated in KC tears; they were placed gently on top of the cellular monolayer, with the concave surface facing upwards and totally immersed in the culture medium.

2.3. Healthy and pathological tears cytotoxicity

To analyze *in vitro* the cytotoxicity of tears from healthy subjects and patients affected by keratoconus, epithelial cells were incubated in tears collected by reflex lacrimation as described in the paragraph *Collection of tear samples*. Although epithelial corneal cells would represent the most adequate model for this study, for experimental reasons we used TsA cells which present a similar behavior under oxidative stress [25]. TsA (human kidney epithelial) cells, derived from HEK 293 cell line, were cultured in DMEM/F12 medium containing 10% fetal bovine serum (FBS) and 2 mM glutamine (Sigma St. Louis, Mo, USA) and incubated at 37 °C in a humidified atmosphere with 5% CO_2. TsA cells were seeded onto 24-well tissue culture plates at density 45×10^3 cell/well. After 24 h, the medium was substituted with 300 µL of a serum-free medium or healthy tears or pathological tears. Viability test was performed after 18 h by trypan blue (0.4% w/v) exclusion test according to the procedure described in Thermofisher user manual: dead cells that incorporated the colorant were observed by microscope and counted.

To confirm that the ability to counteract cell death was provided by the unique properties of LF, control experiments were performed by inducing oxidative stress in TsA cells with 250 µM H_2O_2, as reported in Pastori et al. [25], and by evaluating the effects of CLs incubated in phosphate buffer, albumin solution (2 mg/mL) or apoLF solution (2 mg/mL) on cell viability. CLs loaded with buffer or albumin were not able to protect cells against oxidative stress, confirming that the improvement in cellular viability observed in our experiments was due to lactoferrin activity (Table 1).

2.4. Oxidative stress determination in human tears

TsA cells were seeded onto 24-well tissue culture plates at density 45×10^3 cell/well. After 48 h cells were incubated for 30 min with 20 µM DCFDA (2′,7′–dichlorofluorescin diacetate), a fluorogenic dye that measures hydroxyl, peroxyl and other reactive oxygen species (ROS) activity within the cells [29]. After incubation cells were treated with healthy or keratoconic tears, or 250 µM H_2O_2 as positive control, and their fluorescence intensity was measured by flow cytometer (CytoFlex, Beckman Coulter Inc.) at different times (5, 10, 20, 30, 40 and 60 min). Data analysis was performed by CytExpert software and Excel.

2.5. Indirect determination of iron content in tears

TsA cells were seeded onto 24-well tissue culture plates at density 45×10^3 cell/well. After 24 h, the medium was substituted with a serum-free medium and the cells were incubated with apoLF 1.2 mg/mL and different $FeSO_4$ concentrations (1.2, 2.4, 4.8, 9.6, 35 or 96 µg/mL) in order to obtain a calibration curve for the evaluation of iron concentration in healthy tears. For the estimation of iron from the patient's tears, cells were incubated with "model KC tears" composed by 50% of free serum medium and 50% of healthy tears, to reduce LF content as described in literature, and by different $FeSO_4$ concentrations (9.6, 35 or 96 µg/mL). Viability test was performed after 18 h as previously described.

Table 1
Incubation of CLs with buffer or albumin did not protect cells against the oxidative stress induced by 250 µM H_2O_2.

	cell mortality (%) with 250 µM H_2O_2
native CLs	36,2 ± 4,7
apoLF CLs	10,5 ± 1,2
buffer CLs	45 ± 5
albumin CLs	45 ± 11

2.6. Data analysis

Data are presented as mean ± s.e.m. In the figure captions n represents the number of wells which have been tested. For the experiments with healthy tears, 3–4 wells were analysed with tears collected from each subject; with 4 healthy subjects we performed a total of 12–15 experiments. For the experiments with KC tears, 5–6 wells with the tears of each patient were tested. Statistical evaluations were obtained using the one-way analysis of variance (ANOVA), followed by the Tukey post hoc test, or non parametric Mann-Whitney test.

3. Results

To evaluate *in vitro* tear cytotoxicity, epithelial TsA cells were incubated in tears collected by reflex lacrimation from four subjects without ocular pathologies. The use of TsA cells as a model of corneal epithelium was justified by a previous work that demonstrated the similar behavior under oxidative stress between TsA and cornea epithelial cells [25].

By viability test, here we demonstrate that TsA cells maintained for 18 h in healthy tears showed an equivalent mortality to serum free medium (8.6 ± 1.2% vs. 8.9 ± 0.93%, p = 0.98) (Fig. 1a). Thus, it was possible to conclude that reflex tears from healthy subjects exerted no toxic effects on epithelial cells.

Successively, TsA cells were incubated with reflex tears collected from two subjects affected by keratoconus. In these conditions cell mortalities were significantly higher than for the healthy tears: 27.7 ± 3.95% (p = 0.0003) for KC1, and 17.6 ± 0.95% (p = 0.014) for KC2 (Fig. 1a). These results demonstrated that tears of both patients affected by keratoconus were more cytotoxic than tears of subjects without ocular pathologies.

To evaluate reactive oxygen species content in cells, TsA incubated with DCFDA were successively treated with healthy or keratoconic tears. The analysis was performed at different times (5, 10, 20, 30, 40 and 60 min) by flow cytometer (Fig. 1b). The obtained results showed that in cells incubated in KC tears intracellular ROS content was about 40% higher than in cells treated with healthy tears, confirming that cytotoxicity caused by KC tears could be related to a significant increase of ROS.

In literature the iron concentration in tears is not reported, but the increment of this ion in the corneal epithelium and in tears of subjects affected by keratoconus is known [8]. For this reason we analysed mortality of cells exposed to different $FeSO_4$ concentrations to estimate iron levels in healthy and pathological tears. Incubating TsA cells with a tear model, consisting in serum-free medium, apoLF 1.2 mg/mL and different $FeSO_4$ concentrations, and measuring cell mortality in relation to iron levels, we obtained the data points for a calibration curve which showed a linear trend (R = 0.95) only at low $FeSO_4$ concentrations (Fig. 2); instead, at higher iron concentrations, the cell mortality of our model stabilized at 15%. Using the line equation (y = 0.89673x + 4.4894) we estimated an iron concentration in healthy tears of 4.58 µg/mL.

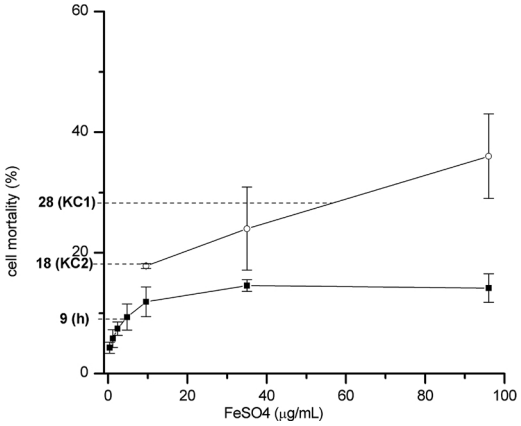

Fig. 2. Estimation of iron concentration in tears. The graph represents the measure of cell mortality in function of $FeSO_4$ concentrations. For the estimation of iron concentration in healthy tears (h), TsA cells were incubated with apoLF 1.2 mg/mL and different $FeSO_4$ concentrations (1.2, 2.4, 4.8, 9.6, 35, or 96 µg/mL) for 18 h (squares, n = 12); the concentration extrapolated from the curve for 8.6% cell mortality was 4.58 µg/mL. For the estimation of iron concentration in keratoconic tears (KC1 and KC2), TsA cells were incubated with a model of keratoconic tears and different $FeSO_4$ concentrations (9.6, 35, or 96 µg/mL) for 18 h (circle, n = 6); iron concentration extrapolated from the calibration curve was 56.28 µg/mL for 27.7% cell mortality (KC1), and 8.7 µg/mL for 17.6% cell mortality (KC2). The number of dead cells was evaluated by trypan blue test. Data are presented as mean ± s.e.m.

Using the model of keratoconic tear described in Methods, the data points we obtained were interpolated with a line (R = 0.99) (Fig. 2). Using the line equation (y = 0.21x + 16.18) we estimated an iron concentration in keratoconic tears of 56.28 µg/mL for KC1, and of 8.7 µg/mL for KC2.

Our results showed that, by incubating epithelial cells in tears of KC patients, we were able to reproduce a simple model of ocular surface. Thus this system was used to study the ability of contact lenses, loaded with lactoferrin, to counteract oxidative stress. Silicone-hydrogel filcon V contact lenses were loaded with apolactoferrin for 5 h and then deposed on the epithelium model. After 18 h, viability test showed that contact lenses significantly reduced cell mortality at 12.4 ± 3.4% vs. 27.7 ± 3.9%, p = 0.035, counteracting cytotoxicity of patient KC1 tears and restoring the viability as with healthy tears (12.4 ± 3.4% vs 8.6 ± 1.2%, p = 0.14) (Fig. 3). These results were confirmed by adding lactoferrin to epithelial cells incubated with tears of KC2 patients. After 18 h incubation with 30 µg of lactoferrin, cell mortality was reduced at 11.3%. Because of the limited volume of tears that KC2

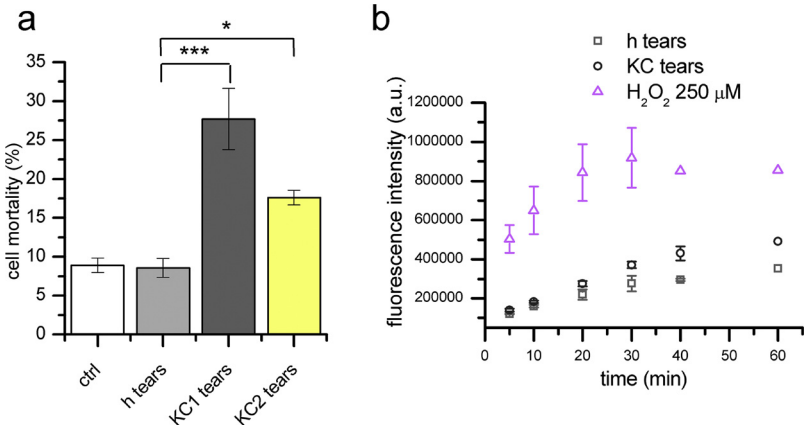

Fig. 1. Healthy and keratoconic tear cytotoxicity. (a) TsA cells were incubated with healthy tears (h tears, 4 subjects, n = 15) and keratoconus patients' tears (KC1 tears, n = 6, and KC2 tears, n = 5) for 18 h. The number of dead cells was evaluated by trypan blue exclusion test; the control condition (ctrl, n = 12) is represented by cells in serum free medium. Data are presented as mean ± s.e.m. Statistical evaluations were obtained using ANOVA or Mann-Whitney test (*p < 0.05, ***p < 0.001). **(b)** TsA cells were incubated with DCFDA to evaluate intracellular ROS content produced with healthy or keratoconic tear treatment, or with 250 µM H_2O_2 as positive control. Fluorescence intensity was measured by flow cytometer at different times (5, 10, 20, 30, 40 and 60 min). The tears of two healthy subjects and two KC patients were used; for positive control two independent experiments were performed. Data are presented as mean ± s.e.m.

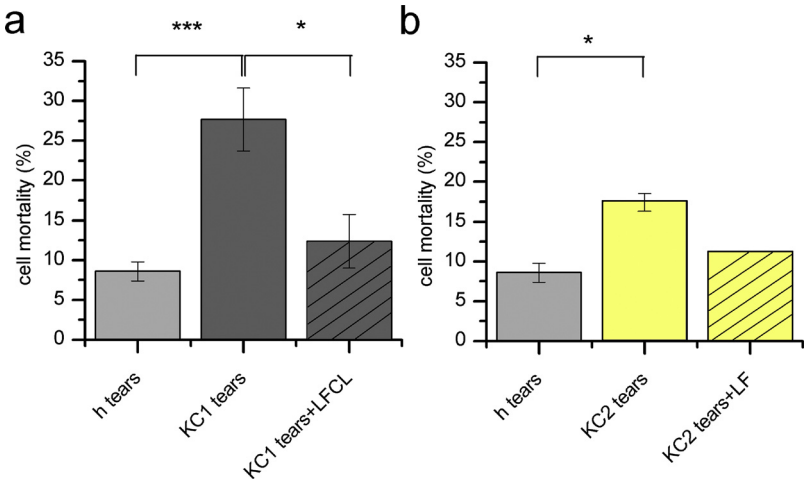

Fig. 3. Lactoferrin protected from oxidative stress cytotoxicity induced by keratoconus patients' tears. **(a)** Cell mortality of cells maintained in KC1 tears (n = 6) was significantly reduced by incubation with lactoferrin-loaded contact lenses (LFCL). No significant difference was observed between healthy tears (n = 15) and KC1 tears + LFCL (n = 6). **(b)** Cell mortality of cells maintained in KC2 tears (n = 5) was reduced by incubation with lactoferrin (LF) (n = 1).The number of dead cells was evaluated by trypan blue test. Data are presented as mean ± s.e.m. Statistical evaluations were obtained using ANOVA or Mann-Whitney test (*p < 0.05, ***p < 0.001).

patient could collect, it was not possible to replicate the experiments with LF-loaded contact lenses. These data demonstrate that contact lenses, which release lactoferrin, could supply an antioxidant potential in the tears of a patient affected by keratoconus, protecting epithelial cells against the effects of oxidative stress.

4. Discussion

Keratoconus is a progressive corneal disorder, which manifests in the second or third decade of life, and leads to ocular discomfort and altered vision quality. Although the exact etiopathogenesis remains unknown, protein and lipid peroxidation, keratocyte apoptosis, iron deposition, and the alteration of the antioxidative machinery within the cornea and the tear film suggest that oxidative stress is involved [4].

Thus the possibility to counteract oxidative stress at the ocular surface might be a strategy to contain disease progression. In Pastori et al. we demonstrated that contact lenses, loaded with the tear protein lactoferrin, could reduce the toxicity that hydrogen peroxide caused at the level of epithelial cells [25]. Thus, in this work we verified if this prototype of therapeutic contact lenses could reproduce the same beneficial effect on TsA epithelial cells incubated with the tears collected from two KC patients. Four healthy subjects provided tears as control. The mortality of cells incubated in healthy tears was not significantly different from the mortality of cells incubated in the culture medium, and we could conclude that tears from subjects without ocular pathologies have no cytotoxic effects. On the contrary, when incubated in the patient's tears, epithelial cells showed a significant increase in mortality, suggesting as literature reports that patients' tears should contain reduced oxidative stress defences, decreased lactoferrin levels, and increased iron concentration. In fact, cells incubated for 40–60 min in KC tears showed about 40% increase of reactive oxygen species compared to cells in healthy tears, and the deposition of LF-loaded contact lenses over the cells was able to suppress cytotoxicity, restoring the same cellular viability we determined with incubation in healthy tears. These results suggest that contact lenses loaded with LF could represent a device to counteract oxidative stress in patients with a diagnosis of keratoconus.

Contact lenses are the mainstay of vision correction for KC patients. Even if soft contact lenses have been specifically developed for KC, their use is limited to early forms of the pathology, since they provide inferior visual performance then rigid contact lenses because of their limited capacity to mask irregular astigmatism [30]. It is our future aim to verify the possibility of inserting LF in hybrid contact lenses, by exploiting new technologies in ocular drug delivery, such as molecular-imprinting, liposome-coating, polymer micelle- and nanoparticle-loading [31–34]. In fact, in the recent years the application of contact lenses has expanded since the new concept of therapeutic contact lenses was introduced [21,35–37].

In our work LF released by the CLs compensated its deficit in tears of KC patients, and counteracted cytotoxicity by the multiple activities that it exerts. We suppose that LF reduced oxidative stress probably by binding ferric ions present in the tears and/or through the internalization in epithelial cells by its receptors, as suggested [38], and that it protected the cells against the inflammatory processes that have been recognized in KC patient tears [39].

Moreover, in this study we tried to indirectly estimate tear iron concentrations in healthy and KC subjects, providing for the first time an indication for this parameter. In fact, these concentrations have not been determined yet, probably because of the complex composition of human tears. Electron Paramagnetic Resonance (EPR) analysis could not provide a reliable measure and we preferred not to include it in this paper. Probably the novel combination of EPR and SQUID magnetometry, recently reported by Kumar et al. [40] to quantify iron in human brain tissue, might be employed for the precise determination. Thus for the healthy subjects, the value of iron concentration we extrapolated was about 5 μg/mL. Considering that the mean LF level reported in literature is about 1.2 mg/mL, the iron concentration we determined was coherent with the quantity of iron bound per mg of protein which is cited in the article of Nojima et al. [41]. Instead, as expected, the iron we estimate from KC tears was more concentrated, about ten folds higher than in healthy tears.

By incubating epithelial cells in human tears, we show for the first time the possibility to reproduce *in vitro* a model of ocular surface, adequate for designing strategies to control oxidative damage at the KC corneal epithelium, and which might be also employed for studying other pathologies of the ocular surface related to oxidative stress.

Finally, the involvement of subjects with different KC levels of severity would be useful to define if LF-loaded contact lenses could represent a valid therapeutic approach not only for the moderate form of the pathology. Moreover, considering the immunoregulatory properties of LF, due to its ability to modulate the function of immune cells and to inhibit the classical complement pathway [42], this work might also suggest a potential strategy to treat inflammation and/or infections at the ocular surface.

Conflict of interest

The authors report no conflicts of interest and have no proprietary interest in any of the materials mentioned in this article.

Acknowledgements

We are very grateful to Dr Mara Arnaboldi, Dr Alice Garibaldi and Dr Arianna Villa for instructing patients in collecting their tears, and to

Prof. Marcella Rocchetti for technical support with fluorimetric analysis. This work was supported by FONDO DI ATENEO PER LA RICERCA (ATE-0096) to ML.

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