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# Lactoferrin as a biomarker of ocular diseases and contact lens discomfort

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**Summary.** — Lactoferrin (Lf) is frequently described as a diagnostic marker for different ocular diseases, such as dry eye (DE), Sjögren's syndrome, and diabetic retinopathy. We therefore performed a meta-analysis on the average Lf concentration in healthy subjects and those affected by ocular diseases. The results suggest that Lf level is a good candidate as a DE syndrome diagnostic biomarker, even though there is still a need for further development of standardized protocols of tear collection, processing, and storage. One of the risk factors for DE is contact lens (CL) wear and DE prevalence is known to be higher in CL wearers than non-wearers. To investigate Lf as a biomarker for CL discomfort, we developed a diagnostic method based on terbium fluorescence to detect Lf directly into human tears. Lf concentration was found to be unchanged after a period of CL wear and after a period of CL suspension. Instead, the results reveal a significant change in Lf affinity for terbium upon CL wear. It is known that an alteration of the protein conformational state or of its substrate-binding site leads to protein inactivation and triggers an inflammatory response. Indeed, high levels of protein denaturation have been found to be correlated with adverse effects, such as papillary conjunctivitis and CL discomfort.

 

## 1. – Ocular diseases

Lactoferrin (Lf), which is also referred to as lactotransferrin, is a nonheme protein that is able to bind iron and belongs to the family of transferrins. It is present in the majority of mucosal secretions, such as tears, saliva and milk [1]. Lf has a multifunctional character, with antimicrobial and immunomodulatory activities being the main ones [2, 3]. For this reason, during the last decades, Lf quantification in tears has gained more and more interest. Different techniques have been employed so far, including gel electrophoresis, liquid chromatography, mass spectrometry, enzyme-linked immunosorbent assay (ELISA), and diagnostic test kits, among others [4]. Several studies focused on patients affected by ocular diseases, such as the dry eye (DE) syndrome [5-7] and keratoconus (KC) [8, 9], reporting a lower concentration of Lf compared with healthy volunteers. Thus, an alteration in Lf concentration has been suggested to represent a good biomarker candidate for the diagnosis of ocular diseases. To further validate this conclusion, it is necessary to verify whether the difference in Lf amount between patients with ocular diseases and healthy controls reported in the literature is statistically significant and reliable. In this view, a meta-analysis has been performed, exploiting the evidence available in the literature and providing an estimate of the mean difference (MD) of Lf concentration between healthy and pathological states [4]. Most studies were focused on keratoconjunctivitis sicca (KS), which is the condition of having DE. Other studies measured Lf concentration in presence of Sjögren's syndrome (SS), diabetic retinopathy (DR), KC, corneal melting and chronic conjunctivitis.

The MD was represented by the value $\overline{x}_{healthy} - \overline{x}_{ill}$ and the standard error was calculated as $\sqrt{\frac{s^2_{healthy}}{n_{healthy}} + \frac{s^2_{ill}}{n_{ill}}}$, where $x$ is the mean value, $s^2$ is the standard deviation and $n$ is the sample size, for healthy and ill subjects, respectively.

For each study on ocular diseases with at least three MD values, the pooled mean difference (pMD) and its 95% CI were estimated by applying the fixed model and the random effect model of DerSimonian and Laird. Between-studies heterogeneity was evaluated by the Cochran's Q test and the $I^2$ index. In case of high heterogeneity, random effect model estimates were reported. To calculate the CI in case of high heterogeneity and less than five studies, the Hartung Knapp Sidik Jonkman method was applied. Considering the DE syndrome, the random effect model provided a pMD of 0.62 (95% CI, 0.35–0.89), highlighting that Lf concentration is significantly lower in DE patients compared to healthy subjects. Nonetheless, high heterogeneity was present ($I^2 = 97.14\%$). Different potential sources of heterogeneity were considered in the stratified analysis, which included tear sampling methods, Lf measurement technique, diagnostic criteria, age, gender balance, geographic area, and sample size. The pMD of Lf concentration was found to be significantly different for the geographic area and for the sample size. In particular, studies that took place in European and Asian countries presented greater pMDs (1.07 and 0.59, respectively) compared with studies conducted in the United States ($-0.03$). As far as the sample size is concerned, large groups of subjects ($> 62$) presented a greater pMD (0.86 *vs.* 0.23). In addition, influence analysis, cumulative analysis and Egger's test were performed to examine robustness of the findings, the effect of sample size on the pMD, and the publication bias, respectively. The results suggested that a single study affected neither the pMD nor heterogeneity. Statistically significant pMDs were observable only when the sample size was larger than 220 subjects, with the pooled estimate tending to stabilize only when the number of subjects outreached 600. Considering the other ocular pathologies, no statistically significant difference was reported

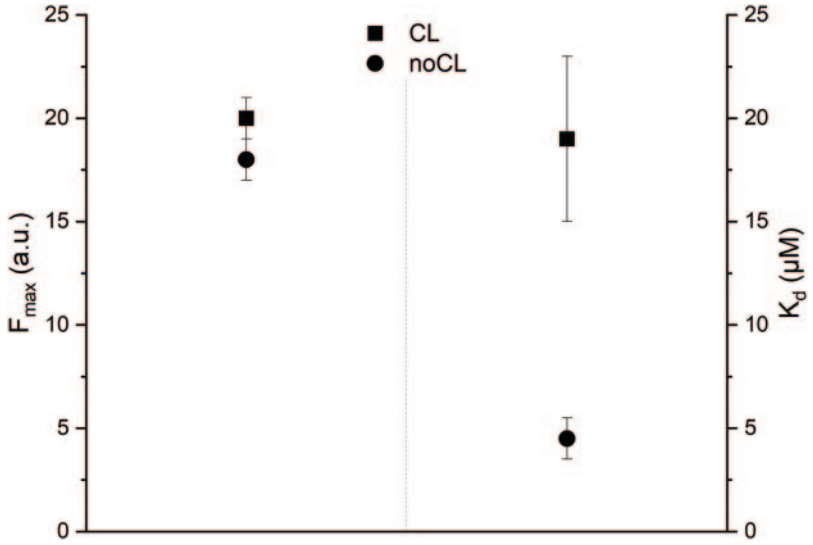


Fig. 1. – Lf-$Tb^{3+}$ binding properties after one week with (grey dots) and without CLs (black squares). $F_{max}$ is the fluorescence intensity under excess of $Tb^{3+}$ compared to Apo-Lf (saturation conditions), and $K_d$ is the dissociation constant of the complex. The error bars represent the standard deviation of four experimental sets for each condition.

between SS or DR patients compared with healthy controls. High heterogeneity was evidenced for both diseases. This meta-analysis suggested Lf concentration as a good candidate as a DE biomarker. Interestingly, the DE syndrome is characterized by an inflammation process, which is thought to be associated with oxidative stress. The natural counteraction is represented by Lf and its ability to chelate iron [10, 11]. One of the risk factors for DE is contact lens (CL) wear and DE prevalence is known to be higher in CL wearers than non-wearers [12, 13]. Also, contact lens (CL) wear can induce alterations of the tear film [13]. Depending on the physico-chemical properties of the CL materials, the tear biomolecules can interact and even penetrate into the CLs matrix in the order of a few micrograms [14-17]. Upon adsorption, tear proteins may go through structural changes that can affect their biological activity, leading to physiological dysfunctions [18]. Tear Lf is mainly present in its unsaturated form (Apo-Lf) and can bind terbium ($Tb^{3+}$) in its iron-binding sites [19]. The resulting complex is fluorescent and can be employed in unprocessed tears for specific protein detection [20]. A recent work assessed Lf concentration and functionality upon hydrogel CL wear [21]. Fluorescence analysis was performed by employing an excitation wavelength of 295 nm and by monitoring the emission in the range 450–575 nm. Volunteers were asked to daily wear disposable Etafilcon A hydrogel CLs for a time period of one week and not to wear any kind of CLs for the following week. Tear collection was performed at the end of each week by the microcapillary method [22-24]. The results showed that CL wear did not significantly change Lf concentration, consistently with previous reports that highlighted a significant change only for the prolactine-induced protein [25]. Nonetheless, CL wear caused an alteration in apparent affinity of Lf for $Tb^{3+}$ (fig. 1).

This alteration is likely to reflect Lf structural changes that affected the metal-ion binding sites of Lf, with possible dysfunctional effects in all the different Lf protective

activities. The results are consistent with previous reports on lysozyme, the only tear protein that was studied in terms of concentration and functionality during CL wear [18, 26-29]. Further studies are needed to expand the understanding of Lf implication in response to ocular diseases and CL wear, taking into account both the estimate of the concentration and the protein conformational state.

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