Tear Proteomic Profiling as Potential Non-Invasive Laboratory Test for Rheumatoid Arthritis

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Summary

Tear film resembles other body fluids in many biochemical aspects. As tear sampling is a noninvasive, simple and rapid method, it may be used for diagnostic purposes regarding local or systemic disorders. Rheumatoid arthritis (RA) is an autoimmune disease that causes chronic inflammation of the joints. It may also cause Sjogren's syndrome, an inflammation of the lachrymal and salivary glands, leading to decreased production of saliva and tears and possible development of a dry mouth and dry eyes. Therefore, lachrymal and accessory glands can become a target of the immune system and show signs of inflammation, leading to possible changes in tear physiology and biochemistry.

30 RA patients and 30 controls (aged 45-55 years) were selected to enter the study. A number of analytical methods including electrophoresis, reverse-phase high-performance liquid-chromatography (HPLC) fractionations; enzyme linked immunosorbant assay (ELISA) were used to separate and display the differential tear proteome. Total tear proteins and biological activities of some important enzymes, lysozyme, lactoferrin and tyrosinase were measured using known chemical assay methods.

Changes in electrophoreograms and chromatographic tear proteomic profiling patterns were observed and characterized in tears of RA patients compared to healthy volunteers. Total tear proteins and lysozyme were decreased in tear samples obtained from RA patients while lactoferrin and immunoglobulins showed about a 10% increase compared to healthy controls.

Based on the results obtained from this study, it is concluded that a rheumatologist should to be aware of the association between RA and inflammatory eye disease. Changes in tear proteins and enzymatic activity may, in most cases, give rise to impaired response of the eye to external and internal factors.

Keywords: Tear protein; noninvasive diagnostic test; lysozyme; Rheumatoid arthritis.

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Introduction

Rheumatoid arthritis (RA) is a disabling inflammatory arthritic condition in which combination of genetic and environmental factors promotes disease susceptibility. Being a chronic, systemic disorder of unknown etiology, RA primarily involves the joints but can also cause multiple extra-articular manifestations. It is the most common autoimmune disease, affecting 1–1.5% of the population worldwide [1-5]. The global existence of RA and its economic and social burdens on the patient, calls for finding the cause and a curative therapeutic strategy for it [6]. In addition, it has been found that in many cases the rate of disability increases progressively [7]. Rheumatoid arthritis (RA) is characterized by synovitis and autoantibody formation (Rheumatoid Factor, RF). The hallmark feature of the disease is persistent symmetric polyarthritis (synovitis) that affects the hands, wrists and feet, although almost all diarthrodial joints may become involved. In addition to articular manifestations, systemic involvement may cause constitutional symptoms (such as weight loss, low-grade fever, malaise), rheumatoid nodules, serositis and vasculitis. The severity of RA may fluctuate over time, but chronic RA most commonly results in the progressive development of various degrees of joint destruction, deformity, significant decline in functional status and a premature death [8, 9]. RA is one of the many chronic autoimmune diseases that predominates in women. The ratio of female to male patients is approximately 2.4:1 [10]. The basis of the gender differences is not known but presumably is related to effects of the hormonal milieu on immune function. Throughout the world, ethnic groups like the North American Pima Indians and southeast Alaskan Indians have a much higher incidence of RA [10]. The incidence of RA rises dramatically during adulthood and peaks in individuals aged 40–60 years.

Routinely, diagnosis of rheumatoid arthritis is based on physical and clinical examinations followed by some biochemical laboratory tests, X-ray radiology and magnetic resonance imaging (MRI). Most of the methods require referring the patients to radiology clinic and diagnostic laboratories, usually for several times. These methods may either be invasive such as using blood sampling or they can cause side effects, e.g. exposure to X-ray. The rheumatoid factor (RF) test is primarily used to help diagnose RA or Sjögren's syndrome and to help distinguish them from other forms of arthritis or other conditions that cause similar symptoms. However, in the case of serological tests, a negative RF does not rule out RA [11]. This condition is sometimes called seronegative arthritis which occurs in 15% of patients [11]. About 20% of people with RA will be persistently negative for RF and/or may have very low levels of RF. During the first year of illness, rheumatoid factor is more likely to be negative with some individuals converting to seropositive status after about 18 months [5]. A positive RF is also seen in other illnesses, for example Sjögren's syndrome, Scleroderma, Dermatomyositis, Sarcoidosis [12] and in approximately 10% of the healthy population [12]. Therefore, the test is not very specific and, for correct diagnosis, other tests are needed to confirm RF results. On the other hand, radiological techniques are able to show signs of destruction and inflammation on ultrasonography. For example, among its many other diagnostic applications, MRI is able to show these symptoms in the second metacarpophalangeal joint to help diagnosis of rheumatoid arthritis.

It has long been known that radiology plays a key role in diagnosis and management of RA. Radiological assessment of RA has been used for diagnosis and grading x-ray film during the course of therapy [13]. Magnetic resonance imaging (MRI) is also a reliable imaging modality because it depicts soft-tissue changes and damage to cartilage and bone at an early stage. Ultrasound and conventional radiography are more readily available but they have their limitations in that they do not show the entire spectrum of the disease. However, knowledge of the imaging findings is needed to accurately select the most helpful imaging technique [14]. In any case, several visits and repeated examinations and investigations are often necessary before the doctor can be certain of the diagnosis.

Tears as a diagnostic tool

Among other disorders arising from rheumatoid arthritis, it may also cause Sjogren's syndrome. One of the most important consequences of this syndrome is inflammation of the lachrymal gland that may lead to decreased production of tears and development of dry eyes [15]. Therefore, lachrymal and accessories glands can become a target of the immune system and show signs of inflammation, leading to possible changes in tear physiology and biochemistry.

The lachrymal gland plays a crucial role in the immunological protection of the ocular surface. This gland, which serves as the predominant source of tear SIgA antibodies, is the primary tissue in the secretory immune defense. The gland also contains an extraordinarily high density of IgA- and IgD-positive plasma cells, which originate primarily from the tonsils and adenoids, migrate through the cervical lymph nodes and make the lachrymal gland as one of the best factories for IgA production in the body. The mechanism by which these lymphocytes home to the lachrymal gland is unclear, but does not appear to depend on mucosal cell-adhesion. Once produced, SlgA antibodies are secreted into the tear film to defend against antigenic challenge; this role is important during prolonged eye closure (e.g. at night), when the levels of SlgA reach almost 80% of the total tear protein [16]. Lachrymal gland epithelial cells also synthesize and secrete numerous enzymes and proteins that play a significant role in ocular immune defense. However, perturbations in the proteolytic processing and sorting of cellular antigens (e.g. muscarinic acetylcholine receptor) could lead to epithelial cell presentation of autoantigens and immune activation. Such a process, coupled with the expression of atypical glandular adhesion molecules (e.g. vascular-cell adhesion molecule 1, PNAd), might contribute to the lachrymal gland inflammation observed in Rheumatoid arthritis and Sjögren's syndrome. The lachrymal gland might be influenced by various disorders, leading to stimulation of local autoimmunity, and activation of local antigen-presenting cells, followed by T-cell infiltration. This results in inflammation of the ocular surface and glandular structures with reduced exocrine function (Fig. 1). Rheumatoid arthritis is one of the most common autoimmune disorders influencing the lachrymal gland. Release of auto-antibodies into the lachrymal gland could, in turn, disturb the normal function of lachrymal gland leading to various changes in biochemistry of tear fluid.
Fig. 1. The lachrymal and meibomian glands might be influenced by various disorders, leading to stimulation of local autoimmunity, and activation of local antigen-presenting cells, followed by T-cell infiltration [16].

The tear film provides the human cornea and conjunctiva with a highly specific system of defenses containing a variety of proteins that alter antimicrobial activity by a variety of mechanisms. The tear film is a complex biological mixture, containing electrolytes, proteins, lipids, mucins, some small organic molecules and metabolites. It consists of 3 layers, an inner mucin layer; a middle aqueous layer containing electrolytes, proteins and various metabolites; and an outer lipid layer [17]. The aqueous layer which is mainly secreted from the lachrymal gland contains a wide range of biochemicals including proteins. Tear proteins are among the most important components with total concentration of ranging from 6 to 10 mg/mL [18]. It should be emphasized that in any tear analysis studies, composition of tear film may slightly vary depending on the collection method, analysis procedure, the analytical instruments used and some other variables such as subject related factor. During 1990s more than 100 different tear proteins had been identified and reported. However it was suggested at the time that there might be much more proteins in human tear fluid [19]. In a more recent study, using a mass spectrometry-based proteomic approach, 491 proteins have been identified in tear fluid [20] the most important of which include lysozyme, lactoferrin, secretory immunoglobulin A (sIgA), serum albumin, lipocalin (previously called tear-specific prealbumin) and lipophilin [21]. Human tear fluid plays an important role in protecting, nourishing and lubricating the cornea and external eye. Normal tears contain approximately 5-6 mg/ml total protein, of which 35-45% is comprised of lysozyme, lactoferrin, lipocalin and IgA [22]. Tear lipocalin is an unusual lipocalin member, because of its high promiscuity for relative insoluble lipids and binding characteristics that differ from other members [23].
Although it acts as the principal lipid binding protein in tear fluid, a more general physiological function has to be proposed due to its wide distribution and properties. Tear lipocalin is the predominant phosphoprotein in human tear fluid. The protein was originally described as a major protein of human tear fluid and thought to be tear specific. Using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) we suggested the presence of tear lipophilin that was rarely attracted attention in previous literature. Lipophilin is a heterodimeric protein in human tear fluid and is belonged to the uteroglobin superfamily [24].

Recently, many efforts have been made to explore tear proteins using proteomic approaches [25, 26]. Therefore, the proteomic analysis of tear fluid is of fundamental interest in eye research as its potential to identify new protein constitution in the tear film. There is no doubt that proteomics will provide a comprehensive approach for cataloguing the tear proteins. This will, in turn, help to elucidate ocular involved disease pathogenesis, make clinical diagnoses and evaluate the influence of medications on the structure, composition and secretion of tear proteins. As human tear fluid is secreted mostly from the lacrimal glands, it may also be influenced by autoimmune disorders such as RA. As a result, its biochemical composition, mostly various proteins in tear fluid, could show significant alterations in response to local autoimmunity. This results in inflammation of the ocular surface and glandular structures with reduced exocrine function. Abnormalities of the tear film, affecting the constituents or the volume, can rapidly result in serious dysfunction of the eyelids and conjunctiva and ultimately affect the transparency of the cornea. This paper provides an overview on laboratory methods for the analysis of proteins in the tear film. Apart from total tear proteins, a few important tear antibacterial and antioxidant proteins (lysozyme, lactoferrin and immunoglobulins) were also quantified in tear fluid of RA patients and control subjects. This was aimed to assess the performance of tear protein profiling as potential alternative noninvasive laboratory test for diagnosis of autoimmune disorders such as RA.

Materials and methods

Materials
Dopamine hydrochloride was purchased from Sigma Aldrich Chemical Company. Sodium mono- and di-phosphates, were purchased from Merck representative in Iran. Electrophoresis and HPLC materials, protein molecular weight markers, and ammonium sulphate were purchased from Sigma and were of analytical grade. Assay kits, Lactoplate®, radial immunodiffusion kit for human lysozyme, ‘NL’ NANORID™ and RID kit for immunoglobulins were purchased from the Binding Site, Birmingham.

Subjects
Thirty patients (aged 45-55 years) who confirmed RA diagnosis according to the international criteria [27] were selected by experienced rheumatologist based on their history and severity of disease. They had been followed up at Department of Rheumatology in Razi Medical University Hospital, Rasht and their clinical history was obtained from medical records. They all had at least 5 years of the clinical history with regular checking. Those with severe clinical presentation of RA complicated with cardiac involvement and those remitted and free from the clinical signs and symptoms of
RA during the last three years were excluded from the study. With kind help of an ophthalmologist, all subjects underwent eye examination before tear sampling and the excluded subjects were selected under his supervision. Subjects with any evidence or history of ocular disease or other inflammatory and immune disorders were excluded. RA patients suffering from severe dry eye syndrome were excluded from the study, as the syndrome not only affects tear volume, but it also alters tear composition.

The control group was 30 healthy non-contact lens wearers (45-55 years), without any inflammatory and immune disorders related to the eye or other parts of the body. A very specific effort was made to also exclude subjects with Sjogren's syndrome. A clinical dry eye assessment was conducted on the RA group and a parallel profiling was performed on control group. RA patients with severe dry eye were excluded and the members of control group were selected so that, apart from being healthy, did not show any sign of dry eye syndrome.

**Tear collection**

Onion vapor stimulated tear samples from patients and controls were collected in sterile capillary tubes. Each micropipette, 12 cm long and 1 mm wide, were calibrated with a mark at 10 microlitres. During tear collection, the patient was positioned on a slit lamp. The slit beam was narrowed to a small beam to avoid bright illumination and reflex tearing. With the patient’s lower lid gently everted, the operator then gently placed the micropipette from a lateral direction at an angle onto the inferior fornices cul-de-sac area. The contact between the tear meniscus and the micropipette tip then facilitated the tear collection via capillary action. All tear samples from patients and controls were collected within 5 minutes from each patient without the need of topical anesthetics. At least 3 tear samples (about 10 µl each) were collected from each individual and they were added together before any examination. Immediately after sampling, tears were diluted 1:10 using sterile saline and stored at -20 °C to be examined later. The total storage time for each sample did not exceed 10 days, in order to prevent possible changes in protein composition of the tears. Each assay and instrumental procedure was performed for each sample from patient and control with 2 repeats. All tear–collection procedures were performed by the same operator throughout the study. Any maneuvers which had caused irritation to the conjunctiva were recorded during tear collection and the specimens were discarded. Human ethics approval for tear collection was obtained from the Gilan Medical University Human Research Ethics Committee.

**Total protein in tears**

Total tear protein in each tear sample was measured spectrophotometrically using a modified Bradford micro assay method [28] and further necessary dilutions were made for the corrections of the measurement. The sensitivity of the method and consistency of results was determined on the basis of three repetitive readings.

**Electrophoresis of tear proteins**

As shown in the result section, total tear protein was about 500mg/100ml, which was diluted to about 50 mg/ml. Therefore, about 0.05 µg total tear protein were present in each µl of diluted tear sample. By considering the above explanation, about 2.5 µg of
total tear protein in volume of 5µl of each tear sample was mixed with equal volume of sample buffer, denatured in boiling water for 3 min and then applied to the polyacrylamide gel. Electrophoresis conditions and instrument have been discussed previously [29]. At the end of electrophoresis run, the gels were stained by either Coomassie Blue or silver nitrate [30].

**Fractionation of tear proteins by reverse-phase HPLC (RP-HPLC)**

About 25 µg of total tear protein in total volume of 50 µl of diluted tear samples were injected into the HPLC system for fractionation. The HPLC system was a 2600 Kauuer (Germany) equipped with a UV detector and pump 1100. The fractionation was achieved on a column C18 (5 µm particle size, 300 Å pore size, 250 x 4.6 mm, Kauuer) at a flow rate of 0.7 mL/min. Mobile phase A was 0.01% trifluoroacetic acid (TFA) in water/acetonitrile(95/5) and mobile phase B was 0.01% TFA in water/acetonitrile (5/95). A linear gradient from 5% B to 25% B was used in the first 11 minutes, followed by 25% B to 40% B in the next 35 minutes, and 40% B to 50% B in the next 60 minutes, followed by 50% B to 85% B in the next 90 minutes, and finally from 85% to 90% B in the last 10 minutes achieving a HPLC run of 100 minutes in total. Nylon was used for filtration of HPLC samples prior to injection [31]. The eluent was monitored at 280nm and 254nm, and the individual peaks were identified and each separated fraction was collected according to the characteristic UV emission spectrum and retention time for further electrophoresis as above.

**Assay of tear lysozyme**

A radial immunodiffusion (RID) kit from NANORID™ was used for lysozyme assay in tears. The samples were diluted as recommended by the kit to values of about 0.01-0.017 mg/ml. The plates were kept at room temperature for 10 minutes and warmed up before the application of the samples. Each well was checked for the presence of any moisture or dust and 10ml of samples were applied to each well very carefully to avoid contamination. The lid was closed tightly and the plate was stored flat at room temperature (not more than 22°C) for 96 hours. After the diffusion was completed the ring diameters were measured to the nearest 0.1mm using a jewellers’ eye piece (Binding Site, code: D040). The sample concentrations were then calculated directly from the calibration curve obtained by plotting the ring diameter squared against the concentrations of the standards. A calibration curve was obtained using the ring diameter squared and the concentrations of lysozyme standard provided by the kit from which lysozyme concentration was calculated in each sample.

**Assay of tear lactoferrin**

Lactoferrin EIA Kit (Human Plasma)™ was used to measure lactoferrin in diluted tear samples. The kit was an in vitro diagnostic device that is also used for the measurement of lactoferrin concentration in human tears as an aid in the diagnosis of some disorders of the eye keratoconjunctivitis sicca (KCS) and to assess lachrymal gland (acinar cell) function [32]. The filter paper provided by the kit was well soaked into tear samples the excess fluid was removed by blotting and was carefully placed to the reagent gel. The gel was transferred to its closed container and left at room temperature for 3 days. The sample diffused into the gel during this period reacting with the reagent and leaving an
opaque ring whose size is related to the concentration of the lactoferrin in the sample using a freshly prepared standard lactoferrin solution. Using the following relationship, the concentrations of the lactoferrin in the unknown solutions were calculated by the following formula:

\[ C = 0.0105 \times D^2 \]

where \( C \) was the concentration in mg/ml and \( D \) the diameter in mm.

**Determination of tear immunoglobulins**

A radial immunodiffusion kit, RID™, kit for immunoglobulins was used according to the procedure provided. The plate was placed at room temperature uncovered before the sample application. 10\( \mu \)l of diluted tear sample was applied to each well; the lid was closed and was incubated at room temperature for exactly 18 hours. The diameter of the ring was proportional to the concentration of the immunoglobulin in the sample as in lysozyme assay above. A series of standards provided by the kit were used to obtain a calibration straight line from which the concentration of tear immunoglobulins was determined. It is known that assay of individual immunoglobulins is more reliable than total tear immunoglobulins [33]. For example, Tear IgG measurement might provide a marker for eye involvement in patients with rheumatoid arthritis. However, the appropriate kits were not available at the time of this study and more detail immunoglobulins studies are the future aim of our research.

**Statistics**

Each assay was repeated triplicate and the results were presented as mean ± SD values. Statistical difference between groups was compared by un-paired t-test, \( p \) values less than 0.05 were retained as significant. The calculations were performed PRISM (version 4.0) for windows (GraphPad Software Sandiego, CA, USA).

**Results**

**Total tear protein and electrophoresis**

The demographic characteristics of the study populations together with their tear proteins are summarized in Table 1.

| Table 1. Demographic characteristics and RA patients and normal subjects. |
|-----------------------------------------------|-----------------|------------------|-----|
|                               | Controls \( n = 30 \) | RA \( n = 30 \) | \( P \) values* |
| Age (years)                   | 4605 ± 3.5      | 52.5 ± 3         | NS  |
| Sex ratio (male to female)    | M-12, F-18      | M-10, F-20       | NS  |
| Body Mass Index (BMI)         | 28.1 ± 2.4      | 28.6 ± 2.3       | NS  |
| Tear Volume collected (µl)   | 12 ± 2.0        | 10 ± 2.0         | 0.05|
| Tear Total Protein (mg/100ml) | 643.8± 5.4      | 536.5 ± 4.6      | 0.06|
| Tear Lysozyme (mg/100ml)      | 202.4 ± 2.2     | 188.7 ± 1.7      | 0.03|
| Tear Lactoferrin (mg/100ml)   | 164.5 ± 1.4     | 192.6 ± 1.7      | 0.03|
| Tear Immunoglobulin (mg/100ml)| 28.6 ± 0.4      | 39.9 ± 0.5       | 0.01|

*Values presented as Mean ± SD.* \( P \) values were compared by t-test; NS – no significance
There was no significant difference in age, sex ratio and body mass index (BMI) between RA patients and control subjects. The average tear volume collected and total protein content in the tear samples was significantly decreased in RA patients when compared with control subjects. The decreased total tear protein in RA patients was about 10%. However, for all experiments, three tear samples collected from each individual, were added together brought to final volume of 30µl (to minimize the effect of reduced volume in RA patients) prior to 1:10 dilution.

Electrophoresis showed alterations in protein profiling pattern in RA patients compared to healthy controls (Fig. 2). Comparing the pattern between two groups, the separated protein bands from control tear samples was less intensive than that from RA tear samples, though the same total tear protein amounts were loaded.

Fig. 2: Tear proteome profiling by electrophoresis. A typical electrophoregram from tear proteins of a healthy control subject (volunteer no.02) and RA patients (Nos. 01, 13, 27 severe RA patients and Nos. 05 and 35 from mild RA patients) are shown. Lane 1 in the left is the protein molecular weight markers. Arrows indicate increased protein band intensity in the lanes.

Looking into the separate protein bands in details, the intensity of the separated protein band with the lowest molecular weight at around 14.0 kDa (lysozyme) in control sample was higher than that in RA samples. The intensity of the remained protein bands with higher molecular weights at around 68 kDa and 35 kDa (representing lactoferrin and possibly the heterodimeric protein, lipophilin respectively) in RA samples was higher that that in control sample. It is worth reminding here that all of these 3 proteins posses antimicrobial properties. Interestingly, there was also a wide spread protein bands from 25 to 35 kDa in RA samples and its intensity was increased with the severity of the diseases condition in RA. This range of molecular weight could be related to some of denatured tear proteins such secretory immunoglobins.
The more severe RA clinical manifestation, the higher was intensity of the 25-35 kDa protein bands. The intensity of protein bands in electrophoregrams were obtained using a densitometer and compared to the standard protein band.

**Reverse-phase HPLC fractionation**

RP-HPLC separated intact proteins in the tear sample into a number of protein fractions.

In total, 11 protein fractioned peaks were collected. The first high prominent peak appeared within 10 minutes, multiple small peaks between 35 to 50 minutes, then a big and wide peak just before 80 minutes, and at last another high peak before 90 minutes. Compared with the protein fractioned peaks with control samples, most of the peak heights and areas were significantly higher in RA samples, except the last 2 peaks at 80 and 90 minutes (Table 2). Each fraction was then collected and concentrated for further electrophoresis for validations. By comparing their relative molecular weights with standard protein markers, the 1st peak, the 2nd last peak and the last peak were identified as lysozyme, lactoferrin and possibly, respectively. Lysozyme in RA samples was significantly lower than in control samples whilst lactoferrin and lipocalin in RA samples was significantly higher than in control samples.
### Table 2. Chromatographic tear proteome fractionation in RA.

<table>
<thead>
<tr>
<th>Peak Labels (left to right)</th>
<th>Control samples $(n = 30)$</th>
<th>RA samples $(n = 30)$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Retention Time (Min)</td>
<td>Peak Height* (cm)</td>
</tr>
<tr>
<td>1</td>
<td>10±1.5</td>
<td>6.1±0.2</td>
</tr>
<tr>
<td>2</td>
<td>37±2.0</td>
<td>0.8±0.0</td>
</tr>
<tr>
<td>3</td>
<td>35±3.0</td>
<td>1.7±0.01</td>
</tr>
<tr>
<td>4</td>
<td>40±1.0</td>
<td>0.4±0.05</td>
</tr>
<tr>
<td>5</td>
<td>42±2.0</td>
<td>1.7±0.01</td>
</tr>
<tr>
<td>6</td>
<td>44±2.0</td>
<td>0.4±0.0</td>
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<tr>
<td>7</td>
<td>49±1.0</td>
<td>0.3±0.0</td>
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<tr>
<td>8</td>
<td>51±1.0</td>
<td>0.1±??</td>
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<tr>
<td>9</td>
<td>65±0.0</td>
<td>0.2±0.0</td>
</tr>
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<td>10</td>
<td>80±2.0</td>
<td>0.8±0.05</td>
</tr>
<tr>
<td>11</td>
<td>88±3.0</td>
<td>2.5±0.1</td>
</tr>
</tbody>
</table>

* p<0.05, ** p<0.001

### Quantification of individual tear proteins

As the differential lysozyme, lactoferrin and immunoglobulins in RA tear samples are the most important proteins in tear film and their concentrations are higher enough to be measurable by simple analytical methods. We had employed the commercial available RID kit to quantify the concentration of each protein in tear samples from RA patients and compared with control subjects. The concentrations of lactoferrin and immunoglobulins were significantly higher in RA patients. On the other hand, the antibacterial tear enzyme, lysozyme, was significantly decreased. In this study, the presence of lipocalin and lipophilin in tear samples was only detected by SDS-PAGE. It is suggested that some more specific tests in each case are required to measure their quantitative amount. In this way, it would then be possible to comment on their alternations due to an auto-immune disorder such as RA.

### Discussion

Alterations in tear protein pattern due to internal and external factors have been reported by various investigators. It has been shown that external factors such as contact lens wear could affect tear protein pattern [34]. Statistically significant differences in tear protein levels have been reported during one day, which was not highly affected by collection method [35]. External factors such as smoking and air pollution could also affect tear protein pattern and activity of some important enzymes [36, 37]. The specific and non-specific tear proteins have shown age related variations using ELISA technique [38]. Internal diseases [39] and eye related disorders can also cause changes in tear proteins [40].
It is known that rheumatoid arthritis is associated with scleritis, scleromalacia perforans and corneal melt [41]. Traditionally, it has been demonstrated that the cornea is an immunologically privileged site due to paucity of blood vessels and lymphatics [42]. According to more recent literature [39] it has been stated that immune cells may in fact pass between the eye and the systemic vascular and lymphatic circulations but, through a process of apoptosis, become inactivated. It is also known that peripheral ulcerative keratitis is complication of RA that can lead to rapid corneal destruction and perforation with loss of vision [41]. In several pathological instances, the lachrymal gland can become a target of the immune system and show signs of inflammation [43]. It has been demonstrated that the protein profile in a single teardrop can reliably differentiate patients with Sjogren's syndrome from those with, for example, dry eye or other ocular disease and from healthy controls [44]. Tear proteomic profile has 87% sensitivity, 100% specificity, and a positive predictive value of 100%. The technique can therefore be thought as a suitable replacement for traditional keratoconjunctivitis sicca tests and lachrymal-gland biopsy. The goal of this study was to identify changes due to RA in tear proteome using a simple proteomic approach.

According to the results obtained from this research, it was demonstrated that tear volume and total tear proteins in RA patients were significantly decreased. On the other hand, tear lactoferrin and immunoglobulins levels were significantly increased while lysozyme and most other separated proteins were decreased. The functions of the tear film include lubrication, protection from invasion of microorganisms and nutrition of the cornea. It also plays a critical role in the optical properties of the eye. Normal tear volume is around 6 µL and the secretion rate is about 1.2 µL per minute, with a turnover rate of approximately 16% per minute [45]. It has been reported that total tear volume and tear flow rate is normally lower in RA patients [46]. Reduction in tear volume was observed almost among all RA patients, this shows that the defense capacity of tear fluid has decreased due to an antiinflammatory response of tear fluid to RA.

Lactoferrin plays an immunoregulatory and antiinflammatory role in RA [47, 48]. In particular, the lactoferrin-mediated reduction of tumor necrosis factor-a (TNF-a) release, a cytokine which plays a pivotal pathogenic role in rheumatoid synovitis, might have a beneficial effect. On the other hand, it is well known that specific granules of neutrophil granulocytes (PMN) are responsible for synovial injury by secreting oxygen free radicals. It has been shown that lactoferrin decreases the generation of extracellular hydroxyl radicals by PMN through rapidly chelating iron in a noncatalytic form [49]. It is found recently that lactoferrin may also affect mononuclear phagocytes [50].

Based on these data, the marked increase in lactoferrin concentration may be due to it protective role at the site of inflammation in RA patients. As lactoferrin is also stored in specific granules of neutrophil granulocytes (PMN), it is released following their activation. On the other hand, lactoferrin modulates inflammatory and immune responses, it decreases the release of interleukin-1 (IL-1), TNF-a, IL-2 and enhances monocyte cytotoxicity and natural killer cell activity [51].

In contrast, the lachrymal gland inflammation due to rheumatoid arthritis, may lead to lachrymal gland dysfunction and decreased production of defense enzymes such as lysozyme. It has been shown that tear volume decreases significantly with increasing age [38] and in some eye disorders as well as systemic inflammatory diseases [46].
It is also known that there is a linear and related decline of tear volume, concentration of lysozyme and lactoferrin with age. IgA levels gradually decline, while caeruloplasmin and IgG both increase after the fifth decade. The results suggest that tear IgG and caeruloplasmin are probably transudates from the serum conjunction that IgA is secreted independently of tear volume, and that lysozyme and lactoferrin are secreted at the same site but independently of tear volume.

In conclusion, an adequate knowledge of physiological variation is important for valid comparative studies of tear proteins. The lachrymal gland is the main contributor to the aqueous layer of the tear film. It secretes proteins, electrolytes and water, which helps to nourish and protect the ocular surface. Lachrymal gland secretion is primarily under neural control, which is achieved through a neural reflex arc. Stimuli to the ocular surface activate afferent sensory nerves in the cornea and conjunctiva. This in turn activates efferent parasympathetic and sympathetic nerves in the lachrymal gland to stimulate secretion [43]. Therefore, based on the results obtained from this study, it could be emphasized that a rheumatologist should be aware of the association between RA and inflammatory eye disease, with particular reference to the site of the anatomical lesion of the eye, in terms of diagnosis and response to therapy.

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