Tear Interferon-Gamma as a Biomarker for Evaporative Dry Eye Disease

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Submitted: April 18, 2016
Accepted: July 27, 2016
Citation: Jackson DC, Zeng W, Wong CY, et al. Tear interferon-gamma as a biomarker for evaporative dry eye disease. Invest Ophthalmol Vis Sci. 2016;57:4824–4830. DOI:10.1167/iovs.16-19757

PURPOSE. To assess whether tear hyperosmolarity, being diagnostic of dry eye disease (DED), is associated with specific alterations to the cytokine content of human tears that may provide a biomarker for DED.

METHODS. In this prospective, cross-sectional, clinical study, participants (n = 77) were recruited from a single clinical site and categorized into groups based upon tear osmolarity status (n = 62 hyperosmolar, n = 15 normo-osmolar). Comprehensive anterior eye clinical assessments were undertaken. Concentrations of seven cytokines (IL-2, IL-4, IL-6, IL-10, IL-17A, IFN-γ, and TNF-α) in basal tears were assayed using multiplex cytometric bead array. The main outcome measure was difference in cytokine concentration between groups. Group comparisons were undertaken using 2-tailed t-tests. Cohen’s effect size was calculated for each finding. Spearman correlations between cytokine concentrations, clinical symptoms, and clinical parameters of DED were calculated.

RESULTS. Tear hyperosmolarity was specifically associated with increased tear IFN-γ levels (13.3 ± 2.0 vs. 4.4 ± 1.4 pg/mL, P = 0.03). Cohen’s effect size was large (0.8) for changes to tear IFN-γ levels. Significant correlations were observed between IFN-γ concentration and each of: tear osmolarity (r = 0.34; P = 0.007), total ocular surface staining (r = 0.56, P < 0.0001), and Schirmer test score (r = −0.33, P = 0.003).

CONCLUSIONS. Tear hyperosmolarity is specifically associated with higher levels of the proinflammatory cytokine IFN-γ, which correlate with key clinical parameters of DED. The calculated effect size (0.8) suggests that this assay has diagnostic power as a biomarker for evaporative DED.

Keywords: dry eye, diagnosis, cytokine, inflammation, osmolarity, tear, interferon-gamma

Tear hyperosmolarity is a consequence of reduced lacrimal secretion and/or excessive tear evaporation, and is recognized to be a key pathogenic mechanism in dry eye disease (DED).1–4 While historically the clinical assessment of tear osmolarity has been hindered by difficulties associated with tear collection methods and access to laboratory facilities, technological advancements have enabled tear osmolar determinations to become clinically viable. Measurement of tear osmolarity has been suggested to be the single best objective indicator of DED severity.5 A diagnostic cut-off value of 316 mOsmol/L has been described to be highly specific for diagnosing clinically significant DED, yielding a sensitivity of 73% and a specificity of 90%.6

In DED, abnormal elevation of the osmolarity of the tear fluid is proposed to induce epithelial injury through the activation of inflammatory events at the ocular surface, resulting in the liberation of inflammatory mediators that can induce epithelial cell apoptosis.1,7 Short-term exposure of cultured human corneal epithelial cells to an hyperosmolar medium can induce IL-6 expression.8 In an animal model of ocular surface disease, tear hyperosmolarity resulted in conjunctival goblet cell death and tear film instability.9 Furthermore, several studies have described changes to proinflammatory cytokines and chemokines in tear fluids from individuals having heterogeneous clinical classifications of DED.10–17 Supporting the role of inflammation in the pathogenesis of DED is a body of clinical evidence that anti-inflammatory therapies, including corticosteroids and cyclosporine, reduce inflammatory markers, and provide symptomatic improvement.18–21

The aim of the study was to characterize tear composition in humans having hyperosmolar tears, to determine whether tear hyperosmolarity is associated with specific changes to tear cytokines that could be useful as a diagnostic clinical marker for DED.

METHODS

This research project was conducted in accordance with the tenets of Declaration of Helsinki and was approved by the University of Melbourne Human Research Ethics Committee.
Tear Interferon-Gamma in Dry Eye Disease

(Health Sciences subcommittee). All participants provided written informed consent to participate.

**Study Participants**

This cross-sectional study involved 77 adults (26 males, 51 females). Dry eye disease participants were being screened for participation in a dry eye intervention study. A target sample size of 75 participants, comprising of n = 60 with tear hyperosmolarity and n = 15 controls, returned 85% power to detect a difference of 20% in tear cytokine levels, given a SD of 20%.

Exclusion criteria included any of: contact lens wear within the past month, anterior segment surgery in the past 6 months, a history of keratorefractive procedures, history of ocular disease other than DED, current pregnancy, nursing or lactation, use of topical medications other than ocular lubricants within the previous 6 months, use of systemic immunomodulators, tetracyclines, or corticosteroids within the past 6 months and punctal occlusion or ocular trauma within 6 months of enrollment. Participants underwent a comprehensive bilateral ocular examination including: dry eye symptom assessment (using the standardised and validated Ocular Surface Disease Index, OSDI), tear osmolarity measurement, basal tear collection, slit-lamp examination (grading of meibomian gland capping, sodium fluorescein tear break-up time [NaFl TBUT], ocular surface staining with NaFl and lissamine green [LG] and Schirmer test with anesthesia).

Participant groups were defined by tear osmolarity status. The ‘hyperosmolar’ tear group (n = 62) had tear osmolarity greater than or equal to 316 mOsm/L; this criterion has been shown to have a predictive diagnostic accuracy of 89% for clinically significant DED. The ‘normo-osmolar’ tear (n = 15) group had bilateral tear osmolarity less than or equal to 308 mOsm/L. For all participants, the eye with higher tear osmolarity was defined as the ‘study eye’ and used for all subsequent analyses.

**Clinical Examination Procedures**

**Tear Osmolarity.** Tear osmolarity was measured from the inferior lateral tear meniscus using the TearLab (TearLab Corp., San Diego, CA, USA) system. The instrument was calibrated daily according to the manufacturer’s instructions. Room temperature was strictly maintained between 20°C and 24°C. Participants using lubricant eye drops were instructed not to instil these for at least 2 hours prior to examination and this was confirmed prior to taking measurements. The same diagnostic pen was used for all assessments. Right eyes were measured first.

**Tear Collection.** Basal (unstimulated) tear fluid, approximately 4 to 5 µL per eye, was collected by capillary flow from the inferior lateral meniscus of each eye using disposable microcapillary tubes (20 µL MicroCaps; Drummond, Broomall, PA, USA). Tear flow rate was monitored to exclude dilution effects caused by reflex tearing. Only samples with a flow rate of 1 to 3 µL/min were used (i.e., any 3-µL sample collected in less than 60 seconds was excluded). Tear samples were stored at −80°C for subsequent analysis.

**Meibomian Gland Evaluation.** The eyelids were examined at a slit-lamp biomicroscope for the degree of meibomian fluid into the eye. Dry Eye Test strips show improved measurement reliability and enhanced precision compared with conventional strips. A DET was moistened with nonpreserved saline and applied to the superior bulbar conjunctiva. One minute after instillation, NaFl TBUT was measured at a slit-lamp biomicroscope using ×10 magnification with cobalt blue illumination and a Wratten 12 yellow-barrier filter. Participants were asked to gently blink twice and then to hold their eyes open for as long as possible. The interval between the second blink and the appearance of the first dark spot or discontinuity in the precorneal fluorescein-stained tear layer was noted. Three NaFl TBUTs were recorded for each eye and averaged.

**Total Ocular Surface Staining.** Corneal NaFl staining, being a measure of corneal epithelial damage, was examined at the slit-lamp biomicroscope using ×16 magnification with cobalt blue illumination and a Wratten 12 yellow-barrier filter, 2 minutes after instillation of NaFl. The degree of staining was assessed in each eye with the five-step diagrammatic Oxford scale, with the clinician subjectively using 0.1-grading increments to maximize precision. Nasal and temporal conjunctival LG staining was assessed using the Oxford scale, 1 minute after instillation of LG (GreenGlo, Sigma Pharmaceuticals, LLC, North Liberty, IA, USA). A “total ocular surface staining” score (ranging from 0.0–15.0) was determined as the sum of the individual NaFl corneal, LG temporal conjunctival, and LG nasal conjunctival scores.

**Schirmer Test.** The Schirmer test, with topical anesthesia, was undertaken to measure tear production. One drop of 0.5% proxymetacaine hydrochloride (Alcon Laboratories, New South Wales, Australia) was instilled into the lower conjunctival sac. After 4 minutes, the folded end of a sterile Schirmer strip (EagleVision, Memphis, TN, USA) was placed between the middle and lateral third of the inferior lid margin. Participants were instructed to close their eyes, in the dimly lit room, and the strip wetting was measured (and recorded in millimeters), after 5 minutes.

**Tear Cytokine Analyses**

Tear cytokine concentrations were measured from the collected basal tear samples. Levels of IL-2, IL-4, IL-6, IL-10, IL-17A, IFN-γ, and TNF-α were determined using a Cytometric Bead Array (CBA) Human Th1/Th2/Th17 kit (BD Biosciences, San Diego, CA, USA) according to the manufacturer’s instructions, with the exception that samples were diluted 1 in 8.333 in a final volume of 25 µL and a total of 1 µL of each capture bead was used in 25 µL of tear sample. Samples were analyzed on a Becton Dickinson FACSCanto II flow cytometer (Franklin Lakes, NJ, US) and data analyzed using Becton Dickinson FCAP Array software.

The average concentration (pg/mL) of the seven cytokines in the multiplex assay was calculated for each tear sample. To assess for the potential effect of unintended reflex tearing causing a generalized dilution of tear proteins, the relative concentration of each tear cytokine (i.e., IL-2, IL-4, IL-6, IL-10, IL-17A, IFN-γ, TNF-α) was calculated as a ratio of the overall average cytokine concentration.

**Statistical Analyses**

Data were analyzed using the GraphPad Prism 5 software package (GraphPad Software, San Diego, CA, USA). Descriptive statistics are summarized as mean ± SEM. A Kolmogorov Smirnov test was used to assess for normality of continuous variables. Comparisons between groups were undertaken using either a t-test or Mann-Whitney U test, as appropriate. A χ² test was used to compare discrete variables. Pairwise
correlations were explored using Spearman’s correlation coefficient (r). Cohen’s effect size was calculated, as the difference of the means of the two groups divided by the weighted pooled SDs of these groups, as a quantitative measure of the substantive impact of each finding. Effect size magnitude (d) is reported, as recommended by Cohen, and described using standard nomenclature as: negligible (<0.20), small (0.20–0.49), medium (0.50–0.79), large (0.80–1.29), or very large (>1.30). An alpha value of 0.05 was adopted for statistical significance.

RESULTS

Participant Characteristics

Table 1 summarizes the demographic and clinical findings for each study group. The normo-osmolar and hyperosmolar tear groups were of similar age and sex. Dry eye symptoms (OSDI score) and total ocular surface staining were higher in the hyperosmolar group, while NaFl TBUT was significantly shorter (P < 0.05 for each comparison). Consistent with the DED population being predominantly of the evaporative subtype, there was no significant difference in Schirmer test findings between groups (P > 0.05) and a higher grade of meibomian gland capping in the hyperosmolar tear group (Table 1, P < 0.05).

Tear Cytokines

Table 2. Absolute Tear Cytokine Concentrations (pg/mL), and the Magnitude of the Effect Size, in Each Study Group

<table>
<thead>
<tr>
<th>Cytokine Level, pg/mL</th>
<th>Normo-osmolar, n = 15</th>
<th>Hyperosmolar, n = 62</th>
<th>P Value</th>
<th>Effect size, d</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>3.3 ± 1.1</td>
<td>5.6 ± 1.1</td>
<td>0.33</td>
<td>0.3</td>
</tr>
<tr>
<td>IL-4</td>
<td>2.4 ± 0.7</td>
<td>1.5 ± 0.2</td>
<td>0.12</td>
<td>0.02</td>
</tr>
<tr>
<td>IL-6</td>
<td>45.1 ± 6.2</td>
<td>52.8 ± 18.1</td>
<td>0.84</td>
<td>0.1</td>
</tr>
<tr>
<td>IL-10</td>
<td>1.6 ± 0.3</td>
<td>1.3 ± 0.2</td>
<td>0.46</td>
<td>0.2</td>
</tr>
<tr>
<td>TNF-α</td>
<td>10.7 ± 3.7</td>
<td>7.5 ± 2.0</td>
<td>0.47</td>
<td>0.2</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>4.4 ± 1.4</td>
<td>13.3 ± 2.0</td>
<td>0.03</td>
<td>0.8</td>
</tr>
<tr>
<td>IL-17A</td>
<td>34.3 ± 9.5</td>
<td>36.1 ± 8.1</td>
<td>0.92</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SEM. Bold numbers indicate statistically significant P values at P < 0.05.

DISCUSSION

This study sought to assess whether tear hyperosmolarity, being a ubiquitous feature of DED, is coupled with changes to tear cytokine levels. Tear hyperosmolarity was found to be specifically associated with elevated tear concentrations of the proinflammatory cytokine, IFN-γ. Cohen’s effect size was large (0.8) only for changes to IFN-γ levels, suggesting its potential...
body of evidence supports the role of T-helper (Th) CD4\(^+\) cells in mediating chronic inflammation in DED. CD4\(^+\) cells have been shown to infiltrate the conjunctival tissues of murine models of dry eye, localized ocular surface inflammation was mediated by CD4\(^+\) T cells. Importantly, activated Th-1 cells, in addition to T-cytotoxic and natural killer (NK) cells, secrete the proinflammatory cytokine, IFN-\(\gamma\), which inhibits the production of IFN-\(\gamma\) by Th-1 cells.32 has been shown to reduce tear osmolarity in chronic ocular surface disease.35 By defining the study groups dichotomously on the basis of tear osmolarity (i.e., normo-osmolar versus hyperosmolar), differentiation was indirectly achieved for most dry eye clinical parameters. The tear hyperosmolarity group had significantly more symptoms (measured using OSDI score), more substantial ocular surface staining, shorter NaFl TBUTs and greater meibomian gland capping than the normo-osmolar tear group (Table 1). Of note, Schirmer test scores did not differ between groups. This finding is in agreement with previous work that has demonstrated a lack of consistent relationships between common signs and symptoms of DED.44,45 This constellation of clinical findings (i.e., reduced NaFl TBUT, tear hyperosmolarity, meibomian gland obstruction, and a Schirmer score within the normative range), implies that the DED in our population was predominantly evaporative in etiology, rather than being due to insufficient aqueous production.

In evaporative subtypes of DED, tear hyperosmolarity occurs as a consequence of an insufficient and/or poor quality tear lipid layer; this promotes tear instability and more rapid evaporation of the tear film from the ocular surface, even in the presence of a normal tear volume.1 This subtype is distinct from aqueous-deficient DED, which results from impaired lacrimal secretion. Given the etiological differences in dry eye subtypes, it is unclear whether our findings (from a clinical population with predominantly evaporative DED) can be extrapolated to aqueous-deficient DED. Nevertheless, the findings of higher conjunctival expression of IFN-\(\gamma\) in patients with non-Sjögren and Sjögren syndrome-associated tear film dysfunction,43 lend support to the concept of this potentially being a generalized feature of DED.

The clinical phenotypes of study populations are likely to contribute to at least some of the differences in our findings compared with earlier work. In particular, we did not observe any difference in tear levels for the other tested cytokines, IL-2, IL-4, IL-6, IL-10, IL-17A, and TNF-\(\alpha\), between our age- and sex-matched groups. The observed similarity in these cytokines between normo-osmolar tears and the hyperosmolar group (with primarily evaporative DED) supports the findings of Boehm and colleagues.37 In contrast, Massingale et al.44 found elevation in a range of proinflammatory cytokines, including IFN-\(\gamma\), within a small sample (\(n = 7\)) of DED patients. This study included only female participants, with and without Sjögren's syndrome, which may have contributed to the relatively higher levels of inflammatory markers detected. Interestingly, although IL-6 levels did not differ between the study groups, across the entire study population levels of this tear cytokine negatively correlated with aqueous tear produc-

It is therefore noteworthy that we find elevated tear osmolarity to be specifically associated with increased tear concentrations of IFN-\(\gamma\) and that this finding has a large Cohen's effect size, indicating the importance of the finding independent of sample size. In addition, there was a significant positive correlation between increasing tear osmolarity and higher tear IFN-\(\gamma\) levels (\(r = 0.34; P = 0.007\)). Although the association between elevated tear osmolarity and ocular surface inflammation is well recognized,\(^{19,36}\) this study is the first to demonstrate a specific link between increasing hyperosmolarity and this proinflammatory cytokine. Moreover, the effect size calculations suggest that tear IFN-\(\gamma\) assay could have diagnostic power as a biomarker for DED.

Our findings suggest that tear IFN-\(\gamma\) assay could be of value for identifying clinical cases of DED with a significant inflammatory overlay, which would therefore benefit from anti-inflammatory intervention. It also follows that tear osmolarity measures may provide a useful surrogate clinical marker for tear IFN-\(\gamma\) levels and could therefore be useful for monitoring the effects of anti-inflammatory treatments on the chronic inflammatory overlay that characterizes DED. Indeed, the immunomodulatory agent cyclosporine-A, which inhibits the production of IFN-\(\gamma\) by Th-1 cells, has been shown to reduce tear osmolarity in chronic ocular surface disease.35

\[\text{Figure 1. Graph showing the ratio of tear IFN-\(\gamma\) concentration, relative to the average concentration of the seven assayed cytokines.} \]

\[\text{Open symbols designate the normo-osmolar tear group and filled symbols designate the hyperosmolar tear group.} \]

\[\text{*Statistically significant difference (} P < 0.05\).} \]
These findings suggest that lower tear production, even within a physiological range, may be associated with measurably higher levels of this proinflammatory cytokine. Lam and colleagues, who defined participant groups using strict clinical criteria but did not measure tear osmolarity, reported an overall increase in tear IL-6, IL-8, and TNF-α levels in patients with dysfunctional tear syndrome. In this study, different tear chemokine/cytokine profiles were observed in individuals with and without meibomian gland dysfunction, being the leading cause of evaporative DED. Tear IFN-γ concentration positively correlated with corneal fluorescein and conjunctival LG staining scores, consistent with our findings.

We acknowledge that an inherent limitation of studies involving tear fluid collection is the possibility that some participants may experience an extent of reflex tearing during sampling. To minimize the potential confound of this effect, we carefully monitored tear flow rate and excluded any samples that exceeded our threshold collection rate for basal tears. Another analytical approach involves the quantification of cytokine levels relative to total tear protein or constitutive proteins. Although the measurement of total tear protein was

### Table 3. Correlation Between Tear Cytokines and Clinical Parameters of DED (n = 77 Participants)

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Tear Osmolarity</th>
<th>OSDI Score</th>
<th>NaFl TBUT</th>
<th>Meibomian Gland Capping</th>
<th>Total Ocular Surface Staining</th>
<th>Schirmer Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>−0.08 (P = 0.47)</td>
<td>0.21 (P = 0.07)</td>
<td>−0.11 (P = 0.35)</td>
<td>0.14 (P = 0.26)</td>
<td>−0.05 (P = 0.68)</td>
<td>0.08 (P = 0.49)</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.03 (P = 0.78)</td>
<td>−0.05 (P = 0.64)</td>
<td>0.05 (P = 0.66)</td>
<td>−0.14 (P = 0.24)</td>
<td>0.03 (P = 0.83)</td>
<td>0.04 (P = 0.69)</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.07 (P = 0.55)</td>
<td>−0.12 (P = 0.29)</td>
<td>−0.03 (P = 0.83)</td>
<td>−0.21 (P = 0.08)</td>
<td>0.07 (P = 0.55)</td>
<td>−0.23 (P = 0.04)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>−0.11 (P = 0.62)</td>
<td>−0.12 (P = 0.29)</td>
<td>−0.07 (P = 0.53)</td>
<td>−0.17 (P = 0.13)</td>
<td>0.05 (P = 0.68)</td>
<td>−0.006 (P = 0.96)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.34 (P = 0.007)</td>
<td>0.12 (P = 0.30)</td>
<td>−0.14 (P = 0.20)</td>
<td>0.03 (P = 0.77)</td>
<td>0.56 (P &lt; 0.0001)</td>
<td>−0.33 (P = 0.003)</td>
</tr>
<tr>
<td>IL-17A</td>
<td>0.11 (P = 0.55)</td>
<td>−0.15 (P = 0.20)</td>
<td>0.001 (P = 0.99)</td>
<td>−0.08 (P = 0.49)</td>
<td>−0.008 (P = 0.94)</td>
<td>−0.13 (P = 0.27)</td>
</tr>
</tbody>
</table>

Numbers represent Spearman correlation coefficients. Significant (P < 0.05) correlations are shown in bold.

![Figure 2](http://tvst.arvojournals.org/)

**Figure 2.** Tear IFN-γ concentration (pg/ml) versus (A) tear osmolarity (mOsmol/L) and (B) total ocular surface staining score (measured using the Oxford grading scale, /15.0). **Open symbols** designate the normo-osmolar tear group and **filled symbols** designate the hyperosmolar tear group.
limited by the volume of collected tears (~4 μL/eye) in this study, we quantified the relative concentration of each tear cytokine as a ratio of the overall average cytokine concentrations, in order to assess for generalized dilution effects. Importantly, this analysis confirmed the findings reported for absolute tear cytokine levels, whereby the only significant difference between study groups was for IFN-γ (Fig. 1), where there was a 9-fold higher relative concentration of this cytokine in the tear hypersmolar tear group. Confirmation of an elevation in tear IFN-γ level using relative cytokine measures, in addition to the absolute cytokine quantification, provides greater confidence in this finding.

In conclusion, this study demonstrates, for the first time, that tear hyperosmolarity is specifically associated with higher tear levels of the proinflammatory cytokine IFN-γ, which correlate with clinical parameters of DED. Further investigation is warranted regarding the use of tear IFN-γ level as a biomarker for evaporative DED, in particular in relation to the modulation of this cytokine in response to therapy.

**Acknowledgments**

The authors thank Laura Deinema, who collected clinical data and tear samples used in this study.

Supported by a grant from the Rebecca I. Cooper Medical Foundation (LED, 2015; Sydney, NSW, Australia). DCJ is a recipient of a National Health and Medical Research Council of Australia (NH&MRC) Research Fellowship.

Disclosure: D.C. Jackson, None; W. Zeng, None; C.Y. Wong, None; E.J. Mifsud, None; N.A. Williamson, None; C.-S. Ang, None; A.J. Vingrys, Allergen Pty Ltd. (F), CooperVision Pty Ltd. (F), Alcon Pty Ltd. (F); LE. Downie, Allergan Pty Ltd. (F); CooperVision Pty Ltd. (F), Alcon Pty Ltd. (F)

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