

Lower Vitamin D Level and Distinct Tear Cytokine Profile Were Observed in Patients with Mild Dry Eye Signs but Exaggerated Symptoms

Rohit Shetty^{1,*}, Swaminathan Sethu^{2,*}, Priyanka Chevour^{2,*}, Kalyani Deshpande¹, Natasha Pahuja¹, Harsha Nagaraja¹, Narendra Pindipapanahalli¹, and Arkasubhra Ghosh²

¹ Cornea and Refractive Surgery Division, Narayana Nethralaya Eye Hospital, Bangalore, India

² GROW Research Laboratory, Narayana Nethralaya Foundation, Bangalore, India

Correspondence: Arkasubhra Ghosh, PhD, and Swaminathan Sethu, GROW Research Laboratory, Narayana Nethralaya Foundation, #258/A, Narayana Nethralaya, Hosur Road, Narayana Health City, Bom-mansandra, Bangalore, India, 560099. e-mail: arkasubhra@narayananeethralaya.com

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Purpose: Dry eye is associated with inflammation, pain, and discomfort. Vitamin D is known to modulate immune responses and pain. This study investigates the level of serum vitamin D and tear-inflammatory proteins with relation to exaggerated symptoms in patients with mild dry eye.

Methods: Patients with mild dry eye signs (Dry Eye Workshop [DEWS] severity grade 1) but with exaggerated symptoms and healthy controls ($n = 19$, each) were recruited for this cross-sectional study. Schirmer's Test I (mm), tear film break-up time (TBUT; secs), and ocular surface disease index (OSDI) score were recorded. Serum vitamin D level and tear cytokine levels were measured.

Results: The mean OSDI score in the patient cohort (46 ± 3) was significantly higher than controls (8.4 ± 1.6). TBUT was lower (7.6 ± 0.3 secs) in patients compared with controls (11.0 ± 0.9 secs). Mean Schirmer's Test I value in patients (19.3 ± 1.4 mm) was lower than in controls (30.6 ± 1.9 mm). An inverse correlation was observed between serum vitamin D levels and OSDI score ($r = -0.569$; $P = 0.01$). Significantly higher levels of interleukin (IL)-17A/F, interferon (IFN)- γ , monocyte chemoattractant protein (MCP)-1, intercellular adhesion molecule (ICAM)-1, IL-4, IL-10, and decreased IL-2 concentrations was observed in the tears of patients compared with controls ($P < 0.05$).

Conclusion: Decreased serum vitamin D was associated with exaggerated symptoms in dry eye patients with mild dry eye signs. In addition, altered tear cytokine profile was also observed in these patients.

Translational Relevance: Vitamin D measurements would aid in the diagnosis and management of dry eye.

Introduction

Dry eye disease (DED) has emerged as one of the common and significant public health issues with 5.5% to 33.7% affected worldwide and is associated with discomfort, visual disturbance, tear-film instability, tear hyperosmolarity, and ocular surface inflammation.¹ Recommended strategies to manage DED are dependent on its severity grade, which is structured based on both signs and symptoms.² Lack of concordance between the signs and symptoms³ would influence severity grading and hence affecting treatment planning

and therapeutic outcomes.^{4,5} A subset of dry eye patients with adequate tear production do present severe symptoms of dry eye such as ocular surface grittiness, pain, discomfort, or irritation, but with mild (Dry Eye Workshop [DEWS] severity grading scheme) or no observable signs of dry eye disease, as those discussed by Rosenthal et al.⁶ and Galor et al.⁷ Clinically, this group presents a major challenge as they do not always respond favorably by resolution of pain symptoms to the current standard management strategies. Hence, the need for further exploration of additional factors that can contribute to the etiology of dry eye with exaggerated symptoms is warranted.

A multitude of factors have been reported to either play a role or are associated with the etiopathogenesis of DED including aging, autoimmune disease, use of contact lens, LASIK, medication, environmental, and occupational hazards. In addition, dietary and nutritional status especially essential fatty acids and very recently vitamin D^{8,9} has been associated with DED. Vitamin D regulates calcium homeostasis, immune responses, and cell fate.¹⁰ Due to its pleiotropic nature, the level of active forms of vitamin D has been associated with various diseases including those related to the eye.^{10,11} Furthermore, there is growing evidence regarding the association of vitamin D with chronic pain.^{9,12} An increase in the levels of inflammatory cytokines has been reported in DED^{13,14} and some with the ability to modulate pain sensation.¹⁵ However, it is not known whether these cytokine levels are differentially regulated in mild dry eye patients with exaggerated symptoms. Therefore, we investigated the status of serum vitamin D level and tear cytokine profile in patients with mild dry eye signs but with severe symptoms.

Methods

Study Design and Clinical Examination

The cross-sectional study was approved by the Narayana Nethralaya institutional review board (EC Ref. No.: C/2015/05/05) and the guidelines followed were in accordance with the tenets of the Declaration of Helsinki. Subjects were recruited for the study only after obtaining informed written consent as per institutional and ethics board guidelines. The subjects were selected from the patients referred to the Cornea Clinic at Narayana Nethralaya, Bangalore, India. After thorough clinical history to rule out ocular and systemic comorbidities, visual acuity assessment, refraction, detailed slit-lamp examination, fundus evaluation, and dry eye investigations (Schirmer's test I, tear film break-up time [TBUT], corneal and conjunctival staining) were performed. Patient discomfort and visual disturbance was graded using ocular surface disease index (OSDI) questionnaire (Allergan, Irvine, CA). Patients with mild dry eye, yet exaggerated symptoms (i.e., dry eye patients with signs indicative of Grade 1 but with symptoms profile indicative of grade 3 or 4 as stated in the DEWS dry eye severity grading scheme¹⁶) were included in the study cohort. The severity of the symptoms was assessed based on OSDI scores and mild dry eye patients with OSDI score greater than 23 (moderate

to severe) were considered to have exaggerated symptoms of dry eye. The inclusion and exclusion criteria used to recruit the study subjects are listed in Table 1. A total of 19 dry eye patients fulfilling the criteria and equal number of age and gender matched healthy controls without any signs and symptoms of ocular pathologies or systemic morbidities were included in the study (Table 1). Serum and tear fluid samples were collected from every subject.

Serum and Tear Sample Collection

Serum was isolated from peripheral venous blood by using BD Vacutainer Plus Plastic Serum Tubes (BD, Franklin Lakes, NJ). Tear samples were collected using Schirmer's strips by following Schirmer's test I protocol. Tear analytes were extracted from Schirmer's strips by cutting them into small pieces, agitation in sterile phosphate buffer solution (PBS) for 2 hours at 4°C followed by centrifugation.

Measurement of Serum Vitamin D

Total vitamin D – 25 (OH) vitamin D levels in the serum was measured by automated direct competitive chemiluminescent enzyme linked immunoassay (AD-VIA Centaur Vitamin D Total; Siemens Healthcare Diagnostics, Inc., Tarrytown, NY) that detects both 25 (OH) vitamins D₂ and D₃.

Cytokine and Chemokine Measurements

The levels of various inflammatory proteins in the tears were measured using cytometric bead array (BD CBA Human Soluble Protein Flex Set System, BD Biosciences, Haryana, India) on a flow cytometer (BD FACSCalibur, BD Biosciences). The CBA for this study was designed for simultaneous detection and quantification of interleukin (IL)-1 α , IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-9, IL-10, IL-12/IL-23p40, IL-12p70, IL-13, IL-17A, IL-17F, IL-21, chemokine ligand 2 (CCL2)/monocyte chemotactic protein (MCP)-1, C-X-C motif chemokine 10 (CXCL10)/IP-10, intercellular adhesion molecule 1 (ICAM1), interferon (IFN)- γ , and vascular endothelial growth factor (VEGF).

Statistical Analyses

All statistical analyses were performed with GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA). Shapiro-Wilk normality test was used to check distribution of the data set. Pearson correlation analysis, ANOVA with Tukey's multiple comparisons, and unpaired *t*-test were used to analyze normally distributed data. Spearman correlations

Table 1. Inclusion and Exclusion Criteria

Inclusion Criteria	Exclusion Criteria
<p>Patients presenting with signs of mild dry eye (DEWS severity grade) but with exaggerated symptoms (OSDI score > 23). Control group includes subjects with no signs of ocular surface conditions with no appreciable discomfort or symptoms (OSDI score < 23)</p>	<p>Sjogren's syndrome (primary, secondary); chronic inflammatory and autoimmune diseases.</p> <p>Lacrimal gland disorders (congenital, secondary); Lacrimal gland destruction secondary to trachoma, Oculocicatrical pemphigoid, Steven Johnson syndrome, trauma; decreased reflex tear secretion</p> <p>Eye lid abnormalities (blinking abnormality, low blink rate, palpebral aperture abnormalities, disorder of lid aperture, lid surface abnormalities); clinical evidence of meibomian gland dysfunction</p> <p>Contact lens use and medications – systemic medications; topical eye drops</p> <p>Surgical – secondary to ocular surgeries including refractive surgery</p> <p>Allergy; ocular infections (e.g., herpetic, fungal) and other systemic inflammatory or allergic conditions; uveitis; other ophthalmic diseases such as corneal erosions and keratoconus</p> <p>Endocrine and metabolic disorders; pregnancy; vitamin A deficiency and general malnutrition</p> <p>Environment and occupation induced dry eye symptoms.</p>

analysis, Kruskal-Wallis, and Mann-Whitney *U* test were used to analyze data sets that were not normally distributed. G*Power v3.1.9.2 was used for power calculation. The mean value of the individual groups was reported as mean \pm standard error of the mean (SEM). *P* less than 0.05 was considered to be statistically significant.

Results

The clinical characteristics of the study cohort is shown in [Table 2](#). Mean OSDI score of patients in this patient cohort (46 ± 3) was significantly ($P < 0.0001$) higher in controls (8.4 ± 1.6) and OSDI scores ranged from 25 to 73 in patients and are categorized to have either moderate or severe dry eye symptoms. Of patients, 26.3% (5/19) had moderate and 73.7% (14/19) had severe ocular surface disease symptoms. The OSDI scores in controls ranged between 0 and 22 with 68.4% (13/19) and 31.5% (6/19) of controls were grouped as either normal or with mild ocular surface disease, respectively. Mean Schirmer's Test I value in patients (19.3 ± 1.4 mm) was significantly lower than controls (30.6 ± 1.9 mm) and the values ranged between 10 and 32 mm within the patient cohort. It was observed that 36.8% (7/19) patients recorded less than or equal to 15 mm and 63.2% (12/19) recorded greater than 15 mm of wetting in 5 minutes. The values in the control group also

exhibited a similar range (10–35 mm) but with 10.5% (2/19) of controls exhibiting wetting less than 15 mm and 89.5% (17/19) with greater than 15 mm in 5 minutes. TBUT in the patients was observed to be significantly lower (7.6 ± 0.3 secs) than controls (11.0 ± 0.9 secs), and it ranged between 5 and 10 seconds in the patient cohort and between 5 and 18 seconds in healthy controls. TBUT was less than 10 seconds in 89% (17/19) of patients and it was equal to 10 seconds in 11% (2/19) of the patients. However, the TBUT was less than 10 seconds in 26.3% (5/19) and it was greater than or equal to 10 seconds in 73.6% (14/19) in the controls. No corneal or conjunctival fluorescein staining pattern based on Oxford schema was observed in the patients and controls. Mean serum vitamin D level in patient cohort was 16.1 ± 2 ng/mL and 5.3% (1/19) were normal (>30 ng/mL), 36.8% (7/19) were insufficient (>20 and <30 ng/mL), and 58% (11/19) were deficient (<20 ng/mL) in vitamin D. The mean serum vitamin D level (20 ± 1.6 ng/mL) in the healthy control cohort was not significantly different from that of the patient cohort. In the current study, we categorized the subjects based on their vitamin D status as less than or equal to 10 ng/mL; 11 to 20 ng/mL and greater than 20 ng/mL to further understand the association between vitamin D status and dry eye indices.

An inverse correlation ($r = -0.569$; $P = 0.0110$) was observed between serum vitamin D levels and OSDI score in the patient cohort as shown in [Figure 1A](#).

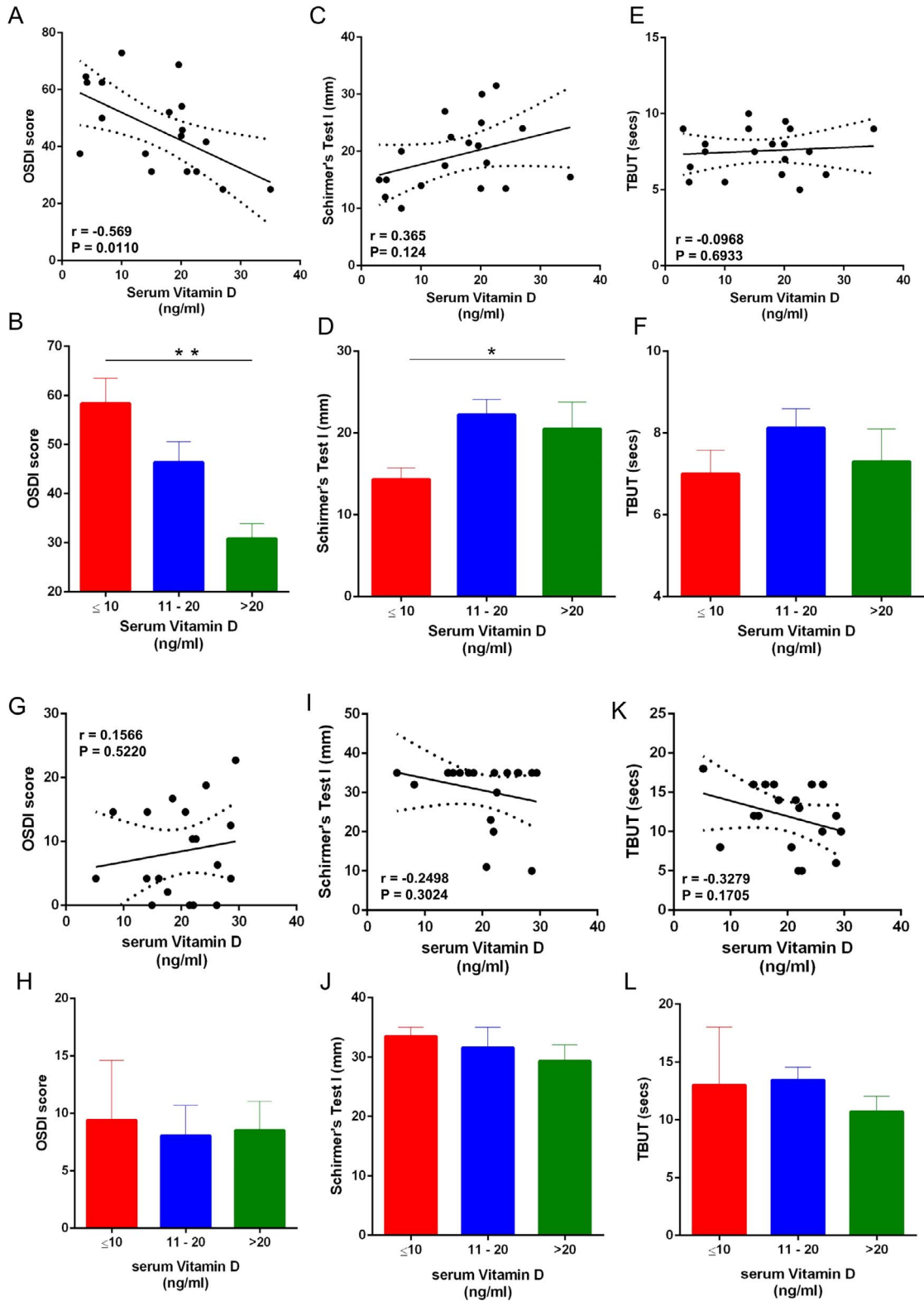


Figure 1. Correlation between serum vitamin D and clinical indices in mild dry eye patients with exaggerated symptoms and controls. (A) Correlation between serum vitamin D and OSDI score of patients ($n = 19$). (B) Mean \pm SEM OSDI score of patients with serum vitamin D level less than or equal to 10 ng/mL, 11 to 20 ng/mL, and greater than 20 ng/mL. (C) Correlation between serum vitamin D and Schirmer's test I in patients ($n = 19$). (D) Mean \pm SEM Schirmer's test I values of dry eye patients with serum vitamin D level less than or

Post-hoc calculation based on the correlation coefficient determined that the power of detection was close to 80%, and hence the sample size was adequate for the observation made. Mean OSDI score of patients with serum vitamin D level less than or equal to 10 ng/mL (58.3 ± 5.1), 11 to 20 ng/mL (46.4 ± 4.2), and greater than 20 ng/mL (30.8 ± 3.0) were significantly (ANOVA, $P = 0.0032$) different (Fig. 1B). Tukey's multiple comparisons test showed a significant difference between the OSDI score of patients with serum vitamin D less than or equal to 10 ng/mL and greater than 20 ng/mL (Fig. 1B). No correlation ($r = 0.1566$; $P = 0.5220$) was observed between serum vitamin D and OSDI scores in the controls (Fig. 1G) and the mean OSDI score of controls among the various serum vitamin D level groups were not significant either (Fig. 1H).

No correlation ($r = 0.365$; $P = 0.124$) was observed between serum vitamin D levels and Schirmer's test I (mm) in the patients (Fig. 1C). However, the mean Schirmer's test I values of patients with serum vitamin D level less than or equal to 10 ng/mL (14.3 ± 1.4), 11 to 20 ng/mL (22.3 ± 1.8), and greater than 20 ng/mL (20.5 ± 3.3) were significantly (ANOVA, $P = 0.042$) different (Fig. 1D). Tukey's multiple comparisons test shows a significant difference between Schirmer's test I values of patients with serum vitamin D less than or equal to 10 ng/mL and 11 to 20 ng/mL (Fig. 1D). No correlation ($r = -0.2498$; $P = 0.3024$) was observed between serum vitamin D levels and Schirmer's test I (mm) in the controls (Fig. 1I) and the mean Schirmer's test I values among the groups based on serum vitamin D levels did not exhibit any difference (Fig. 1J).

There was no correlation between serum vitamin D and TBUT in both patients ($r = -0.0968$; $P = 0.6933$) and control ($r = -0.3279$; $P = 0.1705$) cohorts (Figs. 1E, 1K, respectively). In addition, the mean TBUT was not significantly different in patients and healthy controls with less than or equal to 10 ng/mL; 11 to 20 ng/mL and greater than 20 ng/mL (Figs. 1F, 1L, respectively). These observations indicate that the exaggerated symptoms but not signs observed in

patients with mild dry eye are associated between lower serum vitamin D levels.

The levels of cytokines, chemokines, secreted cell adhesion molecules, and proangiogenic factor in the tears of control and dry eye patients were studied using cytometric bead array. Of the various inflammatory proteins level quantified IL-1 α , IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12/IL-23p40, IL-12p70, IL-17A, IL-17F, CCL2/MCP-1, CXCL10/IP-10, ICAM1, IFN γ , and VEGF were in the detectable range in the tears of both the healthy controls and patients. The tear levels of IL-1 α , IL-1 β , IL-6, IL-8, IL-12/IL-23p40, IL-12p70, CXCL10, and VEGF were not significantly different between the control and patient cohorts (Fig. 2). IL-17A, IFN γ , ICAM1, MCP1, IL-10, and IL-4 were significantly ($P < 0.05$) higher in patients with mild dry eye signs but with exaggerated symptoms compared to controls (Fig. 3). IL-17F was also markedly higher though not significant ($P = 0.06$) in the tears of these patients (Fig. 3). In contrast, IL-2 was observed to be significantly ($P < 0.05$) lower in the patient cohort compared with that controls (Fig. 3). No significant correlations were observed between serum vitamin D and tear cytokine levels in the current patient cohort.

Discussion

Corneal pain indicative of dry eye but without supporting clinical signs often results in diagnostic and therapeutic uncertainty.^{6,7} Therefore, it is essential to delineate aberrant nociception versus neuropathic pain in such scenario, followed by identification of potential etiological factors that could be contributing to the symptoms for an effective management strategy. Serum vitamin D and tear inflammatory factors level were measured in patients with mild signs of dry eye but with disproportionate symptoms in order to comprehend the pathobiology underlying this clinical presentation. The current study indicates that a subset of dry eye patients with mild signs of dry eye but with exaggerated symptoms could be suffering from corneal

equal to 10 ng/mL, 11 to 20 ng/mL, and greater than 20 ng/mL. (E) Correlation between serum vitamin D and TBUT in patients ($n = 19$). (F) Mean \pm SEM TBUT of patients with serum vitamin D level less than or equal to 10 ng/mL, 11 to 20 ng/mL, and greater than 20 ng/mL. (G) Correlation between serum vitamin D and OSDI score of healthy controls ($n = 19$). (H) Mean \pm SEM OSDI score of healthy controls with serum vitamin D level less than or equal to 10 ng/mL, 11 to 20 ng/mL, and greater than 20 ng/mL. (I) Correlation between serum vitamin D and Schirmer's test I in healthy controls ($n = 19$). (J) Mean \pm SEM Schirmer's test I values of healthy controls with serum vitamin D level less than or equal to 10 ng/mL, 11 to 20 ng/mL, and greater than 20 ng/mL. (K) Correlation between serum vitamin D and TBUT in healthy controls ($n = 19$). (L) Mean \pm SEM TBUT of healthy controls with serum vitamin D level less than or equal to 10 ng/mL, 11 to 20 ng/mL, and greater than 20 ng/mL. Schirmer's test I and TBUT values are average of the right and left eyes of a subject. These values were not significantly different between right and left eyes in the same subject. * $P < 0.05$; ** $P < 0.01$.

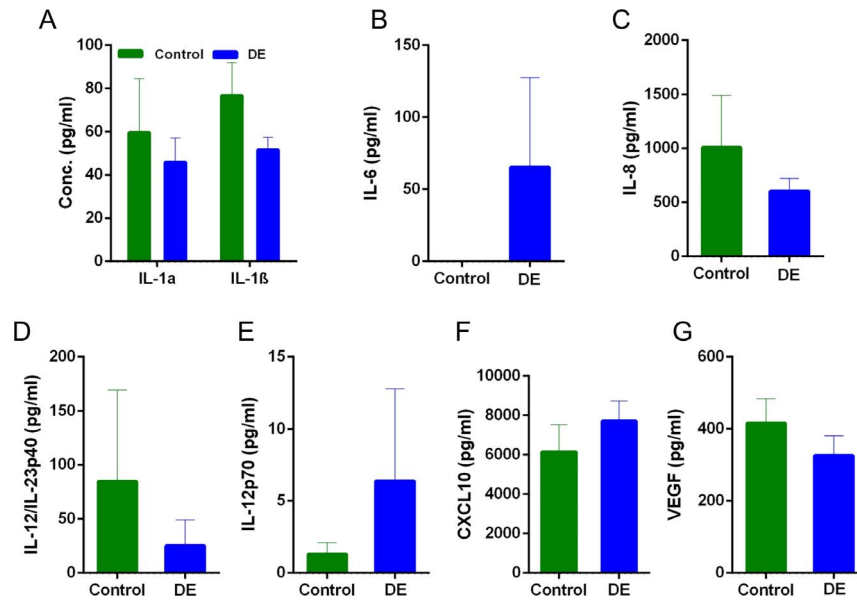


Figure 2. Absence of deregulation in the levels of IL-1 α/β , IL-6, IL-8, IL-12, CXCL10, and VEGF in the tears of mild dry eye patients. The *graphs* indicate the levels of IL-1 α/β (A), IL-6 (B), IL-8 (C), IL-12p40/70 (D, E), CXCL10/IP10 (F), and VEGF (G) quantified by cytometric bead array in the tears of control ($n = 10$) and dry eye patients ($n = 13$). *Bar graphs* indicate mean \pm SEM.

hyperalgesia along with increased tear inflammatory factors. We observed that the symptoms exhibited an inverse correlation with serum vitamin D level (Fig. 1). However, no correlation was observed between vitamin D and Schirmer's test I or TBUT (Fig. 1) in these patients. The current findings is in line with a report that suggests higher serum vitamin D levels correlated with a decrease in dry eye symptoms,⁸ but in contrary

to the observations made in a study on vitamin D deficient subjects with dry eye symptoms.¹⁷ Since vitamin D deficiency is associated with neuralgia and chronic pain^{9,12}, it is plausible that low vitamin D could contribute to the severity of ocular surface symptoms by either directly influencing nociceptive mechanisms or by altering inflammatory cytokines.

A distinctive tear cytokine/chemokine profile

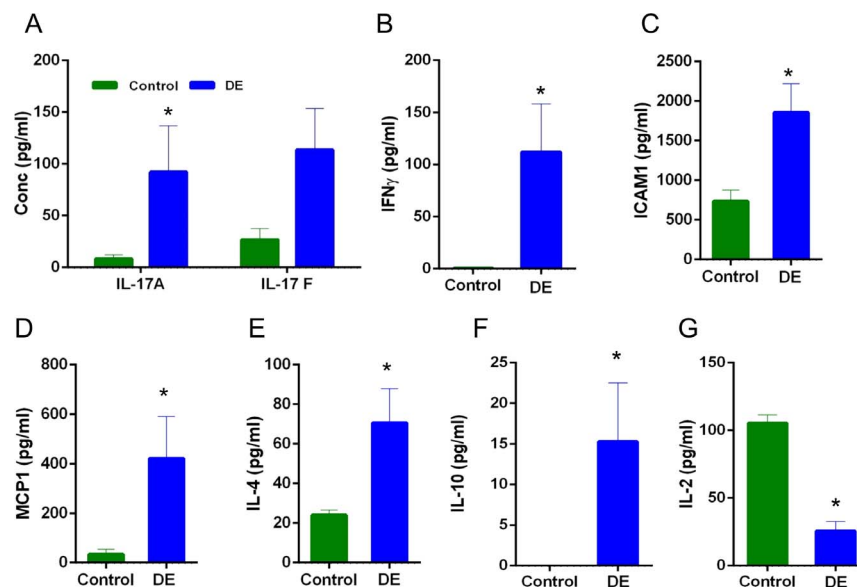


Figure 3. Altered tear levels of IL-17A/F, IFN γ , ICAM1, MCP1, IL-4, IL-10, and IL-2 in the tears of mild dry eye patients. The *graphs* indicate the levels of IL-17A/F (A), IFN γ (B), ICAM1 (C), MCP1 (D), IL-4 (E), IL-10 (F), and IL-2 (G) quantified by cytometric bead array in the tears of control ($n = 10$) and dry eye patients ($n = 13$). *Bar graphs* indicate mean \pm SEM. * $P < 0.05$.

Table 2. Clinical Characteristics of Study Cohort

	Controls	Patient
Sample size (male/female)	$n = 19$ (11/8)	$n = 19$ (10/9)
Age (mean \pm SEM)	27.8 ± 1.3 y	38.8 ± 3.4 y
OSDI score (mean \pm SEM)	8.4 ± 1.6	46 ± 3
Schirmer's Test I (mean \pm SEM)	30.6 ± 1.9 mm	19.3 ± 1.4 mm
TBUT (mean \pm SEM)	11.9 ± 0.9 secs	7.6 ± 0.3 secs
Serum vitamin D (mean \pm SEM)	20 ± 1.6 ng/mL	16.1 ± 2 ng/mL

(Figs. 2, 3) with potential to alter nociception observed in the patients may contribute to the exaggerated symptoms. IL-17, well known for its pathologic role in inflammatory disorders is also involved in nociception by mediating mechanical allodynia by altering the expression of neuronal TRPV4 channels essential for transduction of pain stimulus^{18,19} was found to be higher in the tears of patients (Fig. 3). Furthermore, a clinical trial (NCT01250171, www.clinicaltrials.gov/ct2/show/NCT01250171) reports a decrease in OSDI score in dry eye patient cohort on IL-17A blockers compared to IL-1 β blockers or placebo controls. Similarly, an increased IFN γ , MCP1 and ICAM1 observed in the patients (Fig. 3) could exacerbate the ocular symptoms, as they are reported to mediate pain.²⁰⁻²² Increased anti-inflammatory analgesic cytokines, IL-4, and IL-10 observed in the patients (Fig. 3) could be a compensatory mechanism to counter the pronociceptive effects. However, it should be noted that IL-4 can stimulate ICAM1 expression²³ that was also found to be elevated in patients (Fig. 3). IL-2 known for reducing chronic neuropathic pain²⁴ was found to be significantly reduced in the tears of patients (Fig. 3), indicating a loss of the potential antinociceptive role of IL-2. We also note that known cytokines such as IL-1 β , IL-6, and IL-8 reported earlier to be elevated in the tears of DED patient were not found to be significantly altered in the current patient cohort. Pain symptoms or hyperalgesia observed could be due to the direct effects of these inflammatory mediators by decreasing the sensory nerve thresholds in the ocular surface.

Vitamin D is well known for modulating the expression of various inflammatory cytokines in various cells, including corneal epithelial cells²⁵ and vitamin D deficiency has been shown to be associated with increased inflammatory cytokines. Hence, we speculate that vitamin D deficiency mediated dysregulation of cytokines with pronociceptive potential could result in severe pain symptoms in these patients.

Moreover, a recent case report states that correction of vitamin D deficiency reversed symptoms in a case of corneal neuralgia.⁹ Low vitamin D and ocular inflammatory mediators along with symptoms consistent with that of dry eye can also be associated with a variety of conditions. Hence, an exhaustive exclusion list as stated in Table 1 was adhered to ensure a more uniform group of patients whose primary characteristic was with mild dry eye signs but with severe and/or frequent ocular surface pain and/or irritation. This is one of the studies that report the association between serum vitamin D status and dry eye. In addition to determining the association of serum vitamin D and DED in larger cohorts, it is essential to obtain mechanistic insights into the aetiopathologic role of vitamin D deficiency and aberrant inflammatory cytokines in ocular surface health and corneal pain for targeted management of dry eye.

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*Contributed equally to this article.

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