

Dysregulated Tear Fluid Nociception-Associated Factors, Corneal Dendritic Cell Density, and Vitamin D Levels in Evaporative Dry Eye

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PURPOSE. The purpose of this study was to study the status and association among tear-soluble factors, corneal dendritic cell density, vitamin D, and signs and symptoms in dry eye disease (DED).

METHODS. A total of 33 control subjects and 47 evaporative dry eye patients were included in the study. DED diagnosis and classification was based on the 2017 Report of the Tear Film & Ocular Surface Society International Dry Eye Workshop (TFOS DEWS II). DED workup, including tear film break-up time (TBUT), Schirmer's test I (STI), corneal and conjunctival staining, ocular surface disease index (OSDI) scoring, and in vivo confocal microscopy (to assess corneal dendritic cell density [cDCD] and subbasal nerve plexus [SBNP] features) was performed in the study subjects. Tear fluid using Schirmer's strip and serum were collected from the subjects. Multiplex ELISA or single analyte ELISA was performed to measure 34 tear-soluble factors levels including vitamin D.

RESULTS. Significantly higher OSDI discomfort score, lower TBUT, and lower STI were observed in DED patients. cDCD was significantly higher in DED patients. No significant difference was observed in SBNP features. Tear fluid IL-1 β , IL-17A, MMP9, MMP10, MMP9/TIMP ratio, and VEGF-B were significantly higher in DED patients. Significantly lower tear fluid IL-2, IP-10, NPY, VEGF-A, and vitamin D was observed in DED patients. These dysregulated tear factors showed significant associations with DED signs and symptoms.

CONCLUSIONS. Altered tear fluid soluble factors with potential to modulate nociception exhibited a distinct association with ocular surface discomfort status, TBUT, STI, and cDCD. This implies a functional relationship between the various tear-soluble factors and dry eye pathogenesis, indicating new molecular targets for designing targeted therapies.

Keywords: dry eye disease, corneal dendritic cells, nociception, inflammation, vitamin D

Dry eye disease (DED) remains one of the common public health concerns, with a worldwide prevalence of 5% to 50%.¹ DED is currently defined as a multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film and accompanied by ocular symptoms, in which tear film instability, hyperosmolarity, damage due to ocular surface inflammation, and neurosensory abnormalities play etiologic roles.² The significant economic burden posed by DED is due to reduced quality of life, loss of professional productivity, and psychologic affliction due to ocular surface pain and vision disturbance.¹ The major morbidity associated with DED is ocular surface discomfort or pain. Hence, there is a need to better understand the etiology underlying it to develop newer

and more effective treatment modalities for managing pain and discomfort in DED.

A range of factors including aging, autoimmune disease, use of contact lens, refractive surgery, medication, environment, occupation, and nutritional status are involved in the etiopathogenesis of DED.^{3–6} Regardless of the type of etiologic factor and the sequence of events in DED pathogenesis, it is apparent that DED is characterized by chronic ocular surface inflammation and/or immune response. Human and animal studies have shown an increase in proinflammatory cytokines in DED conditions.^{7–9} Similarly, an altered proportion of ocular surface or corneal immune cells such as neutrophils, T-cell subsets, and dendritic cells has also been reported in animal models of DED.¹⁰ The sustained presence of these inflamma-



tory factors and immune cells would result in disruption of corneal epithelial barrier function and ocular surface homeostasis, resulting in discomfort and vision disturbance.^{4,11} Hence, the current management of DED includes certain immunomodulators in addition to lubricants. However, there is a subgroup of patients who may not always respond favorably to the current standard management strategies.^{12,13}

Despite the available knowledge on the pathobiology of DED, there remain gaps in understanding the mechanisms contributing to DED symptoms. In particular, the discomfort or pain in DED cannot solely be explained by tear film metrics due to the lack of concordance between signs and symptoms in patients.^{14,15} A subset of patients without major abnormalities in tear parameters still present with ocular surface grittiness, pain, discomfort, or irritation and may not respond to conventional treatment.^{2,16,17} Hence, there is an absolute need to further our understanding pertaining to the status of additional factors in DED patients that can impact nociception or neurosensory components resulting in ocular surface discomfort. Evidence does point to dysregulation in the levels of tear soluble factors (with ability to modulate nociception), corneal immune cells, and dysfunction in ocular somatosensory nerves to ocular surface discomfort in DED patients.^{18–20} Furthermore, dietary and nutritional factors, especially vitamin D, have been associated with DED.^{21–25} In addition to the well-known anti-inflammatory role of vitamin D, its association with chronic pain^{6,26} warrants its investigation in the context of ocular surface discomfort in DED. Although the current literature does have previously published data on such factors, those are results from independent studies in different patient cohorts, and therefore the data are fragmented, making clinically relevant correlations across clinical, imaging, and molecular parameters difficult. Therefore, this multiparametric study investigated the status and association among DED signs and symptoms, corneal dendritic cell density, and tear-soluble factors that can impact nociception and vitamin D in DED.

METHODS

Study Design and Clinical Examination

The current cross-sectional study was approved by the Narayana Nethralaya Institutional Review Board (EC Ref. No.: C/2016/10/04). Subject recruitment and sample collection procedures were conducted as per the institutional ethics board guidelines and in accordance with the tenets of the Declaration of Helsinki. Written informed consent was obtained prior to subject recruitment. Subjects for the study were selected from patients with signs and symptoms of DED referred to the Cornea Clinic at Narayana Nethralaya, Bangalore, India. Detailed clinical history, visual acuity assessment, refraction, slit-lamp examination, and fundus evaluation were performed to rule out any other ocular and systemic comorbidities. DED diagnosis and classification were based on the 2017 Report of the Tear Film & Ocular Surface Society International Dry Eye Workshop (TFOS DEWS II).^{2,27} DED investigations including Schirmer's test I (STI), tear film breakup time (TBUT), and corneal and conjunctival fluorescein staining were conducted, and observations were recorded. Schirmer's test without anesthetic was performed using sterile Schirmer's strips (5 × 35 mm²; ContaCare Ophthalmics and Diagnostics, Vadodara, Gujarat, India). TBUT and corneal and conjunctival staining were determined using fluorescein strips (ContaCare Ophthalmics and Diagnostics). Meibomian gland status was examined using infrared meibography (Oculus, Wetzlar, Germany). The symptoms were graded based on the discomfort scale and visual disturbance scale using the ocular

surface disease index (OSDI) questionnaire (Allergan, Dublin, Ireland).²⁸ The corneal dendritic cell density and subbasal nerve plexus features were also determined using in vivo confocal microscopy as described later. Patients presenting with signs and symptoms of dry eye were included in the DED group ($n = 47$). The control group includes subjects ($n = 33$) with no signs and symptoms of ocular surface conditions. DED subjects presenting with STI > 10 mm/5 min, TBUT < 10 seconds, and symptoms were included. Furthermore, DED subjects with STI value < 10 mm/5 min were excluded to avoid enrolling subjects who present DED signs and symptoms as a part of underdiagnosed/yet to be diagnosed systemic conditions such as autoimmune conditions. Additional exclusion criteria included the use of contact lenses; presence of allergy; ongoing ocular or systemic diseases with ocular manifestations such as Sjögren's syndrome, rheumatoid arthritis, and diabetes mellitus; subjects with lacrimal gland or lid disorders including clinically evident meibomian gland dysfunction; subjects who have recently undergone ocular surgery including those for refractive correction; and subjects on any form of topical medication.

Corneal Dendritic Cell Density and Subbasal Nerve Plexus Assessments

In vivo confocal microscopy (IVCM) imaging was performed using Rostock Corneal Module/Heidelberg Retina Tomograph II (RCM/HRT II; Heidelberg Engineering GmbH, Dossenheim, Germany) to determine corneal dendritic cell density (cDCD) and subbasal nerve plexus (SBNP) features in the study subjects as described earlier.²² Both eyes were included for IVCM-based investigations in the subjects of DED cohort, whereas only one eye (right) was included for the control group. Proparacaine drops (0.5%) were used prior to the procedure to anaesthetize the cornea. cDCD (cells/mm²) and dendritiform structures were quantified using Cell Count software (Heidelberg Engineering GmbH) as described earlier.^{22,29} IVCM image-based quantitative SBNP analyses were performed using Automatic CCMetrics software, version 1.0 (University of Manchester, Manchester, UK). The parameters quantified include corneal nerve fiber density (CNFD), the total number of major nerves per square millimeter; nerve fiber length (CNFL), the total length of all nerve fibers and branches (millimeters per square millimeter); nerve branch density (CNBD), number of branches emanating from major nerve trunks per square millimeter; total branch density (CTBD), the total number of branch points per square millimeter; the nerve fiber area (CNFA); and the total nerve fiber area per square millimeter and the nerve fiber width (CNFW), the average nerve fiber width per square millimeter.

Tear Fluid and Serum Collection

Tear fluid samples were collected from the study subjects using Schirmer's strips by following the Schirmer's test I protocol and stored in microcentrifuge tubes at -80°C until further processing. Tear proteins were extracted from Schirmer's strips by agitation in 300- μL sterile 1× PBS for 2 hours at 4°C . The tear fluid was eluted by centrifugation and stored in -80°C until further analyses. The tear samples after elution in 300- μL PBS from each eye of a subject were combined to obtain a total volume of 600- μL tear samples for every study subject, which were then used for quantification of the various soluble factors. Serum was isolated from peripheral venous blood by using BD Vacutainer Plus Plastic Serum Tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

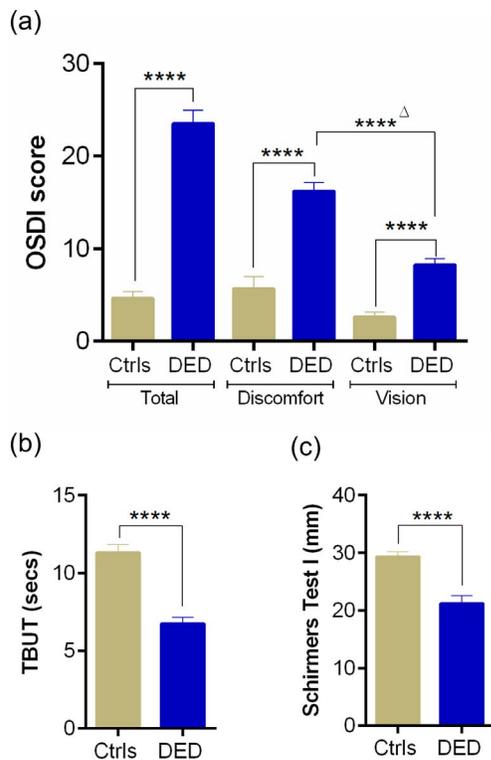


FIGURE 1. Increased ocular surface discomfort and decreased TBUT and STI in DED patients. The graphs indicate ocular surface discomfort index score including total, discomfort, and vision-related scale (a), TBUT (b), and STI value (c) in control subjects ($n = 33$) and DED patients ($n = 47$). Bar graphs indicate mean \pm SEM; **** $P < 0.0001$, Mann-Whitney test; Δ Wilcoxon matched-pairs signed rank test.

Tear-Soluble Factor Measurements

The levels of various secreted factors in the tears were measured using multiplex ELISA or single analyte sandwich ELISA. Simultaneous quantification of interleukin (IL)-1 α , IL-1 β , IL-2, IL-4, IL-8, IL-9, IL-10, IL-17A, IL-17E, TNF α , interferon (IFN) α , IFN γ , CCL2/monocyte chemoattractant protein 1 (MCP1), CXCL10/interferon-gamma inducible protein 10 (IP-10), CCL4/macrophage inflammatory protein 1 beta (MIP1 β), regulated on activation, normal T cell expressed and secreted (RANTES), intercellular adhesion molecule 1 (ICAM-1), and vascular endothelial growth factor (VEGF)-A was done by multiplex ELISA using Cytometric Bead Array (BD CBA Human Soluble Protein Flex Set System; BD Biosciences, San Jose, CA, USA) on a flow cytometer (BD FACSCantoII, BD Biosciences). These analytes were spread across three separate plexes for measurements. Fifty microliters of tear sample was used for each plex as per the manufacturer's instructions. BD FACSDiva software (BD Biosciences) was used to acquire the beads and record signal intensities. FCAP array version 3.0 (BD Biosciences) was used to determine absolute concentration of the analytes using respective standards. Similarly, matrix metalloproteinases (MMP)2, MMP3, MMP7, MMP9, MMP10, MMP12, MMP13, tissue inhibitor of metalloproteinases (TIMP)-1, TIMP-2, TIMP-3, and TIMP-4 were simultaneously measured by multiplex ELISA using Milliplex Map Magnetic bead panel kit (Merckmillipore, Darmstadt, Germany) according to the manufacturer's instructions and measured on the Magpix system (Luminex Corp., Austin, TX, USA). These analytes were also spread across three separate plexes for measurements. Twenty-five microliters of tear sample was used each plex as per the

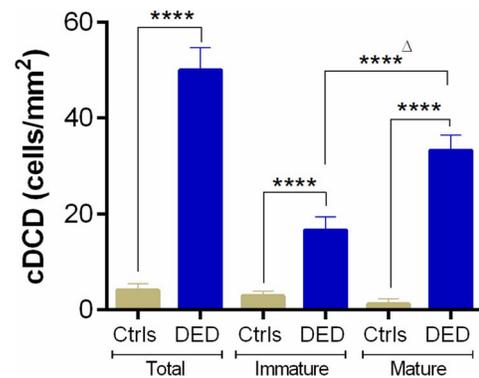


FIGURE 2. Increased cDCD in DED patients. The graphs indicate cDCD including total, immature, and mature forms of DCs in control subjects ($n = 33$) and DED patients ($n = 47$). Bar graphs indicate mean \pm SEM; **** $P < 0.0001$, Mann-Whitney test; Δ Wilcoxon matched-pairs signed rank test.

manufacturer's instructions. Absolute concentration was determined based on respective standards using Bio-Plex manager 6.1 software (Bio-Rad Laboratories, Hercules, CA, USA). VEGF-B was measured by the Human Vascular Endothelial Cell Growth Factor B ELISA Kit, (Abnova, Ltd., Cambridge, UK) with the use of 50- μ L tear sample as recommended by the manufacturer's protocol. Calcitonin gene-related peptide (CGRP) was measured by the CGRP Human EIA Kit (Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA) with the use of a 50- μ L tear sample as recommended by the manufacturer's protocol. Neuropilin-1 was measured by Human Neuropilin-1 DuoSet ELISA (R&D systems, Minneapolis, MN, USA) with the use of a 100- μ L tear sample as recommended by the manufacturer's protocol. Neuropeptide Y (NPY) was measured by the NPY Human EIA Kit (Phoenix Pharmaceuticals, Inc.) with the use of a 50- μ L tear sample as recommended by the manufacturer's protocol. Colorimetric measurements were recorded using a multimodal reader (Tecan Spark; Tecan Austria GmbH, Grödig, Austria). The absolute concentration of these analytes was obtained using respective standards using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). The wetting length of the Schirmer's strip during tear collection and tear elution buffer volume were used to calculate the dilution factor to derive the normalized concentration of the tear analytes.

Measurement of Tear Fluid and Serum Vitamin D

Total vitamin D - 25(OH)vitamin D levels in the serum and tear fluid were measured by direct competitive chemiluminescent enzyme linked immunoassay using 25-hydroxyvitamin D ELISA Kit (Enzo Life Sciences, Lausen, Switzerland) as described previously.⁵⁰ Ten microliters of tear sample/serum diluted with 90 μ L dissociation buffer was used as recommended by the manufacturer's protocol.

Statistical Analyses

Statistical analyses were performed with either GraphPad Prism 6.0 (GraphPad Software, Inc.) or MedCalc Version 12.5 (MedCalc Software bvba, Ostend, Belgium). The distribution status of the dataset was determined by the Shapiro-Wilk normality test. The unpaired t -test with Welch's correction, Mann-Whitney test, Wilcoxon matched-pairs signed rank test, and Spearman correlations analysis were used to analyze datasets. $P < 0.05$ was considered statistically significant.

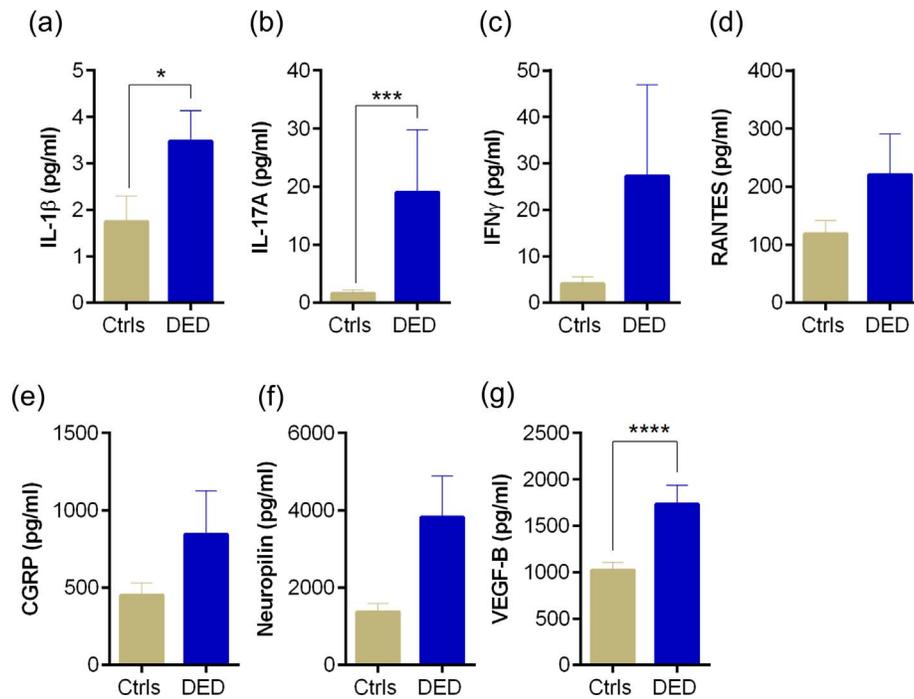


FIGURE 3. Higher tear-fluid soluble factors levels in DED patients. The graphs indicate the levels of IL-1 β (a), IL-17A (b), IFN γ (c), RANTES (d), CGRP (e), Neupilin (f), and VEGF-B (g) in control subjects ($n = 33$) and DED patients ($n = 47$). Bar graphs indicate mean \pm SEM; * $P < 0.05$; *** $P < 0.001$; **** $P < 0.0001$, Mann-Whitney test.

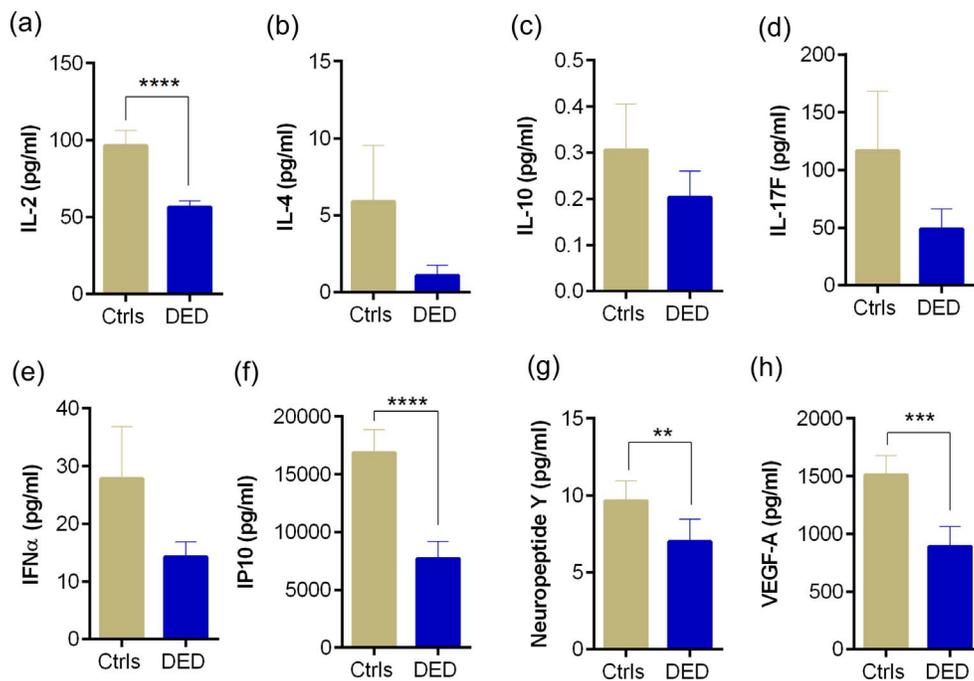


FIGURE 4. Lower tear-fluid soluble factors levels in DED patients. The graphs indicate the levels of IL-2 (a), IL-4 (b), IL-10 (c), IL-17F (d), IFN α (e), IP-10 (f), Neuropeptide Y (g), and VEGF-A (h) in control subjects ($n = 33$) and DED patients ($n = 47$). Bar graphs indicate mean \pm SEM; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$, Mann-Whitney test.

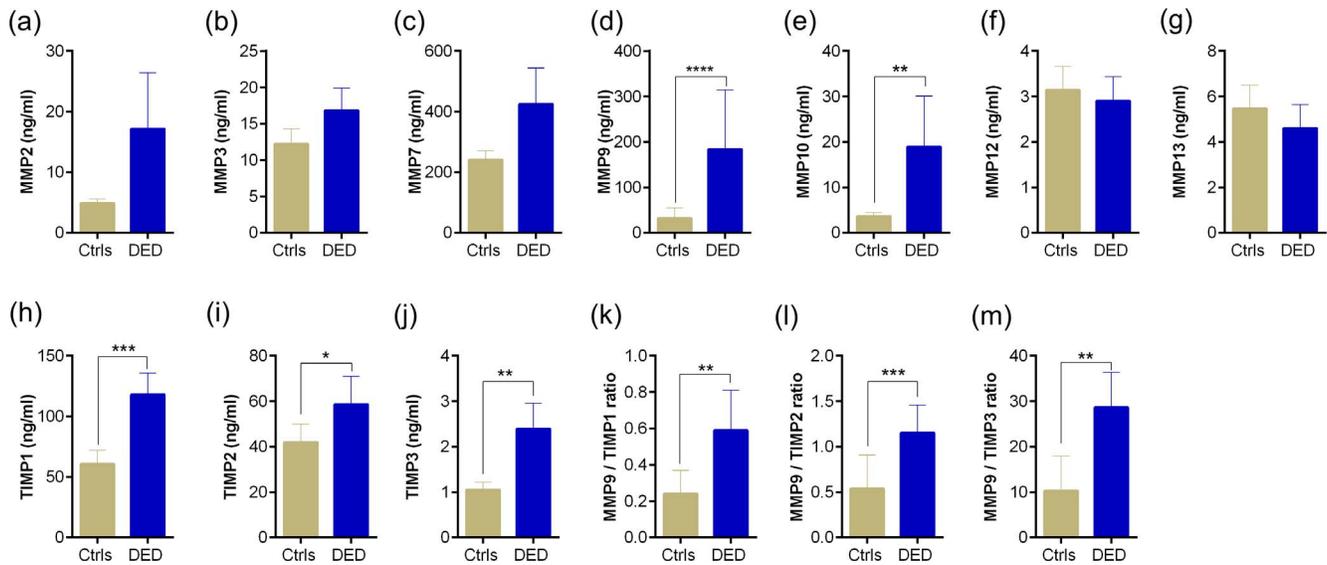


FIGURE 5. Altered tear fluid MMP levels in DED patients. The graphs indicate the levels of MMP2 (a), MMP3 (b), MMP7 (c), MMP9 (d), MMP10 (e), MMP12 (f), MMP13 (g), TIMP1 (h), TIMP2 (i), TIMP3 (j), MMP9/TIMP1 ratio (k), MMP9/TIMP2 ratio (l), and MMP9/TIMP3 ratio (m) in control subjects ($n = 33$) and DED patients ($n = 47$). Bar graphs indicate mean \pm SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$, Mann-Whitney test.

RESULTS

DED Signs and Symptoms

The controls and DED subjects in the study cohort were age and sex matched. The mean \pm standard error of the mean (SEM) (median) age of control and DED subjects was 32.7 ± 0.9 (32) and 37.6 ± 1.9 (33) years, respectively ($P > 0.05$). The control group included 15 males and 18 female subjects, whereas the DED group had 24 males and 23 females. The total OSDI score including both the discomfort scale and vision scale were observed to be significantly higher in DED patients compared with controls (Fig. 1a). Ocular surface discomfort was observed to be the major contributor to the total OSDI scores in DED patients (Fig. 1a). In addition, TBUT and ST1 values were significantly lower in the DED group compared

with controls (Figs. 1b, 1c). However, the ST1 values were >10 mm/5 min in all the subjects including DED patients. Collectively, these observations suggest the study patients had an early form of evaporative dry eye-associated ocular surface discomfort.

cDCD and SBNP Features in DED

IVCM analysis revealed that cDCD was significantly higher in DED patients compared with controls (Fig. 2). A significant increase in both the mature and immature forms of DCs was observed in DED patients (Fig. 2). The density of mature form of DCs was observed to be higher than the immature form in DED patients (Fig. 2). SBNP quantification showed no significant differences in features such as CNFD, CNFL, CNBD, CTBD,

TABLE 1. Association Status of Dry Eye Signs, cDCD, and Tear-Soluble Factors With OSDI Score

	OSDI					
	Total		Discomfort		Vision	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
TBUT, s	-0.437	0.0001	-0.342	0.0019	-0.307	0.0056
STI, mm	-0.213	0.0574	-0.145	0.2001	-0.039	0.7295
Total cDCDs, cells/mm ²	0.595	<0.0001	0.508	<0.0001	0.477	<0.0001
Immature cDCDs, cells/mm ²	0.357	0.0011	0.315	0.0044	0.259	0.0203
Mature cDCDs, cells/mm ²	0.645	<0.0001	0.543	<0.0001	0.537	<0.0001
IL-17A	0.340	0.002	0.279	0.0124	0.309	0.0052
MMP9	0.287	0.01	0.203	0.0708	0.126	0.2642
MMP10	0.297	0.0076	0.293	0.0084	0.097	0.3917
TIMP1	0.268	0.0164	0.221	0.0493	0.117	0.303
TIMP3	0.291	0.0089	0.199	0.0768	0.244	0.0289
VEGF-B	0.332	0.0026	0.335	0.0024	0.137	0.2258
VEGF-A	-0.310	0.0051	-0.282	0.0113	-0.153	0.1755
IL-2	-0.382	0.0005	-0.234	0.0365	-0.303	0.0064
IP-10	-0.327	0.0031	-0.311	0.0049	-0.125	0.2704

Bold entries indicate statistically significant observations. $n = 80$. *r*, Spearman correlation coefficient.

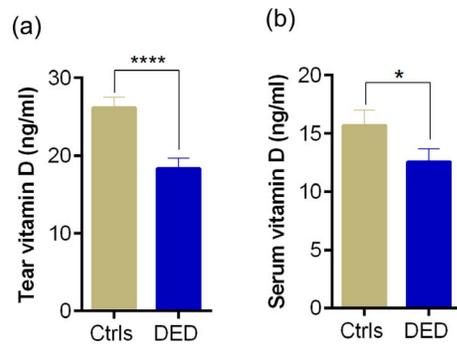


FIGURE 6. Lower tear fluid and serum vitamin D levels in DED patients. The graphs indicate the levels of 25(OH) vitamin D in the tear fluid (a) and serum (b) of control subjects ($n = 33$) and DED patients ($n = 47$). Bar graphs indicate mean \pm SEM. **** $P < 0.0001$, Unpaired t -test with Welch's correction; * $P < 0.05$; Mann-Whitney test.

CNFA, and CNFW between DED patients and controls (Supplementary Fig. S1).

Tear Fluid Cytokines, Chemokines, and Neuropeptides in DED

Marked differences were observed in the levels of proinflammatory factors and nociception modulators in the tear fluid of DED patients (Figs. 3, 4). The levels of tear fluid IL-1 β *, IL-17A*, IFN γ , RANTES, CGRP, Neuropilin, and VEGF-B* were higher in the DED patients compared with controls (* $P < 0.05$; Fig. 3). Conversely, the levels of IL-2*, IL-4, IL-10, IL-17E, IFN α , IP-10*, Neuropeptide Y*, and VEGF-A* were observed to be lower in DED patients compared with controls (* $P < 0.05$; Fig. 4). Significant differences were not observed in the levels of IL-1 α , IL-8, IL-9, TNF α , ICAM1, MCP1, and MIP1 β between DED patients and controls (Supplementary Fig. S2).

TABLE 2. Association Status of OSDI Score, STI, cDCD, and Tear-Soluble Factors With TBUT

	TBUT	
	<i>r</i>	<i>P</i>
OSDI (total)	-0.437	0.0001
OSDI (discomfort)	-0.342	0.0019
OSDI (vision)	-0.307	0.0056
STI, mm	0.398	0.0003
Total cDCDs, cells/mm ²	-0.575	<0.0001
Immature cDCDs, cells/mm ²	-0.391	0.0003
Mature cDCDs, cells/mm ²	-0.603	<0.0001
IL-17A	-0.233	0.0373
MMP9	-0.362	0.001
MMP10	-0.243	0.0296
TIMP1	-0.322	0.0036
MMP9/TIMP1	-0.313	0.0047
MMP9/TIMP2	-0.319	0.0039
MMP9/TIMP3	-0.300	0.0112
MIP1 β	-0.251	0.0247
VEGF-B	-0.276	0.013
VEGF-A	0.216	0.0541
IL-2	0.412	0.0001
IP-10	0.272	0.0145
MCP1	0.265	0.0198

Bold entries indicate statistically significant observations. $n = 80$. r , Spearman correlation coefficient.

TABLE 3. Association Status of OSDI Score, TBUT, cDCD, and Tear-Soluble Factors With STI

	Schirmer's Test I	
	<i>r</i>	<i>P</i>
OSDI (total)	-0.213	0.0574
OSDI (discomfort)	-0.145	0.2001
OSDI (vision)	-0.039	0.7295
TBUT, s	0.398	0.0003
Total cDCDs, cells/mm ²	-0.441	<0.0001
Immature cDCDs, cells/mm ²	-0.345	0.0017
Mature cDCDs, cells/mm ²	-0.400	0.0002
IL-17A	-0.233	0.0373
MMP9	-0.362	0.001
MMP10	-0.243	0.0296
TIMP1	-0.322	0.0036
MMP9/TIMP1	-0.504	<0.0001
MMP9/TIMP2	-0.595	<0.0001
MMP9/TIMP3	-0.549	<0.0001
MIP1 β	-0.251	0.0247
VEGF-B	-0.276	0.013
VEGF-A	0.216	0.0541
IL-2	0.412	0.0001
IP-10	0.272	0.0145
MCP1	0.265	0.0198

Bold entries indicate statistically significant observations. $n = 80$. r , Spearman correlation coefficient.

Tear Fluid MMPs in DED

A panel of MMPs and endogenous inhibitors of MMPs, TIMPs, were measured in the tear fluid of the study subjects. The levels of MMP2, 3, and 7 were higher although not significant in DED patients compared with controls (Figs. 5a-5c). Significantly higher levels of MMP9 and MMP10 were observed in DED patients compared with controls (Figs. 5d, 5e). MMP12 and MMP13 levels were similar between the two study groups (Figs. 5f, 5g). TIMP1, 2, and 3 were detected in the tear fluid of the study subjects, whereas TIMP4 levels in the tear fluid were below the detection limit. TIMP1, 2, and 3 were significantly higher in the DED patients than in controls (Figs. 5h-5j). An increase in TIMP is often observed as a tissue response to increases in MMP. Hence, the MMP/TIMP ratio indicates the balance between MMP and its endogenous inhibitor TIMP in the tear fluid in every subject. DED patients exhibited higher MMP9/TIMP1, MMP9/TIMP2, and MMP9/TIMP3 level ratios in the tear fluid compared with controls (Figs. 5k-5m). We also observed the MMP9/TIMP3 ratio was higher than the MMP9/TIMP2 and MMP9/TIMP1 ratio in the study subjects (Figs. 5k-5m).

Tear Fluid and Serum Vitamin D Levels in DED

The level of tear fluid vitamin D was significantly lower in DED subjects compared with controls (Fig. 6a). Despite the subnormal levels of serum vitamin D in the study cohort including control subjects, it was also observed that the serum vitamin D levels were significantly lower in DED patients compared with controls (Fig. 6b).

Association of cDCD and Tear-Soluble Factors With Dry Eye Signs and Symptoms

DED symptoms as measured by OSDI discomfort scale were observed to have a positive relationship with cDCD (both mature and immature for DCs) and a negative relationship

TABLE 4. Association Status of Dry Eye Signs and Symptoms and Tear-Soluble Factors With cDCD (cells/mm²)

	Corneal DC Density (cells/mm ²)					
	Total		Immature		Mature	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
OSDI (total)	0.595	<0.0001	0.357	0.0011	0.645	<0.0001
OSDI (discomfort)	0.508	<0.0001	0.315	0.0044	0.543	<0.0001
OSDI (vision)	0.477	<0.0001	0.259	0.0203	0.537	<0.0001
TBUT, s	-0.575	<0.0001	-0.391	0.0003	-0.603	<0.0001
STI, mm	-0.441	<0.0001	-0.345	0.0017	-0.400	0.0002
IL-1 β	0.325	0.0032	0.294	0.0082	0.289	0.0093
IL-17A	0.420	0.0001	0.370	0.0007	0.408	0.0002
MMP9	0.422	0.0001	0.329	0.0029	0.389	0.0004
MMP10	0.337	0.0022	0.289	0.0093	0.322	0.0036
TIMP1	0.365	0.0009	0.323	0.0035	0.344	0.0018
TIMP3	0.238	0.0337	0.164	0.1473	0.28	0.0119
MMP9/TIMP1	0.299	0.007	0.210	0.061	0.267	0.0166
MMP9/TIMP2	0.357	0.0011	0.253	0.0237	0.315	0.0044
MMP9/TIMP3	0.301	0.0109	0.283	0.017	0.242	0.0424
VEGF-B	0.516	<0.0001	0.478	<0.0001	0.465	<0.0001
VEGF-A	-0.288	0.0095	-0.166	0.1412	-0.277	0.013
IL-2	-0.510	<0.0001	-0.391	0.0003	-0.497	<0.0001
IL-4	-0.288	0.0095	-0.232	0.0388	-0.268	0.0161
IP-10	-0.613	<0.0001	-0.562	<0.0001	-0.536	<0.0001
MCP1	-0.240	0.0354	-0.141	0.2220	-0.281	0.0132
NPY	-0.420	0.0001	-0.387	0.0004	-0.360	0.001

Bold entries indicate statistically significant observations. $n = 80$. r , Spearman correlation coefficient.

with TBUT (Table 1). Tear-soluble factors with pronociceptive potential such as IL-17A, MMP10, and VEGF-B were observed to have a positive association with OSDI discomfort scores (Table 1). Tear-soluble factors with antinociceptive potential such as VEGF-A, IL-2, and IP-10 showed a negative association with OSDI discomfort scores (Table 1). TIMPs (1 and 3) also had a direct relationship with OSDI scores (Table 1). In addition to TBUT exhibiting an inverse relationship with OSDI scores, it also showed a positive relationship with STI values (Table 2). Further, an inverse relationship was observed between TBUT and cDCD (both mature and immature forms of DCs) as shown in Table 2. Furthermore, TBUT had a positive relationship with VEGF-B, IL-2, IP-10, and MCP1 but a negative relationship with IL-17A, MMP9, MMP10, MMP/TIMP ratio, MIP1 β , and VEGF-B (Table 2). However, MIP1 β and MCP1 levels were not observed to be significantly different between control and DED subjects. Similar associations were observed between STI values, tear factors, and cDCD (Table 3). cDCD was seen to have a direct relationship with the tear levels of IL-1 β , IL-17A, MMP9, MMP10, TIMP1, TIMP3, MMP/TIMP ratio, and VEGF-B and an inverse relationship with tear levels of VEGF-A, IL-2, IL-4, IP-10, MCP1, and NPY (Table 4).

A distinct association was observed with dry eye signs, symptoms, cDCD, and tear-soluble factors with tear vitamin D rather than serum vitamin D levels (Table 5). Tear fluid vitamin D showed a direct association with TBUT and STI but was inversely associated with OSDI discomfort score and cDCD (Table 5). Furthermore, MMPs, TIMPs, MMP/TIMP ratio, and VEGF-B levels were inversely associated with tear vitamin D. The levels of key antinociceptive factors such as IL-2, IL-10, IP-10, IL-17E, NPY, VEGF-A, and other cytokine or chemokines were directly associated with tear vitamin D levels (Table 5).

DISCUSSION

Poor response or lack of symptom relief to conventional therapeutic options with the persistence of ocular surface pain and discomfort in a group of patients poses a major challenge in the management of DED. Hence, identifying additional molecular factors that contribute to ocular surface discomfort and altered tear fluid metrics in DED would help devise novel means to modulate these factors to improve DED management. Despite the availability of knowledge on altered tear-soluble factors in DED, information regarding nociceptive potential of many dysregulated tear-soluble factors in DED is yet to be reported. Therefore, this study has used the OSDI discomfort scale as a measurable parameter for ocular surface pain and correlated the scores with tear soluble factor levels to identify potential soluble factors that may have pro- or antinociceptive functions in DED. In the current study, a select set of tear-soluble factors was identified to have association with the clinical parameters including discomfort. Dysregulation in the molecular and functional components in ocular neurosensory pathways results in corneal hyperalgesia or neuropathic pain.¹⁸ Inflammatory factors are known to sensitize polymodal, thermoreceptors, and mechano-nociceptors by altering the expression or conformation of ion channels that result in hyperexcitability of the neurons and reduces the threshold to pain stimuli.^{18,31} Hence, we categorized the tear-soluble factors based on the potential to modulate nociception as potential pro- or antinociceptive factor in DED.

A distinct dysregulated profile of tear cytokine, chemokine, growth factor, neuropeptides, and MMP was observed in evaporative dry eye patients (Figs. 3-5). We observed an increase in the levels of pronociceptive factors (IL-1 β , IL-17A, IFN γ , RANTES, CGRP, Neuropeilin, VEGF-B, MMP2, MMP7, MMP9, and MMP10) and decrease in the levels of antinociceptive factors (IL-2, IL-4, IL-10, IL-17E, IFN α , IP-10, Neuropeptide Y, and VEGF-A). We therefore speculate that the patients might

TABLE 5. Association Status of Vitamin D Levels With Dry Eye Signs and Symptoms, cDCD, and Tear-Soluble Factors

	Vitamin D			
	Tear Fluid		Serum	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
OSDI (total)	-0.259	0.0203	-0.203	0.071
OSDI (discomfort)	-0.228	0.0416	-0.144	0.2028
OSDI (vision)	-0.091	0.4227	-0.191	0.0888
TBUT, s	0.371	0.0007	0.282	0.0112
STI, mm	0.738	<0.0001	0.117	0.302
Total cDCDs, cells/mm ²	-0.351	0.0014	-0.18	0.1108
Immature cDCDs, cells/mm ²	-0.204	0.0697	-0.062	0.5877
Mature cDCDs, cells/mm ²	-0.347	0.0016	-0.208	0.0638
MMP2	-0.434	0.0001	-0.172	0.1269
MMP3	-0.31	0.0052	-0.082	0.4714
MMP7	-0.34	0.002	-0.155	0.1694
MMP9	-0.581	<0.0001	-0.051	0.6523
MMP10	-0.543	<0.0001	-0.089	0.4318
TIMP1	-0.608	<0.0001	-0.069	0.5421
TIMP2	-0.271	0.0151	-0.041	0.7201
TIMP3	-0.499	<0.0001	-0.111	0.3271
MMP9/TIMP1	-0.455	<0.0001	-0.014	0.9029
MMP9/TIMP2	-0.565	<0.0001	-0.029	0.7965
MMP9/TIMP3	-0.487	<0.0001	0.074	0.5397
VEGF-B	-0.289	0.0093	0.082	0.4697
VEGF-A	0.555	<0.0001	0.051	0.6513
IL-2	0.373	0.0007	0.222	0.0483
IL-10	0.272	0.0148	0.288	0.0096
IP-10	0.484	<0.0001	-0.14	0.2149
IL-17F	0.345	0.0017	0.03	0.7893
NPY	0.226	0.044	-0.062	0.5872
IL-1 α	0.341	0.0019	-0.041	0.7201
IFN α	0.446	<0.0001	0.294	0.0082
IFN γ	0.297	0.0074	0.06	0.5989
MCP1	0.330	0.0034	0.107	0.3559
MMP13	0.229	0.0407	0.063	0.5812

Bold entries indicate statistically significant observations. $n = 80$. *r*, Spearman correlation coefficient.

be experiencing corneal hyperalgesia or abnormal nociceptive response due to disruption in the pro- and antinociceptive factor balance on the ocular surface. IL-1 β , a major proinflammatory cytokine, activates nociceptors to generate action potentials and induce pain.³² IL-17A, a key factor in inflammatory disorders, is also involved in nociception as its receptors are expressed by nociceptor neurons. Thus, IL-17A can mediate mechanical allodynia by altering the expression of neuronal TRPV4 channels essential for transduction of pain stimulus.³³⁻³⁵ CGRP, a neuropeptide, plays a critical part in nociceptive pathways of both peripheral and central nervous system.³⁶ The detailed understanding of CGRP function in the pathophysiology of migraine had led to its development as a therapeutic target for migraine.^{36,37} MMPs, such as MMP2, MMP9, and MMP10, in addition to their major role in extracellular matrix turnover, are also involved in the initiation and propagation of pain including migraine and neuropathic pain.^{38,39} VEGF, a well-known angiogenic factor, is also known to modulate pain. Isoform VEGF-B is reported to augment nociception through selective activation of VEGF receptor 1 expressed on the sensory neurons.⁴⁰ Splice variants of isoform VEGF-A were shown to have antinociceptive potential.⁴¹⁻⁴³ We did observe an increase in VEGF-B and decrease in VEGF-A in the DED cohort, suggesting their aberrant levels may contribute to ocular discomfort in these patients. IL-2, a key

immunoregulatory cytokine, is known to render antinociceptive response by its engagement of opioid receptors.⁴⁴⁻⁴⁶ Anti-inflammatory cytokines IL-4 and IL-10 are also documented for their potent antinociceptive function and are being harnessed in the management of pain.⁴⁷⁻⁵⁰ Chemokine CXCL10/IP-10 is being investigated for their role in pain pathways and were reported to facilitate opioid-mediated antinociception.⁵¹ NPY is a peptide known for its central role in antinociceptive signaling during inflammatory and neuropathic pain.^{52,53} Hence, in the current study it is apparent that there is an imbalance in nociceptive factors favoring ocular surface discomfort in patients of DED. Regaining nociceptive factor balance in the DED patients may therefore be beneficial in reducing symptoms suggesting a role for targeted immunomodulatory or biologic therapies.

IVCM images demonstrate an increase in cDCD in DED patients. However, no change in the SBNP features was observed in this cohort, suggesting that morphologic changes in the corneal nerves may be occur only in advanced stages of disease. Increased corneal DCs have been reported in inflammatory conditions.^{22,54-57} SBNP feature changes in DED may be diverse^{22,58-60} due to the status of underlying disease or ocular surface discomfort severity. Our observations were similar to an earlier report where no significant differences in the SBNP features were observed in the cohort when OSDI score was less than 23.²² A positive association was observed between OSDI discomfort score and cDCD, suggesting the potential role of cDCD in mediating DED symptoms. Studies have implicated the role of dendritic cells in modulation of nociception and pain.^{61,62} The associations of tear factors and cDCD observed, needs to be explored in the context of corneal biology and nociception.

Multiple studies have implicated the relationship between vitamin D deficiency and DED prevalence.^{5,6,21-25} However, all these reports included the levels of serum vitamin D with varying relationships with DED signs and symptoms. Since it is now known that vitamin D can be synthesized, and active forms can be produced locally by the ocular surface cells,⁶³ and that the vitamin D can be measured in the tear fluid,^{30,64} we studied the relationship of both serum and tear fluid vitamin D with molecular factors and DED parameters. Unlike serum vitamin D, the normative range for tear vitamin D had not been previously ascertained. We and other have shown the concentration of vitamin D in tear fluid is higher than in serum,^{30,64} which could account for a lack of a robust association between serum vitamin D and DED as reported in various studies.^{5,6,21-25} We now have observed a significantly lower level of tear fluid and serum vitamin D in DED patients. Tear vitamin D was observed to have more effective biologically relevant association with the various molecular factors and clinical indices compared with serum vitamin D levels in this study. This highlights the relevance of tear vitamin D measurements while investigating ocular surface health. Vitamin D is well known for its immunomodulatory and anti-inflammatory role, and its deficiency is often associated with elevated inflammatory factors. Tear vitamin D would more relevant in ocular surface discomfort because vitamin D modulates the expression of inflammatory cytokines and protects corneal epithelial barrier function.⁶⁵⁻⁶⁷ One of the key immunoregulatory role of vitamin D is its tolerogenic action on DCs, regulating their migration and maturation.⁶⁸ This could explain the inverse relationship observed between cDCD and tear vitamin D in this study. There is emerging evidence on the role of vitamin D in nociceptive response and management of pain in various conditions including corneal neuralgia.^{6,26,69} The negative association between tear vitamin D and OSDI, particularly the discomfort scale, suggests a causal role of vitamin D in modulating pain in DED. The possible

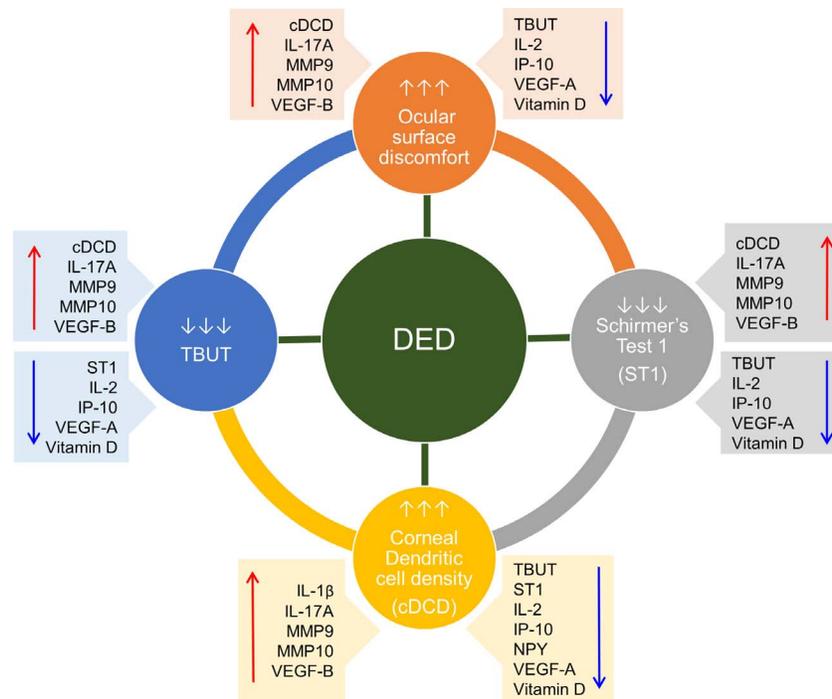


FIGURE 7. Schematic representation summarizing the relationship among tear-soluble factors, cDCD, and dry eye signs and symptoms. The schema illustrates that status of signs and symptoms of dry eye patients and the associations among them and with tear-soluble factors and cDCD. As indicated in the schema, an increase in ocular surface discomfort, reduced TBUT, reduced tear production, and increased cDCD was observed in DED patients. Further, schema also illustrates the associations between the various tear-soluble factors (significantly different in DED patients) with dry eye signs and symptoms and cDCD as listed in Tables 1 to 5. This implies the plausible functional relationship between the various tear-soluble factors and dry eye pathogenesis. Hence, targeting or modulating the levels of these tear-soluble factors would alleviate DED signs and symptoms. The observation also suggests the potential for topical (eye drops) vitamin D supplementation in the management of DED.

mechanisms for vitamin D in nociception regulation are by its anti-inflammatory effects by modulating the levels cytokines with nociceptive potential or via nociceptive neurotransmitters like nitric oxide or serotonin.⁷⁰⁻⁷² Vitamin D is reported to reduce the expression and activation of MMPs⁷³ and VEGF⁷⁴ thus their nociceptive potential. However, the isoform-specific VEGF modulation by vitamin D is yet to be studied to validate the association observed in the current study (i.e., vitamin D levels had a positive and a negative relationship with tear VEGF-A and VEGF-B levels, respectively). These observations are clinically relevant because oral vitamin D supplementation is being explored in DED management, which shows favorable outcomes by enhancing the efficacy of topical treatment and improving various DED signs and symptoms, particularly in those with vitamin D deficiency or those refractory to conventional treatment.⁷⁵⁻⁷⁷

To summarize, an imbalance in nociception modulators with an increase in pronociceptive factors and a decrease in antinociceptive factors was observed (Fig. 7) to be significantly associated with signs and symptoms in DED patients. This implies the plausible role of these factors on the ocular surface neurosensory components in mediating or mitigating symptoms in DED. In addition, tear vitamin D was observed to have a clinically favorable association with these nociceptive factors, DED signs, and symptoms. Hence, restoring ocular surface homeostasis by regaining nociceptive and inflammatory factors balance by augmenting vitamin D-mediated cellular response would prove beneficial.

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Amended October 22, 2019: Figure 7 was replaced with a version that changes “MM10” to “MMP10.”