The Relationship between Corneal Nerve Morphology and Inflammatory Mediators and Neuropeptides in Healthy Individuals

Maria Markoulli, PhD, MOptom, FAAO,¹ Luisa H. Colorado, PhD, BAAppSc(Optom),² and Katie Edwards, PhD, BAAppSc(Optom)²

SIGNIFICANCE: This study set out to explore the relationship between the ocular surface immune and nervous systems by exploring corneal nerve structure and the presence of inflammatory mediators and neuropeptides in the tear film.

PURPOSE: The purpose of this study was to determine the association between corneal nerve morphology and tear film inflammatory mediators and a neuropeptide in healthy individuals.

METHODS: Flush tears were collected from both eyes of 21 healthy participants aged 39.7 ± 9.9 years (10 females, 11 males) and analyzed for substance P, matrix metalloproteinase-9, tissue inhibitor of matrix metalloproteinase-1 (TIMP-1), tumor necrosis factor α, and interleukin 6. In vivo central corneal confocal microscopy was performed on the right eye, and eight images were captured. Variables measured were corneal nerve fiber length (CNFL), corneal nerve density (CNFD), corneal nerve branch density, fiber total branch density, corneal nerve area, corneal nerve fiber width (CNFW), and corneal nerve fractal dimension (CNFrac). For each eye, the average across the images and the maximum and minimum values were determined for each variable. Pearson correlation analysis was performed to test for associations.

RESULTS: Substance P correlated with CNFrac (max) (r = −0.48, P = .03) and CNFW (min) (r = −0.52, P = .02). TIMP-1 correlated with CNFD (average) (r = −0.53, P = .03), CNFL (average) (r = −0.49, P = .05), CNFrac (max) (r = −0.49, P = .05), and CNFD (min) (r = −0.55, P = .02). Interleukin 6 correlated with CNFW (average) (r = −0.49, P = .05), the standard deviation of CNFL (r = −0.51, P = .04), CNFL (max) (r = −0.50, P = .04), CNFrac (max) (r = −0.50, P = .04), and CNFW (min) (r = −0.55, P = .02). Tumor necrosis factor α, matrix metalloproteinase-9, and its ratio with TIMP-1 did not correlate with any corneal nerve parameters.

CONCLUSIONS: Both inflammatory mediators and neuropeptides correlated with measures of corneal nerve morphology, supporting the link between the inflammatory and nervous systems.

Corneal nerves are commonly imaged in vivo to gain insight into the impact of both ocular and systemic disease. In diabetes, for example, there is evidence of corneal nerve degeneration using in vivo corneal confocal microscopy, in the absence of any other evidence of peripheral neuropathy.¹ Corneal nerves express biologically active neuropeptides such as substance P, which can also be measured in the tear film.² The reduction in nerve density in diabetes has been associated with a concurrent reduction in the concentration of substance P in the tear film,³ suggesting that changes to corneal nerve morphology are reflected in the changes to tear film biochemistry. Substante P is widely distributed in many tissues and body fluids⁴–⁶ and systemically is involved in physiological processes such as pain modulation, smooth muscle contraction, blood pressure control, kidney function, and water homeostasis.⁷ At the ocular surface, substance P promotes epithelial migration and proliferation⁸–¹⁰ and may be involved in corneal nerve regeneration.¹¹ It is also involved in the modulation of inflammation through its binding with the neurokinin-1 receptor, a receptor found on nerves, immune cells, and the epithelium.¹² Studies assessing neuropeptide levels in association with corneal nerve morphology may shed light into the pathophysiological mechanism of corneal nerve changes observed in disease. The relationship between tear film substance P and corneal nerve morphology in healthy eyes has not been described.

A possible interaction has also been reported between the ocular immune and nervous system.¹²–¹⁴ Studies have shown an association between the two systems, although the direction of the relationship is still being established.³,¹⁵,¹⁶ An increased density of corneal dendritic cells in ocular surface disease has been shown to correlate with a decrease in subbasal corneal nerve fiber density,¹² supporting the suggestion that the two systems are interlinked. Given recent findings that the peripheral nervous system regulates innate immune reactions against pathogens through hormonal and neuronal routes,¹⁷ we hypothesize that there will be an association between the ocular neural and immune systems in a healthy population as measured using in vivo corneal confocal microscopy and tear film inflammatory mediators, respectively.

Matrix metalloproteinases are a family of collagen-degrading enzymes that are regulated by endogenous tissue inhibitors of metalloproteinase.¹⁸ The ratio of matrix metalloproteinase-9 to tissue inhibitor of metalloproteinase-1 is important in maintaining homeostasis anywhere in the body,¹⁹ and in the tear film, excess matrix metalloproteinases have been shown to lead to inflammatory...
dry eye,\textsuperscript{20} corneal ulceration and erosion,\textsuperscript{21} and perforation in extreme cases,\textsuperscript{22} conditions that are also impacted by neural dysfunction.\textsuperscript{23} Tumor necrosis factor α is an inflammatory cytokine and a potent mediator of leukostasis,\textsuperscript{24} and interleukin 6 is involved in the regulation of matrix metalloproteinases\textsuperscript{25,26} and the propagation of the inflammatory cascade in dry eye disease.\textsuperscript{27} The aim of this study therefore was to determine if there is an association with corneal nerve morphology and the expression of neuropeptide substance P and inflammatory mediators (matrix metalloproteinase-9, tissue inhibitor of metalloproteinase-1, interleukin 6, and tumor necrosis factor α) in the healthy tear film to establish baseline relationships.

\section*{MATERIALS AND METHODS}

\section*{Participants}

This study received ethical approval from the Queensland University of Technology human research ethics committee (approval no. 1400000945) and adhered to the tenets of the Declaration of Helsinki (2013). The sample size was calculated to test the hypothesis that there is a significant association between corneal nerve fiber density and substance P. The sample size was calculated using the PS program (Nashville, TN)\textsuperscript{28} and was based on prior data\textsuperscript{3} indicating that the standard deviation of corneal nerve fiber density is 7.0 mm/mm\textsuperscript{2} and the standard deviation of substance P is 3000 ng/mL. Assuming that the true slope of the line obtained by regressing substance P against corneal nerve fiber density is 281.5 ng/mL, which equates to a correlation of 0.66 between density and substance P, a sample size of 20 subjects was required at 95% confidence and with 80% power.

Participants with active ocular surface disease were excluded, as were those using eye drops and pregnant or lactating women. In addition, individuals with diabetes were excluded from the study, as were those taking doxycycline or corticosteroids. Rigid lens wearers and those with self-described dry eye disease were also excluded. Soft contact lens wearers were eligible to participate but were required to discontinue lens wear for at least 24 hours before each visit.

\section*{Study Design}

Participants attended two visits to allow for corneal nerve migration rate to be measured (data not presented here), each visit being 3 weeks apart.\textsuperscript{29,30} Both visits were conducted at approximately the same time of day, between 10 AM and 2 PM, to minimize diurnal variation in the tear proteins analyzed.\textsuperscript{31} Measurements were corneal nerve fiber density (the total number of main nerves and nerve branches per square millimeter) (no./mm\textsuperscript{2}), corneal nerve fiber length (the total length of main nerves and nerve branches per square millimeter) (μm/mm\textsuperscript{2}), corneal nerve fiber area (the total area of the nerves and branches per square millimeter) (μm\textsuperscript{2}/mm\textsuperscript{2}), corneal nerve branch density, nerve fiber total branch density, nerve branch length, nerve fiber total branch length, nerve fiber area, fractal dimension, and nerve fiber width.\textsuperscript{42}

\section*{Clinical Methods}

Participants were asked to complete a questionnaire on their age, ethnicity, and recent colds or flus to exclude other factors contributing to inflammation.\textsuperscript{32} Participants were also questioned on hours of sleep per night and days per week where they exercised for more than 30 minutes because of their reported effect on the lacrimal system and corneal sensitivity.\textsuperscript{33,34} Visual acuity was measured, and slit-lamp biomicroscopy was performed to exclude any preexisting conditions such as recent or current infection or inflammation. The Cornea and Contact Lens Research Unit scale\textsuperscript{35} was used to evaluate bulbar and limbal redness and to identify preexisting corneal disease. Corneal staining with fluorescein was assessed before and after \textit{in vivo} corneal confocal microscopy to confirm corneal integrity. The tests were ordered so as to minimize the impact on the ocular surface before tear collection: visual acuity was assessed first, followed by slit-lamp biomicroscopy with white light, followed by tear collection, fluorescein assessment, and then confocal microscopy. Corneal integrity was confirmed with fluorescein subsequent to confocal microscopy. Assessments were undertaken at the Anterior Eye Laboratory, Institute of Health and Biomedical Innovation, Queensland University of Technology, Australia.

\subsection*{In Vivo Confocal Microscopy}

Scanning laser \textit{in vivo} corneal confocal microscopy was conducted by the same investigator (KE) to image corneal nerve morphology using the Heidelberg Retinal Tomograph III with the Rostock Corneal Module (Heidelberg Engineering GmbH, Heidelberg, Germany) according to standard technique.\textsuperscript{36} To assess corneal nerve fiber morphology, the participant was asked to observe a central target, and section scans were taken on the central cornea, each image being 400 μm in width by 400 μm in height. Eight images that did not overlap by more than 20% were analyzed, as described previously.\textsuperscript{37,38} Images were captured from the right eye only because corneal nerve parameters have been shown to be symmetrical between healthy eyes.\textsuperscript{39} Images were analyzed by the same investigator (MM) with ACC metrics (version 2.0; Center for Imaging Sciences, The University of Manchester, Manchester, UK; \url{https://www.click2go.umip.com/i/s_w/accmetrics_v2.html})\textsuperscript{40,41} (Fig. 1). Standard variables\textsuperscript{40} determined based on measurements were corneal nerve fiber density (the total number of main nerves per square millimeter) (no./mm\textsuperscript{2}), corneal nerve fiber length (the total length of main nerves and nerve branches per square millimeter) (μm/mm\textsuperscript{2}), corneal nerve branch density (the total number of main branches per square millimeter) (no./mm\textsuperscript{2}), and corneal nerve branch length (the total length of main branches and branch sprouts per square millimeter) (μm/mm\textsuperscript{2}).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1}
\caption{Representative image of the central cornea. Corneal nerve parameters were quantified using automated analysis software (ACCMetrics; University of Manchester, Manchester, UK).\textsuperscript{40,41} The software automatically identifies main nerves (highlighted in the red), branches (highlighted in the blue), and branch points (green squares). From this, the following parameters are derived: nerve fiber density, nerve branch density, nerve fiber length, nerve fiber total branch length, nerve fiber area, fractal dimension, and nerve fiber width.\textsuperscript{42} Each image frame is 400 × 400 μm.}
\end{figure}
millimeter) (mm/mm²), corneal nerve branch density (the total number of main nerve branches per square millimeter) (no./mm²), corneal total branch density (the total number of branch points per square millimeter) (no./mm²), corneal nerve fiber area (the total nerve fiber area per square millimeter) (mm²/mm²), corneal nerve fiber width (the average nerve fiber width per square millimeter) (mm/ mm²), and corneal nerve fractal dimension (the ratio of the change in detail to the change in scale).

For each subject, the mean ± standard deviation of each variable was determined from eight images. In addition, for each individual, we identified the image with the highest value (max) for each parameter and the image with the lowest value (min) to capture the variability in corneal nerve distribution between the eight images. The means across the sample population were then used for analysis. The max and min values were included because the pattern in which nerves deteriorate in the subbasal nerve plexus is not yet fully understood. These parameters were an attempt to capture different aspects of the nerve plexus, which may potentially better reveal nerve deterioration or regeneration.

**Tear Collection**

Four samples were collected from each participant: one from each eye at both visits. Tears were collected by the same investigator (MM) as described previously before in vivo corneal confocal microscopy. Eye order was randomized. Briefly, a 20-μL drop of unit dose sterile saline (sodium chloride for injection 0.9%, AstraZeneca; Clifford Hallam Pharmaceuticals, North Ryde, Australia) was instilled into the inferior palpebral fold using an Eppendorf pipette. The participant was asked to gently close both eyes to allow the saline to mix with the tears. Upon opening the eyes, tears were collected using a 10-μL micropipettory tube (Blaubrand intraMark, Wertheim, Germany) for 1 minute. If reflex tearing was detected, tear collection ceased, and the time taken to collect tears was recorded. The volume collected in that period was monitored as an additional means to track reflex tearing. Tear samples from each eye were stored separately.

For the treatment of tear samples, tears were centrifuged at 4000 revolutions per minute at 4°C for 20 minutes. The supernatant was then aliquoted into separate 1.5-mL siliconized polypropylene microcentrifuge tube (Sigma-Aldrich, Steinheim, Germany) and stored on ice until processing. Tear samples from each eye were stored separately. The right eye samples from visit 1 were assessed for total protein content and substance P. The right eye samples from visit 2 were assessed for matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1, and the left eye samples from visit 2 were assessed for tumor necrosis factor α and interleukin 6.

**Tear Analysis**

All tear analysis was conducted within 2 months by the same investigator (MM) in conjunction with Duoset Ancillary Reagent Kit 2 (Duoset kit; R&D Systems Inc., Minneapolis, MN) containing 1% bovine serum albumin in phosphate-buffered saline and pre-made buffers including stop solution, plate-coating buffer, reagent diluent concentrate, and wash buffer.

**Total Protein Content**

Total protein content was determined using the bicinchoninic acid method and using Pierce reagents and flat-bottom Nunc-F Maxisorp 96-well microplates (Thermo Fisher Scientific, Rochester, NY). A standard curve from 0.1 to 2 mg/mL was generated using serial dilutions of bovine serum albumin, in triplicate. Tear samples were loaded at a 1:10 dilution in MilliQ water. Tears were analyzed in duplicate, and a 10-μL volume was added to each well. Solution A and solution B were combined in respective volumes of 20 and 0.4 mL. This mixture was then added at a volume of 200 μL per well and incubated for 30 minutes. The optical density was read with the Benchmark Plus Microplate Spectrophotometer System using the Microplate Manager 5.2 (Bio-Rad, Hercules, CA) at 595 nm, and a standard curve was generated using the bovine serum albumin as a reference.

**Substance P**

Substance P concentration was determined using a competitive enzyme-linked immunosorbent assay kit for substance P (Cayman Chemical Company, MI). Per the manufacturer, the assay range is 3.5 to 500 pg/mL, and sensitivity is approximately 8 pg/mL. The absorbance is inversely proportional to the amount of substance P tracer bound to the well. Tears were loaded in duplicate in a 1:50 dilution in the buffer provided, and the enzyme-linked immunosorbent assay was performed according to the instructions of the manufacturer.

**Inflammatory Mediators**

**Matrix Metalloproteinase-9**

Total matrix metalloproteinase-9 concentration was determined using sandwich enzyme-linked immunosorbent assay with Quantikine kit (R&D Systems Inc.). This was performed according to the directions of the manufacturer. Samples were loaded in a 1:25 dilution in reagent diluent in duplicate. This assay recognizes the 92-kDa latent and 82-kDa active forms of matrix metalloproteinase-9. The sensitivity of the matrix metalloproteinase-9 assay is less than 0.156 ng/mL according to the R&D Quantikine kit. In six cases where samples were below the detection limits of the standard curve, they were allocated a value of zero.

**Tissue Inhibitor of Metalloproteinase-1**

The concentration of tissue inhibitor of metalloproteinase-1 was determined using enzyme-linked immunosorbent assay with DuoSet kit (R&D Systems Inc.). This was performed according to the directions of the manufacturer. This kit recognizes natural and recombinant tissue inhibitor of metalloproteinase-1. The sensitivity of Quantikine kit (DTM100; R&D Systems Inc.) is reported by the manufacturer as less than 0.08 ng/mL. Tears were analyzed in duplicate in a dilution of 1:200 in reagent diluent.

**Tumor Necrosis Factor α**

The concentration of tumor necrosis factor α was determined using enzyme-linked immunosorbent assay (DuoSet kit; R&D Systems Inc.). This was performed according to the directions of the manufacturer. Tears were analyzed in a dilution of 1:10 in reagent diluent. In eight cases where samples were below the detection limits of the standard curve, they were allocated a value of zero.

**Interleukin 6**

The concentration of interleukin 6 was determined using enzyme-linked immunosorbent assay (DuoSet kit; R&D Systems Inc.). This was performed according to the directions of the manufacturer. Tears were analyzed in a dilution of 1:20 in reagent diluent.
TABLE 1. Coefficients of repeatability between days for all corneal nerve parameters measured

| Variable                                      | Mean of differences | Standard deviation of differences | Coefficient of repeatability | \( P \) |
|-----------------------------------------------|---------------------|----------------------------------|------------------------------|--------|
| Corneal nerve fiber density (no./mm\(^2\))    | 1.7                 | 6.6                              | 13.0                         | .30    |
| Corneal nerve branch density (no./mm\(^2\))   | −4.9                | 17.4                             | 34.1                         | .23    |
| Corneal nerve fiber length (mm/mm\(^2\))      | −0.2                | 2.9                              | 5.7                          | .71    |
| Corneal total branch density (no./mm\(^2\))   | −9.3                | 22.0                             | 43.0                         | .008   |
| Corneal nerve fiber area (mm\(^2\)/mm\(^2\)) | −0.00018            | 0.0016                           | 0.00329                      | .65    |
| Corneal nerve fiber width (mm/mm\(^2\))       | 0.0001              | 0.0009                           | 0.0017                       | .63    |
| Corneal nerve fractal dimension (ratio)       | 0.0016              | 0.0279                           | 0.0546                       | .81    |

\( P \) values relate to the differences between days for each parameter.

diluent. In 10 cases where samples were below the detection limits of the standard curve, they were allocated a value of zero.

Data Analysis

Results are presented as means ± standard deviation. Agreement between days for corneal nerve values were evaluated using Bland-Altman analysis.\(^{44}\) The differences between the two variables were plotted against their means. Coefficients of repeatability (1.96× the standard deviation of mean difference between replicates)\(^{45}\) were calculated. Repeated-measures ANOVA was used to compare corneal nerve morphology parameters with the days as the within-subjects factor. Age and sex were between-subject factors. The assumption of sphericity was verified using Mauchly test. Where main effects were significant, pairwise comparisons with Bonferroni correction were used post hoc, and all procedures were conducted with IBM SPSS Statistics 22 (Chicago, IL).

TABLE 2. Corneal nerve morphology characteristics

|                      | Corneal nerve fiber density | Corneal nerve branch density | Corneal nerve fiber length | Corneal total branch density | Corneal nerve fiber area | Corneal nerve fiber width | Corneal nerve fractal dimension (ratio) |
|----------------------|-----------------------------|-----------------------------|---------------------------|-------------------------------|--------------------------|--------------------------|---------------------------------------|
| Overall mean         | 29.1 ± 6.9                  | 40.0 ± 20.1                 | 16.6 ± 3.2                | 57.3 ± 28.5                   | 0.006 ± 0.001            | 0.020 ± 0.001            | 1.50 ± 0.02                           |
| Females (mean)       | 27.5 ± 7.9                  | 31.9 ± 13.8                 | 15.9 ± 3.6                | 46.9 ± 17.6                   | 0.005 ± 0.001            | 0.020 ± 0.001            | 1.50 ± 0.03                           |
| Males (mean)         | 30.8 ± 6.0                  | 48.6 ± 23.0                 | 17.5 ± 3.0                | 68.8 ± 34.3                   | 0.006 ± 0.002            | 0.020 ± 0.001            | 1.51 ± 0.02                           |
| Maximum (mean)       | 39.9 ± 10.0                 | 62.5 ± 26.8                 | 19.7 ± 3.2                | 85.4 ± 40.7                   | 0.008 ± 0.001            | 0.022 ± 0.002            | 1.53 ± 0.02                           |
| Minimum (mean)       | 20.5 ± 7.9                  | 16.1 ± 13.9                 | 12.9 ± 3.9                | 22.6 ± 18.3                   | 0.003 ± 0.001            | 0.018 ± 0.001            | 1.46 ± 0.05                           |

Corneal nerve parameters are averaged between 2 days. For each individual, the image with the highest value (max) for each parameter and the image with the lowest value (min) were identified to capture the variability in corneal nerve distribution between the eight images. The means across the sample population were then used for analysis. Corneal nerve branch density and total branch density were significantly greater in males (\( P > .05 \)). All units for corneal nerve morphology parameters are as indicated in Table 1.

TABLE 3. Tear collection rate (\( \mu \text{L/min} \))

|                      | \( \mu \text{L/min} \) |
|----------------------|-------------------------|
| Mean ± standard deviation | Right eye Day 1 | 14.0 ± 11.4 |
|                      | Day 2                  | 11.9 ± 6.3  |
|                      | Left eye Day 1         | 12.7 ± 10.0 |
|                      | Day 2                  | 11.0 ± 4.7  |
| Coefficient of repeatability | Right eye Day 1 vs. day 2 | 17.7  |
|                      | Left eye Day 1 vs. day 2 | 17.8  |
|                      | Day 1 Right vs. left   | 12.9  |
|                      | Day 2 Right vs. left   | 8.8   |
|                      | Left eye Day 1 vs. day 2 | .18   |
|                      | Day 1 Right vs. left   | .41   |
|                      | Day 2 Right vs. left   | .41   |

Missing data for the tear analysis for visit 2 from two participants were estimated using the quantitative variables in the data set. The missing data were for tumor necrosis factor \( \alpha \) and interleukin 6 for each of these two participants (hence, a total of four data points). Because this is a very low proportion of missing data, single imputation of missing data using the expectation maximization algorithm in IBM SPSS Statistics was performed under the assumption of normality. Little’s missing completely at random (MCAR) test showed that the assumption of data missing completely at random was tenable. Any negative values generated by the process were replaced with zeros. Pearson correlation analysis was performed to test for associations between corneal nerve morphology and tear film proteins.

RESULTS

Informed consent was obtained from 21 healthy participants aged 39.7 ± 9.9 years (age range, 24.8 to 63.0 years) with 10 females and 11 males. Only one contact lens wearer was recruited, and she adhered to the 24-hour contact lens-free period.

Ocular Surface Characteristics

There was no significant difference between days for either the right or left eye, respectively, in terms of bulbar (\( P = .84 \) and \( P = .82 \)) or limbal redness (\( P = .26 \) and \( P = .39 \)).
Corneal Nerve Parameters

Bland-Altman plots were constructed for all corneal nerve parameters between days, and none showed significant size effects. Table 1 shows the coefficients of repeatability between days. Corneal nerve fiber length showed the lowest "between-day" variability, whereas corneal total branch density showed the highest between-day variability. Corneal nerve parameters were not significantly different between days for any of the corneal nerve parameters measured (Table 1). The data from the 2 days were therefore averaged for each individual for subsequent analysis, with the means shown in Table 2. The only corneal nerve parameters that differed between males and females were corneal nerve branch density, which was higher in males (48.6 ± 23.0 no./mm^2) than females (31.9 ± 13.8 no./mm^2, P = .04) and corneal nerve total branch density (68.8 ± 34.3 in males vs. 46.9 ± 17.6 no./mm^2 in females, P = .04).

Tear Collection

Tear Collection Rate

Mean tear collection rates, coefficients of repeatability, and P values are shown in Table 3. Coefficients of repeatability were greater between days than between eyes. There were no significant differences between right and left eyes or collection days in terms of tear collection rate. Accordingly, tear samples from each eye and each day were used for separate protein analyses.

Tear Proteins

Mean concentration of each tear film protein analyzed is shown in Table 4. There were no differences between males and females for any of the tear proteins analyzed (P > .05).

Sleep and Exercise

Participants reported an average of 7.5 ± 1.0 hours of sleep per night that did not change between the 2 days attended. Participants also reported 4.0 ± 2.4 days of exercise per week, which also did not change between days. Sleep correlated moderately with corneal nerve fiber density (max) (r = 0.57, P = .007) and the standard deviation of corneal nerve fiber density (r = 0.49, P = .02). Exercise correlated with corneal nerve fiber width (max) (r = 0.53, P = .01) (Fig. 2).

![Figure 2](https://www.optvissci.com/OptomVisSci2020/Vol97(3)/149)

**FIGURE 2.** Correlations between corneal nerve parameters and sleep and exercise. Corneal nerve parameters were averaged between days. CNFD = corneal nerve fractal dimension; CNFW = corneal nerve fiber width.

| Protein | Gender | Concentration (mean ± standard deviation) | As a percentage of total protein content (mean ± standard deviation) |
|---------|--------|-------------------------------------------|---------------------------------------------------------------|
| Total protein content (mg/mL) | Average | 2.5 ± 1.2 | 100% |
| | Males | 2.6 ± 1.3 | 100% |
| | Females | 2.5 ± 1.1 | 100% |
| Substance P (ng/mL) | Average | 208.9 ± 277.3 | 0.007 ± 0.008 |
| | Males | 142.0 ± 210.5 | 0.005 ± 0.000 |
| | Females | 269.8 ± 324.5 | 0.009 ± 0.010 |
| MMP-9 (ng/mL) | Average | 12.0 ± 28.1 | 0.0003 ± 0.0010 |
| | Males | 2.3 ± 3.4 | 0.0009 ± 0.0002 |
| | Females | 23.1 ± 39.5 | 0.001 ± 0.002 |
| TIMP-1 (ng/mL) | Average | 78.7 ± 26.9 | 0.004 ± 0.004 |
| | Males | 72.6 ± 21.6 | 0.002 ± 0.007 |
| | Females | 84.2 ± 31.2 | 0.005 ± 0.003 |
| MMP-9/TIMP-1 ratio | Average | 0.20 ± 0.53 | |
| | Males | 0.03 ± 0.04 | |
| | Females | 0.39 ± 0.76 | |
| Tumor necrosis factor α (pg/mL) | Average | 52.7 ± 175.1 | 0.002 ± 0.005 |
| | Males | 96.2 ± 254.7 | 0.002 ± 0.007 |
| | Females | 14.1 ± 31.5 | 0.0005 ± 0.0010 |
| Interleukin 6 (pg/mL) | Average | 36.4 ± 62.9 | 0.001 ± 0.002 |
| | Males | 41.0 ± 58.9 | 0.001 ± 0.002 |
| | Females | 32.3 ± 69.5 | 0.001 ± 0.003 |

MMP-9 = matrix metalloproteinase-9; TIMP-9 = tissue inhibitor of metalloproteinase-9.

**TABLE 4.** Tear film protein concentrations

MMP-9 = matrix metalloproteinase-9; TIMP-9 = tissue inhibitor of metalloproteinase-9.
**Correlations**

**Correlations between Corneal Nerve Parameters and Tear Proteins**

Proteins were considered as their individual concentration as well as their percentage of the total protein content. Corneal nerve parameters and tear flow rate were averaged between days for each individual. Correlations are shown in Appendix (available at http://links.lww.com/OPX/A443) with significant correlations highlighted and presented in Fig. 3 and Fig. 4. P values presented are unadjusted. All P values were >.05 after adjustment for multiple comparisons.

**Correlations with Neuropeptide Substance P**

Substance P correlated negatively and moderately with corneal nerve fiber width (min) \( (r = -0.52, P = .02) \) and corneal nerve fractal dimension (max) \( (r = -0.48, P = .03) \).

**Correlations with Inflammatory Mediators**

In general, a parameter had the same direction of correlation for its average, max, and min values. Interleukin 6 correlated negatively and moderately with corneal nerve fiber width (average) \( (r = -0.49, P = .05) \), the standard deviation of corneal nerve fiber length \( (r = -0.51, P = .04) \), corneal nerve fiber length (max) \( (r = -0.50, P = .04) \), corneal nerve fractal dimension (max) \( (r = -0.50, P = .04) \), and corneal nerve fiber width (min) \( (r = -0.55, P = .02) \). Because of the number of undetectable samples, it should be noted that the sample size of interleukin 6 was low. Tissue inhibitor of metalloproteinase-1 correlated negatively and moderately with corneal nerve fiber density (average) \( (r = -0.53, P = .03) \), corneal nerve fiber length (average) \( (r = -0.49, P = .05) \), corneal nerve fractal dimension (max) \( (r = -0.49, P = .05) \), and corneal nerve fiber density (min) \( (r = -0.55, P = .02) \). Tumor necrosis factor α, matrix metalloproteinase-9, and its ratio with tissue inhibitor of metalloproteinase-1 did not correlate with any corneal nerve parameters.

**DISCUSSION**

This study explored the relationship between corneal nerve morphology and tear film proinflammatory cytokines and neuropeptide substance P in the healthy ocular surface. Corneal nerve parameters measured in this healthy group were analogous to those found in previous studies, as were tear protein concentrations when compared with studies using the same methods.

The association between corneal nerve parameters of fiber width and fractal dimension and substance P in this study supports the theory that substance P is a marker of neural health. Nerve fiber width is thought to indicate nerve regeneration, where a wider nerve indicates a nerve that is regenerating as a result of disease duress after inflammatory insult. Thicker nerves have been reported in established disease such as diabetic neuropathy and in Sjögren’s syndrome. The negative association found in this study between substance P and corneal nerve fiber width supports the concept that a thinner nerve is a healthier nerve, with a greater substance P presence.

Corneal nerve fractal dimension was also found to correlate negatively with substance P concentration. Fractal dimension is a measure of the spatial loss of nerve fibers, and it is a ratio of the change in detail to the change in scale, where a higher value indicates an evenly distributed nerve fiber pattern belonging to a healthy individual. Chen et al. have characterized a high corneal nerve fractal dimension as corresponding to a healthier and evenly distributed complex nerve fiber structure, whereas a lower corneal...
nerve fractal dimension corresponds to fewer distorted nerve fibers, potentially reflecting abnormality. Intuitively, a positive relationship would be expected between substance P and fractal dimension, but the reverse was found in this study, suggesting that further work is required to understand this relationship.

Unlike a previous study, which demonstrated a positive association between substance P and corneal nerve fiber density in patients with diabetes, no relationship was found between these measures in this healthy population, possibly because of the little variability in the corneal nerve fiber density of a healthy and young population studied here. This may also indicate that the levels of substance P in the healthy tear film are protective rather than proinflammatory.

Significant negative correlations were found between corneal nerve parameters and inflammatory mediators tissue inhibitor of metalloproteinase-1 and interleukin 6 but not between corneal nerve parameters and matrix metalloproteinase-9 or tumor necrosis factor α, possibly because of the negligible concentration present in the tear film. Tissue inhibitor of metalloproteinase-1 has been found to be expressed at higher concentrations during waking hours than its substrate, matrix metalloproteinase-9, hence being more easily measured in the tear film. Tissue inhibitors of metalloproteinase form 1:1 noncovalent complexes with matrix metalloproteinases, inhibiting their action. Four tissue inhibitor of metalloproteinase proteins have been found in humans, with tissue inhibitor of metalloproteinase-1 having the greater affinity for matrix metalloproteinase-9. The tissue inhibitor of metalloproteinase-1 gene transcription is induced by proinflammatory cytokines such as interleukin 1, interleukin 6, and tumor necrosis factor α, a relationship that was supported in this study by the negative associations between corneal nerve morphology and both tissue inhibitor of metalloproteinase-1 and interleukin 6. In addition to inhibiting matrix metalloproteinases, tissue inhibitors of metalloproteinase also have growth factor and antiangiogenic properties, independent of matrix metalloproteinase activity, and tissue inhibitor of metalloproteinase-1 also has antiapoptotic effects. Up until now, matrix metalloproteinase-9 has been measured as a marker of corneal inflammation, for example, in dry eye disease. Given the association found between tissue inhibitor of metalloproteinase-1 and corneal nerve parameters and the ease with which it is found in the tear film compared with matrix metalloproteinase-9, tissue inhibitor of metalloproteinase-1 may provide an alternate measure for ocular surface inflammation and requires further exploration. This also suggests that, in the healthy ocular surface, tissue inhibitor of metalloproteinase-1 predominates the tear film, and matrix metalloproteinase-9 has an insignificant effect on corneal nerve health.

A weak negative association was found between corneal nerve parameters and interleukin 6. Interleukin 6 plays a key role in the defense against infection and injury. In physiological conditions, interleukin 6 is present in negligible levels in serum, and in the
The presence of inflammation, it can increase by up to 100,000-fold.\textsuperscript{63} Excess production of interleukin 6 has been implicated in autoimmune disease as well as chronic inflammatory diseases and cancers.\textsuperscript{63} In the tear film, interleukin 6 is elevated during sleep,\textsuperscript{64} suggesting that eye closure results in a state of subclinical inflammation with enhanced responsiveness to proinflammatory cytokines. It is also increased in dry eye disease and has been found to be associated with the severity of disease.\textsuperscript{27,65} In keratoconus, proinflammatory mediators such as interleukin 6, interleukin 1β, and interferon γ have been found to be overexpressed, suggesting an inflammatory pathogenesis.\textsuperscript{66,67} These inflammatory mediators further contribute to the inflammatory cascade in keratoconus by tumor necrosis factor α–induced matrix metalloproteinase-1 expression.\textsuperscript{26}

The negative association found between tissue inhibitor of metalloproteinase-1 and interleukin 6 and corneal nerve parameters supports the hypothesis that the ocular nerves and immune systems are interlinked. Dysfunction of the peripheral nervous system may result in proinflammatory immunological responses, termed \textit{neurogenic inflammation}.\textsuperscript{68} In support of this, Cruzat et al.\textsuperscript{14} performed \textit{in vivo} confocal microscopy on eyes with unilateral bacterial keratitis and showed that both eyes had a reduced corneal nerve density, and both had an increased dendritic cell density. Moreover, these two measures showed a strong correlation, even in this healthy population that had relatively little variability, further supporting the idea that the ocular nerves and immune systems are interlinked. In an animal model of diabetes, another study demonstrated an increase in dendritic cells with a concurrent reduction in the density of nerves in the subbasal nerve plexus, implicating the immune system in the nerve fiber damage seen in diabetes.\textsuperscript{69} Also, in support of our findings, Yamaguchi et al.\textsuperscript{15} collected tears and corneal nerve parameters from patients with bacterial keratitis and showed a weak, albeit significant, relationship between interleukin 6, interleukin 1β, and interleukin 8 with corneal nerve fiber density.

The significant difference found between males and females in corneal nerve branch density and total branch density was unexpected. We found no significant differences between sex in terms of age, exercise, hours of sleep, or other ocular surface measures and hence have no explanation for this. This finding therefore warrants further investigation with a larger sample size to explain the difference in corneal nerve branch density found between males and females.

This study also found an association between exercise and sleep and corneal nerve parameters. Vigorous exercise and acupuncture have been found to provide temporary relief in those experiencing neuropathic pain,\textsuperscript{70} with the proposed mechanism being an increase in neurotrophic factors.\textsuperscript{71,72} The association shown here between corneal nerve structure and these modifiable behaviors reinforces the need for greater understanding into the impact that exercise, in terms of intensity, frequency, and type, as well as the impact of sleep on the nervous system.

Assessment of corneal nerve morphology is very much in its infancy, and the manner in which nerve deterioration occurs is unknown. Estimating the average nerve coverage of the central cornea, which is the approach currently used in all studies, may be too crude to detect subtle, early changes to the nerve plexus, which are particularly relevant in studies on young healthy individuals, which are included in this study. The fact that some of these variables showed relationships confirms that this hypothesis should be explored more in future studies.

In conclusion, both inflammatory mediators and neuropeptide substance P correlated with several measures of corneal nerve morphology, supporting the link between the inflammatory and nervous systems in healthy individuals. Future work should determine how aging and disease states such as peripheral neuropathy and neuropathic pain influence these relationships.

ARTICLE INFORMATION

Supplemental Digital Content: The Appendix, available at http://links.lww.com/OPX/A443, highlights all the correlations explored between corneal nerve parameters and tear film inflammatory mediators and neuropeptides. Highlighted bold italics represent significant associations.

Submitted: August 15, 2018

Accepted: December 8, 2019

Funding/Support: None of the authors have reported funding/support.

Conflict of Interest Disclosure: None of the authors have reported a financial conflict of interest.

Author Contributions and Acknowledgments: Conceptualization, MM, KE; Data Curation, MM, LHC, KE; Formal Analysis, MM, LHC, KE; Funding Acquisition, KE; Investigation, MM, LHC, KE; Methodology, MM, LHC, KE; Project Administration, MM, LHC, KE; Resources, KE; Writing – Original Draft, MM, LHC, KE; Writing – Review & Editing, MM, LHC, KE.

The authors would like to thank Dr. Alex Hui for the statistical guidance, and Dr. Nancy Briggs for statistical guidance.

REFERENCES

1. Pritchard N, Edwards K, Dehghani C, et al. Longitudinal Assessment of Neuropathy in Type 1 Diabetes Using Novel Ophthalmic Markers (Landmark): Study Design and Baseline Characteristics. Diabetes Res Clin Pract 2014;104:248–56.
2. Markoulli M, Gokhale M, You J. Substance P in Flush Tears and Schirmer Strips of Healthy Participants. Optom Vis Sci 2017;94:527–33.
3. Markoulli M, You J, Kim J, et al. Corneal Nerve Morphology and Tear Film Substance P in Diabetes. Optom Vis Sci 2017;94:726–31.
4. Jessell TM. The Role of Substance P in Sensory Transmission and Pain Perception.Adv Biochem Psychopharmacol 1981;28:189–98.
5. Mroz EA, Leeman SE. Substance P. Vitam Horm 1977;35:209–81.
6. Perow B. Substance P. Pharmacol Rev 1983;35:85–141.
7. Steinhoff MS, von Mentzer B, Geggotti P, et al. Tachykinins and Their Receptors: Contributions to Physiological Control and the Mechanisms of Disease. Physiol Rev 2014;94:265–301.
8. Reid TW, Murphy CJ, Iwashkiw CK, et al. Stimulation of Epithelial Cell Growth by the Neuropeptide Substance P. J Cell Biochem 1993;52:476–85.
9. Garcia-Hirschfeld J, Lopez-Brones LG, Belmonte C. Neurotrophic Influences on Corneal Epithelial Cells. Exp Eye Res 1994;59:597–605.
10. Nishida T, Nakamura M, Ofuji K, et al. Synergistic Effects of Substance P with Insulin-like Growth Factor-1 on Epithelial Migration of the Cornea. J Cell Physiol 1996;169:159–66.
11. Bignami F, Rama P, Ferrari G. Substance P and Its Inhibition in Ocular Inflammation. Curr Drug Targets 2016;17:1265–74.
12. Yamaguchi T, Hamrah P, Shimazaki J. Bilateral Alterations in Corneal Nerves, Dendritic Cells, and Tear Cyto-kine Levels in Ocular Surface Disease. Cornea 2016;35 Suppl. 1:S65–70.
13. Ferrari G, Chauhan SK, Ueno H, et al. A Novel Mouse Model for Neurotrophic Keratopathy: Trigeminal Nerve Stereotactic Electrolysis through the Brain. Invest Ophthalmol Vis Sci 2011;52:2532–9.
14. Cruzat A, Witkin D, Baniassad N, et al. Inflammation and the Nervous System: The Connection in the Cornea in Patients with Infectious Keratitis. Invest Ophthalmol Vis Sci 2011;52:5136–43.
15. Golebiowski B, Cho C, Stapleton F, et al. Corneal Nerve Morphology, Sensitivity, and Tear Neuropeptides in Contact Lens Wear. Optom Vis Sci 2017;94:534–42.
16. Yamaguchi T, Calvacanti BM, Cruzat A, et al. Correlation between Human Tear Cytokine Levels and Cellular Corneal Changes in Patients with Bacterial Keratitis by \textit{in Vivo} Confocal Microscopy. Invest Ophthalmol Vis Sci 2014;55:7457–66.
17. Tracey KJ. Reflex Control of Immunity. Nat Rev Immunol 2009;9:418–28.
Gill SE, Parks WC. Metalloproteinases and Their Inhibitors. Regulators of Wound Healing. Int J Biochem Cell Biol 2008;40:1334–47.

Nagase H, Woessner JF, Jr. Matrix Metalloproteinases. J Biol Chem 1999;274:21491–9.

Chotikavanich S, de Paiva CS, Li de Q, et al. Production and Activity of Matrix Metalloproteinase-9 on the Ocular Surface Increase in Dysfunctional Tear Syndrome. Invest Ophthalmol Vis Sci 2009;50:3203–9.

Garrana RM, Zieske JD, Assouline M, et al. Matrix Metalloproteinases in Epithelia from Human Recurrent Corneal Erosion. Invest Ophthalmol Vis Sci 1999;40:1267–9.

Berman M, Dohltman CH, Gnadinger M, et al. Characterization of Collagenolytic Activity in the Ucercating Cornea. Exp Eye Res 1971;11:255–7.

Lambiasi A, Sacchetti M, Mastropasqua A, et al. Corneal Changes in Neurologically Induced Neurotrophic Keratopathy. JAMA Ophthalmol 2013;131:1547–53.

de Luca C, Olesfjky JM. Inflammation and Insulin Resistance. FEBS Lett 2008;582:97–105.

Kusano K, Miyaura C, Inada M, et al. Regulation of MMP Induction with Bone Resorption. Endocrinology 2012;53:1479–84.

Kaptchuk TJ. Acupuncture: Theory, Efficacy, and Appropriate Use of Neurostimulation of the Spinal Cord. Neuromodulation 2015;18:515–22.

Ferrari S, Bombelli M, Pantini E, et al. Clinical and Structural Alterations of the Corneas of Patients with Diabetes. Cornea 2006;25:769–75.

Poyraz C, Irkec M, Mocan MC. Elevated Tear Film Inflammatory Mediators in Patients with Keratoconus. Anterior Eye 2013;36:140–5.

Camacho-Flynn L, Lass JH, Sethi A, et al. Risk Factors for Corneal Inflammtory Events during Continuous Wear of Silicone Hydrogel Contact Lenses. Invest Ophthalmol Vis Sci 2010;51:5421–30.

Li SM, Jing K, Zhou J, et al. Sleep Deprivation Disrupts the Lactrical System and Induces Dry Eye Disease. Exp Mol Med 2018;50:e451.

Dieckmann G, Goyal S, Hamrah P. Neuropathic Corneal Pain: Approaches for Management. Ophthalmology 2017;124:S34–47.

Cornea and Contact Lens Research Unit (CCLRU), CCLRU Grading Scales, Sydney, Australia: University of New South Wales, 1996.

Zhivotovski, Svetlana Blazevic, et al. In Vivo Confocal Microscopic Evaluation of Langerhans Cell Density and Distribution in the Normal Human Corneal Epithelium. Graefes Arch Clin Exp Ophthalmol 2005;243:1056–61.

Efron N, Edwards K, Roper N, et al. Repeatability of Measuring Corneal Subbasal Nerve Fiber Length in Individuals with Type 2 Diabetes, Eye Contact Lens 2010;36:245–8.

Vagenas D, Pritchard N, Edwards K, et al. Optimal Image Sample Size for Corneal Nerve Morphometry. Optom Vis Sci 2012;89:812–7.

Petropoulos IN, Almar J, Fadavi H, et al. Corneal Nerve Loss Detected with Corneal Confocal Microscopy Is Symmetrical and Related to the Severity of Diabetic Polynuropathy. Diabetes Care 2013;36:3646–51.

Dabbah MA, Graham J, Petropoulos IN, et al. Dual-model Automatic Detection of Nerve-fibres in Corneal Confocal Microscopy Images. Med Image Comput Assist Interv 2013;16:300–7.

Malik RA, Kallinikos P, Abbott CA, et al. Corneal Confocal Microscopy: A Non-invasive Surrogate of Nerve Fibre Damage and Repair in Diabetic Patients. Diabetologia 2003;46:683–8.

Van den Steen PE, Dubois B, Nelissen I, et al. Biochemistry and Molecular Biology of Gelatine B or Matrix Metalloproteinase-9 (MMP-9). Crit Rev Biochem Mol Biology 2002;37:375–50.

Gomez DE, Alonso DF, Yoshii H, et al. Tissue Inhibitors of Metalloproteinases: Structure, Regulation and Biological Functions. Eur J Cell Biol 1999;74:111–22.

Roderfeld M, Graujanz G, Biese G, et al. Latent MMP-9 Is Bound to TIMP-1 before Secretion. Biochem 2007;388:1227–34.

Van den Steen PE, Dubois B, Nelissen I, et al. Biochemistry and Molecular Biology of Gelatine B or Matrix Metalloproteinase-9 (MMP-9). Crit Rev Biochem Mol Biology 2002;37:375–50.

Kishimoto T, Kang S, Tanaka T, et al. A New Era for the Treatment of Autoimmune Inflammatory Diseases. In: Nakao K, Minato N, Uemoto S, eds. Innovative Medicine: Basic Research and Development. Tokyo, Japan: Springer; 2015:131–47.

Thakur A, Willcox MD, Stapleton F. The Proinflammtory Cytokines and Arachidonic Acid Metabolites in Human Overnight Tears: Homeostatic Mechanisms. J Clin Immunol 1998;18:61–70.

Zhang J, Yan X, Li H. Analysis of the Correlations of Mucins, Inflammatory Markers, and Clinical Tests in Dry Eye. Cornea 2013;32:928–32.

Sorkhabi R, Ghorbaniaghaghi A, Taheri N, et al. Tear Film Inflammatory Mediators in Patients with Keratococcus. Int Ophthalmol 2015;35:467–72.

Liu R, Gao C, Chen H, et al. Analysis of TIM-1-Associated Cytokines and Clinical Correlations in Patients with Dry Eye Disease. PLoS One 2017;12:e0173301.

Scholz J, Wolf CJ. The Neuropathic Pain Triad: Neurons, Immune Cells and Glia. Nat Neurosci 2010;13:1061–83.

Leppin K, Behrendt AK, Reichard M, et al. Diabetes Mellitus Leads to Accumulation of Dendritic Cells and Nerve Fiber Damage of the Subbasal Nerve Plexus in the Cornea. Invest Ophthalmol Vis Sci 2014;55:3603–15.

Kaptchuk TJ. Acupuncture: Theory, Efficacy, and Practice. Ann Intern Med 2002;136:374–83.

Deer TR, Mekhail N, Provenzano D, et al. The Appropriate Use of Neurostimulation of the Spinal Cord and Peripheral Nervous System for the Treatment of Chronic Pain and Ischemic Diseases: The Neuromodulation Appropriateness Consensus Committee. Neuro modulation 2014;17:515–50.

Almeida C, DeMann A, Kusuda R, et al. Exercise Therapy Normalizes Bdnf Upregulation and Glial Hyperactivity in a Mouse Model of Neuropathic Pain. Pain 2015;156:504–13.