The relationship between corneal dendritic cells, corneal nerve morphology and tear inflammatory mediators and neuropeptides in healthy individuals.

Luisa H. Colorado, PhD,1 Maria Markoulli, PhD,2 Katie Edwards, PhD.1

1Institute of Health and Biomedical Innovation, School of Optometry and Vision Science, Queensland University of Technology, Kelvin Grove, Australia

2School of Optometry & Vision Science, University of New South Wales, Sydney, Australia

Correspondence to: Luisa H. Colorado, Room Q-504, Level 5, Institute of Health and Biomedical Innovation, Queensland University of Technology, 60 Musk Avenue, Kelvin Grove, Queensland 4059, Australia.

Phone: +61 3138 6404

luisa.holguincolorado@qut.edu.au

The authors declare that they have no conflicts of interest.

Grant Information: none

Keywords: Corneal dendritic cell density, corneal nerves, tear proteins.
Abstract

PURPOSE. To determine the association between corneal dendritic cell (DC) density and corneal nerve morphology and tear film inflammatory mediators and neuromediators in healthy individuals.

METHODS. Flush tears were collected from 21 healthy participants aged 39.7 ± 9.9 years and analyzed for total protein content (TPC), substance P, matrix-metalloproteinase-9 (MMP-9), tissue inhibitor of MMPs-1 (TIMP-1), tumor necrosis factor-a (TNF-α) and interleukin-6 (IL-6). In vivo confocal microscopy was used to assess DC density and corneal nerve morphology. Corneal nerve variables measured were corneal nerve fiber length (CNFL), fiber density (CNFD), branch density (CNBD), fiber tortuosity (CNFT), fiber total branch density (CTBD), fiber area (CNFA), fiber width (CNFW) and fractal dimension (CNFrac).

RESULTS. Participants with DC density over 50 cells/mm² correlated with CNBD-average \((r = 0.7, p = 0.02)\), CNBD-high \((r = 0.6, p = 0.02)\), CNBD-low \((r = 0.6, p = 0.02)\) CTBD-average \((r = 0.7, p = 0.01)\), CTBD-high \((r = 0.6, p = 0.03)\), CTBD-low \((r = 0.7, p = 0.01)\), CNFA-average \((r = 0.7, p = 0.00)\), CNFA-high \((r = 0.7, p = 0.01)\), CNFA-low \((r = 0.8, p < 0.001)\), CNFrac-SD \((r = -0.6, p = 0.04)\), CNFrac-low \((r = 0.6, p = 0.04)\) and CNFL-low \((r = 0.7, p = 0.02)\). The percentage of MMP-9 correlated with DC density in the entire cohort \((r = 0.47, p = 0.03)\).

CONCLUSIONS. Corneal nerve measures showed a strong correlation with higher DC density, suggesting that the number of cells maybe be modulated by the corneal nerves in the central cornea. MMP-9 also showed a moderate correlation with DC, supporting an inflammatory role.
INTRODUCTION

The cornea is densely innervated with sensory nerve fibers derived from the ophthalmic branch (V1) of the 5th (trigeminal) cranial nerve. The nerve fibers travel in a radial manner between the corneal basal epithelium and Bowman's layer and converge slightly infero-nasally at the inferior whorl, forming the sub-basal nerve plexus (SNP). Imaging of in-vivo human corneal nerves is possible due to the transparency of the cornea and the parallel arrangement in relation to the ocular surface using corneal confocal microscopy. The presence of distinct morphologic attributes of the nerve bundles, such as length, density, tortuosity area and width have been used as markers to determine treatment efficacy and diagnose and monitor the progression of ocular and systemic diseases.

In addition to assessing the corneal SNP, corneal confocal microscopy is also capable of detecting dendritic cells (DCs). These cells have a distinctive hyper-reflective shape and are located at the same depth of the SNP, at approximately a 50 µm depth from corneal epithelium. Hence, the assessment of these two important ocular structures in a single image offers useful insight into the association and interaction between both; the nervous and immune systems of the ocular surface.

There is a body of evidence suggesting that the mean DC density in central cornea of healthy individuals ranges from approximately 9 to 57 cells/mm² and an increased number of these cells in the central cornea have been considered as an indicator the ocular immune response activation.

Despite many studies evaluating corneal immune cells and nerve density in parallel in disease, there have been few reports using corneal confocal microscopy to study the correlation between the immune and nervous systems in the cornea. In a mouse model, higher corneal infiltration with DCs have been observed in diabetes compared to non-diabetic
controls, as well as a significant negative correlation between the quantity of DCs and corneal nerve fiber density (CNFD), suggesting that DCs may play a role in the development of corneal nerve damage in diabetes. The density of DCs has also been shown to correlate negatively with CNFD in neurotrophic and infectious ocular surface diseases in human eyes, however, no evidence of nerve attributes other than CNFD have been reported in association with DC counts.

To better understand the communication between these structures it is important to consider the role of substances that act as mediators in order to transmit the signalling between the nervous and immune system. Inflammatory mediator and neuromediator tear concentration increases in ocular surface inflammation. A recent report identified the presence of at least 25 inflammatory mediators in the tears of healthy subjects.

Tissue inhibitors of metalloproteinase (TIMPs) are in charge of regulating collagen-degrading enzymes Matrix metalloproteinases (MMPs). Homeostasis is mediated by the ratio of MMP-9 to TIMP-1. During inflammatory dry eye, elevated concentration of MMPs have been found in tears, corneal ulceration and erosion. Tumor necrosis factor-α (TNF-α) is an inflammatory cytokine and interleukin-6 (IL-6) regulates the propagation of the inflammatory cascade in dry eye disease. Substance P is involved in pain modulation and promotes epithelial proliferation and cell regeneration and may be involved in corneal nerve regeneration.

This study aimed to determine whether the density of corneal DCs is associated with corneal nerve morphology and tear film inflammatory mediators (matrix metalloproteinase-9 – MMP-9; tissue inhibitor of MMPs-1 – TIMP-1; tumor necrosis factor-α – TNF-α and interleukin-6 – IL-6) and neuromediator substance P in healthy individuals.

MATERIALS AND METHODS
Participants

Participants were recruited from the staff and student population of the Queensland University of Technology, Brisbane, Australia. Ethical clearance was provided by the Queensland University of Technology Research Ethics Committee (approval number 140000945) and written informed consent was obtained from all participants before enrolment. The research was conducted in accordance with the principles of the Declaration of Helsinki.

Individuals were not eligible if they had active ocular surface disease, treatment for any systemic condition that may affect the ocular surface, use of anti-inflammatory medication or were pregnant or lactating. Contact lens wearers were asked to discontinue lens wear for at least a day prior to each visit. There were no rigid lens wearers.

The correlation between corneal nerve attributes, tear proteins and dendritic cell density in a healthy cohort has not been done before, and for this reason this study is considered as an experimental pilot study. Previous studies have suggested that 10 to 30 individuals constitute a reasonable sample size for a pilot study and therefore 21 healthy participants were recruited and correlations were considered statistically significant for P values < 0.05.

Study design

This was a prospective, observational, clinical study where participants attended two visits, each visit being three weeks apart to allow for corneal nerve migration rate quantification (data not presented here). Both visits were conducted between 10 AM and 2 PM to minimize diurnal variation in the tear proteins analyzed and measurements were obtained from both eyes, the order of eyes being randomized at each visit.

Clinical methods
Participants were asked to complete a questionnaire on their age, ethnicity, recent colds or flus, hours of sleep per night and hours of exercise per week. Visual acuity was measured and slit lamp biomicroscopy was performed in order to exclude any pre-existing conditions such as recent or current infection or inflammation. The tests were ordered so as to minimise the impact on the ocular surface prior to tear collection.

**Slit-lamp biomicroscopy**

The Cornea Contact Lens Research Unit (CCLRU) scale\(^{27}\) was used to evaluate bulbar and limbal redness and to identify pre-existing corneal staining using white light prior to tear collection. Any white light staining of grade 2 and above in extent was considered an exclusion factor. Corneal staining with fluorescein was assessed prior to and following in vivo confocal microscopy.

**In vivo corneal confocal microscopy**

Laser scanning in vivo confocal microscopy was conducted to determine corneal nerve morphology and DC density. The Heidelberg Retinal Tomograph III with the Rostock Corneal Module (Heidelberg Engineering GmbH, Heidelberg, Germany) was used and set up according to standard technique\(^ {28}\).

In order to assess corneal nerve fiber morphology and corneal dendritic cells, the participant was asked to observe a central target and section scans were taken on the central cornea. Images were captured from the right eye only as corneal nerve parameters and DCs have been shown to be symmetrical between eyes ((Figure 1)). The average of eight and five images, that did not overlap by more than 20% were analyzed for corneal nerve morphology\(^ {29}\) and dendritic cells,\(^ {4}\) respectively. Images were captured from both eyes and analyzed with ACC metrics (Version 2.0, Center for Imaging Sciences, The University of
Manchester, United Kingdom), each image being 400 µm in width by 400 µm in height.

Variables measured were corneal nerve fiber density (CNFD – the total number of main nerves per square millimeter) (no./mm²), corneal nerve fiber length (CNFL – the total length of main nerves and nerve branches per square millimeter) (mm/mm²), corneal nerve branch density (CNBD - the total number of main nerve branches per square millimeter) (no./mm²), corneal total branch density (CTBD - the total number of branch points per square millimeter) (no./mm²), corneal nerve fiber area (CNFA - the total nerve fiber area per square millimeter) (mm/mm²), corneal nerve fiber width (CNFW - the average nerve fiber width per square millimeter) (mm/mm²) and corneal nerve fractal dimension (CNFrac - the ratio of the change in detail to the change in scale). The high CNFrac values correspond to a healthier and evenly distributed complex nerve fiber structure, while a lower values correspond to fewer distorted nerve fibers, potentially reflecting abnormality. Corneal nerve fiber tortuosity (CNFT – corneal nerve fibre tortuosity is a factor mathematically derived from the images where lower values correspond to healthy nerve fibre structure).

The image with the highest and lowest value for each corneal nerve parameter was identified for each individual in order to capture the variability in the nerve plexus distribution. The means and standard deviation were then used for analysis. These parameters were included because the pattern in which nerves varies at the infero-nasal whorl relative to the central cornea is not yet fully understood. These parameters were used in order to capture the distribution of the nerve plexus.

**Tear collection**

Tears were collected as described previously prior to corneal confocal microscopy. The volume collected was monitored as an additional means to track reflex tearing. Tear samples from each eye were labelled and stored separately at 4°C until processing, for a maximum of two hours.

**Treatment of tear samples**
Tears were centrifuged at 4,000 rpm at 4°C for 20 minutes. The supernatant was then aliquoted into separate tubes for each protein to be analysed and stored at -80°C until analysis.

Four samples were collected from each participant: one from each eye at both visits. The right eye samples from visit 1 were assessed for TPC and substance P. The right eye samples from visit 2 were assessed for MMP-9 and TIMP-1 and the left eye samples from visit 2 were assessed for TNF-a and IL-6.

**Tear analysis**

Tear analysis was conducted in conjunction with the Duoset Ancillary Reagent Kit 2 (DY008) 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 (TPC) was determined using the bicinchoninic acid method (BCA) and using Pierce reagents and flat-bottom Nunc-F Maxisorp 96-well microplates (Thermo Fisher Scientific, Rochester, NY). Serial dilutions of bovine serum albumin in phosphate buffered saline (BSA) were used as standard. This was loaded in triplicate starting at 2 mg/mL down to 0.1 mg/mL in MilliQ water and 10 µL was added to each well. 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 (BCA Protein Assay Reagent, Pierce, Thermo Fisher Scientific, Rochester, NY) and Solution B (BCA Protein Assay Reagent B, Pierce, Thermo Fisher Scientific, Rochester, NY) were combined in respective volumes of 20 mL and 0.4 mL. This mixture was then added at a volume of 200 µL per well. The optical density was
read with the Benchmark Plus Microplate Spectrophotometer System (Bio-Rad, Hercules, CA, USA) using the Microplate Manager 5.2 (Bio-Rad, Hercules, CA, USA) at 595 nm after 30 minutes and a standard curve generated using the BSA bovine serum albumin as a reference.

**Substance P**

Substance P concentration was determined using an with the Cayman Chemical Company ELISA enzyme linked immunosorbent assay kit for substance P (Cayman Chemical Company, Michigan, USA, catalog number 583751). This is a competitive ELISA enzyme linked immunosorbent assay with an assay range of 3.5-500 pg/mL and a sensitivity of 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 buffer and the ELISA enzyme linked immunosorbent assay was performed according to the instructions of the manufacturer.

**Inflammatory mediators**

**MMP-9**

Total MMP-9 concentration was determined using sandwich enzyme-linked immunosorbent assay (ELISA) with the Quantikine RnD Systems Inc kit (Minneapolis, MN, USA). This was performed according to the directions of the manufacturer. Samples were loaded in a 1:25 dilution in duplicate. This assay recognizes the 92 kDa latent and 82 kDa active forms of MMP-9. The guidelines state that TIMP-1 interferes at concentrations greater than 1.56 ng/mL. Other MMPs are not known to cross-react or interfere when tested at 50 ng/mL. The sensitivity of the MMP-9 assay is less than 0.156 ng/mL according to the RnD Quantikine
kit. Where samples were below the detection limits of the standard curve, they were allocated a value of zero.

TIMP-1

The concentration of TIMP-1 was determined using ELISA with the RnD Systems Inc DuoSet kit (Minneapolis, MN, USA). This was performed according to the directions of the manufacturer. This kit recognizes natural and recombinant TIMP 1. The manufacturer’s guidelines state that there is no cross-reactivity or interference with MMP-1, -2 or -3 or TIMP-2 when tested at 50 ng/mL. MMP-9 is known to interfere at concentrations greater than 100 ng/mL in the RnD Quantikine kit. The sensitivity of the quantikine kit (DTM100 RnD Systems Inc, Minneapolis, MN, USA) is reported by the manufacturer as less than 0.08 ng/mL. Tears were analyzed in duplicate in a dilution of 1:200.

TNF-α

The concentration of TNF-α was determined using ELISA (RnD Systems Inc DuoSet kit, Minneapolis, MN, USA). This was performed according to the directions of the manufacturer. Tears were analyzed in a dilution of 1:10 in reagent diluted.

IL-6

The concentration of IL-6 was determined using ELISA (RnD Systems Inc DuoSet kit, Minneapolis, MN, USA). This was performed according to the directions of the manufacturer. Tears were analyzed in a dilution of 1:20.

Data analysis

Results are presented as mean ± standard deviation (SD). The average of right and left eyes was computed for ocular surface assessment parameters of staining, bulbar and limbal redness. Agreement between visits for DC counts was evaluated using Bland-Altman analysis. The
differences between the two visits were plotted against the means. Coefficient of repeatability
(1.96 x SD of mean differences between the visits). To test the hypothesis that the mean of
all the variables measured at visit 1 and 2 were equal, a dependent sample t-test was performed.
Prior to conducting the analysis, the assumption of normally distributed difference scores was
determined. The assumption was considered satisfied, as the highest skew and kurtosis levels
for all the pairs were estimated at -1.10 and 1.52, respectively, which is less than the maximum
allowable values for a t-test (i.e., skew < 2.0 and kurtosis < 9.0). The null hypothesis of equal
mean values for all the variables tested at visits 1 and 2 was accepted (p > 0.05). Thus, the
mean average value of visit 1 and 2 was used to determine associations between dendritic cells,
corneal nerve morphology, and tear film mediators using Pearson’s correlation analysis. SPSS
for Windows Version 16 (SPSS Sciences, Chicago, IL) was used for all statistical analysis and
a two-tailed α = 0.05 level of significance was applied.

RESULTS

Participant demographics

Self-reported sleep hours (7 ± 1 hours); and days of exercise of 30 minutes or more per week
(4 ± 2 days) were averaged and did not change between the two visits (p = 0.08 and p = 0.33,
respectively).

Ocular surface characteristics

The average score of bulbar and limbal hyperaemia between right and left eyes using the
CCLRU grading was computed at visit one (2.2 ± 0.3 and 1.9 ± 0.3) and visit two (2.0 ± 0.4
and 2.0 ± 0.4), respectively. There was no significant difference between the visits with respect
to bulbar and limbal hyperaemia scores (p = 0.83 and p = 0.29). Corneal staining grading was
less than 2.0 for all participants at each visit before performing in vivo confocal microscopy.
Dendritic cells and corneal nerve attributes

Corneal confocal microscopy detected at least one DC in at least one of the averaged frames from the central cornea in all healthy participants. A Bland-Altman plot was constructed and demonstrated a size effect of DC counts between the visits indicating that the higher the DC count the greater the variation between visits three weeks apart. The coefficient of repeatability between the two visits was 43 cells/mm². A paired sample t-test was used to determine significant differences between DC densities and corneal nerve attributes measures at each visit. Table 1 shows the characteristics and paired-correlations of the variables between the two visits. None of the variables showed significant differences between visits and all pairs correlated between visits for DC counts and corneal nerve parameters. Therefore, these measurements were averaged for the subsequent association analysis.

Tear collection

Flow-rate and protein concentration

In order to explore differences in tear collection, flow-rate between the two visits, a paired-sample t-test was conducted. Table 2 shows the mean ± SD of tear flow rate from right and left eyes at each visit as well as the paired-correlation values between visits per eye and between eyes at each visit. No significant differences between right and left eyes or tear collection flow rate were found. Total protein content, inflammatory mediators (MMP-9, TIMP-1, MMP-9:TIMP-1 ratio, TNF-a and IL-6) and neuropeptide (Substance P) concentrations are shown in Table 3. Tear samples from each eye were used for separate protein analysis.

Correlations

Corneal DC density of the 21 healthy participants did not correlate with the number of sleep hours or days of exercise per week. However, sleep hours correlated with CNFD-high ($r =$
0.57, \( p = 0.007 \)) and the SD of CNFD (\( r = 0.49, p = 0.02 \)). The number of hours of exercise per week correlated with CNFW-high (\( r = 0.53, p = 0.01 \)). There were no correlations between DC density and bulbar and limbal redness.

**Correlations between corneal dendritic cells and nerve parameters**

Visits were averaged for DC densities and all corneal nerve parameters. During the data analysis, it was observed that DC density showed a distinct scattered pattern when correlating with corneal nerve parameters in those individuals with mean DC density above 50 cells/mm\(^2\) (Figure 2). Due to the distinct split in the data, the data was separated into those individuals with DC density above and below this value. There were 12 individuals with mean DC density over 50 cells/mm\(^2\) with a mean and standard deviation of 99 ± 43 compared to 9 individuals with mean DC density under 50 cells/mm\(^2\) and mean and standard deviation of 15 ± 8 cells/mm\(^2\). These two groups also demonstrate significant differences (\( p < 0.001 \)) when using t-test comparison. When the correlation analysis was performed in the entire cohort we found few significant correlations driven by a single individual. This data is showed in Table 2 and in the supplementary data.

**Correlations between corneal dendritic cells and tear proteins**

When MMP-9 was considered as a percentage of total protein content, there was a correlation with corneal DCs (\( r = 0.47, p = 0.03 \)) using the entire cohort data (N=21). No correlations were found when grouping those individuals that demonstrate cell densities above and below 50 cells/mm\(^2\).

There were no other correlations between DCs and tear proteins. However, substance P showed strong correlations with tear flow rate (\( r = 0.61, p = 0.003 \)) and with total protein content (\( r = 0.65, p = 0.002 \)) and a moderate correlation with IL-6 (\( r = 0.52, p = 0.03 \)).
DISCUSSION

The present study explored the association between the number of DCs and multiple attributes of the SNP as well as the concentration and percentage of inflammatory mediators and neuropeptides in the tear film of healthy participants. The normal variability in DC counts between two time points, 3-weeks apart, was first established. A better agreement between visits at lower values was identified, indicating greater variability with greater DC counts. When the relationships between these DCs and inflammatory mediators and neuropeptide substance P were analyzed, there was significant correlation between DC density and the percentage of tear film inflammatory mediator MMP-9 in the entire healthy cohort but not with TIMP-1, TNF-α, IL-6 or substance P. This study also demonstrated an association between density of corneal DCs and nerve morphology when the mean dendritic cell density of healthy participants was over 50 cells/mm².

The presence of DC in the central cornea of healthy controls has previously been described in in vivo studies. Although there is significant variation in the reported density of DCs in healthy controls. The DC density in the present study (55 ± 58 cells/mm²) is within the range of what has previously been published (49 ± 39, 49 ± 43, 57 ± 70 and a median of 52 cells/mm², in healthy controls). Similarly, the mean average of corneal nerve attributes obtained in this study are in agreement with previous reports. Values of tear film proteins analyzed shown in Table 3 also have been reported and shown similarity with previous work using the same methodology of the present study.

Neurogenic inflammation involves a change in the sensory nerve endings by the release of neuropeptides, because of the activity of inflammatory mediators, modifying the function of sensory neurons. Neurogenic inflammation research has been undertaken in disease processes such as arthritis, colitis, bladder inflammation, and asthma. In the trigeminovascular system,
Neuropeptide release further activates trigeminal afferents, inducing sensitisation. Neuropeptides such as substance P are contained within sensory fibers and these, as well as cytokines, may be released to further modulate inflammatory events. The positive associations found in this study between nerve parameters in the avascular central corneal and DC density in healthy subjects supports that the ocular nervous and immune systems are interlinked, specifically in those individuals undergoing presumed immune activity response evidenced by increased number of DCs migration in the central cornea. It is also hypothesized that these correlations change in direction and are stronger and more significant during inflammation. Reduced nerve density and increased DCs has been demonstrated in dry eye with and without Sjögren syndrome and during infectious keratitis, with strong negative correlations between these parameters not only in the affected but also the fellow eye, indicating a systemic effect.

Substance P is produced by nerves and lymphocytes and is present in many areas of the central and peripheral nervous system. In this study, there was no association found between DC densities and substance P. However, substance P has shown to play an important role in augmenting inflammatory responses principally by regulating the function of DCs, via the expression of the neurokinin-1 receptor. In order to elucidate the expression of this receptor in vivo cultured investigations of human and murine cells have been exposed to bacterial infection. The lack of association found in the present study is potentially because under healthy conditions substance P does not exert any control over the dendritic cell migration. In support of this, Golebiowski et al. found no difference in substance P levels between asymptomatic contact lens wearers and healthy controls.

Even though the aim of this study was to recruit healthy participants with no evidence of systemic diseases, allergies, undertaking medication for any treatment or active infection, there was a number of participants (N=12) who showed an increased number of DCs in the central
cornea. It was decided to further explore the data by subdividing these individuals into those with mean DC values over 50 cells/mm² and those with values below. The rationale of making these two groups to explore the association between corneal nerves and DC densities was determined by the hypothesis that migration of DC into the central cornea is considered pathognomonic of activation of the immune response⁴⁹ and this number of cells is over the mean value reported previously in healthy cohorts.⁸, ²⁸, ⁴³, ⁵⁰

The presence of increased dendritic cells in the central cornea is considered under the activation of the immune response of the ocular surface. However, many other factors are involved during the inflammatory process. For example, during acute inflammation, infiltration of inflammatory cells occur under the influence of cytokines such as TNF-α and interferon (IFN)-γ and cause vessel extravagation of polymorphonuclear leukocytes (PMN). The fully mature PMN is well equipped for these activities, with its complement of enzymes, MMPs, cytokines, and antimicrobial peptides. In neurogenic inflammation, peptides such as substance P can up-regulate limbal mast cells, boost PMN chemotaxis and phagocytosis. Other aspects that modulate ocular inflammation are grown factors such as platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF)-2, which are released when the basement membrane is damaged or degraded as a result of increased MMP activity.⁵¹ In contrast to the acute inflammatory response, the chronic response involves recruitment of an army of cells that will initiate antigen stimulus, this chronic response also involves accumulation of cytokines and other toxic mediators. The results of this investigation showed no differences between inflammatory mediators and neuromediators of individuals with increased number of DCs. This may be an indication of the ocular surface immune system being alert and ready to rapidly activate, rather than undergoing inflammation. This hypothesis have been suggested recently in presence of contact lens wear.⁵²
One individual in this study showed an increased DC density (195 cells/mm²), a level which has previously been reported in individuals who suffer from immuno-mediated corneal inflammation such as, herpes simplex virus or adenoviral keratitis and corneal graft rejection. Intriguingly, this individual also had substantially increased corneal nerve branches (CNBD) and total branch points (CTBD), as compared to the mean average of the study cohort shown in Table 1. There is no known condition that increases corneal nerve density, in fact, most diseases that affect the ocular surface usually reduce corneal nerve density but increase corneal nerve tortuosity. However, epidermal nerve density is increased in inflammatory skin conditions such as atopic dermatitis. The cause of atopic dermatitis is unknown but believed to involve genetics and immune system dysfunction which may explain an effect of any underlying atopic condition in this particular individual. As such, we may speculate that the individual in the present study with the significantly higher DC and nerve attributes may have an unknown previous or current systemic diseases or inflammatory processes, or medication use. Upon further questioning, this healthy individual reported being a vegan from birth and exercising five hours per week every week.

There is a significant body of evidence of the increased number of DCs and inflammatory markers in the presence of inflammation of the ocular surface. However, there are no reports on the association between the number of these cells with inflammatory mediators and neuropeptides. For example, in severe dry eye, IL-6 and TNF-α are elevated compared to healthy subjects. An experimental model of dry eye reported gene expression of MMP-9 and TNF-α. In contact lens wear MMP-9, TIMP-1 and the MMP-9: TIMP-1 ratio seems to be altered during the first-month of adaptation to lens wear. In ocular allergies an increased TNF-α and substance P has been shown in patients with active seasonal allergic conjunctivitis. Increased DC density has extensively been reported in these ocular manifestations mentioned above, however associations between the two have never been tested. Given that the present
study was conducted in healthy participants with no diagnosed local or systemic diseases, it can be stated that correlations between inflammatory markers, neuropeptides and dendritic cells are more likely to occur during acute or chronic inflammation of the ocular surface but not with cell migration as activation of the immune response.

The positive association between MMP-9 and DC density was mainly driven by a single individual, mentioned above, that showed a higher number of DCs and elevated percentage of MMP-9. Recent studies in mice have highlighted the role of MMP-9 in DC migration in vitro and in vivo and have shown that matured cells within inflammatory sites and reporting MMP-9 as indicative of directional migration. This effect may explain the relationship shown between MMP-9 and DC density in this study.

There are some limitations in the present study. Although, the sample size of the population is reasonable for this pilot study, the subgrouping for the correlation analysis between DCs and variables of corneal nerves and mediators may not be adequate and therefore a bigger sample size is suggested in future studies. No evidence of seasonal effect on dendritic cell density has been reported and this may be a factor of variety in cell counts. Further research in this area is needed. The younger age of the participants and the lack of a corneal diseases are other limitations from this study.

This study demonstrates that corneal dendritic cells correlated with measures of corneal nerve morphology and inflammatory mediator MMP-9, supporting the interlink between the immune and nervous systems in the ocular surface. Variables of corneal nerve branching, in particular, showed a stronger correlation with DC density indicating that the number of cells may be modulated by the total number of nerve branches in the central cornea. The stronger association with branch density, rather than other nerve measures, was unexpected and warrants further investigation. This study also demonstrates poor correlation between DC density and
inflammatory mediators in healthy participants. In future studies of ocular surface
inflammation and tear film inflammatory proteins, DC density should be included. Larger
cohorts are needed to test these hypothesis.

ACKNOWLEDGEMENTS

Dr Alex Hui for MMP-9 kit and Mr David Smith for technical assistance.
REFERENCES

1. Müller LJ, Marfurt CF, Kruse F, Tervo TM. Corneal nerves: structure, contents and function. Exp Eye Res. 2003;76(5):521-542. doi:10.1016/S0014-4835(03)00050-2

2. Lagali N, Peebo BB, Germundsson J, Edén U, Danyali R, Rinaldo M, Fagerholm P. Laser-scanning in vivo confocal microscopy of the cornea: imaging and analysis methods for preclinical and clinical applications. In: Lagali N, editor. Laser confocal microscopy: applications in medicine, biology, and the food sciences. InTech Open; 2013. p. 51-80.

3. De Cilla S, Ranno S, Carini E, Fogagnolo P, Ceresara, G, Orzalesi N, Rossetti LM. Corneal subbasal nerves changes in patients with diabetic retinopathy: an in vivo confocal study. Invest Ophthalmol Vis Sci. 2009;50(11):5155–5158. doi:10.1167/iovs.09-3384.

4. Alzahrani Y, Colorado LH, Pritchard N, Efron N. Longitudinal changes in Langerhans cell density of the cornea and conjunctiva in contact lens-induced dry eye. Clin Exp Optom. 2017;100:33–40. doi:10.1111/cxo.2017.100.issue-1.

5. López-de la Rosa A, Martín-Montañez V, López-Miguel, Fernández I, Calonge M, González-Méijome MJ, González-Garcia MJ. Ocular response to environmental variations in contact lens wearers. Ophthalmic Physiol Opt. 2017;37:60-70. doi:10.1111/opo.12338

6. Nicolle P, Liang H, Rebourssin E, Rabut G, Warcoin E, Brignole-Baudouin F, Melik-Parsadanianantz S, Baudouin C, Labbe A, Reaux-Le Goazigo A. Proinflammatory Markers, Chemokines, and Enkephalin in Patients Suffering from Dry Eye Disease. Int. J. Mol. Sci. 2018;19(4):1221. doi:10.3390/ijms19041221.

7. Du J, Liu RQ, Ye L, Ye L, Li ZH, Zhao FT, Jiang N, Ye LH, Shao Y. Analysis of corneal morphologic and pathologic changes in early-stage congenital aniridic keratopathy. Int J Ophthalmol. 2017;10(3):378–384. doi:10.18240/ijo.2017.03.09.

8. Cruzat A, Witkin D, Baniasadi N, Zheng L, Ciolino JB, Jurkunas UV, Chodosh J, Pavan-Langston D, Reza D, Hamrath P. Inflammation and the nervous system: the connection in the cornea in patients with infectious keratitis. Invest Ophthalmol Vis Sci. 2011; 52(59): 5136– 5143. doi:10.1167/iovs.17-23651.

9. Shetty R, Sethu S, Deshmukh R, Deshpande K, Ghosh A, Agrawal A, Shroff R. Corneal dendritic cell density is associated with subbasal nerve plexus features, ocular surface disease index, and serum vitamin D in evaporative dry eye disease. Biomed Res Int. 2016;2016:ID4369750. doi:10.1155/2016/4369750

10. Hamrath P, Huq SO, Liu Y, Zhang Q, Dana MR. Corneal immunity is mediated by heterogeneous population of antigen-presenting cells. J Leukoc Biol. 2003; 74(2): 172–178. doi:10.1189/jlb.1102544.

11. Tavakoli M, Boulton A, N E, Malik R. Increased Langerhan cell density and corneal nerve damage in diabetic patients: Role of immune mechanisms in human diabetic neuropathy. Cont Lens Anterior Eye. 2011;34(1):7-11. Doi:10.1016/j.clae.2010.08.007.
12. Gao N, Yan C, Lee P, Sun H, Yu FS. Dendritic cell dysfunction and diabetic sensory neuropathy in the cornea. J Peripher Nerv Syst. J Clin Invest. 2016;126(5):1998–2011. doi:10.1172/JCI85097.

13. Yamaguchi T, Hamrah P, Shimazaki J. Bilateral Alterations in Corneal Nerves, Dendritic Cells and Tear Cytokine Levels in Ocular Surface Disease. Cornea. 2016; 35 (suppl 1): S65–S70. doi:10.1097/ICO.0000000000000989.

14. de Souza GA, Godoy LMF, Mann M. Identification of 491 proteins in the tear fluid proteome reveals a large number of proteases and protease inhibitors. Genome Biol. 2006;7:R72. doi:10.1186/gb-2006-7-8-r72.

15. Carreno E, Enriquez-de-Salamanca A, Teson M, García-Vázquez C, Stern ME, Whitcup SM, Calonge M. Cytokine and chemokine levels in tears from healthy subjects. Acta Ophthalomol. 2010;88(7):250–258. doi: 10.1111/j.1755-3768.2010.01978.x.

16. Gill SE, Parks WC. Metalloproteinases and their inhibitors: regulators of wound healing. Int J Biochem Cell Biol 2008;40:1334-1347.

17. Nagase H, Woessner JF, Jr. Matrix metalloproteinases. J Biol Chem 1999;274:21491-21494.

18. 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;50:3203-3209.

19. Garrana RM, Zieske JD, Assouline M, Gipson IK. Matrix metalloproteinases in epithelia from human recurrent corneal erosion. Invest Ophthalmol Vis Sci 1999;40:1266-1270.

20. Yoon KC, Jeong IY, Park YG, Yang SY. Interleukin-6 and tumor necrosis factor-alpha levels in tears of patients with dry eye syndrome. Cornea 2007;26:431-437.

21. Reid TW, Murphy CJ, Iwashashi CK, Foster BA, Mannis MJ. Stimulation of epithelial cell growth by the neuropeptide substance P. J Cell Biochem 1993;52:476-485.

22. Garcia-Hirschfeld J, Lopez-Briones LG, Belmonte C. Neurotrophic influences on corneal epithelial cells. Exp Eye Res 1994;59:597-605.

23. Nishida T, Nakamura M, Ofuji K, Reid TW, Mannis MJ, Murphy CJ. Synergistic effects of substance P with insulin-like growth factor-1 on epithelial migration of the cornea. J Cell Physiol 1996;169:159-166.

24. Hill R. What sample size is “Enough” in internet survey research? Interpers Comput Technol. 1998;6:1–10.

25. Isaac S, Michael BW. A collection of principles, methods, and strategies Useful in the planning, design, and evaluation of studies in education and the Behavioral sciences. Handbook in research and evaluation. 1995:34-58.

26. Markoulli M, Papas E, Cole N, Holden B. Effect of contact lens wear on the diurnal profile of matrix metalloproteinase 9 in tears. Optom. Vis. Sci. 2013;90(5):419-429. doi:10.1097/OPX.0b013e31828d7d3b
19.27. Cornea and Contact Lens Research Unit (CCLRU). Grading Scales. University of New South Wales (UNSW): Sydney, Australia, 1996.

19.28. Zhivov A, Stave J, Vollmar B, Guthoff R. In vivo confocal microscopic evaluation of Langerhans cell density and distribution in the normal human corneal epithelium. Graefes Arch Clin Exp Ophthalmol. 2005;243:1056–1061. Doi:10.1007/s00417-004-1075-8.

20.29. Vagenas D, Pritchard N, Edwards K, Shahidi MA, Sampson PG, Russell WA, Malik AR, Efron N. Optimal image sample size for corneal nerve morphometry. Optom Vis Sci. 2012;89(5):12–17. doi: 10.1097/OPX.0b013e31824ee8c9.

21.30. Chen X, Graham J, Petropoulos IN, Ponirakis G, Asghar O, Alam U, Marshall A, Ferdousi M, Azmi S, Efron N, et al. Corneal nerve fractal dimension: a novel corneal nerve metric for the diagnosis of diabetic sensorimotor polyneuropathy Invest Ophthalmol Vis Sci. 2018;59:1113–1118. doi:10.1167/iovs.17-23342.

22.31. Kallinikos P, Berhanu M, O'Donnell C, Boulton AJ, Efron N, Malik RA. Corneal nerve tortuosity in diabetic patients with neuropathy. Invest Ophthalmol Vis Sci. 2004;45(2):418-22. doi:10.1167/iovs.03-0637.

23.32. Efron N, Edwards K, Roper N, Pritchard N, Sampson GP, Shahidi AM, Vagenas D, Russell A, Graham J, Dabbah MA, et al. Repeatability of measuring corneal subbasal nerve fiber length in individuals with type 2 diabetes. Eye Contact Lens 2010;36(5):245-248. doi:10.1097/ICL.0b013e3181ea915.

24.33. Markouli M, Gokhale M, You J. Substance P in flush tears and schirmer strips of healthy participants. Optom Vis Sci. 2017;94(4):527–33. doi:10.1097/OPX.0000000000001040

25.34. Bland JM, Altman D. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet 1986;327:307-310. doi:10.1016/S0140-6736(86)90837-8.

26.35. BSI. Precision of Test Methods. Part 1: Guide for the Determination of Repeatability and Reproducibility for a Standard Test Method. British Standards Institute London; 1979.

27.36. Posten HO, Cheng YH, Owen DB. Robustness of the two-sample t-test under violations of the homogeneity of variance assumption. Commun Stat Theor Methods. 2007;11:109–126. doi:10.1080/0361092820828221.

28.37. Chen X, Graham J, Dabbah MA, Petropoulos IN, Ponirakis G, Asghar O, Alam U, Marshall A, Fadavi H, Ferdousi M, et al. Small nerve fiber quantification in the diagnosis of diabetic sensorimotor polyneuropathy: comparing corneal confocal microscopy with intraepidermal nerve fiber density. Diabetes care 2015;38:1138-1144. doi:10.2337/dc14-2422.

29.38. Brines M, Culver DA, Ferdousi M, Tannemaat MR, Velzen M, Dahan A, Malik RA. Corneal nerve fiber size adds utility to the diagnosis and assessment of therapeutic response in patients with small fiber neuropathy. Sci Rep. 2018;8(1):4734. doi:10.1038/s41598-018-23107-w.
30.39. Barnes PJ. Neurogenic inflammation in the airways. Respir Physiol. 2001;125:145-154. Doi:10.1016/s0034-5687(00)00210-3.

31.40. Bienenstock J, Blennerhassett M, Goetzl E. Autonomic neuroimmunology. CRC Press; 2003.

32.41. Sharav Y, Benoliel R. Orofacial pain and headache: Elsevier Health Sciences; 2008.

33.42. Black PH. Stress and the inflammatory response: A review of neurogenic inflammation. Brain Behav Immun. 2002;16(6):622-653. doi:10.1016/s0889-1591(02)00021-1.

34.43. Tepelus TC, Chiu GB, Huang J, Huang P, Sadda SR, Irvine J, Lee OL. Correlation between corneal innervation and inflammation evaluated with confocal microscopy and symptomatology in patients with dry eye syndromes: a preliminary study. Graefe Arch Clin Exp Ophthalmol. 2017;255(9):1771-1778. doi:10.1007/s00417-017-3680-3.

35.44. Cruzat A, Schrem WS, Schrems-Hoesl LM, Cavalcanti BM, Baniasadi N, Witkin D, Pavan-Langston D, Dana R, Hamrah P. Contralateral clinically unaffected eyes of patients with unilateral infectious keratitis demonstrate a sympathetic immune response. Invest Ophthalmol Vis Sci. 2015;56(11):6612-6620.

36.45. Marriott I, Bost KL. Substance P receptor mediated macrophage responses. Adv Exp Med Biol. 2001;493:247-54. doi:10.1007/0-306-47611-8_30.

37.46. Lambrecht BN, Germonpré PR, Everaert EG, Carro-Muino I, De Veerman M, de Felipe C, Hunt SP, Thielemans K, Joos GF, Pauwels RA. Endogenously produced substance P contributes to lymphocyte proliferation induced by dendritic cells and direct TCR ligation. Eur J Immunol. 1999;29(12):3815-3825. doi:10.1002/(sici)1521-4141(199912)29:12<3815::aid-immu3815>3.0.co;2-%23.

38.47. Marriott I, Bost KL. Expression of authentic substance P receptors in murine and human dendritic cells. J Neuroimmunol. 2001;114(1-2):131-141. doi:10.1016/s0165-5728(00)00466-5.

39.48. Golebiowski B, Chao C, Stapleton F, Jalbert I. Corneal nerve morphology, sensitivity and tear neuropeptides in contact lens wear. Optom Vis Sci. 2017;94(4):534-42. doi: 10.1097/OPX.0000000000001063.

40.49. Hazlett LD, McClellan SA, Rudner XL, Barrett RP. The role of Langerhans cells in Pseudomonas aeruginosa infection. Invest Ophthalmol Vis Sci. 2002;43(1):189-197.

41.50. Lopez Alemany A., Presencia Rendal A. Inflammation oculaire et lentilles de contact. Contactologia 1991;13:14-17.

42.51. McDermott AM, Perez V, Huang AJ, et al. Pathways of corneal and ocular surface inflammation: a perspective from the cullen symposium. The ocular surface 2005;3:S-131-S-138.

43.52. Efron N. Contact lens wear is intrinsically inflammatory. Clin Exp Optom. 2017;100(1):3-19. doi:10.1111/cxo.12487.
Mastropasqua L, Nubile M, Lanzini M, Carpineto P, Ciancaglini M, Pannellini T, Di Nicola M, Dua HS. Epithelial dendritic cell distribution in normal and inflamed human cornea: in vivo confocal microscopy study. Am J Ophthalmol. 2006;142(5):736-744. doi:10.1016/j.ajo.2006.06.057.

Malik RA, Kallinikos P, Abbott CA, van Schie CH, Morgan P, Efron N, Boulton AJ. Corneal confocal microscopy: a non-invasive surrogate of nerve fibre damage and repair in diabetic patients. Diabetologia. 2003;46(5):683-688. doi:10.1007/s00125-003-1086-8.

Edwards K, Pritchard N, Vagenas D, Russell A, Malik RA, Efron N. Standardizing corneal nerve fibre length for nerve tortuosity increases its association with measures of diabetic neuropathy. Diabet Med. 2014;31(10):1205-1209. doi:10.1111/dme.12466

Urashima R, Mihara M. Cutaneous nerves in atopic dermatitis. A histological, immunohistochemical and electron microscopic study. Virchows Arch. 1998;432(4):363-370. doi:10.1007/s004280050

Lam H, Bleiden L, De Paiva CS, Farley W, Stern ME, Pflugfelder SC. Tear cytokine profiles in dysfunctional tear syndrome. Am J Ophthalmol. 2009;147(2):198-205. doi:10.1016/j.ajo.2008.08.032

Meloni M, De Servi B, Marasco D, Del Prete S. Molecular mechanism of ocular surface damage: Application to an in vitro dry eye model on human corneal epithelium. Mol Vis. 2011;17:113-126.

Maggi E, Biswas P, Del Prete G, Parronchi P, Macchia D, Simonelli C, Emmi L, De Carli M, Tiri A, Ricci M. Accumulation of Th-2-like helper T cells in the conjunctiva of patients with vernal conjunctivitis. J Immunol. 1991;146(4):1169-1174.

Jotwani R, Eswaran S, Moonga S, Cutler CW. MMP-9/TIMP-1 Imbalance Induced in Human Dendritic Cells by Porphyromonas gingivalis. FEMS Immunol Med Microbiol. 2010;58(8):314-321. doi:10.1111/j.1574-695X.2009.00637.x.
FIGURES

Figure 1: Representative image of the central cornea. Dendritic cells can be seen located in proximity with the nerve bundles.

Figure 2: Correlations between corneal dendritic cells and corneal nerve parameters. Square data points correspond to dendritic cell counts \( \geq 50 \text{ cells/mm}^2 \) and circles represent dendritic cell counts \( \leq 50 \text{ cells/mm}^2 \).

TABLES

Table 1: Paired-samples correlations and averaged characteristics of corneal dendritic cell counts and corneal nerves attributes between visits

| Variable | Visit 1 (mean ± SD) | Visit 2 (mean ± SD) | Correlation (r) (P value) | Average of 2 Visits (mean ± SD) |
|----------|---------------------|---------------------|--------------------------|--------------------------------|
| DC density (cells/mm\(^2\)) | 68 ± 57 | 63 ± 57 | 0.93 (0.000) | 55 ± 58 |
| CNFD (n/mm\(^2\)) | 30.6 ± 8.2 | 28.9 ± 7.1 | 0.63 (0.004) | 29.1 ± 6.9 |
| CNBD (mm/mm\(^2\)) | 38.0 ± 20.1 | 42.9 ± 24.4 | 0.71 (0.001) | 40.0 ± 20.1 |
| CNFL (n/mm\(^2\)) | 16.8 ± 10.4 | 17.0 ± 3.7 | 0.67 (0.002) | 16.6 ± 3.2 |
| CTBD (n/mm\(^2\)) | 53.2 ± 27.0 | 62.5 ± 35.0 | 0.78 (0.000) | 57.3 ± 28.5 |
| CNFA (mm/mm\(^2\)) | 0.006 ± 0.001 | 0.006 ± 0.002 | 0.59 (0.008) | 0.006 ± 0.001 |
| CNFW (mm/mm\(^2\)) | 0.020 ± 0.001 | 0.020 ± 0.001 | 0.52 (0.024) | 0.020 ± 0.001 |
| CN-fractal dimension (ratio scale) | 1.50 ± 0.03 | 1.50 ± 0.03 | 0.48 (0.039) | 1.50 ± 0.02 |
Table 2: Correlations between corneal dendritic cells and nerve parameters

| Corneal nerve attributes | All data (n = 21) | DC density ≥ 50 cells/mm² (n = 12) | DC density ≤ 50 cells/mm² (n = 9) |
|--------------------------|-------------------|-----------------------------------|-----------------------------------|
| CNBD-average             | r = 0.5*          | r = 0.7*                          | r = 0.2                           |
|                          | p = 0.03          | p = 0.02                          | p = 0.6                           |
| CTBD-average             | r = 0.5*          | r = 0.7**                         | r = 0.3                           |
|                          | p = 0.01          | p = 0.01                          | p = 0.5                           |
| CNFA-average             | r = 0.4           | r = 0.7**                         | r = 0.2                           |
|                          | p = 0.06          | p = 0.00                          | p = 0.5                           |
| CNFrac-sd                | r = 0.3           | r = -0.6*                         | r = -0.0                          |
|                          | p = 0.2           | p = 0.04                          | p = 1.0                           |
| CNBD-high                | r = 0.5*          | r = 0.6*                          | r = 0.2                           |
|                          | p = 0.01          | p = 0.02                          | p = 0.5                           |
| CTBD-high                | r = 0.508*        | r = 0.6*                          | r = 0.3                           |
|                          | p = 0.02          | p = 0.03                          | p = 0.4                           |
| CNFA-high                | r = 0.3           | r = 0.7*                          | r = 0.2                           |
|                          | p = 0.2           | p = 0.01                          | p = 0.5                           |
| CNBD-low                 | r = 0.5*          | r = 0.6*                          | r = 0.2                           |
|                          | p = 0.03          | p = 0.02                          | p = 0.6                           |
| CTBD-low                 | r = 0.5*          | r = 0.7*                          | r = 0.1                           |
|                          | p = 0.01          | p = 0.01                          | p = 0.8                           |
| CNFA-low                 | r = 0.4           | r = 0.8**                         | r = -0.1                          |
|                          | p = 0.08          | p = 0.00                          | p = 0.8                           |
| CNFrac-low               | r = 0.2           | r = 0.6*                          | r = 0.3                           |
|                          | p = 0.3           | p = 0.04                          | p = 0.4                           |
| CNFL-low                 | r = 0.2           | r = 0.7*                          | r = 0.3                           |
|                          | p = 0.4           | p = 0.02                          | p = 0.4                           |

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).
Table 3: Tear film protein collection, concentration and as a percentage of total protein content

| Protein                        | Eye  | Concentration (mean ± SD) | Percentage (mean ± SD) |
|--------------------------------|------|---------------------------|------------------------|
| Total protein content         | left | 2.5 ± 1.2 mg/mL           | n.a                    |
| **Inflammatory mediators**    |      |                           |                        |
| MMP-9                         | right| 12 ± 28 ng/mL             | 0.0003 ± 0.0010        |
| TIMP-1                        | right| 79 ± 27 ng/mL             | 0.0043 ± 0.0044        |
| MMP-9:TIMP-1 ratio            | right| 0.20 ± 0.53               | n.a                    |
| TNF-a                         | left | 53 ± 175 ng/mL            | 0.0016 ± 0.0051        |
| IL-6                          | left | 36 ± 63 ng/mL             | 0.0012 ± 0.0023        |
| **Neuropeptide**              |      |                           |                        |
| Substance P                   | right| 209 ± 277 ng/mL           | 0.0065 ± 0.0077        |