Neuropathic Corneal Pain: Tear Proteomic and Neuromediator Profiles, Imaging Features, and Clinical Manifestations

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• PURPOSE: To investigate the tear proteomic and neuromediator profiles, in vivo confocal microscopy (IVCM) imaging features, and clinical manifestations in neuropathic corneal pain (NCP) patients.

• DESIGN: Cross-sectional study.

• METHODS: A total of 20 NCP patients and 20 age-matched controls were recruited. All subjects were evaluated by corneal sensitivity, Schirmer test, tear break-up time, and corneal and ocular surface staining, Ocular Surface Disease Index and Ocular Pain Assessment Survey questionnaires were administered, as well as IVCM examinations for corneal nerves, microneruomas, and epithelial and dendritic cells. Tears were collected for neuromediator and proteomic analysis using enzyme-linked immunosorbent assay and data-independent acquisition mass spectrometry.

• RESULTS: Burning and sensitivity to light were the 2 most common symptoms in NCP. A total of 188 significantly dysregulated proteins, such as elevated metallothionein-2, creatine kinases B-type, vesicle-associated membrane protein 2, neurofilament light polypeptide, and myelin basic protein, were identified in the NCP patients. The top 10 dysregulated biological pathways in NCP include neurotoxicity, axonal signaling, wound healing, neutrophil degradation, apoptosis, thrombin signaling mitochondrial dysfunction, and RHOGDI and P70S6K signaling pathways. Compared to controls, the NCP cohort presented with significantly decreased corneal sensitivity ($P < .001$), decreased corneal nerve fiber length ($P = .003$), corneal nerve fiber density ($P = .006$), and nerve fiber fractal dimension ($P = .033$), as well as increased corneal nerve fiber width ($P = .002$), increased length, total area and perimeter of microneuromas ($P < .001$, $P < .001$, $P = .019$), smaller corneal epithelial size ($P = .017$), and higher nerve growth factor level in tears ($P = .006$).

• CONCLUSIONS: These clinical manifestations, imaging features, and molecular characterizations would contribute to the diagnostics and potential therapeutic targets for NCP. (Am J Ophthalmol 2024;265: 6–20.

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Neuropathic corneal pain (NCP) is characterized by dysfunctional corneal nerves and ocular symptoms including pain, discomfort, burning, stinging, allodynia, and photophobia, which are typically not relieved by conventional dry eye treatment because of its neurobiological nature.[1] The symptoms of NCP can have a great impact on quality of life and cause a significant psychological and economic burden.[2] However, because of its wide range of causation, absence of correlation between clinical signs and symptoms, and lack of consensus in the diagnostic criteria and treatment protocols, the current understanding of NCP remains limited. There is a need to objectively quantify clinical manifestations, imaging characteristics, and molecular profiles, to improve the diagnosis, monitoring, and therapeutic outcomes.

The pain of NCP is thought to be due mainly to abnormal firing of injured corneal nerve endings, or abnormal connections with adjacent nerve endings that produce spontaneous neuronal activity.[3] With persistent stimulation, spinal and supraspinal nociceptive pathways, as well as remodeled brain cortical areas such as the somatosensory cortex, thalamus, and amygdala, can become sensitized to subsequent stimuli, leading to central sensitiza-

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tion and a state of chronic pain.[4] NCP can be categorized as central, peripheral, and mixed type. In vivo confocal microscopy (IVCM) has been used widely to visualize the corneal nerve plexus.[5] Advances in image analysis may further contribute to the development of standard diagnostic criteria and an imaging metric for the evaluation of NCP. Through IVCM evaluation, Ross et al[6] described findings of decreased nerve densities, activated keratocytes, and a greater number of microneuromas in patients with NCP. Moein et al[7] further reported that the presence of microneuromas on IVCM may be a useful biomarker for the diagnosis of NCP, with a sensitivity and specificity of 100%, to differentiate NCP patients from patients with dry eye disease.

Corneal neuromediators play an important role in maintaining the homeostasis of the ocular surface by regulating neuroinflammatory responses.[8] The majority of studies on neuropathic pain focus on animal models, whereas clinical studies are very scarce. In a rat model, following injury of the spinal dorsal horn in rats, calcitonin gene-related peptide (CGRP) has been shown to serve as an inflammatory mediator in the pathogenesis of neuropathic pain.[9,10] Neurokinin 1 (NK-1), which is the receptor of substance P, has also been implicated in the pathophysiology of pain, and NK-1 receptor antagonists could effectively block peripheral inflammatory responses, as well as inflammation-related nociceptive pain.[11-13] In addition, nerve growth factor (NGF), which is regarded as a multifactorial modulator of neuro–immune–endocrine function mediating pain and inflammation, has been reported to alleviate allodynia and hyperalgesia by weakening reactive astrocytosis and reversing glial morphomolecularin in rat models of NCP.[14,15] In a clinical study, tear NGF and interleukin 9 levels were significantly associated with the development of chronic ocular pain after refractive surgery.[16]

However, on a molecular level, proteomic profiles in NCP have not been investigated, and only a few studies have been conducted on spinal neuropathic pain. In a rat spinal nerve ligation model, downregulation of creatine kinase B-type (KCRB), syanpsin 1, and microtubule-associated protein were reported to contribute to the onset and development of neuropathic pain.[17,18] In recent years, advanced quantitative proteomics has become an emerging tool for clinical studies, especially when analyzing a limited volume of samples, such as tear samples.[19-22] Understanding the molecular basis would help decipher the pathogenesis and provide insight into the potential biomarkers or therapeutic targets for NCP.

At present, the proteomic and neuromediator profiles of NCP, as well as their link with clinical presentation and nerve metrics changes, have not been explored. In this study, we aimed to provide detailed and comprehensive data on the clinical manifestations, corneal nerve imaging features, tear neuromediators, and proteomic profiles for NCP patients, and to compare them with those of control subjects.

METHODS

• **STUDY DESIGN AND POPULATIONS:** This cross-sectional study included 40 eyes of 20 patients who were diagnosed with NCP based on the criteria detailed in Table 1. The type of NCP was categorized based on proparacaine challenge test: patients experienced complete, partial relief, or no response after topical proparacaine administration suggest peripheral, mixed, and central NCP, respectively.[23] The exclusion criteria are listed in Table 1. An additional 40 eyes of 20 age-matched controls who had no history of inflammatory eye diseases, diabetes mellitus, corneal or ocular surgeries, systemic inflammatory diseases, contact lens wear, or use of any eye drops within 1 month were also recruited. Approval for the study was granted by the Institutional Review Board of SingHealth (number 2022/2046), Singapore. The study was conducted in accordance with the Declaration of Helsinki, and informed consent was obtained from the subjects.

Patients' demographic characteristics, medication history, history of ocular surgery and trauma, and comorbidities were recorded.

• **OCULAR SURFACE ASSESSMENTS:** The Schirmer I test was performed,[24] and the results were recorded as the length (mm) of wetting of the strips after 5 minutes. For the tear break-up time (TBUT) evaluation, a sterile fluorescein strip was dipped in the inferior conjunctival fornix to apply fluorescein dye. TBUT was measured 3 times and recorded as the time between the last blink and the first dry spot appearance with cobalt blue illumination.[24] Ocular surface integrity was assessed with fluorescein staining using the Oxford score (0-5), and corneal fluorescein staining was graded with the National Eye Institute (NEI) scale, which ranges from 0 to 3 for each of the 5 areas of the cornea (total score 0-15), with higher scores indicating a greater abnormality. Corneal sensitivity was evaluated by Cochet Bonnet aesthesiometer (Luneau Ophthalmologia) at each quadrant and central area of the cornea. The length (0-6 cm) of filament that resulted in a blink reflex or escape response was taken. The value of the entire cornea thus ranged from 0 to 30 cm.

• **SUBJECTIVE QUESTIONNAIRES:** Ocular pain was assessed using the Ocular Pain Assessment Survey (OPAS), a validated multidimensional questionnaire that includes 6 dimensions for analyzing the following items[25]: eye pain intensity for the last 24 hours and the last 2 weeks, non–eye pain intensity, how much pain has affected quality of life, aggravating factors, and associated factors for pain. Patients were asked to respond to all questions according to the pain scale or frequency between 0 and 10, or 0% to 100% (0 = no pain, 10 or 100% = worst pain ever).[26] The Ocular Surface Disease Index (OSDI), a 12-item questionnaire, was used to assess the symptoms of dry eye (Q1-5),

TABLE 1. Inclusion and Exclusion Criteria for NCP Study Population

Inclusion Criteria
• Persistent ocular pain or pain-like symptoms, including burning sensation, allodynia, photoallodynia, stinging, hyperalgesia, throbbing, shooting, sharp, cramping, gnawing, or a feeling of electric shock, with a minimum score of 30% for more than 3 questions in the OPAS questionnaire for at least 3 months
• Presence of any one of the following corneal nerve abnormalities: microneuromas, beading, nerve tortuosity, decrease in CNFD or CNFL, on IVCM images
• Minimal or no ocular surface fluorescein staining, with the NEI score <2
• No response to conventional dry eye treatments, including topical lubricant eye drops, gel or ointment, topical cyclosporine, punctual occlusion, and eyelid warming/massage/cleaning, for at least 6 months
Exclusion Criteria
• Presence of active ocular surface disease
• Active anterior or posterior blepharitis
• Presence of concomitant ocular disease that may cause ocular pain, such as uveitis or other ocular inflammatory disease

CNFD = corneal nerve fiber density; CNFL = corneal nerve fiber length; IVCM = in vivo confocal microscopy; NCP = neuropathic corneal pain; NEI = National Eye Institute; OPAS = Ocular Pain Assessment Survey.

its impact on vision-related functioning (Q6-9), and the effect of environmental factors (Q10-12). The total score was 0-100 following calculating with the formula: Total score $= \frac{Sum\ of\ scores\ for\ all\ questions\ answered \times 100}{Total\ number\ of\ questions\ answered \times 4}$.[27,28]

• **IVCM SCAN AND IMAGE ANALYSIS FOR CORNEAL NERVES, EPITHELIAL CELLS, MICRONEUROMAS, AND DENDRITIC CELLS:** IVCM (Heidelberg Retina Tomography III, Rostock Cornea Module, Heidelberg Engineering GmbH) was used to examine corneal epithelial cells, sub-basal nerve plexus, corneal microneuromas, dendritic cells and stromal keratocytes.[29] Patients were asked to fixate on a light source, and the scanning depth ranged from the superficial corneal epithelium to the posterior stroma. The sub-basal nerve plexus of the central cornea, as well as 4 quadrants of peripheral areas that were 3 mm away from the corneal apex, were scanned. Five best-focused images were selected from each area and each eye (a total of 25 images), and each nerve (main truck or branched nerve) was selected only once. Each image was analyzed separately for each parameter using ACCMetrics software (University of Manchester).[30] The following nerve parameters were obtained: corneal nerve fiber density (CNFD; the number of fibers/mm^2, each frame area = 0.16033 mm^2), corneal nerve branch density (CNBD; the number of branch points on the main fibers/mm^2), corneal nerve fiber length (CNFL; the total length of fiber [mm/mm^2]), corneal total branch density (CTBD; the total number of branch points/mm^2), corneal nerve fiber area (CNFA; the total nerve fiber area mm^2/mm^2), corneal nerve fiber width (CNFW; average nerve fiber width mm/mm^2), and nerve fiber fractal dimension (CFracDim). CFracDim represents the spatial loss of nerves: a higher CFracDim value corresponds to a more even distribution of corneal nerve fiber.[5,31] The mean value of the 25 selected images was used for statistical analysis.

Microneuromas were manually identified. Spindle microneuromas were hyperreflective fusiform enlargements of the nerves; lateral microneuromas appeared as localized hyperreflective enlargements of a nerve, from which single or multiple tortuous nerves sprout; stump microneuroma were characterized by terminate swelling of the nerves abruptly.[32] The images with microneuroma were manually quantified using Image J (National Institute of Health). The parameters assessed were microneuroma area (μm^2), average length (μm), and perimeter (μm). The images were analyzed by a single experienced and masked investigator (I.X.Y.L).

The AIConfocal Rapid Image Evaluation System (ARIES; ADCIS, S.A.) was used to analyze corneal epithelial cells and dendritic cells.[33] Five best-focused epithelial micrographs selected for each eye were automatically analyzed to obtain the following parameters: epithelial cell count, cell density (cells/μm^2), average cell size (μm^2), and cell circularity. Dendritic cells were defined as small white cells either to be isolated from the nerve or connected to the nerve branch.[34] Five to ten most representative images were selected and analyzed for each eye. The following parameters were obtained: cell count, cell density (cells/μm^2), average length (the mean width of all dendritic cells [μm]), average area in micromillimeters squared (μm^2), and elongation (absolute value of the difference between the major and minor axes divided by the sum of the major and minor axes [μm]).

• **TEAR NEUROMEDIATOR ANALYSIS:** Tear samples were taken from the wetted Schirmer strips, which were cut into small pieces and submerged into 200 μL of ice-cold tear elution buffer containing 0.55M NaCl, 0.33% Tween-20,

0.55% bovine serum albumin and 1x protease inhibitor, and were subjected to sonication, homogenization, followed by 17 hours incubation at 4°C with gentle agitation at 450 rpm.[35] The homogenization step was repeated once on the next day before centrifugation at 11,000 rpm at 4°C for 20 minutes. Each eluted tear protein sample contained clear supernatants, and was subjected to analysis using enzyme-linked immunosorbent assay (ELISA): Substance P (6 × dilution), CGRP (4 × dilution), and NGF (1.5 × dilution), respectively (CGRP from Phoenix Pharmaceuticals; Substance P and NGF from R&D Systems).

• **TEAR PROTEOMIC PROFILE ANALYSIS:** Quantitative tear proteomic analysis was conducted as described previously.[22,36] The small pieces of Schirmer strips and 100 µL of lysis buffer were mixed at 20°C for 1.5 hours. The total protein concentration was detected with Bio-Rad DC Protein assay. A 100-µg quantity of eluted tear proteins was reduced, alkylated, tryptic digested, and desalted, and the total peptide amounts were quantified. All peptide samples were analyzed by coupling to an Orbitrap Exploris 480 Mass Spectrometer via an EASY-Spray Source with an EASY-nLC 1200 system. Liquid chromatography (LC) separation was performed using an Acclaim PepMap 100 C18 as precolumn and a PepMap®RSLC C18 as analytical column (ThermoFisher Scientific). Data independent acquisition (DIA) assay was performed, and a total of 19 Windows of 45.7 Da with 3 Da overlaps were used. A library-free direct-DIA workflow in Spectronaut 15 (Biognosys) was used to process DIA data.

• **STATISTICAL ANALYSIS:** All data were expressed as mean ± SD. The comparison between the data of NCP patients and controls was analyzed using a linear mixed model, to account for the correlation of right–left paired eyes. P values <.05 were considered statistically significant. All statistical analyses were performed using STATA software (version 17, StataCorp). For the proteomic data, the raw protein abundance values were obtained after fragment ions were selected for quantification, and raw abundance data were median normalized and log transformed for statistical analyses. A fold change (FC) ≥2.0 and FC ≤ −2.0 with an adjusted P value of <.05 were considered significant. Gene Ontology (GO) enrichment analysis was performed, and Ingenuity Pathways Analysis (IPA; Qiagen) was used to identify enriched pathways. Benjamini adjusted P values were calculated by modified Fisher exact test, with smaller P values representing a higher chance of enrichment. Custom scripts in R (64-bit version 4.1.1) was used to perform the downstream data analysis and data visualization, and to generate the plots to illustrate the results of protein expression, GO, and IPA analysis. The difference in tear proteomic profiles between NCP patients and controls was evaluated using orthogonal partial least-squares discrimination analysis (OPLS-DA).

RESULTS

• **PATIENT DEMOGRAPHICS:** The mean age of 20 NCP patients and 20 controls were 46.4 ± 13.0 and 41.5 ± 5.5 ($P = .159$). In the NCP cohort, 13 patients (65.0%) were diagnosed with peripheral NCP, 2 (10.0%) with central, and 5 (25.0%) mixed NCP. All the patients presented with bilateral pain. The demographics of the NCP cohort are presented in Table 2. Eleven patients (55.0%) had a history of dry eye disease, 7 (35.0%) had a history of refractive surgery, and 12 patients (60.0%) had co-morbidities such as depression, anxiety and sleep disorders. Six patients received systemic and topical treatment, 14 patients received topical treatment alone, and no patient received systemic treatment alone.

• **OCULAR SURFACE ASSESSMENTS:** At the time of the diagnosis, the NCP cohort presented with significantly decreased corneal sensitivity ($P < .001$), TBUT ($P < .001$), and Schirmer tests ($P = .029$) compared to controls. The mean Oxford and NEI scores in the NCP patients were very minimal at 0.9 ± 0.8 and 0.8 ± 0.9, respectively, and there was no significant difference in these 2 scores between the NCP group and controls ($P = .149$, $P = .108$, respectively) (Table 3).

• **PAIN AND OCULAR SURFACE SUBJECTIVE SYMPTOMS:** On the OPAS questionnaire, the 2 most frequent and bothersome symptoms were "burning" and "sensitivity to light." In all, 60% of NCP patients reported severe pain (pain score >6) for the past 24 hours, and 70% of NCP patients reported severe pain for the past 2 weeks. From 50% to 80% of patients had their quality of life significantly (>60%) affected because of the pain, especially when reading and/or during computer use (80%), and as well as mood (75%). In the control group, the majority of patients reported no or very minimal discomfort scores (pain score ≤1) (Figure 1).

On the OSDI questionnaire, 14 of patients (70%) were sensitive to light, 12 (60%) experienced a sensation of gritiness, and 15 (75%) had painful or sore eyes more than half of the time. In all, 14 patients (70%) reported being sensitive to windy conditions, 11 (55%) felt uncomfortable in areas with low humidity, and 11 (55%) felt uncomfortable in air conditioning more than half of the time. In the control group, no patient reported having symptoms more than half of the time (Figure 2).

• **IVCM FINDINGS FOR CORNEAL NERVES, EPITHELIAL CELLS, MICRONEUROMAS AND STROMAL KERATOCYTES:** The CNFD, CNFL, and CFracDim were significantly decreased in NCP patients compared to controls ($P = .006$, $P = .003$, and $P = .033$, respectively) (Figure 3, A and 6B). NCP patients also had a significantly higher CNFW

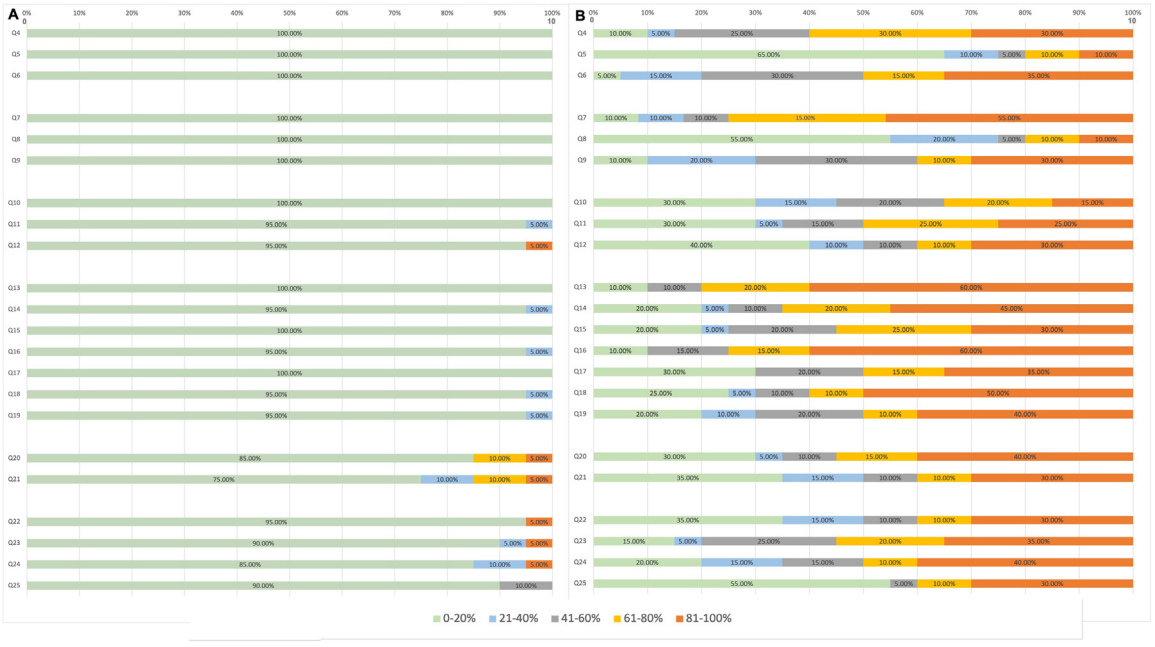

FIGURE 1. Percentage of patients who answer the pain intensity question on each Ocular Pain Assessment Survey (OPAS) questionnaire item. Charts in (A) controls and (B) neuropathic corneal pain (NCP) patients.

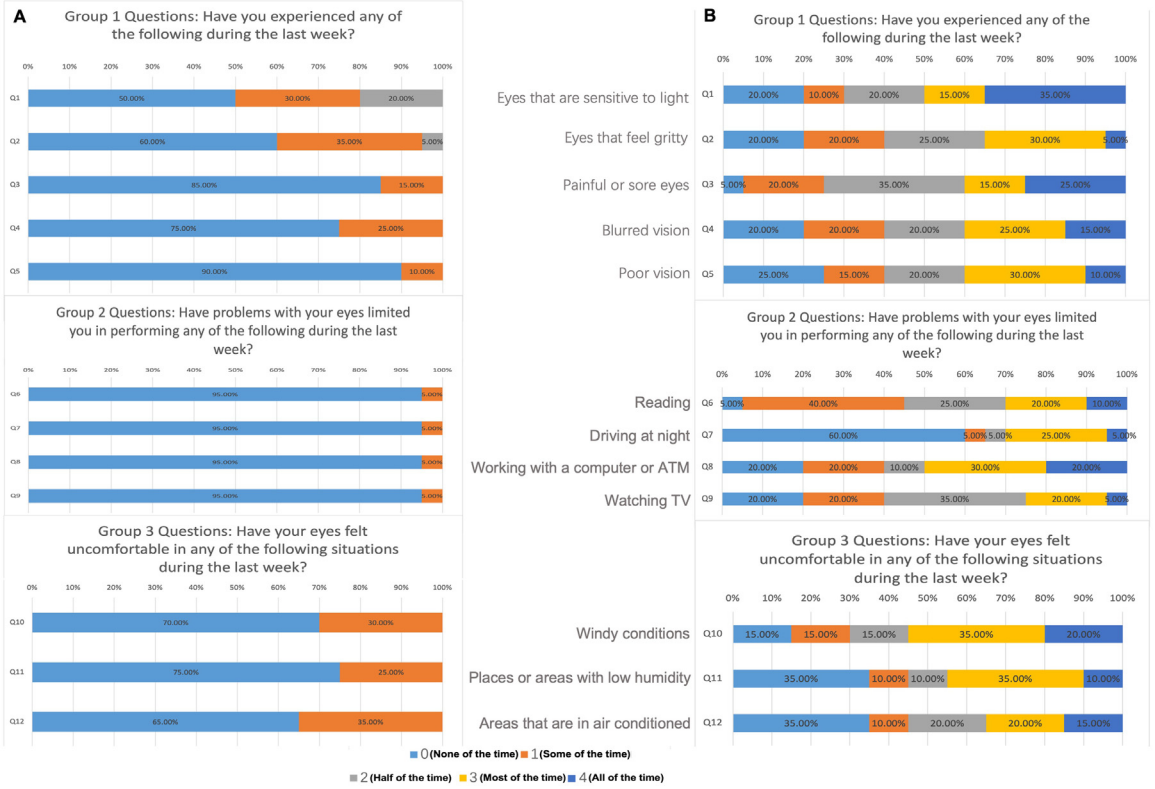

FIGURE 2. Percentage of patients who answer the frequency on each Ocular Surface Disease Index (OSDI) questionnaire item. Charts in (A) controls and (B) neuropathic corneal pain (NCP) patients.

TABLE 2. Characteristics of NCP Patients (N = 20)

Demographics	Patients
Age (y)	46.4 ± 13.0
Sex (male/female)	4/16
Race (Chinese/Indian)	17/3
Central/peripheral/mixed type of NCP	2/13/5
Medical History	
Diabetes	1
Refractive surgery	7
Dry eye disease	11
History of ocular trauma	1
Fibromyalgia	1
Small nerve fiber neuropathy	1
Coronavirus disease 2019 (COVID-19)	1
Comorbidities	
Anxiety/depression	2
Migraine	3
Sleep disorder	9

NCP = neuropathic corneal pain.

TABLE 3. Ocular Surface Assessments of NCP Patients and Controls

Parameters	NCP Patients (n = 40 eyes)	Controls (n = 40 eyes)	*P* Value
Corneal sensitivity (cm)	26.4 ± 6.4	29.0 ± 1.8	**<.001**
TBUT (s)	3.1 ± 2.2	7.1 ± 2.2	**<.001**
Schirmer test (mm)	7.1 ± 2.2	11.4 ± 4.1	**.029**
Oxford score (0-5)	0.9 ± 0.8	0.4 ± 0.7	.149
NEI score (0-15)	0.8 ± 0.9	0.3 ± 0.6	.108

NCP = neuropathic corneal pain; NEI = National Eye Institute; TBUT = tear break-up time. Boldface indicateds significance at $P<.05$.

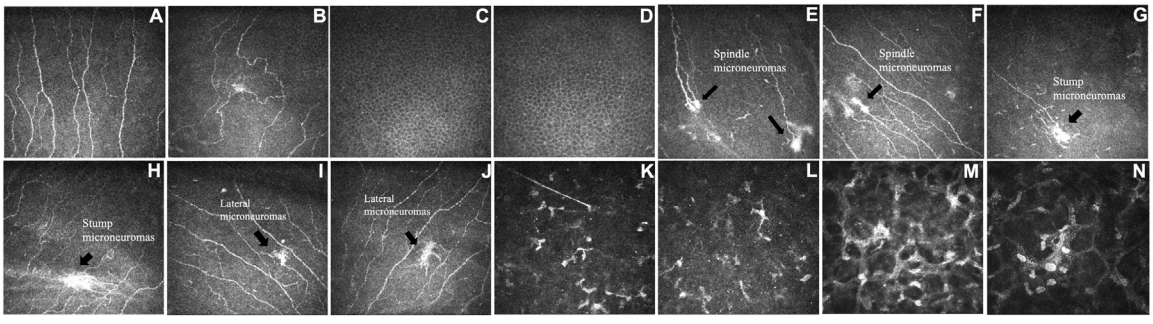

FIGURE 3. Representative in vivo confocal microscopy (IVCM) images in controls and neuropathic corneal pain (NCP) patients, activated stromal keratocytes, and 3 types microneuroma in patients with NCP. A, B. Normal nerve morphology in a healthy individual and representative tortuous nerves with decreased density and the presence of microneuroma in a patient with NCP. C, D. Epithelial cells in a healthy individual and a patient with NCP. E, F. Spindle microneuromas. G, H. Stump microneuromas. I, J. Lateral microneuromas. K, L. Patchy areas of hyperreflectivity. M, N. Honeycomb appearance of the corneal anterior stroma.

TABLE 4. In Vivo Confocal Microscopy Imaging Analysis of NCP Patients and Controls

Parameters	NCP Patients (n = 40 eyes)	Controls (n = 40 eyes)	*P* Value
Nerve Parameters			
CNFD (number/mm^2)	11.1 ± 7.8	15.9 ± 6.2	**.006**
CNBD (number/mm^2)	11.7 ± 11.5	14.9 ± 6.8	.151
CNFL (mm/mm^2)	8.2 ± 3.7	10.5 ± 2.3	**.003**
CTBD (number/mm^2)	21.9 ± 15.5	24.6 ± 9.2	.371
CNFA (mm^2/mm^2)	0.0046 ± 0.0014	0.0046 ± 0.0010	.978
CNFW (mm/mm^2)	0.0227 ± 0.0022	0.0213 ± 0.0010	**.002**
CFracDim	1.40 ± 0.06	1.42 ± 0.03	**.033**
Epithelial Cell Parameters			
Cell count	483.6 ± 116.3	437.1 ± 99.4	.078
Circularity	0.7 ± 0.1	0.7 ± 0.1	.276
Density (cells/μm^2)	0.0079 ± 0.0006	0.0082 ± 0.0038	.681
Average size (μm^2)	128.0 ± 10.4	133.3 ± 7.4	**.017**
Dendritic Cell Parameters			
Cell count	13.4 ± 9.8	11.2 ± 8.0	.298
Density (μm^{-2})	0.0228 ± 0.0019	0.0227 ± 0.0030	.885
Area (μm^2)	44.9 ± 3.5	46.1 ± 3.9	.182
Elongation (μm)	0.7 ± 0.4	0.6 ± 0.1	.490
Average length (μm)	10.7 ± 1.1	11.0 ± 0.7	.094
Microneuroma Parameters			
Total area (μm^2)	428.9 ± 205.2	198.3 ± 236.8	**<.001**
Average length (μm)	104.3 ± 73.8	26.9 ± 43.6	**<.001**
Perimeter (μm)	66.8 ± 67.9	37.1 ± 45.9	**.019**

CFracDim = nerve fiber fractal dimension; CNBD = corneal nerve branch density; CNFA = corneal nerve fiber area; CNFD = corneal nerve fiber density; CNFL = corneal nerve fiber length; CNFW = corneal nerve fiber width; CTBD = corneal total branch density; NCP = neuropathic corneal pain. Boldface indicates significance at $P<.05$.

compared to controls ($P = .002$), suggesting swollen nerve plexus. There was no significant difference in the CNBD, CTBD, and CNFA between NCP patients and controls (Table 4).

For the corneal epithelial evaluation, NCP patients had significantly smaller epithelia ($P = .017$). There was no significant difference in the rest of the corneal epithelial parameters, as well as all of the parameters for dendritic cell analysis, between the NCP patients and controls (all $P > .05$) (Table 4, Figure 3, C and D).

The presence of microneuromas was consistently observed in all eyes of the NCP patients. Among the microneuromas that we detected in NCP patiens, 40% of were spindle microneuromas, 30% were stump microneuromas, and 30% were lateral microneuromas (Figure 3, E-J). NCP patients presented with a significantly greater total microneuroma area ($P < .001$), average length ($P < .001$), and perimeter ($P = .019$) in comparison to controls (Table 4).

NCP patients also demonstrated varying degrees of activated stromal keratocytes. In all, 45% of eyes had patchy areas of high stromal reflectivity, and 15% of eyes showed a honeycomb appearance of the stroma with increasing reflectivity and fusion of cytoplasm (Figure 3, K-N).

• **TEAR NEUROMEDIATOR PROFILES IN NCP:** NCP patients had significantly higher tear NGF concentrations ($P = .006$) in comparison to controls. There was no difference in the tear CGRP and SP concentrations between the 2 groups (Table 5). We also found that the NGF level was significantly correlated with corneal epithelial cell circularity ($r = -0.38$, $P = .048$).

• **TEAR PROTEOMIC PROFILES AND PATHWAY ANALYSIS:** The score plots and back-scaled loading plots of OPLS-DA analysis displayed a clear separation of the tear proteomic profiles of the NCP patients vs controls ($R^2Y = 0.992$) (Figure 4, A). A permutation test showed that the OPLS-DA model was not overfitted, and the OPLS-DA model had a goodness of prediction ($Q^2 = 0.619$). A total of 188 significantly dysregulated proteins were identified in the eyes with NCP (Figure 4, B) compared to control eyes. In the eyes with NCP, metallothionein-2 (MT2) and 40S ribosomal protein S8 (RS8), which are related to neuropathic pain, were significantly increased ($\log_2FC = 2.13$ and $\log_2FC = 1.74$, respectively). Elevated optineurin (OPTN), related to neurodegenerative diseases, was also observed ($\log_2FC = 1.24$). Vesicle-associated membrane protein 2 (VAMP2) and neurofilament light polypeptide (NFL), which are involved in peripheral mononeuropathic

TABLE 5. Tear Neuromediators of Neuropathic Corneal Pain Patients and Controls

Tear Neuromediators	NCP Patients (n = 40 Eyes)	Controls (n = 40 Eyes)	*P* Value
SP (pg/mL)	1560.0 ± 1425.0	1755.0 ± 814.5	.614
CGRP (pg/mL)	1.0 ± 0.9	1.6 ± 1.1	.059
NGF (pg/mL)	60.1 ± 91.1	12.0 ± 14.6	**.006**

SP = Substance P; CGRP = Calcitonin gene-related peptide; NGF = nerve growth factor. Boldface indicates significance at $P < .05$.

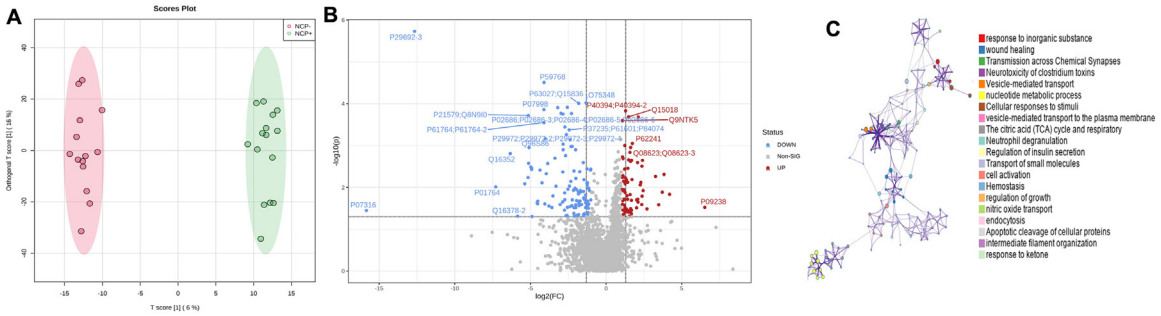

FIGURE 4. Tear proteomic profiles of neuropathic corneal pain (NCP) patients and controls. A. Orthogonal partial least-squares discrimination analysis (OPLS-DA) analysis showing a clear separation of the tear proteomic profiles of the NCP patients vs controls. B. Volcano plots presenting the fold changes (FC) of the tear proteins comparing NCP patients vs controls. Significantly up-regulated (FC > 2.0 and $P < .05$, ie, $\log_2$ FC >1 and $-\log_{10}$, $P > 1.3$) and downregulated (FC ≤2.0 and $P < .05$, ie, $-\log_2$ FC > 1 and $-\log_{10}$, $P > 1.3$) proteins are shown in blue and red, respectively. C. Gene Ontology enrichment analysis based on 188 dysregulated proteins identified in NCP patients.

pain, were significantly downregulated ($\log_2$FC = –1.81 and $\log_2$FC = –2.38, respectively). Bone morphogenetic protein 3 (BMP3) and dihydropyrimidinase-related protein 2 (DPYL2), a marker for axonal growth and nerve development, were also significantly downregulated ($\log_2$FC = –2.56 and $\log_2$FC = –2.29, respectively). Aquaporin-1 (AQP1; $\log_2$FC = –2.62), which is associated with nerve injury, tubulin beta-4A chain (TBB4A; $\log_2$FC = –2.98), a neuron-specific protein, and myelin basic protein (MBP; $\log_2$FC = –2.72), which is prevalent in idiopathic pain states, as well as KCRB ($\log_2$FC = –2.84), synaptosomal-associated protein 25 (SNP25; $\log_2$FC = –2.85), and G-protein coupled receptor family C group 5 member C (GPC5C; $\log_2$FC = –3.20), which is involved in neuropathic pain, were significantly downregulated (Table 6, Figure 5). These significantly dysregulated proteins were related to the following: (1) axonal guidance signaling, (2) p70S6K signaling, (3) soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) signaling, and (4) thrombin signaling mitochondrial dysfunction, which are all pain or neuropathic pain related (Figure 6).

On the GO enrichment pathway analysis, the pathways that the significantly dysregulated proteins were associated with in the eyes with NCP included wound healing (logP = –9.659), neurotoxicity (logP = –8.743), neutrophil degeneration (logP = –6.604), and apoptosis (logP = –4.855) (Table 7, Figure 4, C).

DISCUSSION

In the present study, we described the clinical manifestations and imaging features of patients with NCP. We also demonstrated, for the first time, the tear proteomic profiles and neuromediators of NCP. Clinically, burning sensation and sensitivity to light were the 2 most common symptoms. On IVCM, there was a significant decrease in corneal nerve density and length, accompanied by the presence of microneuromas, activated keratocytes, and decreased epithelial size. In NCP patients, significantly increased tear NGF levels and dysregulated proteins such as MT2, KCRB, VAMP2, NFL, and MBP were observed. Biological reactions, including wound healing, neurotoxicity, neutrophil degeneration, axonal guidance, p70S6K signaling, thrombin signaling mitochondrial dysfunction, and SNARE signaling pathway were significantly altered in NCP.

We found that NCP patients had decreased TBUT, Schirmer values, and corneal sensitivity in comparison to controls, and there was no difference in the corneal and ocular surface staining. Similarly, Kim et al observed decreased tear film stability and tear secretion in NCP patients compared to controls, and no differences were found between central and peripheral NCP in these variables.[37] On the contrary, another study showed there was no difference in

TABLE 6. Top 20 Significantly Upregulated or Downregulated Tear Proteins in Patients With NCP Compared to Controls

	Protein Name	FC	log_2FC	*P* Value		Protein Name	FC	log_2FC	*P* Value
1	Metallothionein-2 (MT2)	4.3797	2.1308	**.0002**	11	Hippocalcin-like protein 1 (HPCL1)	0.1841	–2.4413	**.0004**
2	40S ribosomal protein S8 (RS8)	3.3344	1.7374	**.0009**	12	Bone morphogenetic protein 3 (BMP3)	0.1693	–2.5625	**.0001**
3	BRISC complex subunit Abraxas 2 (ABRX2)	2.8076	1.4894	**.0002**	13	Aquaporin-1 (AQP1)	0.1622	–2.6241	**.0005**
4	All-trans-retinol dehydrogenase [NAD(+)] (ADH7)	2.4459	1.2903	**.0001**	14	Myelin basic protein (MBP)	0.1514	–2.7233	**.0004**
5	Optineurin (OPTN)	2.3570	1.2370	**.0010**	15	Creatine kinase B-type (KCRB)	0.1395	–2.8420	**.0009**
6	Obg-like ATPase 1(PLSI)	2.1308	1.0914	**.0003**	16	Synaptosomal-associated protein 25 (SNP25)	0.1386	–2.8509	**.0002**
7	V-type proton ATPase subunit G 1 (VATG1)	0.3984	–1.3276	**.0001**	17	Tubulin beta-4A chain (TBB4A)	0.1269	–2.9784	**.0002**
8	Vesicle-associated membrane protein 2 (VAMP2)	0.2852	–1.8099	**.0001**	18	G-protein coupled receptor family C group 5 member C (GPC5C)	0.1089	–3.1986	**.0001**
9	Dihydropyrimidinase-related protein 2 (DPYL2)	0.2050	–2.2862	**.0002**	19	Sodium/glucose cotransporter 1 (SC5A1)	0.0617	–4.0189	**.0007**
10	Neurofilament light polypeptide (NFL)	0.1927	–2.3757	**.0007**	20	Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-2 (GBG2)	0.0582	–4.1024	**.0000**

FC = fold change; NCP = neuropathic corneal pain.

TABLE 7. Top 10 Significantly Expressed Pathways Involved by Significantly Upregulated and Downregulated Proteins in NCP Patients vs Controls

Pathway Name	logP Value
Wound healing	–9.659
Neurotoxicity	–8.743
Vesicle-mediated transport	–8.107
Nucleotide metabolic response	–7.070
Cellular response	–7.789
Neutrophil degeneration	–6.604
Cell activation	–5.953
Regulation of growth	–5.895
Endocytosis	–5.599
Apoptosis	–4.855

NCP = neuropathic corneal pain.

Schirmer test results between NCP patients and controls,[38] and NCP patients presented with increased corneal sensitivity.[39] Dry eye disease has been identified as a common underlying cause of NCP.[23,40] However, typically patients' symptoms and signs are disconnected, with no or only minimal ocular surface staining, suggesting a component of neuropathic pain.

Burning sensation and sensitivity to light were the 2 most frequent and bothersome symptoms in our NCP cohort. Aggarwal et al reported that patients with corneal neuropathy may have photoallodynia, which may be a proxy for pain.[15] The mechanism of how light causes pain is not well understood, and 3 biological pathways have been proposed.[41] First, light exposure evokes pain-associated signals in trigeminal afferents and trigeminal nucleus, resulting in ocular vasodilation and subsequent activation of nociceptive neurons in blood vessels, causing the sensation of pain.[42] Second, intrinsically sensitive retinal ganglion cells (ipRGCs), which contain the photopigment melanopsin and can be stimulated by light directly without input from the traditional photoreceptors, have been identified as the central role of photoallodynia pathways.[43] The direct connection between ipRGCs and thalamus pain centers has been proved to be a critical center for somatosensory output, and it could explain the occurrence of photoallodynia.[44] Third, animal experiments have found ipRGCs and melanopsin-containing neurons in the ocular surface and iris, and they may result in pain by activating trigeminal vascular afferents directly without going through the optic nerve.[45]

Significantly greater length, total area, and perimeter of microneuromas were observed in the NCP cohort. Microneuromas occur when an abnormal axon is interrupted and unable to re-establish axonal continuity because of localized nerve damage or degeneration. Schwann cells, axons, and perineural fibroblasts tend to regenerate and form

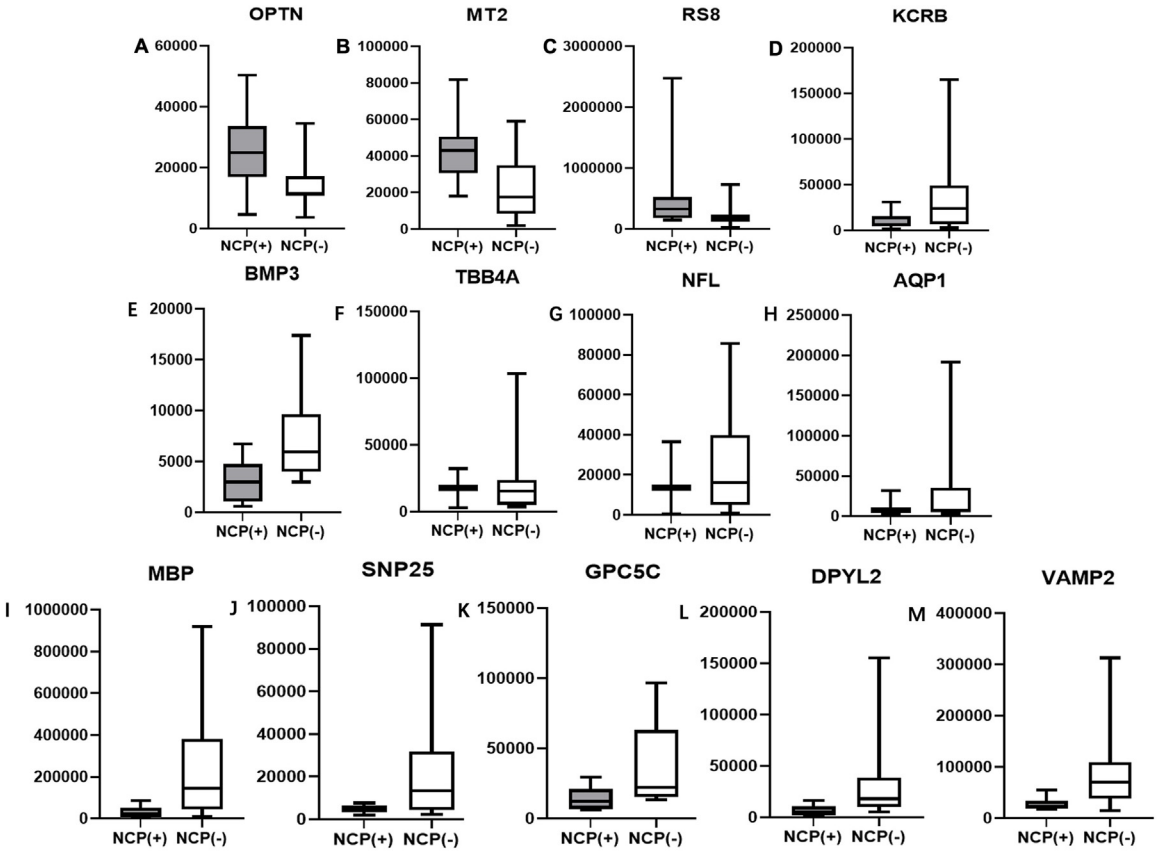

FIGURE 5. Box plots showing the representative tear proteins of neuropathic corneal pain (NCP) patients. A significantly upregulated (A) OPTN, (B) MT2, (C) RS8, and a significantly downregulated (D) KCRB, (E) BMP3, (F) TBB4A, (G) NFL, (H) AQP1, (I) MBP, (J) SNP25, (K) GPC5C, (L) DPYL2, and (M)VAMP2 in patients with NCP, compared to controls.

disorganized and tangled masses, which may lead to severe refractory neuropathic pain.[46–48] Moreover, the regeneration of neurons upregulates the expression of sodium channels, thus altering nerve activity.[49] As a result of altered expression of ion channel proteins in the neuron bodies and regenerative nerve endings, injured nerves begin to regenerate and form microneuromas that exhibit abnormal responsiveness and spontaneous discharges, leading to pain, hyperalgesia, allodynia, and other discomforts.[50] Therefore, microneuromas have been considered as a dynamic marker of pathological recovery of corneal nerves. A previous study has demonstrated that microneuromas were observed in the central cornea of all of the NCP patients, whereas patients with dry eye without pain or normal controls did not present with microneruomas.[16] Other groups have also demonstrated that the presence of microneuromas may potentially serve as a highly sensitive and specific diagnostic biomarker for the diagnosis of NCP.[7]

In the present study, patients with NCP presented with significantly decreased CNFL, CNFD, and CFracDim, and increased CNFW. The difference might be partially due to the inclusion criteria set "presence of any one of corneal nerve abnormalities." The findings were consistent with previous studies, in which reduced numbers of nerves, main nerve trunks, and nerve branches in the NCP cohort, compared to controls, were observed.[6,7] The increased width of nerve fibers might be due to nerve swelling caused by neuroinflammation, stimulating the release of neurotrophic factors, which in turn causes compensatory nerve hypertrophy.[51,52] We also observed activated stromal keratocytes in the majority of NCP patients. Keratocytes transform from a silent state to an activated phenotype influenced by cytokines and growth factors,[53] in response to tissue insult such as neuroinflammation.[54] In NCP patients, corneal epithelial cells were of significantly smaller size. Cell size had been associated with cell differentiation and proliferation.[55] The smaller epithelial cells show a stronger ability for proliferation and differentiation.[56] A previous study has demonstrated that NGF induces corneal epithelial cell cycle progression and proliferation.[57] Hence, higher tear NGF levels in NCP patients may lead to greater proliferation of the corneal epithelium. The epithelial proliferation might also be a compensatory reaction to neuroinflammation in NCP.

We observed significantly higher tear NGF concentrations in NCP patients. The NGF level was also significantly correlated with the corneal epithelial cell circularity. Although NGF was originally known to be a trophic factor

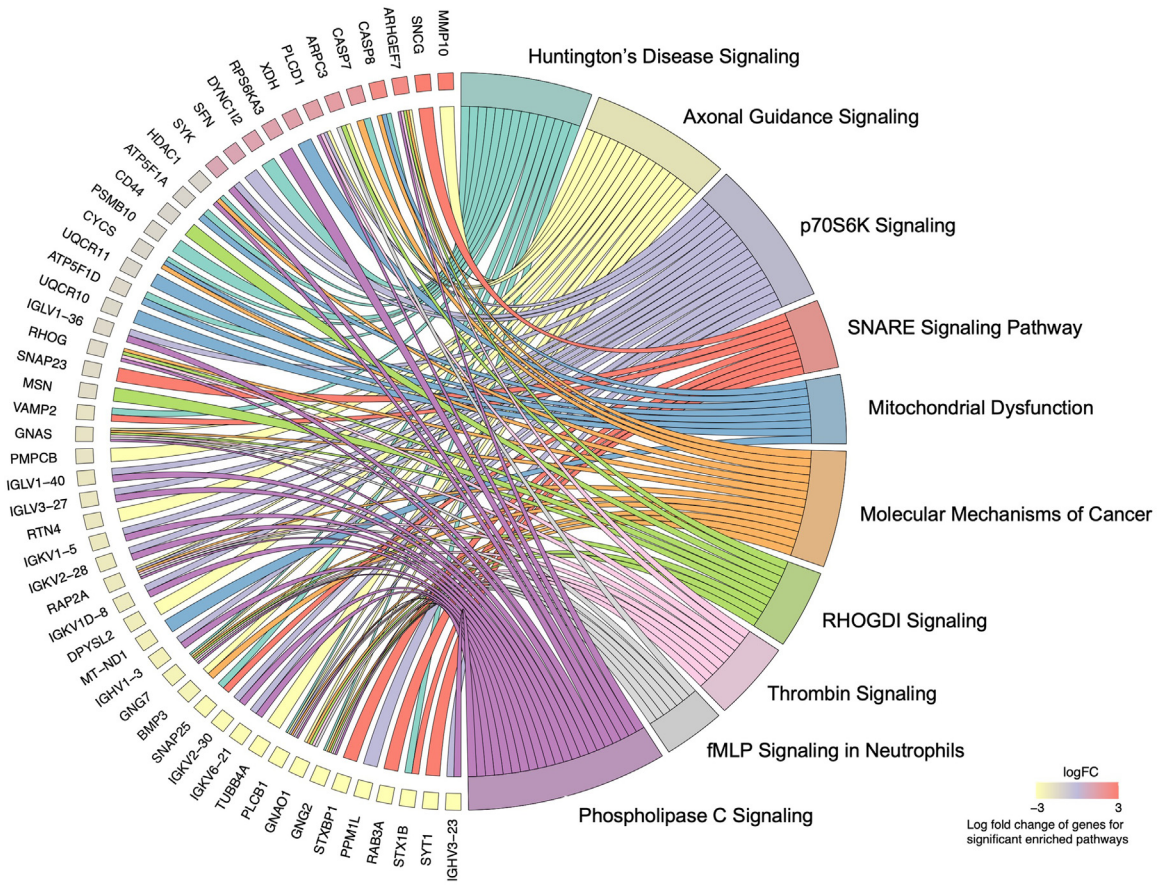

FIGURE 6. Top 10 significantly expressed biological pathways associated with the significantly dysregulated proteins determined by ingenuity pathway analysis (IPA).

for sensory neurons, a growing number of researchers have highlighted the crucial role of NGF in neuropathic pain, with inconsistent findings. It was reported in early years that, in rodent models of diabetic painful neuropathy and chemotherapy-induced peripheral neuropathy, the levels of NGF were decreased in the dorsal root ganglions.[58] Conversely, elevated NGF was observed in the Schwann cells close to the injured sensory neurons in rodent models of neuropathic pain, suggesting that axonal damage can induce the expression of NGF in response to nerve insult, which are thought to promote neuronal regeneration.[59] The mast cells, eosinophils, lymphocytes and macrophages at the location of injured nerves also release NGF.[59] In addition, NGF directly acts on peptidergic C fiber nociceptors that link to pain pathways.[60] Administration of NGF in rats or in humans has been shown to cause significant mechanical and thermal hyperalgesia,[61] and anti-NGF drugs have been shown to be effective in relieving pain. These findings suggest that NGF could be a new therapeutic target for pain relief in NCP.[61]

To identify the proteins involved in NCP, we compared the tear proteomic profiles in the NCP patients and controls. In the NCP patients, MT2 was significantly upregulated. MT2 was reported to be involved in the pathogenesis of pain, taking part in the initiation of inflammatory and neuropathic pain in response to noxious stimuli in the spinal cord.[62] VAMP2, which was significantly downregulated, has been known as the vesicular component of the neuronal SNARE complex. Decreased VAMP2 impairs the efficiency of neurotransmission, resulting in an imbalance of excitatory and inhibitory responses.[63] Numerous microRNAs (miRNAs) have been proved to regulate pain-related genes in chronic pain,[64] and MiR-7a may attenuate neuropathic pain by downregulating NFL,[65] which was observed in our study. We also found dysregulated SNAP-25 in our NCP patients. SNAP-25 was increased in neuropathic pain, and the reverse of the upregulation of SNAP-25 significantly attenuated pain-related behavior in rats.[66] Downregulated expression of BMP3 and AQP1 was observed in our NCP cohort. The former was involved in the regeneration of the peripheral nerves,[67] and the latter has been reported to play a role in the development of neuropathic pain.[68] AQP1 knock-out mice developed impairments in nociceptive responses.[69] Moreover, KCRB GPC5C, TBB4A, MBP, and DPYL2 were downregulated in NCP, which is in agreement with previous animal studies. A KCRB level was observed in the spinal cord in an ischemia-induced allodynia model,[70] whereas a decreased

GPRC5 level was found to participate in spinal neuropathic pain.[71] Downregulated TBB4A, a neuronal marker,[18] suggests neuronal loss, which is in line with our IVCM findings. MBP is an auto-antigen that induces mechanical allodynia in multiple sclerosis,[72] and our data, for the first time, demonstrate its presence in neuropathic pain. Finally, DPYL2 is a marker for axonal growth and nerve development, and its downregulation indicates decreased nerve density and length, which is evidenced by our IVCM results.

Several pathways linked to the above-mentioned proteins have been identified in the IPA and GO pathway analysis. Axon guidance molecules have been shown to mediate mechanical hyperalgesia in pre-clinical inflammatory pain models, and the expression of axon guidance molecules is also modulated by pro-inflammatory processes.[73] The expression of mammalian target of rapamycin (mTOR)–mediated phosphorylation of p70S6K has been reported to be amplified in the spinal cord in rats with peripheral neuropathic pain.[74] Inhibiting mTOR/p70S6K pathway-mediated changes could alleviate neuropathic pain.[75,76] Voltage-gated calcium channels in nerve endings control neurotransmitter release by facilitating vesicular fusion mediated by SNARE complexes, which can be deregulated in response to nerve damage.[77] Thrombin signaling pathway was also identified in NCP patients. It has been reported in a murine model that thrombin produced hyperalgesia and allodynia, which could be relieved by thrombin inhibitors.[78] NCP patients also had significantly dysregulated reactions of wound healing, neurotoxicity, neutrophil degeneration, and apoptosis (Table 7).

There are several limitations in the present study. First, this study was cross-sectional, as our aim was to report characteristics of NCP patients. Second, the number with the central type of NCP is much smaller than that of peripheral NCP, as the majority of the cohort was referred from ophthalmologists. Hence, the comparisons between subgroups for each parameter might be underpowered, but will be included in our future studies. Third, 5 patients had been treated with topical steroids for various periods ranging from 3 days to 3 months before referral, and this might have affected the proteomic results. However, our data reflect the real-world clinical setting in which treatment might have been started before the definite diagnosis of NCP. We have also compared the data between those who received topical treatment only vs those who received systemic and topical treatment concurrently. We did not find significant differences between these 2 subgroups. Whether these 2 groups were truly comparable, or whether the nonsignificant differences resulted from the small sample size, warrants further research.

In conclusion, in this study, we have described the tear proteomic and neuromediator profiles, clinical manifestations, and imaging features of NCP. NCP patients had characteristic microneuromas and activated keratocytes, as well as significant nerve swelling, decreased epithelial cell size, corneal nerve density, and length. Proteins related to neuroinflammation, neurotoxicity, axonal signaling, and wound healing, such as NGF, MT2, VAMP2, NFL, and MBP, were significantly dysregulated. Our study provides more in-depth insights and a better understanding of the pathophysiology and symptomatology of NCP. The molecules identified may also contribute to the exploration of novel biomarkers and new therapeutic targets for NCP.

CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

Chang Liu: Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **Molly Tzu-Yu Lin:** Data curation, Methodology. **Isabelle Xin Yu Lee:** Formal analysis, Methodology. **Jipson Hon Fai Wong:** Investigation. **Daqian Lu:** Methodology. **Thomas Chuen Lam:** Methodology. **Lei Zhou:** Methodology, Writing – review & editing. **Hon Shing Ong:** Writing – review & editing. **Marcus Ang:** Writing – review & editing. **Louis Tong:** Resources, Writing – review & editing. **Yu-Chi Liu:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – review & editing.

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