

Optic Nerve Head Spatial Transcriptomic Change in Nonhuman Primate Early Experimental Glaucoma

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PURPOSE. The purpose of this study was to determine optic nerve head (ONH) laminar collagen and retrolaminar myelin expression change in non-human-primate (NHP) experimental glaucoma (EG) using immunohistochemistry (IHC) and spatial transcriptomics.

METHODS. Unilateral EG NHPs ($n = 3$) were perfusion fixed, ONHs were trephined, embedded in paraffin, and serial sectioned for IHC and spatial transcriptomics. EG versus control eye retinal nerve fiber layer thickness (RNFLT) and axon loss within each region of study were estimated.

RESULTS. CNPase levels decrease in the retrolaminar region of the ONH in early EG by IHC. Multiple myelin genes are decreased in early NHP EG in the retrolaminar region. Inflammatory pathways were upregulated in the retrolaminar region as well. Among the top genes that were altered in the laminar region were collagen-related genes and transforming growth factor beta 1 (*TGFβ1*).

CONCLUSIONS. Spatial transcriptomics analysis revealed a consistent downregulation of multiple myelin-related genes, and IHC confirmed a corresponding decrease in CNPase protein expression. Importantly, spatial transcriptomics identified differential profiles among the prelaminar, laminar, and retrolaminar ONH. Together, these findings highlight early myelin disruption and provide insights into the spatial and molecular dynamics of disease onset in NHP. This work advances our understanding of glaucoma pathogenesis and lays the groundwork for developing novel therapeutic strategies.

Keywords: nonhuman primate (NHP), monkey, experimental glaucoma (EG), transcriptomics, optic nerve head (ONH)

Glaucoma is a common cause of blindness in all populations^{1,2} with an estimated 4.22 million people in the United States who are living with glaucoma.³ The pathophysiology of vision loss in glaucoma includes the retina, optic nerve head (ONH), peri-neural canal sclera, optic nerve (ON), mid-brain projections of the retinal ganglion cell (RGC) axons, and the visual cortex.^{4–9} Multiple studies suggest that the ONH connective tissue deformation and remodeling are primary events in the optic neuropathy of nonhuman primate (NHP) experimental glaucoma (EG). However, the mechanism and initiating pathways of ONH connective tissue remodeling and the mechanisms by which axonal homeostasis is disrupted within the lamina remain unknown.^{10–14} NHP EG models are physiologically similar to humans and allow understanding the pathogenesis.^{15–19} Our laboratory has used NHP EG models¹⁹ and three dimensional (3D) histomorphometry to detect, parameterize, and characterize regional laminar deformation,²⁰ remodeling,²¹ and migration²² in NHP with early EG. We have hypothesized that these connective tissue phenomena contribute to a

disruption of myelin homeostasis in the immediate retrolaminar ON and have demonstrated this disruption by immunohistochemistry (IHC)²³ and scanning block face electron microscopy²⁴ in separate reports. Understanding regional pathology in glaucoma offers advantages toward identifying cellular mechanisms of RGC axon homeostasis breakdown within the prelaminar, lamina, and retrolaminar ONH.²⁵ In this paper, we, for the first time, use carefully regionalized spatial transcriptomics to explore the mechanisms underlying alterations within the prelaminar, laminar, and retrolaminar ONH regions of NHP EG eyes associated with early clinical retinal nerve fiber thickness loss by optical coherence tomography (OCT) and orbital optic nerve axon loss based on automated axon counts.

Several previous studies have used a variety of approaches to study regional and cellular changes within ONH. Tribble et al. 2020 performed RNA-sequencing using microglia mRNA from the ONH in DBA/2J mice before detectable neurodegeneration and identified phagocytosis, inflammatory, and sensory pathways along with mitochon-

drial gene expression alterations.²⁶ Sustained IOP elevation by episcleral vein hypertonic saline injection and extensive injury affecting 50% of axons in rats significantly affected genes involved in cell proliferation, immune response, lysosome, cytoskeleton, extracellular matrix (ECM), and ribosome.²⁷ They also detected significantly altered expression of *Tgfb* receptors and signaling pathway intermediates.²⁷ Monavarfeshani et al. identified 37 distinct cell types in the human eye posterior segment that included ON, ONH, peripapillary sclera, choroid, and retinal pigment epithelium by high throughput single nucleus RNA-sequencing.²⁸ They identified oligodendrocytes in the ONH and ON, with oligodendrocyte progenitor cells being less abundant in the ONH than oligodendrocytes. They reported heterogeneity in fibroblast types and that the posterior segment fibroblasts were similar to those in the anterior segment. A fibroblast type C29 was enriched in the ONH but was also present in the peripapillary sclera and optic nerve. Advances in RNA-sequencing technologies have led to the identification of global transcriptional alterations that are associated with several neurodegenerative diseases including glaucoma. These big data methods allow comprehensive analyses of transcripts, providing tools for hypothesis testing and hypothesis generation. However, they do not provide information on location, microenvironment, or cellular heterogeneity. Spatially resolved transcriptomics was first described in 2019 and has been refined since then to provide cellular level information in spatially heterogeneous diseases like glaucoma.²⁹ Recently, Girkin et al. 2024 used spatial transcriptomic analysis on the ONH from living human eyes after IOP elevation.³⁰

Here, we describe initial results using spatial transcriptomics from anatomically consistent ONH clinical locations (relative to the foveal-BMO axis) and histologic regions (prelaminar, laminar, and retrolaminar) of the EG and control eyes from three NHPs with early levels of EG versus control eyes retinal nerve fiber layer thickness (RNFLT) thinning and orbital optic nerve axon loss. We observed regional differences of gene profiling with multiple myelination genes being downregulated in the retrolaminar region of the ONH, confirming our previously published data.²³ We also detected complement 4b (*C4B*), Apolipoprotein E (*APOE*), and cathepsin B (*CTSB*) genes in the top 10 genes being upregulated in the retrolaminar region. In the laminar region, collagen-related genes and transforming growth factor beta 1 (*TGFβ1*) were increased. In the prelaminar region of the ONH, *BEX3*, *NCR1*, *CACNA1S*, and *SYNPO* genes were among the top genes that were upregulated.

METHODS

Animals

All animals were treated in accordance with the Association for Research and Vision in Ophthalmology (ARVO) statement for the use of animals in research and were approved by the Legacy Institutional Animal Care and Use Committee (IACUC; Protocols 07–09 and 10–07), which is authorized by the United States Department of Agriculture (USDA) license (92-R-0002) and is governed by an assurance with the Office of Laboratory Animal Welfare (A3234-01) as well as Legacy Policy (100.16). The Legacy Department of Comparative Medicine, which supervises all aspects of animal care, also maintains full Association for Assess-

TABLE. Animal Demographics, IOP, Optical Coherence Tomography (OCT) Detected Experimental Glaucoma (EG) Eye Retinal Nerve Fiber Loss and Axon Loss

NHP Number	Sex	Age, Y	Eye	EG or C	Max IOP mm Hg	Cumulative IOP	Average Post Laser IOP mm Hg	Sectoral % RNFLT Change in EG Eyes*	Sectoral EG vs. C % Axon Loss*	Global % RNFLT Change in EG Eyes	Global EG vs. C % Axon Loss
NHP1	M	3	OD	EG	53	1900	30.9	15	37	−4.7	36.0
			OS	C	15.7						
NHP2	M	3	OD	C	15.3	490	30.1	0	21	1.6	15.0
			OS	EG	37						
NHP3	M	3	OD	C	18.3	374	20.6	16	32	−11.8	35.0
			OS	EG	35						

EG eye percent RNFLT change – percent of EG at the last time point before euthanasia/EG at baseline.

% Axon loss EG-C/C.

NHP, nonhuman primate; M, male; y, years of age; OD, right eye; OS, left eye; EG, experimental glaucoma; mm Hg, millimeters of mercury; C, healthy control group; RNFLT, retinal nerve fiber layer thickness.

* Average from 30 degrees of the FoBMO sectors 5, 6, and 7.

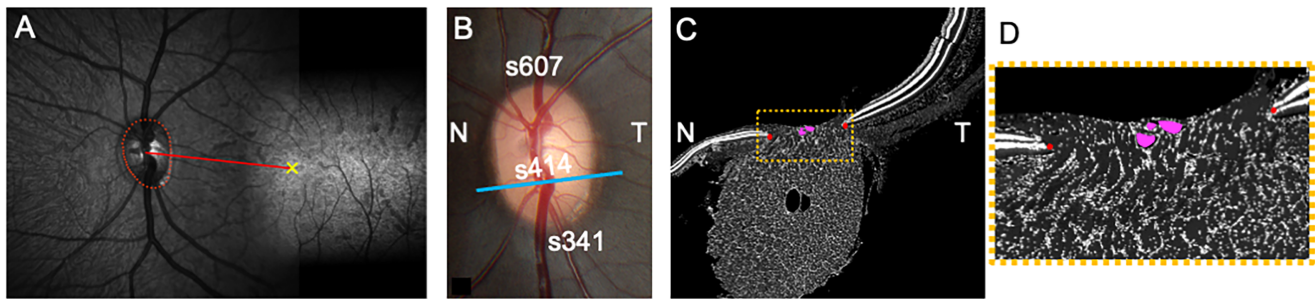


FIGURE 1. Estimating the clinical location of temporal and nasal “cardinal” ONH paraffin sections relative to the OCT foveal-BMO (FoBMO) axis. (A) The OCT-determined foveal to BMO centroid (FoBMO) axis as projected onto the infrared (IR) image of a combined ONH and macular scans which were acquired at the time of OCT image acquisition during the pre-euthanasia imaging session. OS eye is shown here. (B) The location and orientation of the inferior (341) and superior (607) cardinal tissue sections relative to the OCT-determined FoBMO axis is achieved by colocalizing the color fundus image containing their locations to the OCT IR image using the retinal vessels. The nasal/temporal position and S versus I orientation of each individual IHC section is then approximated relative to the cardinal sections by using the section number and fine-tuned using the vessel crossings and BMO points. The *blue line* is the location of the section. (C) Section stained with DAPI. (D) The *orange dotted box* is magnified to show the blood vessels. *Red* indicates the BMO points, and *magenta* marks the blood vessels.

ment and Accreditation of Laboratory Animal Care International (AAALAC) accreditation (Unit #000992). NHP rhesus macaques (*Macaca mulatta*) were housed in a temperature and humidity-controlled room with a 12-hour light/12-hour dark cycle and provided with food and water ad libitum. Animal demographics are shown in the [Table](#).

Unilateral Experimental Glaucoma Model

Our NHP EG model has previously been described in detail.^{19,23,24} Similar to our published reports, early EG was defined to be the onset of ONH surface change as revealed by longitudinal confocal scanning laser tomography (Heidelberg retinal tomography [HRT]; Heidelberg Engineering, Heidelberg, Germany) acquired during standard imaging sessions performed at Baseline (3–5 individual sessions prior to laser) and every 1 to 2 weeks post-laser (i.e. following the onset of unilateral laser to the trabecular meshwork to induce IOP elevation, leading to EG).³¹ Our definition of HRT-detected ONH surface change required confirmation from two subsequent imaging sessions. Longitudinal OCT measurements of retinal nerve fiber layer (RNFLT) thickness (standard 3.5 mm circle scan) were also performed but not used in defining EG onset. Contralateral eyes were used as controls. Eyes from early EG were used in this study ($n = 3$ EG and $n = 3$ control; see the [Table](#)) as determined by the global RNFLT. The 3 EG eyes used for spatial transcriptomics had 15%, 0%, and 16% RNFLT loss near the section as determined by the RNFLT 30-degree sector plots.

Euthanasia and Postmortem Tissue Processing

All animals were euthanized by 4% paraformaldehyde perfusion fixation delivered via a catheter into the left ventricle of the heart while under deep isoflurane anesthesia. Eyes were enucleated after perfusion fixation. For each eye, the orbital tissues were removed and the orbital ON was cut 3 mm behind the globe. Eyes were further immersed in 4% paraformaldehyde for 24 hours. The ONH tissues were trephined (10 mm) using a circular blade similar to our previous publication.²³ The trephined ONH tissue was then embedded in paraffin and serial 5 μ m sections

were cut through the ONH and macula. Two sections were captured and mounted on Superfrost plus glass slide (EMS, Cat #71869-10) and stored at room temperature. The location of the section was determined as described below.²³

Anatomically Consistent EG and Control Eyes ONH Regional and Clinical Location Sampling for Spatial Transcriptomics and IHC

As previously described,²³ we chose anatomically consistent sections from EG and control eyes. The trephine was sectioned from inferior to superior, and the approximate location of the section was determined by locating the transition to open BMO. The location of the section was fine-tuned by section number, BMO points, and blood vessels in the ONH color photograph and OCT determined foveal Bruch’s membrane opening (FoBMO) axis ([Fig. 1](#)).

IHC in EG and Control ONH

For IHC target (CNPase), four adjacent paraffin sections were chosen from anatomically consistent ONH clinical regions from both the EG and control eyes of each animal ([Fig. 2](#)). The four adjacent or near-adjacent paraffin sections from both eyes of 3 NHPs ($n = 24$ sections total) were processed together through the following steps during a 2-day processing period: dewaxed in histoclear, dehydrated in ethanol solutions (100%, 95%, 70%, and 50%); rehydrated with water, then washed in phosphate buffered saline (PBS) for 10 minutes.

The sections were then incubated with 0.1% Triton X-100 and blocked with bovine serum albumin and serum (same species as the secondary antibody). The sections were incubated with CNPase (myelin, 1:50; Cell Signaling Technology catalog 5664, rabbit) primary antibody overnight at 4°C in a humidified chamber. The sections were washed with PBS 3 times, 5 minutes each, and incubated with goat anti rabbit Alexa Fluor 568 (1:200, Thermo Fisher, A11011) in PBS for 2 hours at room temperature. DAPI was used to stain nuclei. The sections were washed and mounted in Prolong Gold (P36934; ThermoFisher).

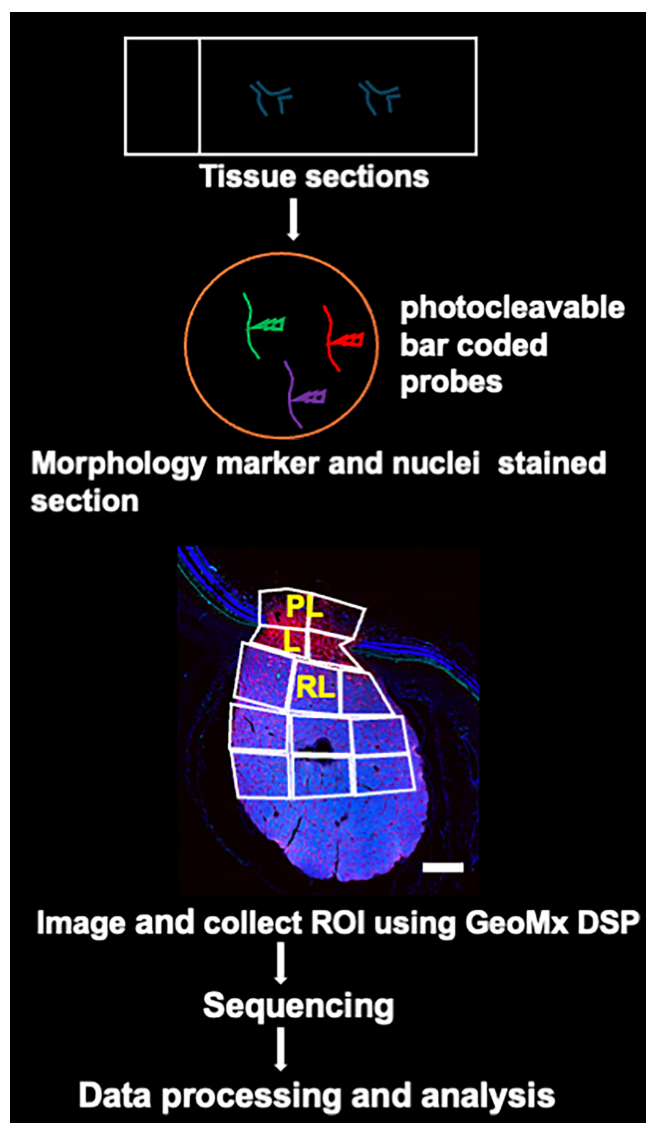


FIGURE 2. Transcriptomics work-flow schematic. Paraffin sections mounted on slides were hybridized with GeoMx human WTA probe mix. After hybridization, fluorescent conjugated Iba1 and GFAP were used as morphological markers and incubated along with SYTO-13 for nuclei staining. ROIs prelaminar (PL), laminar (L), and retrolaminar (RL) were defined and collected in 96-well plates for sequencing. Generated sequencing data were filtered and normalized in DSP and used for data analysis. Scale bar = 500 microns.

Image Acquisition

Zeiss Axioscan 7 slide scanner, a fully automated imaging system, was used for imaging (Zen 3.7 Zeiss software). Fluorescence imaging was done at 40 $\times$ using 0.95 NA Plan-Apochromat. Fluorescent images were acquired by an Orca Flash 4.0 C13440 camera (Hamamatsu). For fluorescent imaging, emission light was generated using the Colibri 7 LED module with excitation wavelengths of 385/30, 469/38, 555/30, and 531/33. The following emission filters were used: for DAPI, bandpass 450/40; for Alexa 488, bandpass filter 525/50; for Alexa 555, bandpass filter 605/70; and for Alexa 647, bandpass filter 690/50. Zeiss Zen blue software version 3.4 was used to export .czi images to .png. The images were analyzed using custom software (ATL 3D Suite; Fortune et al.³²), using the ONH landmarks as described

before.²⁵ Anatomically consistent laminar and retrolaminar regions were made and comparisons for EG versus control eyes were done. CNPase intensity was measured and analyzed in the retrolaminar region as CNPase was not detected in other regions.

EG Versus Control Eye Spatial Transcriptomics Using the DSP GeoMx Whole Transcriptome Atlas Assay

Sections adjacent to those used for CNPase staining were used for spatial transcriptomics (see Fig. 2). Based on the diagram, 13 different regions of interest (ROIs) were analyzed per ON: 2 prelaminar, 2 lamina, and 9 retrolaminar regions. The quality of RNA was determined by isolating RNA from sections on slides by RNA Integrity number (RIN) and DV200 that evaluates the percentage of fragments of >200 nucleotides. RIN value was 2.4 and DV200 30% to 50%. Sections (5 μ m) were baked at 60 $^{\circ}$ C overnight. Tissue samples were deparaffinized in xylene (5 minutes, 2 washes) and rehydrated using a graded series of ethanol washes and finally in PBS. Antigen retrieval using high pH9.0 Tris EDTA was immediately performed at 100 $^{\circ}$ C steamer for 15 minutes. The sections were treated with proteinase K (1 μ g/mL) at 37 $^{\circ}$ C for 15 minutes. The sections were fixed with 10% neutral buffered formalin (NBF) for 5 minutes and washed twice with NBF stop buffer. Tissue samples were then hybridized with GeoMx Human WTA probe mix (#121401102; NanoString Technologies, Inc., cross reactive and high similarity with NHP genes³³) at 37 $^{\circ}$ C for 16 to 18 hours. Samples were washed twice with 50% formamide with 2 $\times$ SSC buffer for 25 minutes to remove unspecific probes. After stringent washes, samples were incubated with anti-Iba1 (E4O4W, XP Rabbit mAb, Alexa Fluor 594 Conjugate, #48934, dilution 1:200), anti-GFAP (Novus, NBP1-05197AF647, dilution 1:250), and Syto13 for nuclei staining for 1 hour at room temperature. ROIs were drawn with size around 500 $\times$ 500 microns (to contain approximately 400 nuclei). ROIs with less than 100 nuclei were excluded to ensure data quality. The sections were scanned on the digital spatial profiler (DSP), and prelaminar, laminar, and retrolaminar regions were identified using staining and morphology in sagittal sections (Supplementary Fig. S1). Each ROI was illuminated with UV light to cleave the barcoded oligonucleotides from each target probe, which were collected in a 96-well plate to generate the sequencing library.

Following collection on the GeoMx DSP instrument, the 96-well collection plate was submitted to the Molecular Biology Core Facilities at Dana-Farber Cancer Institute. PCR amplification was performed according to the GeoMX NGS Readout library prep User Manual (MAN-10117-05). Uniquely dual-indexed libraries were pooled and quantified by Qubit fluorometer, Agilent TapeStation 2200, and RT-qPCR using the Kapa Biosystems library quantification kit, according to the manufacturer's protocols. Sequencing was done on the NovaSeq, Illumina instrument using an S2 flow-cell with paired-end 27 bp reads. Demultiplexing and fastq file generation were performed using Illumina bcl2fastq version 2.20 software. Fastq files were processed further using GeoMx NGS pipeline version 2.0.0.16 software to generate DCC files. Negative control probes, designed using External RNA Controls Consortium (ERCC) sequences, are included in each GeoMx WTA assay to estimate background noise and the limit of quantification (LoQ). For this work,

the LoQ was defined as the negative probe geomean plus the geometric standard deviations of the negative probes. After filtering out genes based on LoQ, the data were normalized in Q3 and batch effect between runs was removed using ComBat-Seq function in Bioconductor's SVA package.³⁴ Normalization effectively minimized technical variation (Supplementary Fig. S2). All ROIs were compared with their contralateral control eye using Aseesa Stars platform.

Statistical Analyses

Statistical analyses were performed using the R software, version 4.0.3 (2020-10-10; The R Foundation for Statistical Computing, Vienna, Austria, downloadable from <http://www.R-project.org/>). For CNPase IHC, the average signal intensity for each tissue region or sub-region was compared between EG and control eyes using linear mixed effects models. Spatial transcriptomics data were analyzed using Aseesa Stars software (version 2.0.0; <https://www.aseesa.com/>).^{35,36} Batch analysis was used for volcano plots, Venn diagrams, graphs, pie charts, and to identify the most changed genes. Aseesa Stars automatically selects the most appropriate significance test for each symbol, rather than using a single test on the entire dataset, thus ensuring a tailored analysis. For every pair of "EG" and "control" gene expression, normality of each distribution was evaluated using the Shapiro-Wilk test ($\alpha = 0.05$). If the gene expression data for both groups pass normality, a pooled-variance Student's *t*-test was applied. If they did not pass normality, then Welch's *t*-test (for unequal variances) or, in the non-normal case, the nonparametric Mann-Whitney *U* test was applied. Genes were considered "most changed" based on an average rank computed across three sorting metrics applied to all groups: ascending *P* values, descending relative change, and descending absolute change. Bonferroni correction, a conservative test, was applied to all pairwise hypothesis tests for each gene. For each gene, we perform every possible comparison between groups, then multiply each raw *P* value by the total number of comparisons. This adjustment ensures that the probability of committing at least one type I error across all tests remains below the nominal significance level. The Benjamini Hochberg procedure was used for controlling false discovery rate (FDR) in multiple hypothesis testing. It is most effective when the tests are independent or exhibit positive dependency (Supplementary Table S1, see www.aseesa.com/documentation for details).

Heat maps were created to show several symbols in a single chart instead of multiple bar charts. Value labels show the group average abundance (based on sequence reads) or average/median $\log_2$ fold change versus the respective control group. Significantly changed values by Welch's *t*-test are enclosed in a box and denoted with *, **, or *** denoting $P < 0.05$, 0.01, and 0.001, respectively. Color intensity indicates the extent of change, with red for increases and blue for decreases. Volcano plots showing changes in gene expression (prelaminar, laminar, and retrolaminar region) were plotted. Volcano plot displaying $\log_2$ fold change in genes versus significance $-\log_{10}(P \text{ value})$ were plotted. Green lines mark *P* value significance thresholds. The Ontology extension detects changes in gene ontology-based functions. The pathways were analyzed using the Ontology extension in Aseesa Stars that enables the identification of functional patterns and pathway-level insights using Gene Ontology (GO) annotations and Reactome pathway data. Reactome

API services are utilized to retrieve pathway-level information, including associated pathways, sub-pathways, related reactions, and symbol-level summaries.^{37,38}

RESULTS

Retrolaminar Region Changes in Protein (IHC) and Gene (Spatial Transcriptomics) Expression

Myelin disruption occurs in early EG, and the retrolaminar and laminar region play a role in initiating events. We interrogate localized changes with IHC and spatial transcriptomics in this paper. First, we examined CNPase intensity in the retrolaminar region up to 750 μm behind the lamina. CNPase intensity decreased in EG versus control in IHC (EG 539.3 ± 216.7 vs. control 1259.0 ± 491.4 [mean $\pm$ SD; $P < 0.001$; Fig. 3A). Consistent with the decrease in CNPase immunolabeling and our previous finding,²³ myelin genes including *CNP* and *MBP* were also significantly decreased in the retrolaminar region of the ONH (using all 9 retrolaminar regions shown in Figs. 2, 3B, 3C). All three NHP showed a decrease in *CNP* and *MBP* when we looked at individual NHP data. Overall, 1000 genes of 6519 were significantly altered in the retrolaminar region. Top 10 genes changed in the retrolaminar is shown in Figure 4A. The top 10 genes changed in expression are *PRCC*, *CTSB*, *HMGCS1*, *C4B*, and *APOE* among others. Heatmaps of change in abundance for known myelin, astrocytes, and immune function genes in the retrolaminar region (Fig. 5, full list of genes provided in Supplementary File S1 and additional information for pie charts Supplementary File S2).

The top 100 changed genes were analyzed for GO categorizing genes based on function; pathway analysis was used to reveal how genes interact within specific biological pathways. The top five ontology analyses provided a broad functional overview that the genes are involved in signal transduction, proteolysis, cholesterol metabolic process, lipid metabolic process, and regulation of DNA-templated transcription. The top five pathways showed the interconnected mechanisms neutrophil degranulation (odds ratio [OR] = 10.5), platelet degranulation (OR = 5.6), neddylation, cholesterol biosynthesis, and activation of gene expression by *SREBF* (*SREBP*), all with OR = 4.5 (Fig. 6; top, Supplementary File S3).

Laminar Region Alterations in Gene Expression

Two of the top 10 genes are collagen-related (*COL1A2* and *COL3A1*). The other top changes included *PI4KA*, *TGF β 1*, *ANXA11*, *SFRP2*, *IGFBP2*, and *FAU* (Fig. 4B). The top 100 genes were used to look at ontology and pathways involved. The top five ontology-based functions were signal transduction, positive regulation of transcription by RNA polymerase II, DNA damage response, apoptotic process, and cell adhesion. The top five pathways involved were ECM proteoglycans (OR = 5), platelet degranulation (OR = 4.3), syndecan interactions (OR = 2.8), assembly of collagen fibrils (OR = 2.8) and SRP-dependent cotranslational protein targeting to membrane (OR = 2.8; see Fig. 6 middle).

Prelaminar Region Alterations in Gene Expression

MRTFB, *CACNA1S*, *GASK1B*, *SYNPO*, *TGIF2*, *CNTNAP3*, and *PREX2* were among the top 10 changes in the prelaminar region (Fig. 4C). The top 100 genes were used to look at

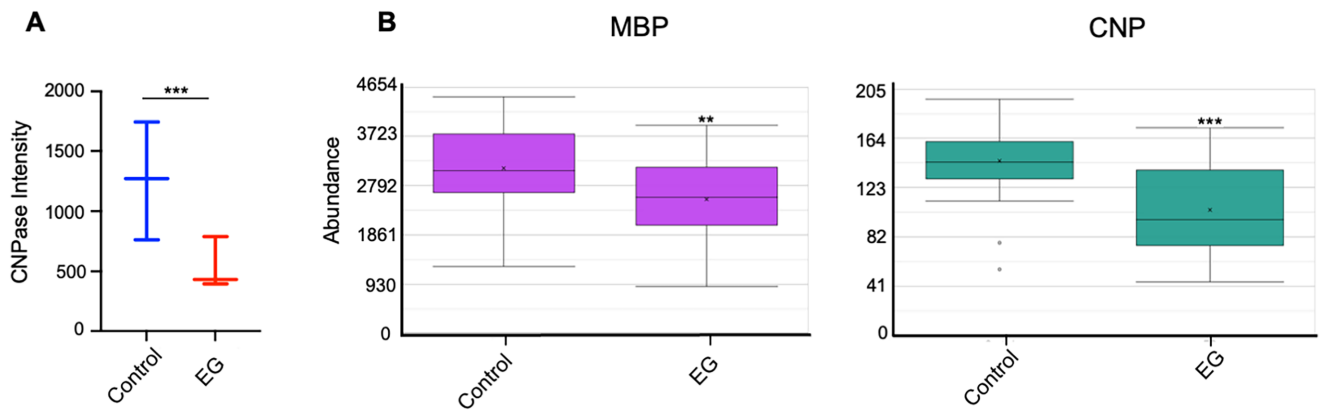


FIGURE 3. CNPase protein and myelin related RNA expression reduction in retrolaminar region. **(A)** Quantitation of IHC for CNPase intensity in the retrolaminar optic nerve ($n = 3$ NHP EG and control). All EG data are statistically significant from the controls using the mixed effects model. Data are shown from minimum to maximum. Pooled results from the region up to 750 μm behind the lamina are shown. **(B)** Box and Whisker plots show relative abundance for *MBP* and *CNP*. *MBP* and *CNP* are both statistically reduced in the retrolaminar region. The *gray dots* are the outliers. The *boxes* represent the interquartile range (IQR) with medians, whiskers extend to $1.5 \times \text{IQR}$, and outliers appear as separate points. *** $P < 0.001$, ** $P < 0.01$.

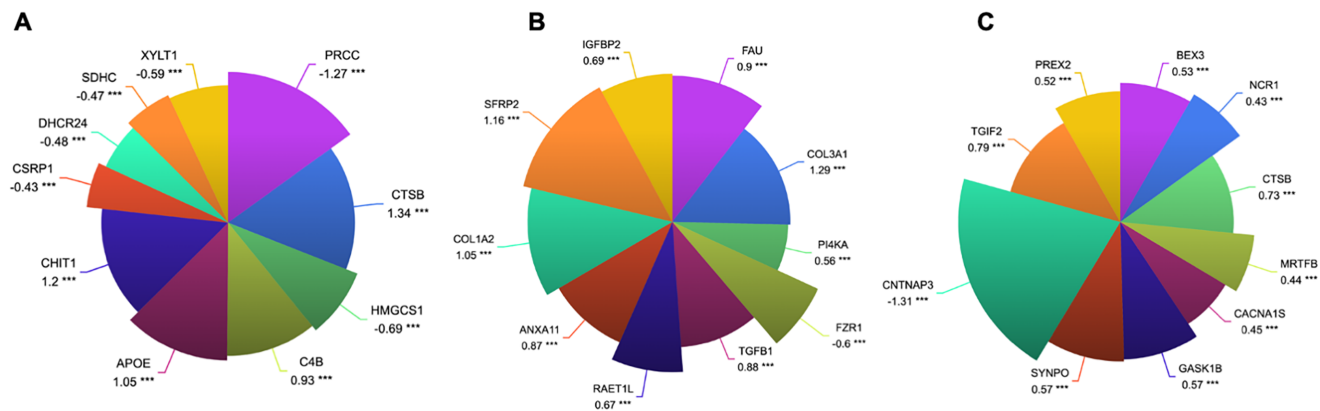


FIGURE 4. Pie chart showing regional alterations for the top 10 gene changes. **(A)** The retrolaminar, **(B)** laminar, and **(C)** prelaminar regions. The arc length of each slice, and consequently its area and central angle, is proportional to the magnitude of log₂ fold change in EG versus controls. *** $P < 0.001$. Each color area shows the amount of change.

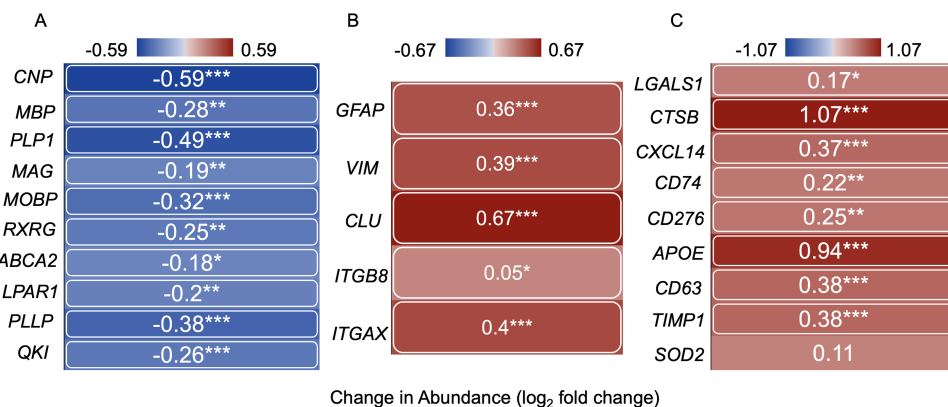


FIGURE 5. Expression alterations at the retrolaminar nerve. **(A)** Myelin and oligodendrocyte related genes are downregulated (*blue*). **(B)** Astrocytic and integrin genes are upregulated (*red*). **(C)** Inflammatory genes are upregulated (*red*). Log₂ fold change is shown. Asterisk indicates significant changes. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

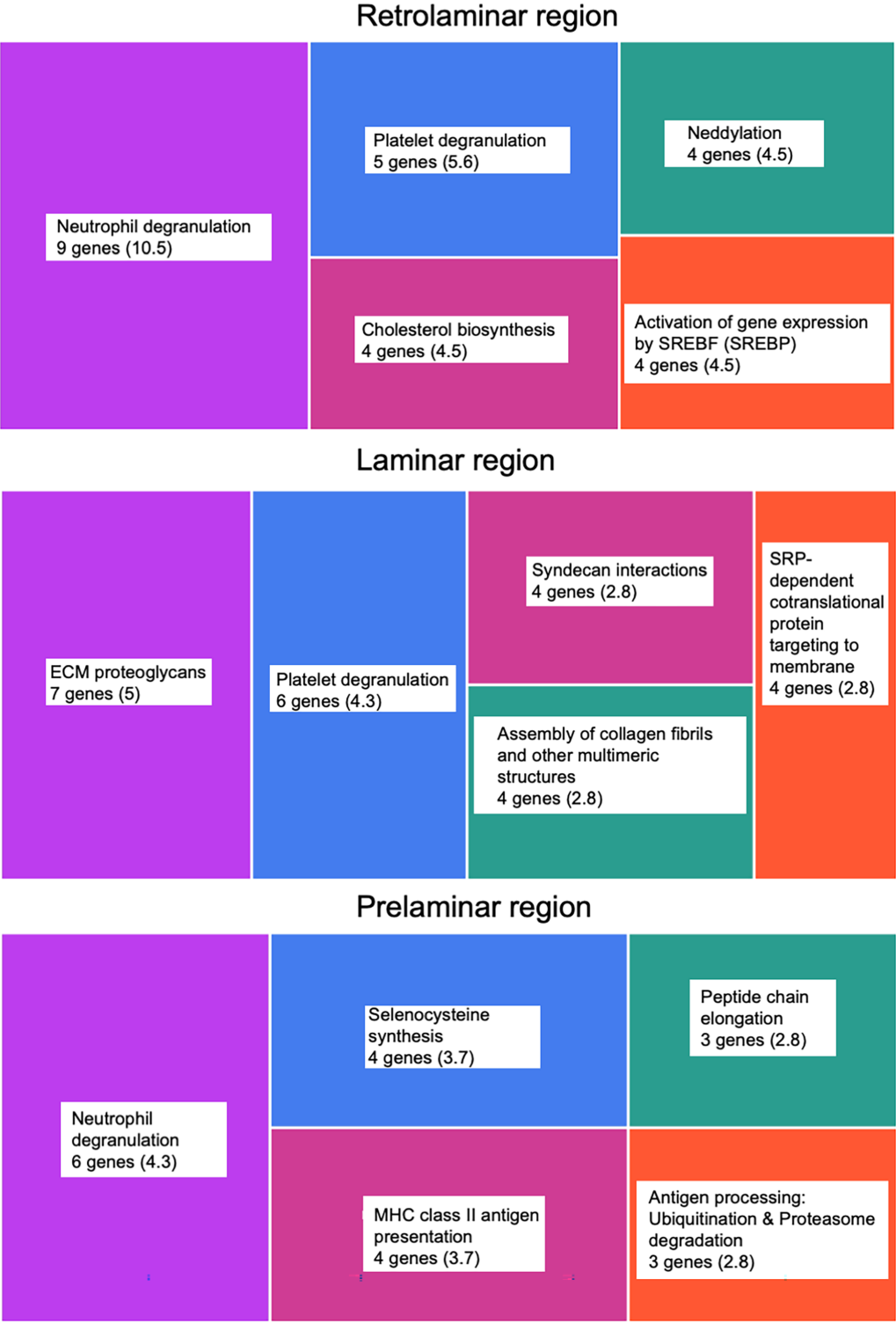


FIGURE 6. Tree Map is provided for ontology results which enables visualization of the pathway and gene ontology results. The odds ratio (OR) is a measure of the strength of association between a specific biological pathway in EG eyes (the size of the *rectangular box* indicates strength). It quantifies whether genes within that pathway are more likely to be found in the “list of interest” (e.g. significant genes) compared to the “background list” (all other genes). OR > 1 shows a positive association. The genes in the pathway are more likely to be enriched in the EG eyes. A higher OR value indicates a stronger association. The top five pathways were picked from a list of genes that were not overlapping as the same genes belong to multiple pathways.

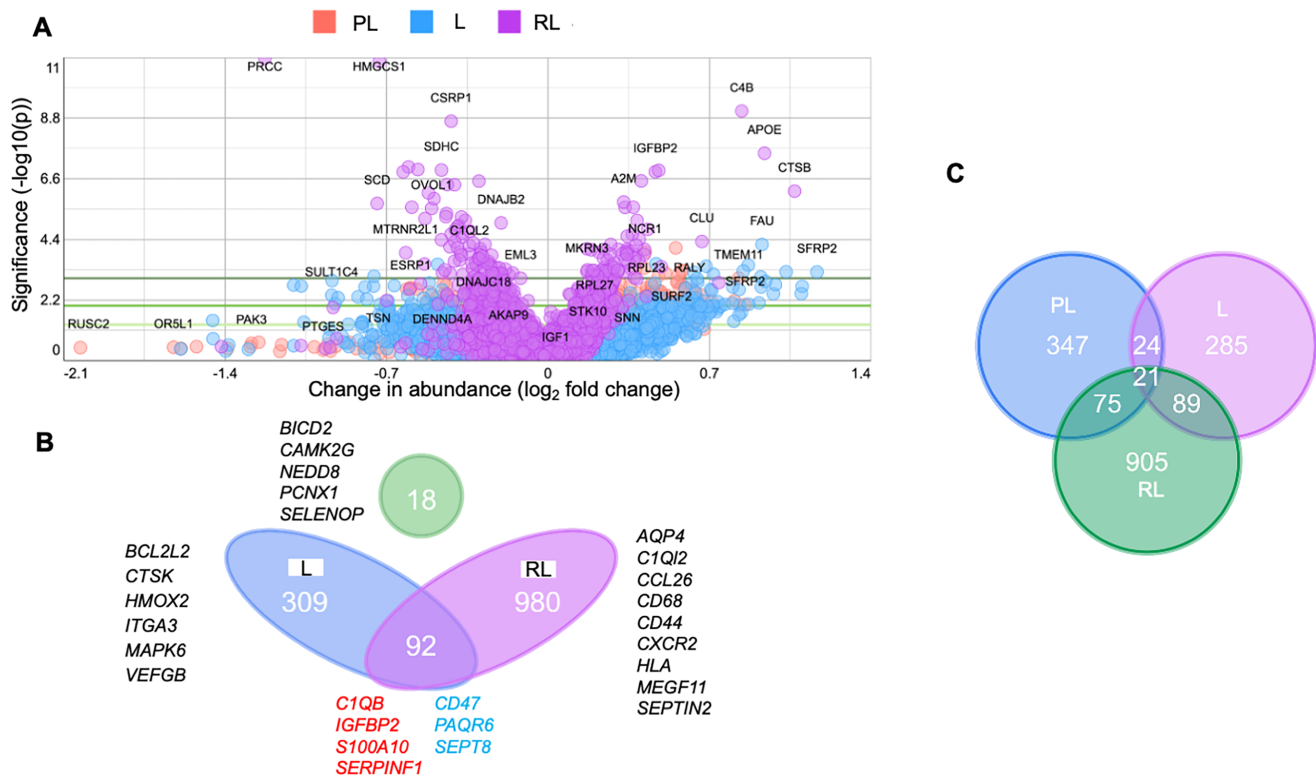


FIGURE 7. Volcano plots and Venn diagrams contrasting significantly changed genes for prelamellar (PL), lamellar (L), and retrolamellar (RL) regions. (A) EG-PL versus C-PL (orange), EG-L versus C-L (blue), and EG-RL versus C-RL (magenta) regions are shown. The green lines show significance $P < 0.001$, $P < 0.01$, and $P < 0.05$, respectively. (B) Venn diagram showing the number of exclusive significantly changed symbols in the same direction (left/right), and the number of shared significantly changed symbols in the same direction (middle) and in opposite directions (top). There were 110 shared genes, out of which 92 genes changed in the same direction and 18 genes that changed in opposite directions in the retrolamellar and lamellar regions. Examples of differentially expressed genes are shown above and below the Venn diagram (red = increased and blue = decreased). (C) Multi Venn diagram shows the number of significant genes between retrolamellar (RL), lamellar (L), and prelamellar (PL) regions. Twenty-one genes are common among all three regions. These include *A2M*, *ACAD9*, *ANXA2*, *CLU*, *COL6A2*, *CPEB3*, *CTSB*, *GPX3*, *IGFBP2*, *IGFBP7*, *ITGAX*, *MARCKS*, *NPD1*, *NXF2*, *PRCC*, *PTGES3*, *RNASEK*, *S100A6*, *SFRP2*, *SPANX1*, and *VIM*. The common shaded area shows the number of genes that are shared in the prelamellar and retrolamellar regions, the prelamellar and lamellar regions, and the lamellar and retrolamellar regions.

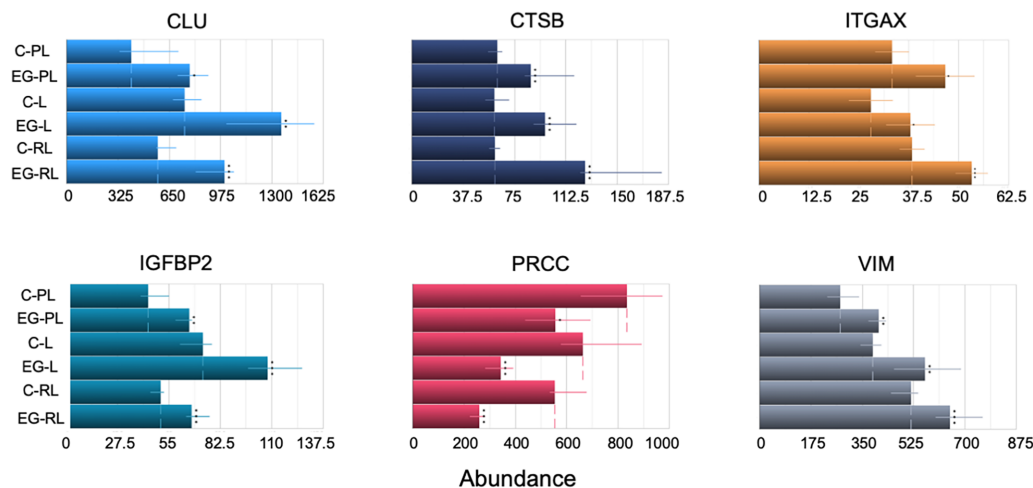


FIGURE 8. *CLU*, *CTSB*, *ITGAX*, *IGFBP2*, *PRCC* and *VIM* transcripts are altered in prelamellar (PL), lamellar (L), and retrolamellar (RL) regions. Graph showing abundance versus regional changes between control and EG eyes. Error bars 95% confidence interval are shown. Significance is shown as *** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$ versus controls.

ontology and pathways involved. The top Five ontology-based functions were signal transduction, cell differentiation, cell adhesion, apoptotic process, and lipid metabolic

process. The top five pathways involved were neutrophil degranulation (OR = 4.3), selenocysteine synthesis (OR = 3.7), MHC class II antigen presentation (OR = 3.7), peptide

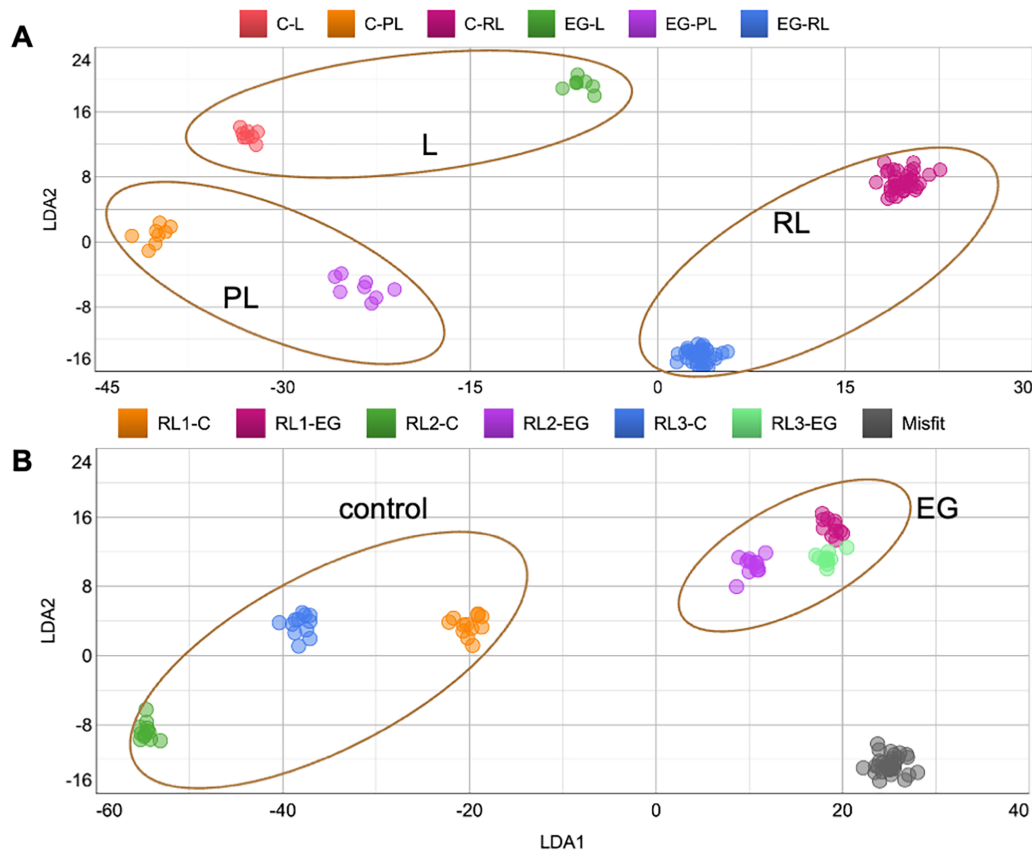


FIGURE 9. LDA reveals regional and disease-specific transcriptional signatures in the ONH. (A) LDA was used to visualize group separation based on gene expression profiles across distinct optic nerve head (ONH) regions using the top 100 differentially expressed genes identified in the retrolaminar (RL) region. LDA was applied to control and EG eyes across laminar (L), prelaminar (PL), and retrolaminar (RL) regions. Each point represents an individual sample projected onto the first two linear discriminants (LDA1 and LDA2). Control samples are shown for laminar (C-L, red), prelaminar (C-PL, orange), and retrolaminar (C-RL, magenta) regions, whereas corresponding EG samples are shown for laminar (EG-L, green), prelaminar (EG-PL, purple), and retrolaminar (EG-RL, blue). The analysis demonstrates robust separation between control and EG eyes, as well as among ONH subregions, highlighting both disease-specific and region-specific transcriptional alterations in early EG. (B) LDA was performed using the top 100 differentially expressed genes identified in the retrolaminar (RL) region to assess transcriptional separation across subregions and conditions. Three ROIs in the top layer (RL1), middle (RL2), and bottom (RL3) layers were compared (see Fig. 2). Control samples are shown for RL1 (orange), RL2 (green), and RL3 (blue), whereas EG samples are shown for RL1 (magenta), RL2 (purple), and RL3 (light green). Misfit samples (gray) indicate cases not clustering with their assigned group, reflecting transcriptional variability or classification uncertainty. The clear separation between control and EG samples, as well as among RL subregions, shows regional heterogeneity in early EG.

chain elongation, and antigen processing (OR = 2.8; see Fig. 6 bottom).

A volcano plot of prelaminar, laminar, and retrolaminar regional comparisons between EG versus control is shown in Figure 7A. A Venn diagram (Fig. 7B) shows differential gene expression between the laminar and retrolaminar regions. *C1QB*, *IGFBP2*, and *S100A10* increased in the laminar and retrolaminar regions. *CD47*, *PAQR6*, and *SEPT8* decreased in the laminar and retrolaminar regions. Some genes, such as *CLU*, *CTSB*, *ITGAX*, *IGFBP2*, *PRCC*, and *VIM*, changed in all prelaminar, laminar, and retrolaminar regions of the ONH, although the majority were differentially regulated in the three regions (Figs. 7C, 8). Further the top 100 significant gene set in the retrolaminar region was chosen to enrich for biologically relevant signals and minimize noise from nonsignificant genes, thereby highlighting disease-associated clustering patterns using Linear Discriminant Analysis (LDA). The separation of prelaminar, laminar, and retrolaminar regions highlights early region-specific transcriptional differences between ONH subre-

gions (Fig. 9A). Figure 9B demonstrates clustering by both disease status and retrolaminar subregion, suggesting that different regions of the ONH undergo different biological changes in EG.

DISCUSSION

Spatial transcriptomic analysis of early EG ONHs revealed a coordinated downregulation of multiple myelin-related genes and an upregulation of inflammatory mediators in the retrolaminar region. These findings are consistent with our previous studies where we have demonstrated loss of myelin in structurally intact axons using electron microscopy and shown reduced MBP and CNP intensities by IHC.^{23,24} In EG, reduced fluorescence may be due to loss or disorganization of CNPase-expressing structures, such as oligodendrocytes or myelinated axons, rather than genuine downregulation within surviving cells.

Among the significantly altered genes, *HMGCS1*, a key enzyme in biosynthesis of cholesterol was dysregulated

in the retrolaminar region. Pathway analysis of the top 100 differentially expressed genes highlighted perturbations in the cholesterol biosynthesis pathway. Thus, our data suggest that cholesterol pathways seem to play an important role in the retrolaminar pathology in early NHP EG. In addition, *APOE* and *C4B* were significantly upregulated in the retrolaminar region indicating changes in lipid transport process and complement activation. Damage-associated molecular patterns (DAMPs) are molecules that are released by damaged cells to alert the immune system.³⁹ Several DAMPS, including *HMGB1*, *S100B*, and *S100A10*, showed altered expression in the retrolaminar region. Other differentially expressed genes including *CD44*, *CD47*, *CD63*, *CD68*, *CD74*, *ITGB8*, *ITGAX*, *VWF*, *SDC3*, *CCL4L1*, *TNFRSF12A*, *TNFRSF18*, *CXCL14*, *C1QB*, *C1QL2*, *CFD*, *VCAN*, *TNC*, and *TIMP1*, show interplay of inflammatory, ECM, and immune pathways in the NHP EG retrolaminar region.

The laminar region of the ONH was delineated to identify region-specific transcriptional changes. Analysis revealed significant alteration of ECM related genes, including *COL3A1*, *COL1A2*, *COL1A1*, *TGFβ1*, *C1QB*, *ITGA3*, *CD47*, *FN1*, *TIMP1*, *ITGAX*, and *VCAN*. Our findings support previous studies that have suggested TGFβ to be an important ECM modulator in the glaucomatous lamina cribrosa.^{40–42} Thus, the enrichment of TGFβ1 associated pathways, including ECM-receptor interactions, proteoglycans signaling, and syndecan mediated interactions, suggest that TGFβ1-driven ECM pathways may represent an early event in initiation of laminar remodeling. Notably, these pathway alterations were specific to the laminar region of the ONH indicating spatial heterogeneity of molecular changes in early EG. The link between TGFβ1 and ECM remodeling is plausible but needs to be investigated further.

Selenocysteine synthesis was one of the top pathways altered in the prelaminar region and in the retrolaminar region when we analyzed pathways using 1000 significant genes. Selenocysteine synthesis is essential for central nervous system (CNS) function. Impairment in this pathway leads to hypomyelination, oligodendrocyte dysfunction, and neurodegeneration, suggesting that this pathway may contribute to early pathogenic process.^{43–46}

RNA technologies have led to the identification of global transcriptional alterations and pathway analysis in glaucoma.⁴⁷ Alterations in genes that are known in glaucoma literature, for example, *C4B*, *GFAP*, *VIM*, *MBP*, and *CNP* lend support for the validity of the spatial transcriptomics study presented here. This study demonstrates that the ONH is a complex tissue in which diverse biological processes change early in the glaucoma, and that these changes may be highly regionalized. Neutrophil degranulation and MHC class II antigen presentation pathways were top pathways altered in the retrolaminar and prelaminar region. These novel pathways need further investigation for developing strategies to modulate inflammation.

Alterations in Other Early Glaucoma Studies

In a comprehensive study, Howell et al. have identified genes and pathways in the ONH in early stages of glaucoma in DBA/2J mice.⁴⁸ Eyes with no detectable glaucoma by conventional morphological assays were grouped into molecular defined stages of glaucoma. The early changes detected by clustering analysis precede morphologically detectable glaucomatous damage. Early-stage expression changes included upregulation of complement cascade,

focal adhesion pathways, apoptosis, ECM receptors interaction, cytokine-cytokine receptors, and endothelin systems. Howell et al. provide a roadmap of early events in glaucoma and find that *Col1a2*, *Col3a1*, *Fn1*, *Cd47*, *Itgb1*, *Vcan*, and *Timp1*, along with *C1q*, *Ccl3*, *Ccl5*, and *Cxcl11* genes were altered in early glaucoma. They also highlight that there are many altered molecular states and asynchronous nature in such a variable disease. In a recent study, Tribble et al. performed RNA-sequencing using microglia mRNA from the ONH in DBA/2J mice before detectable neurodegeneration to identify phagocytosis (TREM2-TYROBP signaling network), inflammatory, and integrin pathways were down-regulated and increased expression in metabolic and mitochondrial functions.²⁶ This study indicates that microglial dysfunction and metabolic stress are earliest detectable molecular events. In addition, Lozano et al.⁴⁹ analyzed ONH RNA after 8 hours of controlled elevation of IOP model in rats. They identified *Angptl4*, *Cebpb*, *Edn1*, *Myd88*, *Nfil3*, *Slc2a1*, and *Srebfl1* genes as significantly regulated genes using a Nanostring panel containing 770 genes. These genes were involved in immune response, response to cytokine, and leukocyte differentiation. Perturbation of cholesterol pathways has been reported in many neurodegenerative diseases,⁵⁰ including retinal tissue in rat experimental glaucoma as early as 18 hours after laser-induced intraocular hypertension.⁵¹ In living human eyes, increased glial reactivity was detected after 30 hours of IOP elevation. Lamina cribrosa exhibited alterations in ECM genes, including *COL1A1*, *COL4A1*, and *COL4A4*, along with alterations in *FN1*, *AQP4*, *VCAN*, and *TGFB2*, among others.³⁰

Myelin and Inflammatory Pathways

Similar to our study, cell proliferation and inflammatory-related genes were upregulated in the ONH in early spontaneous glaucoma in the cat eye.⁵² Early OPC proliferation and late oligodendrocyte loss were also demonstrated in the retrolaminar region in cat spontaneous glaucoma. Contrary to this, Nakazawa et al. have shown early oligodendrocyte loss in a mouse model where laser surgery was used to elevate the IOP.⁵³

The transcriptional profile using bulk RNA-sequencing from isolated microglia identified upregulation of secreted molecules, such as ApoE, Igals3, proinflammatory cytokines (e.g., *Tnf-α* and *Il-1β*), complement (e.g., *C4b*), and potent chemotaxis molecules (e.g., *Ccl2* and *Ccl12*) in the retina.^{54,55}

ECM Remodeling

Concentrations of stress and strain occur within the ONH connective tissues (prelaminar, laminar, retrolaminar, and peri-neural canal scleral) at all levels of IOP, due to the tensile stresses generated by IOP within the sclera and the translaminal pressure difference between IOP and retrolaminar tissue pressure.¹¹ We have previously described profound connective tissue deformation and remodeling within all three ONH regions through the course of NHP EG and have shown that OCT ONH deep neural and connective tissue change preceded or coincided with early OCT detected RNFLT change in most EG eyes.^{20,31} Taken together, our studies strongly suggest that deformation and remodeling of the ONH connective tissues occur early in the optic neuropathy of glaucoma. The outer (posterior) lamina and retrolaminar myelin transition zone is the area where

connective tissue remodeling¹⁴ and demyelination of structurally intact axons²⁴ overlap. However, the mechanisms of connective tissue remodeling, retrolaminar demyelination, and axonal insult remain unknown.

Keuthan et al. 2023 analyzed regional gene expression from the retina, ONH, and ON of mice with optic nerve crush and experimental glaucoma.⁵⁶ Gene expression patterns in the unmyelinated ON showed significant enrichment of the *Wnt*, *Hippo*, *PI3K-Akt*, and *Tgfβ* pathways, as well as ECM-receptor and cell membrane signaling pathways, compared to the myelinated optic nerve in naïve mice. Changes in the ECM are known to play a role in the progression of glaucoma.^{57–61} ECM reorganization is critical for glaucomatous change. *TGFβ* is known to play a role in the anterior chamber of the glaucomatous eye.⁶² Similar findings of increased levels of *TGFβ1* in donor primary open-angle glaucoma lamina cribrosa cells have been reported, though the stage of glaucoma is not known.⁶³ Yang et al. 2007⁶⁴ used translimbal laser photocoagulation to induce IOP elevation in rats and performed microarrays on the retina demonstrating the importance of neuroinflammation and *TNFα* in retinal injury using microarrays.

To our knowledge, this is the first report using a spatial transcriptomics approach in NHP EG to understand local environmental changes and regional heterogeneities. There are a few limitations of the spatial transcriptomics in this study, including a small sample size of three EG and three control young eyes. Findings would likely be more robust if the study included bilateral controls as well as increased numbers of EG and control eyes and validation of differentially expressed genes by IHC and PCR. The study focuses exclusively on early experimental glaucoma using eyes from 3-year-old male NHPs. Although this provides valuable insight into early-stage molecular events, the absence of longitudinal or progressive timepoints limits understanding of how these transcriptional changes evolve with disease severity. Including intermediate and late stages in future work would strengthen mechanistic interpretations and clarify whether observed alterations represent initiating or secondary responses. We could not determine changes in the peri-neural canal sclera due to the limited number of cell nuclei in that region. Another limitation is that the spatial transcriptional changes are likely the consequence of cellular alterations of the ONH, rather than the cause of those alterations. The cellular processes that result in these alterations remain to be determined, and the gene expression changes observed suggest multiple hypothesis worthy of further investigation. We plan to integrate higher-resolution approaches, such as single-cell or multiplexed spatial transcriptomics, for disentangling cell-type-specific contributions to the observed regional patterns.

Conclusions

Preliminary spatial transcriptomics data demonstrates that there are differences in gene profiles at the level of prelaminar, laminar, and retrolaminar regions of the ONH. Mapping pathological changes in the prelaminar, laminar, and retrolaminar regions of the ONH will help in understanding the local environmental changes in glaucoma and open promising avenues for early therapeutic interventions. This study is an expansion of our work toward understanding and identifying the cellular mechanisms of ONH connective tissue remodeling. This work is essential to investigate novel molecular pathways that will identify early inter-

vention points capable of preventing ECM stiffening and irreversible axonal loss. By systematically delineating the sequence of molecular events underlying these pathological changes in EG will aim to define the earliest drivers of ONH remodeling. Understanding these mechanisms will not only illuminate how biomechanical and cellular stress contribute to neurodegeneration but also establish a rational framework for therapeutic targeting of specific pathways. Importantly, this research will lay the groundwork for translational studies testing pharmacological inhibition of the *TGFβ1* signaling pathway, a central regulator of ECM remodeling. Modulating *TGFβ1* activity has the potential to preserve tissue compliance, maintain axonal integrity, and ultimately prevent vision loss in glaucoma.

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Data Availability: Raw and processed data files generated for this study are deposited in the Gene Expression Omnibus (GEO; <https://www.ncbi.nlm.nih.gov/geo>) database under accession number GSE316553.

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