Neurodegeneration Markers Galectin-3 and Apolipoprotein E Are Elevated in the Aqueous Humor of Eyes With Glaucoma

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Purpose: Galectin-3 (Gal-3) and apolipoprotein E (APOE) are markers of activated microglia in neurodegenerative diseases of the central nervous system, whose targeting is protective in mouse models of glaucoma. In this study, we examined levels of Gal-3 and APOE in human aqueous humor (AH) and defined their clinical associations with glaucoma.

Methods: We collected AH from 59 glaucoma patients and 15 controls at the start of planned ophthalmic surgery. Gal-3 and APOE levels were quantified by enzyme-linked immunosorbent assay. Total protein in AH was quantified by bicinchoninic acid assay. Significant associations between Gal-3, APOE, and clinical covariates were defined using univariate and multivariate linear regression models.

Results: Gal-3 and APOE levels were significantly elevated in the AH of glaucoma patients compared to controls (P = 0.004 and P < 0.001, respectively). Gal-3 and APOE were positively correlated across the entire cohort (r = 0.65, P = 6.2E-9). No association was observed between Gal-3 and total protein or APOE and total protein (P = 0.35 and P = 0.50, respectively), indicating that their levels were not increased in glaucomatous AH due to nonspecific protein accumulation. Multivariate linear regression modeling revealed significant associations between Gal-3 and maximum recorded intraocular pressure (P = 0.009) and between APOE and number of past ophthalmic surgeries (P = 0.031).

Conclusions: We demonstrate that Gal-3 and APOE are significantly elevated in the AH of eyes with glaucoma and are associated with a history of poorly controlled disease.

Translational Relevance: Gal-3 and APOE in AH may inform clinical decision-making as quantifiable readouts of microglial activation in eyes with glaucoma.

Introduction

Glaucoma is a chronic neurodegenerative disease and the leading cause of irreversible blindness worldwide, projected to affect 111.8 million people by the year 2040.¹ Glaucoma has been associated with various risk factors and underlying disease mechanisms, including glial reactivity and metabolic dysfunction in the retina and the optic nerve.² Though etiologically complex, these pathologic mechanisms converge on apoptosis of retinal ganglion cells (RGCs) and progressive optic nerve head remodeling.³ Intraocular pressure (IOP) elevation is a common clinical feature of glaucoma and remains to date the only modifiable risk factor for preventing disease progression. However, surgical intervention may prove unsuccessful, and daily eyedrop use can present unpleasant side effects and high cost that promote poor adherence. Furthermore, the disease can progress despite good control of IOP.² Thus, there is a need for improved therapeutics that more directly protect the retina and optic nerve from degeneration.³

Microglia, the resident immune cells of the central nervous system (CNS), have been identified as a critical cell type contributing to RGC loss in glaucoma.⁴ In
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human glaucoma, activated microglia colocalize with proinflammatory cytokines in the optic nerve, including tumor necrosis factor $\alpha$. Furthermore, studies conducted in mice have shown that genetic and pharmacologic suppression of microglial activation protects RGCs both structurally and functionally despite IOP elevation. More recent studies using RNA sequencing have further fine-tuned our understanding of microglial phenotypes in neurodegeneration. In the context of both aging and neurodegeneration, brain microglia transition from a homeostatic phenotype to an activated phenotype characterized by upregulation of proinflammatory cytokines, complement, and secreted factors such as apolipoprotein E (APOE) and galectin-3 (Gal-3). Researchers have named this activated microglial state “MGnD” for microglia in neurodegeneration, or “DAM,” for disease-associated microglia, and they have implicated it in a myriad of CNS degenerations.

Importantly, this microglial phenotype has also recently been described in mouse models of glaucoma and photoreceptor degeneration. APOE (gene nomenclature indicates uppercase font for human alleles [APOE], lower-case font for murine alleles [Apoe], italicization in reference to the gene, and regular font in reference to the protein) is the major apolipoprotein of the CNS, has been identified as critical upstream regulator of microglial activation in neurodegeneration. Recent work has shown that mice with global or microglia-specific deletion of ApoE are protected from RGC loss in glaucoma, demonstrating that this molecule acts cell-autonomously in microglia to drive disease progression. Consistent with this role, APOE has previously been detected in both the aqueous humor (AH) and retinal tissue of human glaucoma samples. Of note, although mice possess only one isoform of ApoE, in humans it is found as three distinct isoforms (APOE2, APOE3, and APOE4). APOE4 is well known as a strong risk factor for Alzheimer’s disease, although it is interestingly associated with a decreased risk of glaucoma. We have recently used humanized mice with ApoE3 and APOE4 alleles to demonstrate that APOE4 acts as a loss-of-function isoform, which preserves RGCs by impairing microglial activation in a manner similar to ApoE deletion. This discovery may explain the protective effect of APOE4 in the human population.

Although APOE is critical for driving RGC loss in glaucoma, it is a ubiquitous apolipoprotein with numerous biological functions in both health and disease and is not an easily druggable target. This obstacle in translation has led to investigation of downstream molecules acting in microglia with greater potential for therapeutic intervention. Gal-3, a secreted, carbohydrate-binding lectin, has been identified as a critical downstream effector of APOE signaling in microglia, whose upregulation is abrogated in both APOE4 and ApoE knockout mice with glaucoma. Interestingly, Gal-3 has been implicated in several other CNS neurodegenerations, such as Alzheimer’s disease, Huntington’s disease, multiple sclerosis, and traumatic optic neuropathy, as well as in systemic inflammatory conditions, including pulmonary fibrosis. Targeting Gal-3 in mouse models of these diseases led to amelioration of anatomic and functional outcomes. Similarly, targeting Gal-3 both genetically and by intravitreal injection of a selective small molecule inhibitor resulted in robust protection of RGCs despite IOP elevation in a mouse model of glaucoma. Therefore Gal-3 inhibition has the potential to become a powerful neuroprotective strategy for the treatment of this blinding disease.

As neuroprotection becomes a more viable therapeutic option for glaucoma, there exists a parallel demand for improved biomarkers that may inform patient care with finer resolution. We hypothesized that Gal-3 and APOE could serve as biomarkers for identifying patients who may benefit from microglia-based neuroprotective therapies, including Gal-3 inhibition. In this pilot study, we demonstrate that Gal-3 and APOE are elevated in the AH of glaucoma patients and correlate with a history of poorly controlled disease. Our findings are the first to detect Gal-3 in glaucomatous AH and suggest that Gal-3 and APOE hold promise as glaucoma biomarkers with potential utility in clinical practice.

**Methods**

This case-control study was approved by the Mass General Brigham Institutional Review Board (2021P000253) and adhered to the tenets of the Declaration of Helsinki. To determine the appropriate sample size to identify a significant difference in AH levels of APOE and Gal-3 between patients with glaucoma and controls, we conducted a power analysis using the `pwr` package in R. Estimating an effect size of $d = 0.70$ (medium-to-large effect) with a planned ratio of cases to controls of $\sim 4:1$, we calculated a sample size of $N = 63$ (51 cases and 13 controls) to achieve 60% power at a two-tailed alpha of 0.05. We recruited 59 cases and 15 controls from Massachusetts Eye and Ear to account for the possible need to exclude approximately 10% of patients. All 74 patients provided written informed consent before AH collection. Case patients included glaucoma patients...
with any glaucoma type undergoing surgery by one specialist (D.S.) as part of their normal treatment plan. Despite some high recorded IOPs, none of the patients displayed clinical signs of retinal ischemia (non-arteritic anterior ischemic optic neuropathy, central retinal vein occlusion, or central retinal artery occlusion). Control patients included 15 patients undergoing routine phacoemulsification with intraocular lens implantation for age-related cataracts by one of three comprehensive ophthalmologists (S.L.W., K.L., and C.S.). Controls underwent comprehensive examination and had no known diagnoses of glaucoma, glaucoma suspect, or ocular hypertension. Controls had normal cup-to-disc ratios, no known history of elevated intraocular pressure, and no known family history of glaucoma. None of the enrolled patients had known diagnosis of neurological disease. Subsequent AH analyses resulted in exclusion of two cases and one control because of poor sample quality, yielding a final cohort of 57 glaucoma samples and 14 control samples.

For each patient enrolled, AH was obtained in the operating room at the start of the planned ophthalmic surgery. Briefly, a blunt cannula on a syringe was inserted into the initial peripheral paracentesis, and 25 to 150 μL of AH was removed. The AH was then carefully transferred to a cryovial, snap-frozen on dry ice, and stored at −80°C until further analysis. For both cases and controls, AH was collected only from the surgically treated eye, accounting for one sample per patient. After AH collection, the planned ophthalmic surgery was performed.

Galectin-3 and APOE levels in AH were quantified using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Galectin-3 ELISA; BG Medicine, Waltham, MA, USA; APOE ELISA, Thermo Fisher Scientific, Waltham, MA, USA). These assays provide high sensitivity and excellent specificity for target proteins in biological samples. Samples were thawed, spun in a centrifuge at 14,000 RPM, diluted, and processed according to assay instructions. Optical density data were plotted against concentration (SoftMax, 4PI) to interpolate Gal-3 and APOE levels from the resulting standard curve. The acceptance criterion was set at a percent coefficient of variation greater than 20%. Samples with higher than acceptable percent coefficient of variation were omitted from the study, and samples with Gal-3 or APOE levels below the assay’s lower limit of detection were approximated as undetectable/zero. These analyses resulted in exclusion of one case and one control because of poor test reliability.

To determine whether elevated Gal-3 and APOE levels in AH were due to nonspecific protein accumulation, the relative amount of total protein per sample was determined using bicinchoninic acid (BCA) assay (BCA Assay, Thermo Fisher Scientific). These assays rely on reduction of a copper-containing substrate by peptide bonds and report a colorimetric readout proportional to the amount of protein in solution. To validate results of the BCA assay, A280 Nanodrop analysis for ultraviolet absorption of aromatic amino acids and C-C bonds was performed on a subset of samples. These analyses resulted in exclusion of one additional glaucoma case as an extreme outlier. Additionally, although Gal-3 and total protein data were obtained for all 57 patients included in the study, APOE was missing from five cases and three controls because of insufficient AH volume.

Demographic and clinical information were obtained by retrospective chart review by investigators masked to AH Gal-3 and APOE levels (K.M.P., C.N.). For both cases and controls, covariates included age, sex, self-reported race, laterality, best corrected visual acuity (BCVA), maximum recorded IOP and preoperative IOP. BCVA was converted to logarithm of the minimum angle of resolution (logMAR) using the conversion logMAR = (−log10[decimal BCVA]). IOP was measured as part of routine care using Goldmann applanation tonometry. Additional information obtained for glaucoma patients included surgery type, glaucoma type, glaucoma stage, number and type of preoperative glaucoma medications, prior ophthalmic surgeries, prior laser history, Humphrey visual field (HVF) mean and pattern standard deviation (obtained with 24-2 Swedish Interactive Threshold Algorithm – Standard test pattern), and retinal nerve fiber layer (RNFL) thickness by Cirrus optical coherence tomography. Glaucoma stage was determined by D.S. using optical coherence tomography and HVF as previously described by Fellman et al. Mean deviation and pattern standard deviation values were missing from three patients (5.3%) and RNFL data from two patients (3.5%) in the glaucoma cohort.

Descriptive statistics and frequencies were calculated for all demographic and clinical variables of interest across the entire patient sample, and between cohorts using χ2 tests of independence and Kruskal-Wallis tests. Pearson’s correlation coefficients were calculated between Gal-3 and APOE, Gal-3 and total protein, and APOE and total protein to determine their mutual associations. A series of univariate t-tests and analyses of variance were then conducted on each covariate within the glaucoma case cohort to determine if significant associations existed with either Gal-3 or APOE levels. Tukey’s test for multiple comparisons was used to identify pairwise differences between those categorical characteristics which had a significant analysis of variance result. Addition-
ally, a series of Pearson’s correlation coefficients were calculated between each continuous covariate of interest and Gal-3 and APOE. These univariate results were used to inform two series of multivariate linear regression models, which modeled average Gal-3 and APOE against demographic and clinical characteristics within the glaucoma case cohort. Normal distribution was assessed using the Shapiro-Wilk test and a log-transformation was performed on both Gal-3 and APOE values to normalize their residuals given their right-skewed distribution, which has been a recommended adjustment in numerous studies.\(^{38,39}\) Fully saturated models were fit first for both Gal-3 and APOE. Final multivariate linear regression models were derived from the fully saturated models via automated stepwise selection utilizing Akaike’s Information Criterion (AIC). AIC is used as a quality metric to evaluate how well a model fits the data, relative to other models, using a weighted log-likelihood formula.\(^{40}\) All analyses were conducted in R statistical programming software (version 3.5.1). We considered \(P < 0.05\) to be statistically significant.

## Results

Table 1 shows demographic and clinical characteristics of the 57 glaucoma patients and 14 control patients included in the study. No significant differences were observed between cases and controls in terms of age, sex, self-reported race, laterality or preoperative IOP. Significant differences were observed in maximum IOP and preoperative logMAR BCVA (\(P < 0.001\) and \(P = 0.008\), respectively). All controls underwent

| Variable               | Control   | Glaucoma  | \(P\) Value\(^{a,b}\) |
|------------------------|-----------|-----------|-----------------------|
| Number of patients     | 14        | 57        | 0.27                  |
| Age                    | 72.6 (8.2)| 68.9 (11.4)| >0.99                |
| Sex                    |           |           |                       |
| Female                 | 9 (64.3%) | 35 (61.4%)|                       |
| Male                   | 5 (35.7%) | 22 (38.6%)|                       |
| Self-Reported Race     |           |           | 0.12                  |
| White                  | 11 (78.6%)| 33 (57.9%)|                       |
| Black                  | 1 (7.1%)  | 13 (22.8%)|                       |
| Hispanic               | 1 (7.1%)  | 10 (17.5%)|                       |
| Asian                  | 1 (7.1%)  | 0 (0%)    |                       |
| Native American        | 0 (0%)    | 1 (1.8%)  |                       |
| Laterality             |           |           | 0.55                  |
| OD                     | 8 (57.1%) | 25 (43.9%)|                       |
| OS                     | 6 (42.9%) | 32 (56.1%)|                       |
| Pre-Op LogMAR BCVA     | 0.52 (0.25)| 0.34 (0.48)| 0.008                |
| Max IOP (mm Hg)        | 15.7 (2.8)| 30.1 (12.8)| <0.001               |
| Pre-Op IOP (mm Hg)     | 15.4 (2.6)| 17.2 (6.3)| 0.51                 |
| Number of preoperative glaucoma medications |           |           | <0.001                |
| 0                      | 14 (100%) | 6 (10.5%)  |                       |
| 1                      | 0 (0%)    | 12 (21.1%)|                       |
| 2                      | 0 (0%)    | 9 (15.8%)  |                       |
| 3                      | 0 (0%)    | 4 (7.0%)   |                       |
| 4                      | 0 (0%)    | 9 (15.8%)  |                       |
| 5                      | 0 (0%)    | 9 (15.8%)  |                       |
| 6                      | 0 (0%)    | 8 (14.0%)  |                       |

OD, right eye; OS, left eye; LogMAR, logarithm of the minimum angle of resolution; Max IOP, maximum intraocular pressure. The data are reported as mean ± standard deviation for continuous variables and frequency count for categorical variables. A significance level of \(\alpha = 0.05\) was used in analysis.

\(^a\)Kruskal-Wallis rank sum test for continuous variables.

\(^b\)Pearson’s \(\chi^2\) test for categorical variables.
Table 2. Clinical and Surgical Characteristics of the Glaucoma Cohort

| Glaucoma type           | Count (Percentage) |
|-------------------------|--------------------|
| Primary open angle      | 24 (42.1%)         |
| Normal tension          | 9 (15.8%)          |
| Mixed mechanism         | 14 (24.6%)         |
| Pseudoexfoliation       | 4 (7.0%)           |
| Chronic angle closure   | 4 (7.0%)           |
| Uveitic                 | 1 (1.8%)           |
| Pigmentary              | 1 (1.8%)           |

| Glaucoma stage          | Count (Percentage) |
|-------------------------|--------------------|
| Suspect                 | 2 (3.5%)           |
| Mild                    | 19 (33.3%)         |
| Moderate                | 15 (26.3%)         |
| Severe                  | 17 (29.8%)         |
| Indeterminate           | 4 (7.0%)           |

| Surgery type            | Count (Percentage) |
|-------------------------|--------------------|
| Phaco                   | 2 (3.5%)           |
| Phaco/MIGS              | 19 (33.3%)         |
| Incisional              | 11 (19.3%)         |
| Phaco/Incisional        | 20 (35.1%)         |
| Other                   | 5 (8.8%)           |

| Class of preoperative glaucoma medications | Count (Percentage) |
|--------------------------------------------|--------------------|
| Prostaglandin analogue                     | 44 (77.2%)         |
| Alpha agonist                              | 29 (50.9%)         |
| Beta blocker                               | 31 (54.4%)         |
| Topical CAI                                | 32 (56.1%)         |
| Oral CAI                                   | 7 (12.3%)          |
| Rho-kinase inhibitor                       | 23 (40.4%)         |

| Number of past ophthalmic surgeries | Count (Percentage) |
|------------------------------------|--------------------|
| 0                                  | 35 (61.4%)         |
| 1                                  | 10 (17.5%)         |
| 2+                                 | 12 (21.1%)         |

| Prior laser procedure | Count (Percentage) |
|-----------------------|--------------------|
| Yes                   | 25 (43.9%)         |
| No                    | 32 (56.1%)         |

| Mean deviation (dB)    | 7.90 (8.92)        |
| Pattern standard deviation (dB) | 4.95 (3.33) |

| RNFL thickness (μm)    | 71.15 (14.05)      |

Phaco = phacoemulsification, MIGS = micro-invasive glaucoma surgery, CAI = carbonic anhydrase inhibitor, RNFL = optical coherence tomography retinal nerve fiber layer. The data is reported as mean ± standard deviation (SD) for continuous variables and frequency count for categorical variables.

We first aimed to determine whether Gal-3 and APOE were significantly elevated in glaucoma cases compared to controls. We found that Gal-3 and APOE were both significantly higher in the AH of glaucoma patients (2.33 ± 2.09 ng/mL and 1.64 ± 1.45 μg/mL, respectively) compared to controls (0.92 ± 0.81 ng/mL and 0.40 ± 0.19 μg/mL, respectively) as determined by the Kruskal-Wallis significance test (P = 0.004 and P < 0.001, respectively; Fig. 1 and Table 3). We next used Pearson’s correlation coefficients to determine the degree of association between AH levels of Gal-3 and APOE, Gal-3 and total protein, and APOE and total protein across both cases and controls. Gal-3 and APOE were moderately positively correlated across the entire cohort (r = 0.65, P = 6.2E-9; Fig. 2A). Importantly, no association was observed between Gal-3 and phacoemulsification with intraocular lens implantation for age-related cataracts and had no prior history of ophthalmic surgeries or laser procedures. Additionally, as expected, controls did not undergo glaucoma testing as a part of their normal care and therefore did not have HVF mean deviation, pattern standard deviation, or RNFL thickness measurements to report.

The 57 patients with glaucoma represented a diverse cohort that underwent various surgical interventions, as summarized in Table 2. On the day of AH collection, 2 (3.5%) had phacoemulsification for age-related cataracts, 19 (33.3%) had phacoemulsification combined with minimally invasive glaucoma surgery, and 20 (35.1%) underwent phacoemulsification combined with incisional glaucoma surgery. These patients also had diverse types of glaucoma: 24 (42.1%) had primary open-angle glaucoma, 9 (15.8%) had normal-tension glaucoma, 14 (24.6%) had mixed mechanism glaucoma, 4 (7.0%) had pseudoexfoliation glaucoma, 4 (7.0%) had chronic angle-closure glaucoma, 1 (1.8%) had uveitic glaucoma, and 1 (1.8%) had pigmentary glaucoma. Their glaucoma was classified as suspect (3.5%), mild (33.3%), moderate (26.3%), severe (29.8%), or indeterminate (7.0%) and was being treated at the time of surgery with a range of glaucoma medications: 44 (77.2%) with prostaglandin agonists, 29 (50.9%) with alpha antagonists, 31 (54.4%) with beta blockers, 32 (56.1%) with topical carbonic anhydrase inhibitors (CAI), 7 (12.3%) with oral CAI, and 23 (40.4%) with rho-kinase inhibitors. These patients had a range of previous ophthalmic surgeries (n = 0–5), and 25 (43.8%) had also undergone previous laser procedures (Table 2). Of patients with history of past laser, 15 (26.3%) had selective laser trabeculoplasty, 3 (5.3%) had YAG capsulotomy, and 7 (12.3%) had laser peripheral iridotomy.
Figure 1. (A) Gal-3 and (B) APOE levels are elevated in the aqueous humor of patients with glaucoma. Significance was determined using the Kruskal-Wallis test and results are shown as mean ± standard error of the mean (SEM). **P < 0.01, ***P < 0.001.

Table 3. Galactin-3, APOE, and Total Protein Measurements in the Aqueous Humor of Control and Glaucoma Patients

| Variable          | Control         | Glaucoma       | P Valuea |
|-------------------|-----------------|----------------|----------|
| Gal-3 in AH (ng/mL) | 0.92 (0.81)     | 2.33 (2.09)    | 0.004    |
| APOE in AH (μg/mL) | 0.40 (0.19)     | 1.64 (1.45)    | <0.001   |
| Total protein (mg/mL) | 10.60 (3.88)   | 11.76 (4.02)   | 0.16     |

The data are reported as mean ± standard deviation (SD). A significance level of α = 0.05 was used in analysis.

a Kruskal-Wallis rank sum test for continuous variables.

total protein (Fig. 2B) or APOE and total protein (Fig. 2C), indicating that Gal-3 and APOE levels were not increased in glaucomatous AH because of nonspecific protein accumulation.

Before multivariate linear regression modeling, we performed a series of univariate analyses within the glaucoma cohort to identify demographic and clinical covariates significantly associated with Gal-3 and APOE levels. These tests revealed significant associations between maximum IOP and Gal-3 (r = 0.58, P = 1.8E-6), maximum IOP and APOE (r = 0.38, P = 0.0055), number of past ophthalmic surgeries and Gal-3 (r = 0.37, P = 0.0042), and number of past ophthalmic surgeries and APOE (r = 0.38, P = 0.0049) (Figs. 3A, 3B, Figs. 4A, 4B). We also found that Gal-3 and APOE were both significantly higher in the AH of patients who had history of past laser (3.11 ng/mL and 2.13 μg/mL, respectively) compared to those who did not (1.72 ng/mL and 1.24 μg/mL, respectively) (P = 0.016 and P = 0.041), whereas no association was observed between Gal-3 or APOE and any laser subtype. Additionally, several covariates showed a trend toward significance (defined as P < 0.1). These included preoperative IOP (r = 0.24, P = 0.072) and number of preoperative glaucoma medications (r = 0.23, P = 0.09) for Gal-3, and number of preoperative glaucoma medications (r = 0.27, P = 0.051) and mean deviation (r = −0.24, P = 0.091) for APOE (Figs. 3C, 3D, Figs. 4C, 4D). Finally, covariates with no association with Gal-3 and APOE were identified. These included mean deviation (r = −0.18, P = 0.18) and RNFL thickness (r = −0.072, P = 0.6) for Gal-3, and preoperative IOP (r = 0.18, P = 0.21) and RNFL thickness (r = −0.011, P = 0.94) for APOE (Figs. 3E, 3F, Figs. 4E, 4F).

We finally sought to adjust for covariates that may influence Gal-3 and APOE levels in AH. Results of the above univariate analyses were used to inform our selection of covariates for priority inclusion in fully saturated multivariate models. For both Gal-3 and APOE, these covariates included mean deviation, number of past ophthalmic surgeries, preoperative IOP, number of preoperative glaucoma medications, and maximum IOP (Table 4; Table 5). Gal-3 and APOE were significantly associated with each other in both final models, with every 10.0% increase in APOE resulting in a 4.6% average increase in Gal-3 (95% CI, 2.0% to 7.1%; P < 0.001). Furthermore, the signif-
Figure 2. (A) There is moderate, positive association between Gal-3 and APOE across the entire cohort ($r = 0.65$, $P = 6.2E-9$). No association was observed between Gal-3 and total protein ($r = 0.10$, $P = 0.35$) (B) or APOE and total protein ($r = 0.09$, $P = 0.50$) (C). Individual circles depict individual participants, and dashed lines represent 95% confidence interval bands. Pearson’s correlation coefficient $r$ is reported.

In this pilot study, we demonstrate that Gal-3 and APOE, markers of activated microglia in neurodegeneration, are elevated in the AH of patients with glaucoma. Gal-3 and APOE were moderately positively correlated across the entire cohort, as expected based on reports demonstrating significant upregulation of these proteins in human glaucomatous retinas and evidence that they act in the same signaling pathway in microglia in mouse models of glaucoma. Furthermore, AH Gal-3 and APOE showed no correlation with AH total protein, indicating that their increase is not a result of nonspecific protein accumulation in disease caused by medication use or prior surgeries.

After controlling for possibly confounding covariates in a fully-saturated multivariate linear regression model, we show that Gal-3 is significantly associated with historic maximum recorded intraocular pressure while APOE is associated with number of past ophthalmic surgeries. These results suggest that Gal-3 and APOE correlate with a history of poorly controlled disease rather than with absolute glaucoma severity, a notion that is further reinforced by their lack of association with traditional glaucoma staging metrics, including RNFL thickness and HVF mean deviation.

Our study builds on a previous report that detected elevated APOE levels in the AH of glaucoma patients, which were weakly correlated with HVF mean deviation. In contrast, our work is the first to examine Gal-3 in AH and to report its clinical associations with glaucoma disease characteristics and with APOE. The correlation between Gal-3 and APOE in our dataset suggests that the combined readout of these two markers may be a useful measure of microglial activation in eyes with glaucoma.

Gal-3 and APOE are upregulated by activated microglia in various neurodegenerative diseases of the CNS, including AD, Huntington’s disease, and multiple sclerosis. Recently, Gal-3 was identified as a cerebrospinal fluid biomarker in Alzheimer’s disease which correlated with markers of neuronal degeneration, synaptic dysfunction, and neuroinflammation. Gal-3 and Apoe are also upregulated in microglia in mouse models of glaucoma and photoreceptor degeneration, as well as in human postmortem glaucomatous tissues. We therefore hypothesize that these molecules may be released from activated retinal microglia into the vitreous chamber, thereby reaching the anterior chamber of the eye. Interestingly, prior literature has reported prolonged peripheral immune infiltration and loss of RGCs following transient IOP elevation in a mouse model of glaucoma.
Figure 3. There is a significant association between Gal-3 and maximum intraocular pressure (max IOP) (A) and Gal-3 and number of past ophthalmic surgeries (B). Trending association was observed between Gal-3 and both preoperative IOP (C) and number of preoperative glaucoma medications (D). No association was observed between Gal-3 and HVF mean deviation (E) or optical coherence tomography RNFL thickness (F). Individual circles depict individual participants and dashed lines represent 95% confidence intervals bands. Green indicates significant association (A-B) ($P < 0.05$), yellow indicates association trending towards significance (C-D) ($P < 0.1$), and red indicates no significant association (E-F) ($P \geq 0.1$). Pearson’s correlation coefficient $r$ is reported.
Figure 4. There is a significant association between APOE and number of past ophthalmic surgeries (A) and APOE and maximum intraocular pressure (max IOP) (B). Trending association was observed between APOE and both number of preoperative glaucoma medications (C) and HVF mean deviation (D). No association was observed between APOE and preoperative IOP (E) or optical coherence tomography RNFL thickness (F). Individual circles depict individual participants and dashed lines represent 95% confidence intervals bands. Green indicates significant association (A-B) ($P < 0.05$), yellow indicates association trending toward significance (C-D) ($P < 0.1$), and red indicates no significant association (E-F) ($P \geq 0.1$). Pearson's correlation coefficient $r$ is reported.
fore postulate that history of ocular insult from high IOP may be sufficient to trigger chronic neuroinflammation and microgliosis in patients with glaucoma, which may explain the strong correlation of Gal-3 and APOE with maximum recorded eye pressure in our study.

The AH is a central reservoir for proteins from both the anterior and posterior segments, including neural tissue, with many prior studies demonstrating associations between the AH proteome and biomarkers of CNS neurodegeneration. Although we propose that Gal-3 and APOE are secreted from retinal microglia in glaucoma, we also cannot rule out contribution from other cellular sources to the accumulation of these proteins in AH. For example, it has been shown that macrophages infiltrate the optic nerve in both human and experimental glaucoma, as well as the retina in response to ocular surgery, suggesting that peripherally-derived immune cells may contribute to the observed pool of AH Gal-3 and APOE in glaucoma. Macrophage infiltration has similarly been reported in the trabecular meshwork of glaucoma patients, where Gal-3 is thought to play a role in glaucomatous fibrotic processes. Additionally, it is possible that AH Gal-3 may be partially cornea derived, where it is known to promote angiogenesis and fibrosis in response to ocular injury. Further experiments will be required to determine the definitive

### Table 4. Multivariate Linear Regression Modeling of Galectin-3 in the Glaucoma Cohort

| Variable                                | Fully Saturated Model | Final Model |
|-----------------------------------------|-----------------------|-------------|
|                                        | Estimates  | 95% CI  | P Value | Estimates  | 95% CI  | P Value |
| Intercept                               | −3.644    | −5.586, −1.702 | 0.001    | −3.842    | −5.597, −2.087 | <0.001  |
| APOE (log-transformed)                  | 0.432     | 0.141, 0.722 | 0.005    | 0.467     | 0.211, 0.724  | 0.001   |
| Mean deviation                          | 0.001     | −0.025, 0.027 | 0.951    |           |           |         |
| Number of past ophthalmic surgeries     | 0.088     | −0.113, 0.289 | 0.379    |           |           |         |
| Preoperative IOP                        | 0.029     | −0.009, 0.068 | 0.125    | 0.023     | −0.007, 0.054 | 0.134   |
| Number of preoperative glaucoma medications | −0.022   | −0.151, 0.107 | 0.731    |           |           |         |
| Maximum IOP                             | 0.022     | 0.003, 0.040 | 0.023    | 0.023     | 0.006, 0.040  | 0.009   |
| Observations                            | 46        |           |         | 46        |           |         |
| R²/R² adjusted                          | 0.520/0.446 |       |         | 0.508/0.473 |       |         |
| AIC                                     | 98.485    |           |         | 93.601    |           |         |

The data are reported as mean ± standard deviation for continuous variables and frequency count for categorical variables. A significance level of α = 0.05 was used in analysis. Statistically significant values in the final model are indicated in bold.

### Table 5. Multivariate Linear Regression Modeling of APOE in the Glaucoma Cohort

| Variable                                | Fully Saturated Model | Final Model |
|-----------------------------------------|-----------------------|-------------|
|                                        | Estimates  | 95% CI  | P Value | Estimates  | 95% CI  | P Value |
| Intercept                               | 6.685     | 5.957, 7.413 | <0.001   | 6.717     | 6.473, 6.961 | <0.001 |
| Gal-3 (log-transformed)                 | 0.436     | 0.143, 0.730 | 0.005    | 0.461     | 0.221, 0.702 | <0.001 |
| Mean deviation                          | −0.009    | −0.035, 0.017 | 0.487    |           |           |         |
| Number of past ophthalmic surgeries     | 0.167     | −0.030, 0.363 | 0.094    | 0.195     | 0.019, 0.371  | 0.031   |
| Preoperative IOP                        | −0.013    | −0.052, 0.026 | 0.511    |           |           |         |
| Number of preoperative glaucoma medications | 0.04   | −0.089, 0.169 | 0.538    |           |           |         |
| Maximum IOP                             | 0.003     | −0.017, 0.023 | 0.763    |           |           |         |
| Observations                            | 46        |           |         |           |           |         |
| R²/R² adjusted                          | 0.459/0.376 |       |         | 0.428/0.402 |       |         |
| AIC                                     | 98.956    |           |         | 93.501    |           |         |

The data are reported as mean ± standard deviation for continuous variables and frequency count for categorical variables. A significance level of α = 0.05 was used in analysis. Statistically significant values in the final model are indicated in bold.
source of these neurodegeneration-associated markers in glaucomatous AH.

Our group has previously demonstrated that Gal-3 and Apoe are critical molecules that drive microglia-induced neuronal loss in glaucoma. Importantly, this same study revealed that Gal-3 can be pharmacologically targeted in the mouse eye, such that intravitreal injections of the selective Gal-3 inhibitor TD139 conferred robust protection of RGCs despite sustained IOP elevation in the microbead glaucoma model. TD139 is a commercially available small molecule inhibitor that has recently shown therapeutic efficacy in a phase 1/2a clinical study for idiopathic pulmonary fibrosis. Furthermore, TD139 has been successfully compounded in eyedrop form for the treatment of murine corneal neovascularization, with good bioavailability and therapeutic results. If Gal-3 inhibition were to become a clinically feasible neuroprotective strategy for the treatment of glaucoma, its detectability as a biomarker may prove critical for identifying those patients who would benefit most from this intervention. Because we investigated a diverse glaucoma case cohort in this study, our findings suggest that Gal-3 and APOE may be broadly useful biomarkers across different glaucoma types and stages.

There were several limitations to our study. Although we adjusted for multiple covariates likely to influence Gal-3 and APOE AH levels, it remains possible that there were other demographic and clinical covariates affecting their levels that we did not examine here. Glaucoma patients in our study had been treated with a variety of ophthalmic procedures and medications as a part of their ongoing care, and we were not adequately powered to investigate the effect of individual IOP-lowering surgeries, glaucoma lasers, or medications on Gal-3 and APOE levels (for example, the commonly used prostaglandin analogues, which are known to be proinflammatory). Furthermore, because of limitations inherent in retrospective chart review, we were not able to ascertain the time of maximum IOP recording for some of the enrolled patients, because their IOP was initially measured by referring practitioners not in our electronic medical record system. Our study also did not consider the effect of APOE genotype, which is known to affect the degree of microglial activation and Gal-3 upregulation in glaucoma. Given that APOE4 has been found to impair the microglial response in glaucoma, future studies should consider the effect of APOE genotype when defining its clinical and molecular associations with glaucoma. Finally, although useful as a proof-of-principle study, AH collection before ophthalmic procedures has limited utility for glaucoma patients being evaluated in clinic. Future studies should examine whether Gal-3 and APOE are detectable in the serum of patients with glaucoma and whether these levels correlate with those found in AH.

Taken together, our study provides the first evidence that Gal-3 and APOE are elevated in the AH of patients with glaucoma and associated with a history of poorly controlled disease. If validated in serum samples, these molecules may provide an accessible way of identifying patients with high levels of microglial activation in the retina who may benefit from microglia-targeted glaucoma treatments in the future.

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