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Original Article

Association of serum TIM-3 and Galectin-9 levels with glaucoma progression: implications for clinical monitoring

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Abstract: Background: Primary open-angle glaucoma (POAG) causes irreversible blindness via retinal ganglion cell loss. Neuroinflammation and immune dysfunction drive its progression, yet the role of the TIM-3/Galectin-9 axis remains unclear. Objective: To investigate the association of serum soluble TIM-3 (sTIM-3) and Galectin-9 levels with the progression of primary open-angle glaucoma (POAG). Methods: This retrospective analysis enrolled POAG patients from our ophthalmology department (January 2022-December 2024). After a minimum 12-month follow-up, participants were categorized as progression or stable based on visual field and optical coherence tomography (OCT) assessments. Healthy controls were age/sex-matched. Recorded clinical variables included IOP, CCT, visual fields, and OCT measures. Baseline concentrations of sTIM-3 and Galectin-9 in serum were determined by ELISA. Multivariate logistic regression assessed independent links between these markers and disease progression, supplemented by trend tests, E-value calculations, and subgroup analyses. Results: The study included 120 POAG patients (52 progression, 68 stable) and 60 healthy controls. Baseline measures were balanced across groups. Both sTIM-3 and Galectin-9 were significantly elevated in progression relative to stable patients and controls (all $P < 0.001$). Multivariate logistic regression identified sTIM-3 (OR = 2.14, P = 0.004) and Galectin-9 (OR = 3.39, $P < 0.001$) as independent factors linked to disease progression. Trend analyses revealed dose-response patterns (P for trend = 0.015 and 0.004, respectively). These findings were further corroborated by E-value and subgroup analyses, supporting their robustness. Conclusions: Higher baseline sTIM-3 and Galectin-9 levels correlate independently with POAG progression risk, showing a dose-response pattern. These biomarkers may serve as adjunctive references for risk stratification, although their utility warrants prospective validation with longitudinal sampling.

Keywords: Primary open angle glaucoma, serum sTIM-3, Galectin-9, E value, analysis of association

Introduction

A leading cause of irreversible blindness, primary open-angle glaucoma (POAG) affects roughly 57.5 million individuals globally, and it is expected that the number of patients will be more than 110 million in 2040 [1]. POAG involves the gradual degeneration of RGCs, leading to optic nerve injury and subsequent visual field impairment. Although lowering intraocular pressure (IOP) is currently the only evidence-based intervention, visual field damage continues to worsen in some patients even when IOP is well controlled [2, 3]. Therefore, it is clinically important to identify clinical monitoring indicators associated with POAG progression.

Recent clinical studies have found that neuroinflammation and immune disorders are associated with the pathogenesis and progression of POAG. For instance, elevated serum levels of inflammatory cytokines (e.g., IL-6, TNF-α) have been found in POAG patients versus healthy individuals, and these levels correlate with visual field loss and optic nerve structural changes [4, 5]. In addition, inflammatory indicators such as neutrophil-to-lymphocyte ratio, derived from routine blood tests, has also been associated with POAG [6]. Changes in the levels of a variety of immune-related molecules have been observed in POAG patients, suggesting that immune status may play an important role in the disease process [7]. In this context, the value of immune-related molecules as potential

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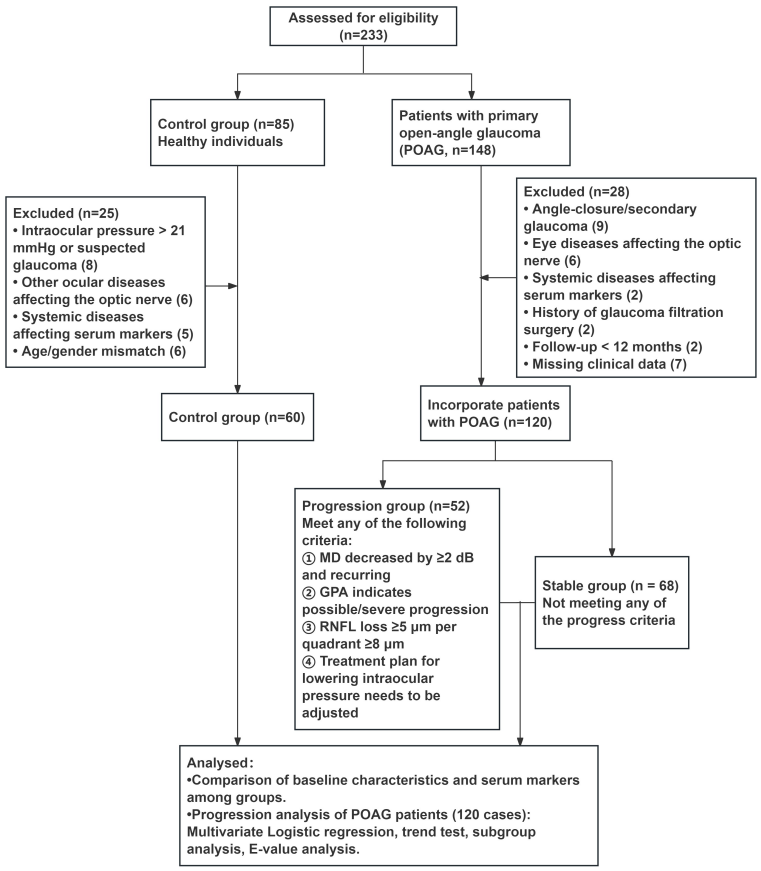

Figure 1. Design flow chart.

biomarkers of POAG has attracted increasing attention.

The immune checkpoint TIM-3 and its ligand Galectin-9 interact to control T-cell function and immunity [8, 9]. Elevated serum levels of sTIM-3 and Galectin-9 are observed during active disease stages and have been clinically linked to adverse outcomes in conditions such as multiple myeloma and systemic mastocytosis [10]. In ophthalmology, Gal-9 expression has been detected in corneal and retinal pigment epithelial cells, with levels rising in response to inflammatory stimuli [11, 12]. In addition, sTIM-3 is a soluble molecule that can be reliably detected in serum. Some reviews have highlighted its potential as a soluble biomarker in a variety of diseases [13]. Nevertheless, the clinical relevance of the TIM-3/Galectin-9 axis in POAG progression remains unknown, and evidence linking serum sTIM-3 and Galectin-9 levels to POAG progression risk is still lacking.

Thus, our hypothesis was that increased sTIM-3 and Galectin-9 independently predict POAG progression, independent of age and sex. To find novel clinical reference tools for POAG, this retrospective cohort assessed the association of baseline sTIM-3 and Galectin-9 with disease progression.

Materials and methods

Study design

This retrospective cohort study retrieved clinical data of POAG patients from our hospital's electronic records (January 2022-December 2024). Patients were categorized into progression or stable subjects based on follow-up visual field and optical coherence tomography (OCT) findings. Healthy controls, matched for age (± 3 years) and sex, were recruited from our hospital's physical examination center. The First Affiliated Hospital of Lishui University, Lishui People's Hospital Ethics Committee approved (Approval number: 2026-06-02) the study and waived the requirement for informed consent, as the analysis was performed retrospectively on anonymized data. This investigation fully complied with the ethical principles set forth in the Declaration of Helsinki and the International Code of Medical Research Ethics [14]. **Figure 1** illustrates the study design and workflow.

Inclusion and exclusion criteria

POAG patients [15]: Inclusion criteria: ① Gonioscopy showed open chamber Angle; ② Signs of glaucomatous damage to the optic nerve, including enlarged cupping, focal rim loss, optic disc bleeding, or RNFL thinning; ③ Characteristic glaucomatous visual field loss, including paracentral or arcuate scotomas and a nasal step; ④ Excluded secondary glaucoma and other eye diseases that may cause the above manifestations; and ⑤ complete regular follow-up for at least 12 months.

Exclusion criteria: ① Angle-closure glaucoma, congenital glaucoma or secondary glaucoma (such as glucocorticoid glaucoma, uveitic glau-

coma, traumatic glaucoma); ② Coexisting eye diseases with potential to affect optic nerve integrity or performance, such as myopic optic neuropathy, ischemic optic neuropathy, or retinitis pigmentosa; ③ Combined with systemic diseases that may affect serum TIM-3/Gal-9 levels, including autoimmune diseases, malignant tumors, chronic active infections, etc.; ④ Previous glaucoma filtration surgery or destructive ciliary body surgery (except selective laser trabeculoplasty); ⑤ Missing key variables of clinical data.

Healthy controls [16]: Inclusion criteria: ① Binocular intraocular pressure (IOP) ≤ 21 mmHg; ② open Angle; ③ Fundus examination showed no nerve damage; ④ No family history of glaucoma.

Exclusion criteria: ① any type of glaucoma diagnosis or suspected glaucoma; ② any eye disease that may affect the optic nerve or retinal structure; ③ any systemic diseases that may affect serum TIM-3/Gal-9 levels.

Sample size evaluation

Taking serum sTIM-3 level as the main observation index, referring to the results of our previous pilot study and previous research [17, 18], we used a two-sided significance threshold of $\alpha = 0.05$ and set the statistical power at $1-\beta = 0.80$. The expected difference in sTIM-3 levels between the stable and progression groups was approximately 1.5 ng per milliliter (estimated as mean values of approximately 3.9 ng per milliliter in the progression group and 2.4 ng per milliliter in the stable group), with a standard deviation of approximately 1.2 ng per milliliter. Based on the comparison of the mean sample sizes of two independent groups, the required sample size was estimated to be approximately 25 cases per group, yielding a total of approximately 50 cases for the study. Accounting for an approximate 20% attrition rate, the required minimum sample size was calculated as roughly 60 individuals (30 per arm). This study included 120 POAG patients (52 with progression disease, 68 with stable disease) and 60 healthy controls. The above minimum sample size requirements were met, indicating that the current sample size had sufficient statistical power to support the main study conclusions.

Treatment options

All POAG patients received routine IOP lowering treatment after enrollment and during the follow-up period, and the specific plan was formulated by the attending physician according to the baseline IOP level of the patient, the degree of optic nerve damage, and the recommendations of the Chinese Glaucoma Guidelines (2020) [15]. The first-line treatment is mainly IOP-lowering eye drops, commonly used drugs include prostaglandin analogues (such as latanoprost, travoprost), β-blockers (such as timolol), α2 receptor agonists (such as brimonidine) and carbonic anhydrase inhibitors (such as brinzolamide). Single or combined drug regimens are used according to the control of intraocular pressure. If the drug treatment could not achieve the target intraocular pressure, trabeculectomy was used according to the patient's wishes and conditions. During the follow-up period, if the patient needed to increase the IOP-lowering regimen due to the progression of the disease (such as additional drugs, conversion to surgical intervention), the event was recorded and used as one of the progression criteria.

Evaluation indicators

Main observation indicators: Fasting peripheral venous blood (5 mL) was drawn from each subject at baseline (morning). Samples were left at room temperature for 30 min, then centrifuged (3,000 rpm, 15 min) to obtain serum, and aliquots were stored at -80°C. Serum concentrations of sTIM-3 and Galectin-9 were measured using commercial double-antibody sandwich ELISA kits (R&D Systems, DY2365 for sTIM-3, DY2045 for Galectin-9) according to the manufacturer's protocols.

Ophthalmic examination indicators: All subjects underwent the following eye examinations at enrollment (baseline) and during follow-up (every 6 months):

(1) IOP: Goldmann applanation tonometer was used to measure IOP, and each eye was measured three times. (2) Central corneal thickness (CCT) was assessed using an ultrasonic pachymeter. (3) Visual field examination: Static perimetry was performed using the Humphrey Field Analyzer (Carl Zeiss Meditec, Germany) with the 24-2 SITA-Standard protocol. Mean

deviation (MD) and pattern standard deviation (PSD) were recorded. Examinations were considered reliable if false-negative, false-positive, and fixation loss rates were ≤ 33% [19]. (4) OCT examination: The Cirrus HD-OCT system (Carl Zeiss Meditec, Germany) was employed to assess mean peripapillary RNFL thickness, RNFL thickness in the four quadrants, and disc parameters (e.g., cup-to-disc area ratio).

Criteria for progression group and stable group: After 12 months of follow-up, two independent ophthalmologists (both with ≥ 5 years of clinical experience in glaucoma) assessed whether POAG patients had disease progression according to the following criteria. If the results were inconsistent, a consensus was reached through consultation: if any of the following items were met, the patient was classified as having progression [20-23]: ① The mean deviation of visual field decreased ≥ 2 dB from baseline, and it was repeated in two consecutive reexaminations; ② Glaucoma progression analysis indicated "possible progression" or "significant progression"; ③ Loss of OCT-RNFL average thickness ≥ 5 μm, or loss of any quadrant ≥ 8 μm, and exceeding the device test-retest variability; ④ Adjustment of IOP lowering therapy (e.g., additional drugs, conversion to laser or surgery) due to disease progression. Patients who did not meet any of these criteria were included in the stable group.

Statistical analysis

SPSS (version 26.0) was used for all statistical analyses. Prior to group comparisons, all continuous variables were assessed for normality using the Shapiro-Wilk test and for homogeneity of variances using Levene's test. Normally distributed data with homogeneous variances were presented as mean ± SD and compared using one-way ANOVA with LSD post-hoc comparisons. Normally distributed data with unequal variances were analyzed using Welch's ANOVA with Bonferroni post-hoc corrections. Non-normally distributed data were presented as median (IQR) and compared using the Kruskal-Wallis H test with Bonferroni-corrected pairwise comparisons. Categorical variables were analyzed using the Chi-square or Fisher's exact test. For multivariate analysis, binary logistic regression was performed with POAG progression as the dependent variable. Serum sTIM-3 and Galectin-9 (as continuous variables) were the primary independent variables, with adjustment for age, sex, baseline IOP, CCT, and baseline MD. Adjusted odds ratios (ORs) with 95% confidence intervals (CIs) were calculated. To evaluate dose-response relationships, participants were categorized into quartiles (Q1-Q4) based on the distribution of sTIM-3 and Galectin-9 among POAG patients (n = 120). The median of each quartile was entered as a continuous variable in the multivariate logistic regression model, with the P for trend calculated. Sensitivity analyses included E-value calculation [24] to quantify unmeasured confounding, and subgroup analyses stratified by sex and age (using the whole-sample median age of 62 years as the cut-off). Interaction effects were tested by including product terms in the full-sample models. With the exception of the trend test, E-value analysis, and subgroup analyses, two-sided tests were employed. Statistical significance was set at $P < 0.05$.

Results

Baseline characteristics

This work enrolled 120 POAG individuals (52 progression, 68 stable) alongside 60 healthy controls, balanced for age and sex. **Table 1** presents the baseline demographic and clinical characteristics for all three groups. There were no significant differences in age ($F = 0.009$, $df_1 = 2$, $df_2 = 177$, $P = 0.991$), BMI ($F = 0.539$, $df_1 = 2$, $df_2 = 177$, $P = 0.584$), or sex distribution ($\chi^2 = 3.53$, $P = 0.172$), indicating that the groups were well balanced at baseline.

No significant difference in baseline IOP was observed between the progression group (18.2 ± 2.5 mmHg) and the stable group (17.9 ± 2.3 mmHg) ($P = 0.482$). Similarly, CCT did not differ significantly between the progression group (542.1 ± 28.6 μm) and the stable group (545.3 ± 30.2 μm) ($P = 0.566$). The median baseline MD was -3.8 dB (IQR: -6.2 to - 2.1 dB) in the progression group, which was significantly worse than that of the stable group (-2.1 dB (IQR: -3.5 to - 1.2 dB)) ($P = 0.003$), suggesting more severe baseline visual field impairment in the progression group. Using the median age of the overall cohort (62 years) as the cutoff value, 58 participants (48.3%) were under the age of 62 years, and 62 individuals (51.7%) were 62 or older.

Table 1. Baseline characteristics of the three groups

Characteristic	Control group (n = 60)	Stable group (n = 68)	Progression group (n = 52)	*P*-value
Age (years), mean ± SD	61.67 ± 7.08	61.63 ± 7.54	61.81 ± 7.56	0.991
Male, n (%)	28 (46.7)	40 (58.8)	33 (63.5)	0.172
BMI (kg/m^2), mean ± SD	23.86 ± 2.68	24.37 ± 2.73	24.23 ± 3.23	0.584
Education (≥ high school), n (%)	35 (58.3)	41 (60.3)	32 (61.5)	0.94
Hypertension, n (%)	14 (23.3)	16 (23.5)	14 (26.9)	0.885
Diabetes, n (%)	5 (8.3)	8 (11.8)	9 (17.3)	0.348
Hyperlipidemia, n (%)	9 (15.0)	13 (19.1)	9 (17.3)	0.827
Cardiovascular disease, n (%)	3 (5.0)	6 (8.8)	3 (5.8)	0.656
Family history of glaucoma, n (%)	1 (1.7)	18 (26.5)	16 (30.8)	< 0.001
Smoking history, n (%)	11 (18.3)	15 (22.1)	11 (21.2)	0.866
Alcohol consumption, n (%)	11 (18.3)	4 (5.9)	12 (23.1)	0.022
Myopia (< -3.0 D), n (%)	3 (5.0)	8 (11.8)	6 (11.5)	0.353

Notes: IQR, interquartile range; SD, standard deviation; BMI, body mass index. Pearson's chi-square test was applied to compare categorical variables. Regarding continuous variables, age (Shapiro-Wilk, W = 0.989, P = 0.201; Levene's homogeneity of variance test, F = 0.167, P = 0.846) and BMI (Shapiro-Wilk, W = 0.991, P = 0.310; Levene test of homogeneity of variance, F = 0.571, P = 0.566) followed a normal distribution and had homogeneous variances, so the data were expressed as Mean± standard deviation (Mean ± SD), One-way ANOVA was used for comparison between groups.

Table 2. Ocular biometrics at baseline across the three study groups (mean ± SD)

Parameter	Control group (n = 60)	Stable group (n = 68)	Progression group (n = 52)	*P*-value
Baseline IOP (mmHg)	16.67 ± 2.37	17.80 ± 2.06	17.85 ± 2.41	0.006
CCT (μm)	553.20 ± 31.18	541.63 ± 27.89	543.33 ± 28.82	0.064
Corneal curvature (D)	43.38 ± 1.67	43.26 ± 1.45	42.99 ± 1.40	0.373
Axial length (mm)	23.45 ± 0.83	23.59 ± 0.87	23.73 ± 0.89	0.232
Anterior chamber depth (mm)	3.13 ± 0.22	3.11 ± 0.28	3.05 ± 0.30	0.271
Spherical equivalent (D)	-0.56 ± 1.23	-0.73 ± 1.71	-0.98 ± 1.79	0.361

Notes: IOP, intraocular pressure; CCT, central corneal thickness; D, diopters; SE, spherical equivalent. For parameters with homogeneous variances (Levene's test P > 0.05), conventional one-way ANOVA was applied; for anterior chamber depth and spherical equivalent, which showed variance heterogeneity (Levene's test P = 0.031 and P = 0.009, respectively), Welch's ANOVA was used instead. Post-hoc comparisons were adjusted using Bonferroni correction when applicable.

Comparison of baseline ocular biological parameters

The results of comparison of baseline ocular biological parameters among the three groups of subjects are shown in **Table 2**. Baseline IOP differed significantly across the three groups, as determined by one-way ANOVA (P = 0.006). Post-hoc LSD comparisons revealed that IOP was significantly lower in controls than in stable (P = 0.005) or progression patients (P = 0.007), whereas stable and progression groups did not differ from each other (P = 0.918). No significant intergroup differences were observed for other ocular biometric parameters (CCT, corneal curvature, axial length, anterior chamber depth, refractive error), with all *P*-values > 0.05.

Baseline visual field and OCT structural parameters

Table 3 reveals significant intergroup differences (all P < 0.001) for all visual field indices (MD, PSD) and OCT structural metrics (average RNFL thickness, superior/inferior quadrant RNFL, cup-to-disc area ratio). From controls to stable patients and then to progression, we observed a stepwise decline in MD, a stepwise rise in PSD and cup-to-disc ratio, and progression RNFL thinning.

Between-group comparison of serum sTIM-3 and Galectin-9 levels

Table 4 summarizes baseline serum sTIM-3 and Galectin-9 levels across the three groups.

Table 3. Baseline visual field and OCT structural parameters across the three groups

Parameter	Control group (n = 60)	Stable group (n = 68)	Progression group (n = 52)	*P*-value
Visual field parameters				
MD (dB), mean ± SD	-0.34 ± 0.90	-2.35 ± 1.42	-4.56 ± 2.11	< 0.001
PSD (dB), mean ± SD	1.67 ± 0.32	3.45 ± 1.09	5.37 ± 1.84	< 0.001
OCT structural parameters				
Average RNFL thickness (μm), mean ± SD	105.67 ± 8.87	84.29 ± 10.49	74.33 ± 8.89	< 0.001
Inferior quadrant RNFL (μm), mean ± SD	124.02 ± 14.60	98.49 ± 16.37	85.96 ± 14.20	< 0.001
Superior quadrant RNFL (μm), mean ± SD	119.52 ± 12.37	94.09 ± 12.33	83.88 ± 12.22	< 0.001
Cup-to-disc area ratio, mean ± SD	0.32 ± 0.10	0.59 ± 0.19	0.63 ± 0.21	< 0.001

Notes: MD, mean deviation; PSD, pattern standard deviation; RNFL, retinal nerve fiber layer; IQR, interquartile range; SD, standard deviation. For MD and PSD, Welch's ANOVA was applied due to variance heterogeneity (Levene's test, both P < 0.001). For RNFL parameters, conventional one-way ANOVA was used. For cup-to-disc ratio, Welch's ANOVA was applied due to variance heterogeneity (Levene's test, P < 0.001). Post-hoc comparisons were adjusted using Bonferroni or LSD methods as appropriate. Glaucoma predominantly affects the superior and inferior quadrants; nasal and temporal quadrant data are not shown. All visual field examinations met reliability standards (false-negative, false-positive, and fixation loss rates ≤ 33%).

Table 4. Serum levels of sTIM-3 and Galectin-9 among the three groups

Parameter	Control group (n = 60)	Stable group (n = 68)	Progression group (n = 52)	*P*-value
sTIM-3 (ng/mL), median (IQR)	1.74 (1.55-1.98)	2.38 (2.01-3.22)	4.29 (3.21-5.56)	< 0.001
Galectin-9 (ng/mL), median (IQR)	4.53 (3.86-5.10)	5.96 (5.18-6.81)	8.88 (7.65-10.11)	< 0.001

Notes: Data are presented as median (IQR) because serum sTIM-3 and Galectin-9 levels in the control and stable groups did not follow a normal distribution (Shapiro-Wilk test: for sTIM-3, Control W = 0.947, P = 0.011; Stable W = 0.893, P < 0.001; for Galectin-9, Control W = 0.948, P = 0.013; Stable W = 0.962, P = 0.036), and the overall data across groups did not uniformly meet parametric assumptions. Group comparisons were performed using the Kruskal-Wallis H test, followed by Bonferroni-adjusted post-hoc pairwise analyses. IQR, interquartile range.

Both markers differed significantly among the groups (P < 0.001 for each). All pairwise comparisons showed that sTIM-3 and Galectin-9 levels were significantly higher in the progression group than in both the stable and control groups, and were also elevated in the stable group compared with controls (all P < 0.05).

Multivariate Logistic regression analysis

Univariate Logistic regression analysis was performed with POAG progression during follow-up as the dependent variable (progression = 1, stability = 0). Significant associations with POAG progression were observed for sTIM-3 (OR = 2.38, P < 0.001), Galectin-9 (OR = 2.80, P < 0.001), and baseline MD (OR = 1.96, P < 0.001). Age, gender, baseline IOP and CCT were not significantly correlated in univariate analysis (all P > 0.05). These variables (age, gender, baseline IOP, CCT, baseline MD) were then entered into a multivariate Logistic regression model. After controlling for potential confounders, serum sTIM-3 (OR = 2.14, P = 0.004) and Galectin-9 (OR = 3.39, P < 0.001) were each independently associated with POAG progression. In the multivariate model, baseline MD remained significantly associated (OR = 2.05, P < 0.001). See **Table 5** for full details.

Trend test (dose-response relationship)

Quartile analysis categorized serum sTIM-3 and Galectin-9 levels based on the 25th, 50th, and 75th percentiles of the whole sample (n = 120) with Q1 (the lowest) serving as the reference (**Table 6**). After adjusting for age, sex, baseline IOP, CCT, and baseline MD, the adjusted ORs for Q2, Q3, and Q4 of sTIM-3 were 1.32 (0.35-4.97), 3.50 (1.00-12.27), and 8.78 (2.17-35.49), respectively (P for trend = 0.015). Galectin-9 showed a similar dose-response trend (P for trend = 0.004), with ORs of 1.68 (0.41-6.95), 5.14 (1.25-21.14), and 17.46 (3.96-76.94) for the upper three quartiles. These findings indicate that higher baseline levels of sTIM-3 and Galectin-9 were associated with a progressively increasing risk of POAG progression, suggesting a dose-response relationship.

Table 5. Univariate and multivariate logistic regression analyses of variables associated with POAG progression

Variable	Univariate analysis		Multivariate analysis	
	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
sTIM-3 (per 1 ng/mL)	2.38 (1.67-3.40)	< 0.001	2.14 (1.28-3.58)	0.004
Galectin-9 (per 1 ng/mL)	2.80 (1.97-3.99)	< 0.001	3.39 (1.89-6.06)	< 0.001
Age (per 1-year increase)	1.00 (0.96-1.05)	0.899	0.95 (0.86-1.06)	0.340
Sex (male vs. female)	1.22 (0.58-2.56)	0.606	3.65 (0.74-17.95)	0.111
Baseline IOP (per 1 mmHg)	1.01 (0.86-1.19)	0.915	0.94 (0.69-1.28)	0.940
CCT (per 10 μm)	1.02 (0.90-1.16)	0.743	0.92 (0.72-1.17)	0.500
Baseline MD (per 1 dB worsening)	1.96 (1.51-2.54)	< 0.001	2.05 (1.40-3.01)	< 0.001

Notes: The multivariate model simultaneously included all listed variables (age, sex, baseline IOP, CCT, and baseline MD). No continuous variables were centered before entry into the model. The OR for baseline MD corresponds to each 1-dB reduction in MD (i.e., worsening of visual field defect).

Table 6. sTIM-3 and Galectin-9: quartile trend testing for POAG progression

Variable	Quartile	Progression group (n)	Stable group (n)	Adjusted OR (95% CI)	P for trend
sTIM-3	Q1 (≤ 2.22 ng/mL)	5	25	1.00 (Ref)	0.015
	Q2 (2.23-3.05 ng/mL)	7	23	1.32 (0.35-4.97)	
	Q3 (3.06-4.36 ng/mL)	16	15	3.50 (1.00-12.27)	
	Q4 (≥ 4.37 ng/mL)	24	5	8.78 (2.17-35.49)	
Galectin-9	Q1 (≤ 5.76 ng/mL)	4	30	1.00 (Ref)	0.004
	Q2 (5.77-7.02 ng/mL)	7	24	1.68 (0.41-6.95)	
	Q3 (7.03-8.70 ng/mL)	13	10	5.14 (1.25-21.14)	
	Q4 (≥ 8.71 ng/mL)	28	4	17.46 (3.96-76.94)	

Notes: ORs were adjusted for age, sex, baseline IOP, CCT, and baseline MD. Due to tied values when dividing continuous biomarkers by percentiles, actual case numbers across quartiles are not strictly equal; this does not affect the validity of the trend test.

Table 7. POAG progression: subgroup analyses for serum sTIM-3 and Galectin-9

Subgroup	Progression group/ Stable group (n)	sTIM-3 OR (95% CI)	*P* for interaction	Galectin-9 OR (95% CI)	*P* for interaction
Sex					
Male	33/40	1.39 (1.09-1.78)	0.682	1.20 (1.01-1.44)	0.351
Female	19/28	1.27 (0.96-1.68)		1.09 (0.92-1.29)	
Age					
< 62 years	22/35	1.34 (1.00-1.80)	0.471	1.17 (0.98-1.40)	0.523
≥ 62 years	30/33	1.45 (1.10-1.91)		1.22 (1.00-1.49)	

Notes: ORs were adjusted for age (when stratified by sex), baseline IOP, CCT, and baseline MD; age was not adjusted in the age-stratified analysis. *P* values for interaction were derived from extended models including product terms (subgroup variable × biomarker).

Subgroup analysis

Subgroup analyses by sex and age (median, 62 years) were performed, and multivariate Logistic regression was used to adjust for the same covariates within each subgroup, and the *P* value for interaction was calculated by including the product term. The results are shown in **Table 7**. The OR of sTIM-3 was 1.39 (95% CI: 1.09-1.78) in men and 1.27 (0.96-1.68) in women. The OR of Galectin-9 was 1.20 (1.01-1.44) in men and 1.09 (0.92-1.29) in women. No significant interaction with gender was observed for sTIM-3 (P = 0.682) or Galectin-9 (P = 0.351), suggesting that the association of both markers with progression was not altered by sex. In the age subgroup, the OR of sTIM-3 was 1.34 (1.00-1.80) in patients < 62 years old

Table 8. Quantifying unmeasured confounding: E-values for sTIM-3 and Galectin-9 linked to POAG progression

Variable	OR (95% CI)	E-value	E-value (95% CI lower bound)
sTIM-3	2.14 (1.28-3.58)	3.70	1.88
Galectin-9	3.39 (1.89-6.06)	6.24	3.19

Notes: The E-value for the point estimate was obtained from the OR estimate, while the E-value for the CI lower bound was derived from the lower limit of the OR's 95% CI. Together, they reflect the minimal confounding strength needed to fully negate the observed association under the most conservative evaluation. Greater E-values indicate a reduced risk that unmeasured confounding has materially affected the results.

and 1.45 (1.10-1.91) in patients ≥ 62 years old. The OR values of Galectin-9 were 1.17 (0.98-1.40) and 1.22 (1.00-1.49), respectively. The interaction *P* values were 0.471 and 0.523, indicating that age did not significantly modify the observed associations.

E-value analysis

For sTIM-3, the main analysis showed an adjusted OR of 2.14 (95% CI: 1.28-3.58). The corresponding E-value was 3.70 for the point estimate and 1.88 for the 95% CI lower bound. For Galectin-9, the OR was 3.39 (95% CI: 1.89 to 6.06), with a point estimated E value of 6.24 and a lower 95% CI E value of 3.19 (**Table 8**). An E-value quantifies the minimum confounding strength required (on the risk ratio scale) to fully negate an observed association. Our point-estimate E-values of 3.70 and 6.24 indicate that any unmeasured confounder(s) would need to be at least 3.70-fold and 6.24-fold associated with both the biomarker level and disease progression to completely explain away our findings. Even with the most conservative estimate of effect (the lower limit of the confidence interval), the required confounding strength was 1.88 and 3.19 times. Considering that the study was adjusted for common clinical confounding variables such as age, sex, baseline IOP, CCT, and baseline MD, the possibility of such a strong association for these unmeasured confounders is low, suggesting that the main results of this study are relatively robust.

Discussion

This retrospective cohort study examined the clinical link between serum sTIM-3/Galectin-9 and POAG progression. The main findings included: (1) Elevated serum sTIM-3 and Galectin-9 were observed in the progression group compared with both the stable group and healthy controls; (2) Multivariate logistic regression identified sTIM-3 (OR = 2.14, P = 0.004) and Galectin-9 (OR = 3.39, P < 0.001) as independent factors correlated with POAG progression; (3) A significant dose-response trend was observed for both sTIM-3 and Galectin-9 with progression risk (P for trend = 0.015 and 0.004, respectively); (4) Subgroup analysis showed that the association was consistent across gender and age subgroups; (5) E-value analysis suggested that the results were less likely to be affected by unmeasured confounders (sTIM-3 E = 3.70, Galectin-9 E = 6.24). This initial evidence suggests sTIM-3 and Galectin-9 may be useful as adjunctive reference markers for baseline risk stratification, though their utility for longitudinal disease monitoring requires prospective validation with repeated measurements.

Elevated sTIM-3 and Galectin-9 levels in the POAG progression group (versus stable patients and controls) paralleled the trend reported by Fujita et al. [17] in adult-onset Still's disease (ASD). Fujita et al. found that ASD patients had markedly higher serum Gal-9 (median 21.57 ng/ml) and sTIM-3 levels than rheumatoid arthritis patients and healthy controls (median Gal-9 in controls: 4.51 ng/ml). After immunosuppressive therapy, both markers fell sharply alongside disease activity, indicating their potential as indicators of inflammatory activity. Despite differing pathophysiologies between POAG and ASD, our stable group already showed higher sTIM-3 and Galectin-9 levels than healthy controls, and the progression group had even higher levels. This pattern parallels the "active > remission > control" gradient observed in ASD. These findings imply that both markers might correlate with the degree of inflammation or immune imbalance in POAG.

Evidence from ophthalmology research indicates that the TIM-3/Galectin-9 pathway is functionally expressed. According to Shimmura-Tomita et al. [25], normal mouse eyes constitutively express Gal-9 in the corneal epithelium, endothelium, and iris-ciliary body; additionally, TIM-3 is expressed on CD8+ T cells that infiltrate corneal grafts. Blocking this pathway

could shorten the survival time of the corneal graft. It is suggested that this pathway plays a protective role in maintaining the immune privilege state of the eye. In recent years, the research focus of this pathway has gradually shifted to the field of retinal diseases. In common retinopathy, galectin family members such as Gal-9 participate in the disease process by regulating the activation of microglia, vascular dysfunction and oxidative stress [26]. Inhibition of Gal-3 can significantly reduce the activation of microglia and delay the progression of retinal degeneration [27]. In addition, TIM-3 was expressed in more than 50% of the intraocular lesion T cells in the herpes simplex virus (HSV)-induced keratopathy model, and Gal-9 treatment reduced the severity of stromal keratitis and neovascularization [28]. These observations offer a histological and biological rationale for the increased serum sTIM-3 and Galectin-9 levels seen in our POAG cohort. In this study, sTIM-3 and Galectin-9 levels were already higher in the stable group than in the healthy control group, and further increased in the progression group, a gradient consistent with the pattern of "inflammatory/active" versus "inactive/control" levels described above in the basic study. It is suggested that the elevation of these two markers may reflect a state of intraocular immune dysregulation or neuroinflammation associated with the progression of POAG. We acknowledge that the absence of aqueous humor measurements is a limitation in establishing a direct correlation between serum sTIM-3/Galectin-9 levels and intraocular neuroinflammation. However, several considerations support the clinical relevance of peripheral detection in this context. First, accumulating evidence indicates that neuroinflammation in glaucoma is not an isolated intraocular event but is accompanied by systemic immune alterations, including changes in circulating cytokine profiles and immune cell subsets [29-31]. Therefore, elevated serum levels of immune-related molecules may reflect systemic immune dysregulation that parallels or contributes to the intraocular pathological process. Second, although aqueous humor provides more direct access to intraocular molecular changes, its invasive collection limits routine clinical application. Serum biomarkers, by contrast, offer practical advantages including non-invasiveness, ease of repeated sampling, and high patient acceptability, making them more feasible for large-scale screening and routine follow-up [5]. Third, previous studies have demonstrated correlations between serum and aqueous humor levels of inflammatory markers in ocular diseases, suggesting that peripheral measurements can provide meaningful information about intraocular immune status [32, 33]. Nevertheless, we fully acknowledge that the lack of paired aqueous humor data limits our ability to definitively attribute the observed serum elevations to local ocular inflammation. Future studies incorporating simultaneous detection of sTIM-3 and Galectin-9 in both serum and aqueous humor, along with correlation analyses, are needed to validate the relationship between peripheral and intraocular levels and to more accurately reflect the intraocular immune microenvironment.

As a kind of chronic neurodegenerative disease, the pathological process of glaucoma involves the activation of microglia, oxidative stress and neuroinflammation. This study found that sTIM-3 and Galectin-9 levels were independently associated with POAG progression, which was consistent with the mode of action of Galectin-9/Tim-3 pathway in basic research on dopaminergic neurodegenerative diseases. In a mouse model of Parkinson's disease induced by MPTP, Peng et al. [27] demonstrated that Gal-9 enhances microglial activation and upregulates pro-inflammatory cytokines such as IL-6, IL-1β, and TNF-α. Knockout of Gal-9 or blocking Tim-3 could alleviate the activation of microglia, reduce the loss of dopaminergic neurons and improve motor function, suggesting that Gal-9/Tim-3 pathway is involved in the pathogenesis of PD by increasing neuroinflammation. Moreover, recent work has shown that Tim-3 knockout ameliorates motor dysfunction and neuroinflammation in MPP^+/MPTP-induced PD models via the NF-κB/NLRP3 pathway, further supporting a proinflammatory role for Tim-3 in Parkinson's disease [34]. In the study of Alzheimer's disease, Ramirez Hernandez et al. [35] reported that ventricular injection of Aβ25-35 peptide could induce learning and memory impairment in rats, accompanied by enhanced oxidative stress and inflammatory response in the hippocampus. Immunohistochemistry revealed that microglial expression of Gal-9 and Tim-3 was markedly increased in the Aβ25-35 group, whereas no such upregulation was observed in

the control group. These findings indicate that Gal-9 and Tim-3 become elevated in activated microglia and may participate in modulating the neuroinflammatory response and neuronal damage triggered by Aβ. While direct evidence of Tim-3/Galectin-9 involvement in glaucoma neuroinflammation is still lacking, the pathogenic role of this pathway in PD and AD models aligns with our observation that higher sTIM-3 and Gal-9 levels correlate with POAG progression. Hence, this pathway could contribute to retinal ganglion cell damage through a similar process.

The prognostic significance of TIM-3 and Galectin-9 has been established across multiple diseases. In oncology, a meta-analysis [36] of 28 studies involving 7,284 cancer patients demonstrated that high TIM-3 expression independently predicted poorer overall survival (HR = 1.54, P = 0.001). High TIM-3 expression correlates with adverse outcomes in osteosarcoma, gastric cancer, liver cancer, esophageal cancer, and lymphoma. In colorectal cancer patients, Zhang et al. [37] reported significantly higher Tim-3 expression scores in tumor tissues than in adjacent normal tissues. This high expression was strongly associated with deeper tumor invasion, vascular infiltration, and advanced clinical stage. Kaplan-Meier analysis revealed that high Tim-3 expression was associated with markedly reduced recurrence-free and overall survival. The prognostic value of Galectin-9, however, depends on the tissue type. So et al. [38] reported that high Gal-9 expression was linked to better overall survival in solid tumors (HR = 0.75, P = 0.002), but to faster disease progression in hematologic malignancies (HR = 2.29, P = 0.007). Although the pathophysiological mechanisms of these diseases and POAG are different, these observations suggest that sTIM-3 and Galectin-9 may serve as universal markers of chronic inflammation or immune dysregulation, and their elevated levels are consistently associated with disease progression and poor prognosis across diseases.

At the methodological level, multi-dimensional validation of the main conclusions was carried out through trend test, subgroup analysis and E-value analysis. The trend test showed that sTIM-3 and Galectin-9 levels showed a monotonically increasing trend with the risk of progression, which strengthened the evidence for their association. Subgroup analysis showed that gender and age did not significantly alter the association between markers and progression (P for interaction > 0.05), suggesting that the association was consistent in patients with different clinical characteristics. E-value analysis showed that an association strength of 3.70-fold (sTIM-3) and 6.24-fold (Galectin-9) for unmeasured confounders would be required to fully explain the observed results, which is unlikely, as the analysis already adjusted for major confounders (IOP, MD, age, and sex). This further supports the robustness of our findings. Regarding medication-related confounding, we acknowledge that data on specific IOP-lowering medication and adherence were not included in the multivariate model due to the retrospective design. However, several factors mitigate this concern. First, baseline IOP was adjusted for in the model, which serves as a summary measure of the net effect of treatment intensity and adherence on the actual IOP level. Second, baseline MD, a strong predictor of glaucoma progression, was also included, reflecting baseline disease severity. Third, the E-value analysis itself provides quantitative evidence that unmeasured confounders-including medication type or adherence-would need to be associated with both the biomarker level and disease progression by at least 3.70-fold (sTIM-3) and 6.24-fold (Galectin-9) to fully negate the observed associations, a strength of association unlikely for these factors in clinical practice. Nevertheless, we fully acknowledge this limitation and have emphasized in the Limitations section that future prospective studies should systematically record medication regimens and adherence to enable more comprehensive adjustment. Although the lack of serial measurements precludes an assessment of dynamic changes, the baseline association observed in this study remains clinically relevant. Blood sampling at initial presentation is logistically feasible and non-invasive, making baseline sTIM-3 and Galectin-9 levels potentially useful for early risk stratification. This information could assist clinicians in identifying patients who may warrant closer follow-up, even though these markers cannot currently serve as real-time monitoring tools.

Clinically, higher sTIM-3 and Galectin-9 levels independently correlated with POAG progression. This association persisted after adjusting for visual field damage (baseline MD) and sev-

eral other confounding variables. At present, the clinical diagnosis of glaucoma progression mainly depends on visual field and OCT morphological function examination, and there is still a lack of simple and quantifiable serological indicators as auxiliary reference. Compared with the above tests, serum marker detection has the advantages of convenient operation, high patient acceptance, and no reliance on expensive equipment. As immune checkpoint related molecules, serum sTIM-3 and Galectin-9 may reflect the potential immune dysregulation in POAG patients and provide new reference information for clinical follow-up. Nevertheless, given the retrospective design of this study, these markers are not intended to replace current examination methods, and their clinical utility must be confirmed through prospective investigations. While our findings support the potential utility of these markers, future studies are needed to establish specific risk stratification thresholds (e.g., optimal cut-off values for sTIM-3 and Galectin-9) and to construct a joint prediction model incorporating traditional risk factors. Such a model could inform a practical clinical screening algorithm-for example, patients with elevated baseline levels could be recommended for more frequent visual field and OCT monitoring. These translational steps are essential to move these biomarkers from correlative findings to actionable clinical tools.

Limitations and future directions

Several limitations must be considered. First, serum sTIM-3 and Galectin-9 were measured only at baseline, without repeated sampling during the 12-month follow-up. Therefore, this study could not evaluate whether longitudinal changes in these markers correlate with the rate of glaucoma progression, and their utility as real-time monitoring indicators remains unestablished. Future prospective studies with serial measurements (e.g., every 6-12 months) are needed to characterize the temporal trajectories of these markers and to determine whether changes in their levels parallel structural and functional deterioration. Second, the retrospective, single-center nature and modest sample size (n = 120) may introduce selection bias, and the study lacks an independent external validation cohort or internal cross-validation to confirm the generalizability of the risk association and dose-response relationship. Hence, these results should be validated in prospective multi-center studies with larger samples. Third, the multivariate regression model did not include the type of IOP-lowering medication or medication adherence, both of which may influence disease progression. This was due to the retrospective study design, where detailed medication records and adherence data were not systematically captured. Although baseline IOP (which reflects the net effect of treatment and adherence) and baseline MD (reflecting baseline severity) were adjusted for in the model, and E-value analyses suggested that residual confounding is unlikely to fully explain the observed associations, the lack of these variables remains a limitation. Future prospective studies should systematically document medication regimens and assess adherence to allow more comprehensive adjustment. Fourth, the corresponding markers in aqueous humor were not detected in this study, and the correlation between serum levels and local intraocular inflammation cannot be definitively determined. While serum biomarkers offer practical advantages for clinical application, the lack of paired aqueous humor data limits mechanistic interpretation. Future studies combining serum and aqueous humor analyses will help clarify the biological relevance of these markers to intraocular neuroinflammation. Fifth, the study subjects were all Han Chinese, and whether the conclusions are applicable to other ethnic groups or regions remains unknown. Sixth, subgroup analyses were limited to age and sex stratification; we did not perform stratified analyses by baseline disease severity (e.g., early vs. moderate vs. advanced glaucoma based on baseline MD) or by IOP-lowering medication class, due to the limited sample size and the retrospective nature of medication records. Whether the predictive value of these markers varies across different disease stages or treatment regimens requires further investigation in larger cohorts. Seventh, although commercially available ELISA kits with established protocols were used, we did not systematically evaluate batch-to-batch variability or inter-assay precision (e.g., inter-operator variability among testing personnel). The lack of repeated measurements of the same samples across different batches may introduce detection bias. Future studies should include quality control measures such as internal controls and replicate testing to ensure assay reliability. Future stud-

ies should focus on the following aspects: ① further validation in prospective, multi-center, large-scale POAG cohorts to confirm the stability and generalizability of these findings; ② incorporation of repeated sTIM-3 and Galectin-9 measurements (e.g., every 6-12 months) to assess the correlation between longitudinal changes in these markers and the progression rate of visual field and OCT structural parameters; ③ construction of risk prediction models combining sTIM-3 and Galectin-9 with other immune-inflammatory indicators (such as cytokines, neurofilament light chain, and glial fibrillary acidic protein) and traditional risk factors (IOP, MD, RNFL thickness) to evaluate incremental predictive value; ④ simultaneous detection of sTIM-3 and Galectin-9 in aqueous humor to analyze the correlation between serum and local intraocular levels, thereby more accurately reflecting the intraocular immune microenvironment; ⑤ validation of the universality of these findings in populations of different races and regions to assess their potential as broadly applicable markers; ⑥ performing internal cross-validation (e.g., bootstrap or k-fold) and external validation in independent cohorts to assess the reproducibility and generalizability of the predictive associations; ⑦ establishing specific risk stratification thresholds (e.g., optimal cut-off values for sTIM-3 and Galectin-9) and developing a joint prediction model integrated with traditional risk factors, alongside a practical clinical screening algorithm - for example, patients with elevated baseline levels could be recommended for more frequent visual field and OCT monitoring to translate these correlative findings into actionable clinical tools.

Conclusions

In summary, our retrospective cohort findings suggest that higher baseline sTIM-3 and Galectin-9 levels are independently associated with an increased risk of POAG progression, exhibiting a dose-response pattern. These biomarkers may serve as adjunctive reference markers for risk stratification at baseline, rather than real-time monitoring tools. Prospective studies with repeated measurements are warranted to validate their clinical utility and to explore whether longitudinal changes in these markers reflect disease dynamics.

Disclosure of conflict of interest

None.

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