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Original Research

Tear Cytokines in Treated Primary Open-Angle Glaucoma: An Exploratory Study

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Abstract

Background: Tear fluid provides a minimally invasive matrix for the study of ocular surface inflammation. In a study conducted over approximately two weeks, we compared tear interleukin-1 beta (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ) concentrations between participants with treated primary open-angle glaucoma (POAG) and healthy controls across three visits. **Methods:** This exploratory matched case-control study included 30 participants: 15 with treated POAG and 15 healthy controls. Sampling was scheduled at baseline, Day 7 ± 2 days, and Day 14 ± 2 days. At each visit, three tear withdrawals from one study eye were pooled, and duplicate measurements of the four cytokines were performed using test cassettes read with the i-ImmunDx Analyzer. Cytokine-specific linear mixed-effects models included group, visit, their interaction, age, sex, and a participant-specific random intercept. The Benjamini–Hochberg procedure was applied to the four primary between-group comparisons. **Results:** Across 90 observations per cytokine, adjusted geometric mean ratios (GMRs) comparing participants with treated POAG with healthy controls were 1.44 for IL-1β (95% confidence interval [CI], 1.33–1.57), 1.39 for IL-6 (95% CI, 1.33–1.44), 1.42 for TNF-α (95% CI, 1.35–1.50), and 1.27 for IFN-γ (95% CI, 1.16–1.40). These ratios correspond to adjusted geometric mean concentrations that were 44%, 39%, 42%, and 27% higher, respectively, in participants with treated POAG than in healthy controls. All four primary comparisons had $q < 0.001$ after false discovery rate correction. Exploratory interaction tests did not establish temporal equivalence. Boundary or singularity warnings and residual diagnostic concerns limited confidence in the precision of the model-based estimates. **Conclusions:** Participants with treated POAG had higher average tear IL-1β, IL-6, TNF-α, and IFN-γ concentrations than healthy controls. These differences do not distinguish glaucoma-related effects from those of topical treatment, preservative exposure, or ocular surface inflammation. Interpretation is further limited by the small sample size and model diagnostic issues. The results are exploratory and require independent validation before disease specificity, reproducibility, or clinical utility can be established.

Keywords: open-angle glaucoma; tears; inflammation mediators; cytokines; biomarkers

1. Introduction

Glaucoma is a neurodegenerative optic neuropathy characterized by progressive loss of retinal ganglion cells and their axons, leading to optic nerve cupping and irreversible visual field loss [1]. Glaucoma is the leading cause of global irreversible blindness. An estimated 76 million people were affected by this disease in 2020, with more than 111 million expected by 2040 [2]. High intraocular pressure (IOP) is the most important modifiable risk factor for glaucoma progression: the treatment, including medical, laser, or surgical interventions, focuses on lowering IOP [3]. However, glaucoma is a multifactorial disease: aging, myopia, family history, thin central corneal thickness (CCT) and low corneal hysteresis, low ocular blood flow (OBF), lamina cribrosa abnormalities, oxidative stress, neuroinflammation, and lifestyle factors, such as diabetes, sleep apnea, diet, and smoking also modulate disease onset and course [4]. In fact, the clinical course of primary open-angle glaucoma (POAG) is heterogeneous, and a subset of patients continues to progress despite apparently adequate IOP control, suggesting that mechanisms other than mechanical pressure-related stress may contribute to glaucomatous neurodegeneration [5].

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The pathophysiology of POAG is complex and involves interactions among ocular hypertension, impaired aqueous humour outflow, vascular dysregulation, oxidative stress, mitochondrial dysfunction, excitotoxicity, impaired axonal transport, glial activation, and immune-mediated mechanisms. In recent years, increasing attention has been directed toward the role of neuroinflammation in glaucomatous damage [6]. Although inflammation in glaucoma is not classically characterized by overt intraocular inflammatory signs, experimental and clinical evidence suggests that chronic low-grade inflammatory activation may contribute to retinal ganglion cell vulnerability and optic nerve head remodeling. Activated Müller cells, astrocytes, microglia, trabecular meshwork cells and ocular surface epithelial cells can secrete cytokines, chemokines, growth factors and matrix-modifying enzymes that can influence neuronal viability, extracellular matrix dynamics, vascular permeability and immune cell recruitment.

Of the inflammatory mediators, particular attention should be paid to tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8/CXCL8), monocyte chemoattractant protein-1 (MCP-1/CCL2), interferon-gamma (IFN-γ), interleukin-10 (IL-10), and vascular endothelial growth factor (VEGF). TNF-α has been linked to retinal ganglion cell death via excitotoxic pathways, oxidative stress, mitochondrial dysfunction, and receptor-mediated apoptotic signaling [7]. IL-1β and IL-6 may represent activation of innate immune mechanisms and stress responses in epithelial or glial cells [8], whereas IL-8 and MCP-1 are involved in chemotaxis and amplification of local inflammatory networks [9].

Tear fluid has emerged as a promising method for investigating ophthalmic biomarkers. It can be obtained by minimally invasive techniques and contains a broad array of molecular and vesicular components that mirror the biology of the ocular surface and anterior segment. Tears can be collected repeatedly with minimal discomfort, making them more suitable for longitudinal biomarker studies than aqueous humour, which requires an invasive intraocular approach and is therefore limited to the surgical environment. However, tear fluid composition is subject to substantial pre-analytical and biological variability due to sampling conditions, ocular and systemic factors, treatment exposure and sample handling. Therefore, consistent collection and analytical methods are essential to produce interpretable and reproducible tear biomarker data [10,11,12].

Multiplex immunoassays allow several inflammatory mediators to be measured simultaneously in small tear volumes [13]. Repeated sampling can help characterize between-group differences and short-term variation, although these measurements alone do not establish biomarker reproducibility or clinical utility.

Previous studies have demonstrated altered tear cytokine profiles in primary open-angle glaucoma (POAG); however the extent and direction of these alterations are not entirely consistent between studies. Gupta et al. [14] evaluated the tear film cytokines in newly diagnosed eye drop-naïve POAG patients and reported that the mean concentrations of most of the measured pro-inflammatory cytokines were lower than those of controls, with a significant reduction in IL-12p70 levels, suggesting that early untreated POAG may be characterized by a different tear cytokine profile. In contrast, studies including treated glaucoma patients have more frequently reported evidence of ocular surface inflammatory activation. Burgos-Blasco et al. [15] evaluated tear and aqueous humour cytokines in POAG and found differences in inflammatory mediator profiles between POAG patients and healthy controls, supporting the concept that tear fluid may reflect glaucoma-related or treatment-related inflammatory changes; Csősz et al. [16] compared cytokine profiles in glaucomatous tears and aqueous humour using multiplex immunobead assays and showed that tear cytokine composition differs from aqueous humour, suggesting that the higher cytokine levels in tear compared to aqueous humour might reflect not only the glaucoma-related, but also the pro-inflammatory conditions induced by eye drops or other factors.

Topical glaucoma therapy represents an important potential confounder in tear cytokine studies, and its association with ocular surface inflammation should be interpreted in relation to the specific treatment formulation and preservative exposure. Long-term exposure to preserved IOP-lowering eye drops, particularly formulations containing benzalkonium chloride, may disrupt ocular surface homeostasis, compromising epithelial integrity and tear-film stability while promoting goblet cell loss and chronic conjunctival inflammation. Martinez-de-la-Casa et al. [17] reported significantly higher levels of several tear cytokines in patients receiving preserved latanoprost than in healthy controls, whereas no statistically significant differences in the cytokines assessed were detected between patients receiving preservative-free latanoprost and healthy controls. Benitez-Del-Castillo et al. [18] reported differences in tear cytokine and chemokine profiles in patients with glaucoma or dry eye disease and also examined differences associated with preservative exposure within the POAG group. These findings should be interpreted within the specific treatment and ocular surface contexts investigated; findings from preservative-exposed groups should not be generalized to all patients receiving treatment for POAG. Therefore, preservative exposure, number of topical medications, treatment duration, and ocular surface status should be documented and considered when interpreting tear cytokine findings in POAG.

Despite increasing evidence supporting the involvement of inflammatory pathways in glaucoma, several gaps remain. First, the interpretation of tear cytokine data in glaucoma remains challenging, largely because differences in clinical context and methodological design limit comparability across studies. Second, most published tear cytokine studies in POAG are cross-sectional and do not adequately characterize short-term intra-individual variability.

Third, few studies have used repeated sampling within the same individual over multiple visits, which is essential to determine whether tear cytokines are sufficiently stable for use as biomarkers. Fourth, many analyses do not fully account for the hierarchical structure of repeated tear measurements, in which multiple samples are nested within visits and visits are nested within subjects. Finally, the relationship between tear cytokine levels and clinical glaucoma severity remains incompletely defined.

Current evidence supports further investigation of tear inflammatory mediators, but interpretation remains constrained by methodological heterogeneity, short-term variability, and treatment-related ocular surface effects. The present exploratory study compared four tear cytokines between participants with treated POAG and healthy controls over three visits. It also described short-term variation and examined cytokine values alongside ocular measurements without testing clinical associations.

2. Materials and Methods

2.1 Study Design and Setting

This prospective observational study used an exploratory matched case-control design with repeated measurements. The primary objective was to compare tear IL-1β, IL-6, TNF-α, and IFN-γ concentrations between participants with treated POAG and healthy controls matched by age and sex. Three visits over approximately two weeks allowed exploratory assessment of variation across visits. Reporting was guided by the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement [19]; a reporting checklist is provided after the references.

2.2 Participants and Clinical Assessment

Participants were recruited from the ophthalmology and glaucoma clinical services of G.B. Morgagni, Catania, Italy, between July and August 2026. The glaucoma group included patients with a clinical diagnosis of POAG, and controls had no glaucoma, ocular hypertension, or optic neuropathy. Healthy controls were frequency-matched to the POAG group by age and sex, with the aim of obtaining similar distributions of these characteristics across groups. No individual case-control pairs were formed. The planned sample was 30 participants, with 15 in each group. Visits were scheduled at baseline, Day 7 ± 2 days, and Day 14 ± 2 days. At each visit, three sequential tear withdrawals from one study eye were pooled before laboratory analysis. Each participant therefore contributed one biological observation per cytokine at each visit, with a maximum of three observations per cytokine overall.

Patients were eligible for inclusion in the glaucoma group if they were aged 40 years or older, had a confirmed clinical diagnosis of POAG, were receiving stable glaucoma therapy for at least 3 months, were willing to attend all scheduled study visits, and provided written informed consent. Healthy controls were eligible if they were aged 40 years or older, had no diagnosis of glaucoma or ocular hypertension, had a normal optic nerve examination, normal IOP, no evidence of optic neuropathy, and provided written informed consent. Exclusion criteria for both groups were active ocular infection, allergic conjunctivitis, moderate or severe dry eye disease, ocular surgery within the previous 6 months, topical anti-inflammatory therapy within the previous 3 months, contact lens use within the previous month, systemic inflammatory disease, autoimmune disease, recent systemic infection, pregnancy or breastfeeding, and inability to comply with the study protocol. These criteria were selected to minimize the impact of ocular surface inflammation, systemic immune activation, and recent surgical or pharmacological confounders on tear cytokine levels.

All participants with POAG had early to moderate glaucoma, with visual field mean deviation values between –2 and –12 dB, and none had undergone glaucoma surgery before enrollment. POAG participants were required to have received stable topical glaucoma therapy for at least three months. This criterion established treatment stability but did not quantify cumulative exposure. Detailed information on medication burden, individual drug classes, preservative exposure, treatment duration, and ocular surface findings was not available for adjustment in the reported analyses.

At every visit, the study protocol specified assessment of best-corrected visual acuity when available, IOP, ocular surface symptoms, the sampling time, and difficulties with sampling. According to the protocol, structural and functional diagnostic information was to be collected from POAG participants along with disease duration, number and class of medications, and exposure to preservatives. For participants with POAG, the eye with the clearest diagnostic documentation and reliable visual field testing was chosen. In controls, the study eye was randomly selected or laterality matched when appropriate.

2.3 Tear Collection and Storage

To reduce pre-sampling variability associated with time of day [20], reflex tearing, topical medication, and ocular surface manipulation, tear collection was standardized. Samples were collected between 9:00 and 11:00 AM, with each participant attending at approximately the same time of day. Participants were instructed to avoid caffeine and smoking for at least two hours before sampling and facial creams and eye makeup on the morning of the visit. Topical eye drops were avoided for two hours before collection, and no diagnostic drops were instilled beforehand. This short interval does not eliminate the effects of chronic topical therapy or preservative exposure.

Basal tear fluid was collected using sterile glass microcapillary tubes without touching the cornea or conjunctiva, a commonly used method for tear sampling in biomarker

research because it allows collection of relatively pure tear fluid while avoiding dilution and contamination.

At each visit, three sequential tear withdrawals of approximately 5 µL each were obtained from the same study eye, with intervals of approximately 2–3 minutes. The next withdrawal was therefore not started immediately after the preceding collection. The three withdrawals were pooled into one participant-specific sample for that visit, yielding an expected total volume of approximately 15 µL. Each pooled sample represented one biological observation per cytokine; individual withdrawals were not analyzed independently. Sampling difficulties, insufficient volume, reflex tearing, and accidental ocular surface contact were recorded. Samples were placed on ice, transferred to sterile low-binding microtubes, and stored at –80 °C until analysis. Repeated freeze–thaw cycles were avoided, and processing time, storage conditions, assay identification, and quality-control indicators were recorded.

2.4 Multiplex Analysis of Tear Cytokines

Tear IL-1β, IL-6, TNF-α, and IFN-γ concentrations were quantified using the i-ImmunDx assay system (reported kit catalogue number: 697297646S03VG; Seinda Biomedical Corporation, Guangzhou, China), with test cassettes read using the standalone i-ImmunDx Analyzer, according to the manufacturer's instructions. According to the manufacturer's platform documentation, the analyzer identifies test cassettes through their barcodes and applies the corresponding test-specific protocols, calibration curves, and detection parameters. The platform provides automated quantitative readout and storage of test results. Measurements did not involve flow cytometric bead-array acquisition. Each pooled sample was analyzed in duplicate. Duplicate measurements were technical replicates and did not increase the number of independent biological observations. Duplicate variability was assessed using the intra-assay coefficient of variation. Samples exceeding predefined quality-control thresholds were reanalyzed when sufficient material was available or excluded from analyte-specific analyses.

2.5 Outcomes

The primary outcome was the between-group difference in the four tear cytokine concentrations, averaged across visits. A secondary exploratory objective was to characterize changes in cytokine concentrations across the three visits. Short-term reproducibility was not formally assessed. Assessment of medication burden, drug class, and preservative exposure had been envisaged, but these effects could not be evaluated with the analyzable information available.

2.6 Statistical Analysis

Statistical analyses included participants with at least one technically valid cytokine measurement. Continuous variables were summarized using means and standard deviations, as appropriate, and categorical variables using frequencies and percentages. The analytical approach specified logarithmic transformation of markedly skewed cytokine concentrations.

For each cytokine, a linear mixed-effects model included study group (treated POAG versus healthy controls), categorical visit (baseline, Day 7, and Day 14), the group-by-visit interaction, age, and sex as fixed effects, with a participant-specific random intercept to account for repeated measurements. Age and sex were included as covariates to adjust for the frequency-matching factors. Because matching was performed at the group level and no individual case-control pairs were formed, no matched-pair term was included. The model specification was: transformed cytokine concentration ~ group × visit + age + sex + (1 | participant). Small-sample inference used the Kenward–Roger approximation.

The primary contrast was the equally weighted average of the three visit-specific between-group differences on the log-concentration scale, calculated as a linear contrast of the model's fixed-effect estimates. Baseline, Day 7, and Day 14 each received a weight of one-third. Results were reported as geometric mean ratios (GMRs), with 95% confidence intervals and two-sided p values. The Benjamini–Hochberg procedure controlled the false discovery rate across the four primary between-group cytokine comparisons. Visit-specific comparisons, temporal effects, and group-by-visit interactions were exploratory and hypothesis-generating. Their p values and confidence intervals were not adjusted for multiplicity and were not used to establish definitive temporal or clinical conclusions.

Information on analyte-specific LOD and LOQ values and sample-level censoring flags was unavailable in the analysis database. Laboratory handling of below-threshold results before database entry could not be verified. Mixed-effects models used available valid observations without single-value imputation. Model diagnostics included assessment of boundary or singularity warnings, heteroscedasticity, residual normality, and extreme standardized residuals. Generalized estimating equations (GEE) and leave-one-subject-out analyses were used as sensitivity analyses. The analysis did not include a formal assessment of short-term reproducibility using intraclass correlation coefficients (ICCs). The near-zero random-intercept estimate for IL-6, the absence of a group-by-visit interaction, and the agreement between model estimates should not be interpreted as evidence of biological stability or reproducibility. Given the sample of 30 participants, interpretation emphasized effect sizes while acknowledging uncertainty in their precision. Analyses were performed using R (version 4.6.1, manufacturer R Foundation for Statistical Computing, Vienna, Austria).

3. Results

3.1 Participants and Available Measurements

All 30 participants (15 with treated POAG and 15 healthy controls) had complete cytokine measurements at all three visits, resulting in 90 observations per analyte and 360 analyte-level observations in total. All enrolled participants completed the three scheduled visits, with no post-enrollment losses. These observations were derived from 30 distinct participants rather than 90 independent individuals per analyte. Each visit's three pooled tear withdrawals represented one biological observation per cytokine, and technical duplicates did not increase the number of independent observations.

Classification relative to the assay-specific LOD and LOQ was unavailable for all four cytokines; consequently, the numbers of measurements below each threshold could not be determined.

Although IOP and retinal nerve fiber layer (RNFL) measurements were available for descriptive graphical assessment in the POAG group, a comprehensive baseline comparison of clinical and treatment characteristics between groups is not presented.

3.2 Primary Between-Group Comparisons

Throughout the visits, the adjusted geometric mean concentrations for all 4 cytokines were higher in the POAG group. The IL-1β geometric mean ratio was 1.44 (95% CI, 1.33–1.57), corresponding to a 44% higher adjusted geometric mean than in controls. The corresponding ratios for IL-6, TNF-α, and IFN-γ were 1.39 (95% CI, 1.33–1.44), 1.42 (95% CI, 1.35–1.50), and 1.27 (95% CI, 1.16–1.40), respectively. After the Benjamini–Hochberg correction, all 4 main comparisons remained statistically significant ($q < 0.001$). Fig. 1 shows descriptive participant-level distributions rather than adjusted model estimates.

3.3 Exploratory Temporal Findings

Visit-specific geometric mean ratios ranged from 1.17 to 1.49 across the four analytes. The group-by-visit interaction tests did not provide clear evidence of differences in temporal patterns between groups, with p values ranging from 0.063 for IFN-γ to 0.949 for IL-6. However, the small sample size (15 participants per group) limited statistical power to detect interaction effects. Consequently, these nonsignificant findings do not demonstrate similar temporal profiles or equivalence between the groups, and potentially meaningful differences in changes over time cannot be excluded.

Exploratory analyses of visit effects averaged across groups showed variation by visit for IL-6 ($p = 0.001$), TNF-α ($p < 0.001$), and IFN-γ ($p < 0.001$), but not for IL-1β ($p = 0.107$). These exploratory comparisons were not adjusted for multiple comparisons. No equivalence analysis was performed.

3.4 Descriptive Ocular Measurements

As illustrated in Fig. 2, the participant-level cytokine summaries were related to either IOP or RNFL thickness in the POAG group. The four cytokines showed no consistent directional pattern. The displays were descriptive, with no adjusted association estimates or measures of predictive performance available. No correlation coefficients, regression estimates, confidence intervals, or hypothesis tests are presented. The plots should not be interpreted as evidence of an association between cytokine concentrations and IOP, structural damage, or glaucoma severity.

3.5 Model Diagnostics and Sensitivity Analyses

The GEE sensitivity analyses produced the same rounded GMRs as the primary mixed-effects analyses, with very similar confidence intervals. Estimated working correlations ranged from –0.029 to 0.173. Nevertheless, all mixed-effects fits generated boundary or singularity warnings. For IL-6, the estimated random-intercept contribution was essentially zero, indicating that this component contributed negligibly to the fitted model. A common random-intercept specification was retained across the four cytokines to reflect the repeated-measurement design, although the near-zero estimate for IL-6 raises questions about the necessity of this component for that analyte.

Residual diagnostics identified heteroscedasticity and extreme standardized residuals for IL-1β and TNF-α, while IFN-γ showed residual non-normality and an extreme standardized residual. These findings raise concerns regarding the assumptions underlying model-based variance estimation and inference. The Kenward–Roger approximation does not, by itself, resolve these concerns.

The geometric mean ratios in leave-one-subject-out analyses ranged from 1.42 to 1.47 for IL-1β, from 1.38 to 1.40 for IL-6, from 1.40 to 1.43 for TNF-α, and from 1.23 to 1.29 for IFN-γ. The omission of individual participants produced relatively small changes in the point estimates. However, these findings do not resolve uncertainty regarding variance estimation, confidence-interval coverage, and biological reproducibility.

4. Discussion

This exploratory case-control study found higher average tear IL-1β, IL-6, TNF-α, and IFN-γ concentrations in participants with treated POAG than in matched healthy controls. The reported adjusted GMRs ranged from 1.27 to 1.44. These observations concern four measured cytokines and do not establish a broader cellular or molecular profile or a disease-specific inflammatory signature. Overall, the present findings are broadly consistent with the existing literature on tear cytokine profiles, although some discrepancies remain. Gupta et al. [14] studied newly diagnosed, eye drop-naïve POAG and found lower mean concentrations for 8 of 10 measured cytokines, with a statistically significant reduction in IL-12p70. Conversely, Martinez-

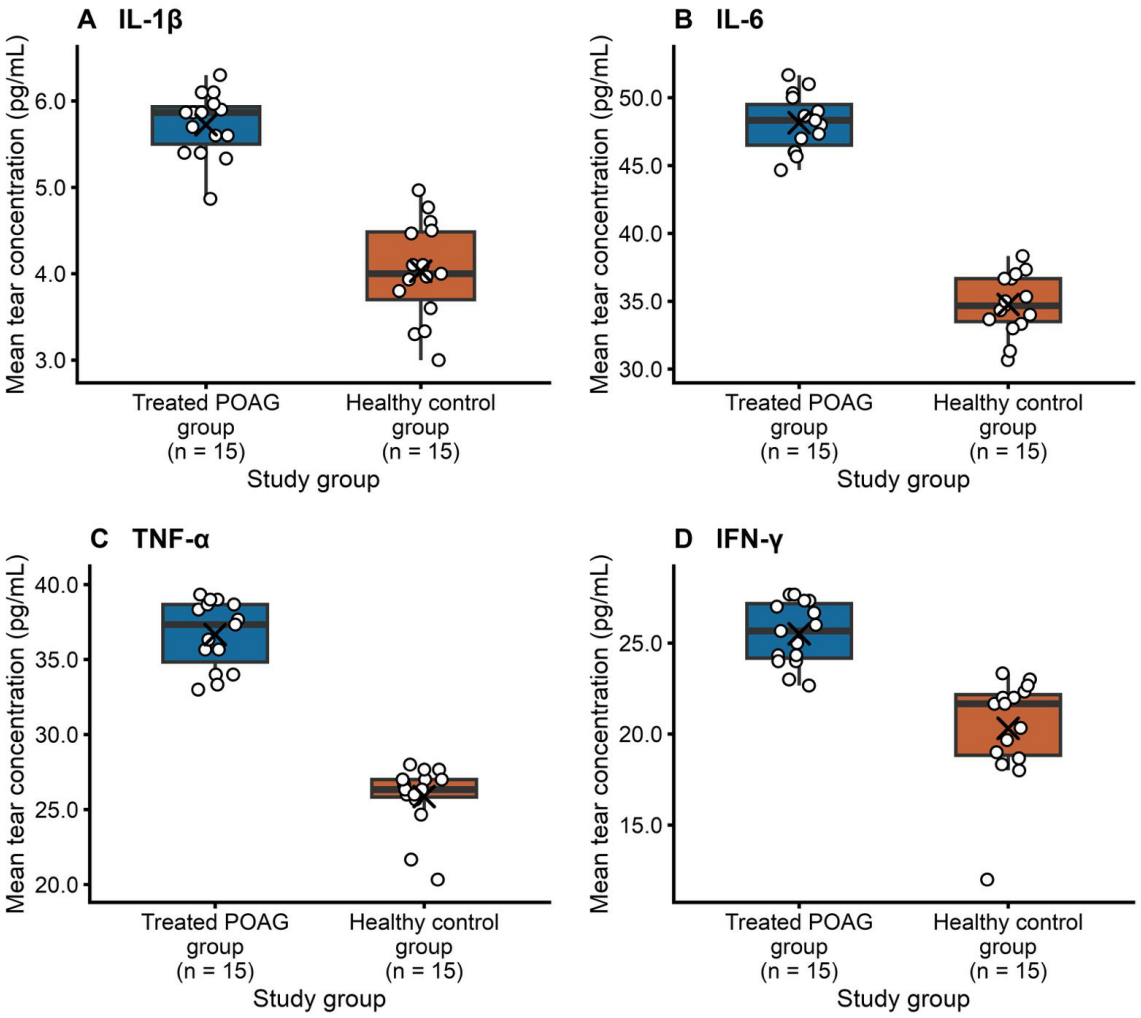


Fig. 1. Agraphical representation of tear cytokines in treated POAG patients and healthy controls. The plots from (A) to (D) demonstrate the distributions of IL-1β, IL-6, TNF-α, and IFN-γ, respectively. The mean participant-level values for each distribution were calculated over three sampling visits. The glaucoma group is represented in blue and the control group in red. The adjusted geometric mean ratios and their confidence intervals are not shown in these descriptive plots. POAG, primary open-angle glaucoma; IL-1β, interleukin-1 beta; IL-6, interleukin-6; TNF-α, tumor necrosis factor-alpha; IFN-γ, interferon-gamma.

de-la-Casa et al. [17] reported higher tear inflammatory cytokines in treated POAG than in healthy controls and observed cytokine differences according to preservative exposure. Mravec Bencurova et al. [21] reported higher tear IL-1β concentrations in patients with glaucoma or ocular hypertension than in healthy controls, consistent with the direction of the IL-1β difference observed in the present study. Direct comparison across these studies is nevertheless limited by substantial differences in treatment exposure, ocular surface status, disease spectrum, analyte panels, collection procedures, laboratory platforms, and statistical approaches. Consequently, the available evidence does not yet define a consistent tear-cytokine signature associated with POAG.

Differences between ocular compartments should also be considered when interpreting cytokine profiles. Burgos-Blasco et al. [15] reported a tear profile in POAG characterized by increases in IL-4, IL-12, IL-15, basic FGF, and VEGF and a decrease in MIP-1α, together with poor correlation between tear and aqueous humour cytokine levels. In paired glaucomatous samples, Csősz et al. [16] likewise found substantially higher concentrations of many cytokines in tears than in aqueous humour and concluded that the two matrices showed distinct inflammatory response profiles. Accordingly, the present tear measurements should primarily be interpreted as an ocular surface/anterior segment signal. TNF-α signaling and other inflammatory pathways have biological plausibility in glaucomatous neurodegeneration [7,8], but this evidence alone cannot determine the origin of the measured tear cytokines.

The between-group comparison combines differences in glaucoma status with differences in topical glaucoma treatment. The presence of increased cytokine concentrations in treated POAG tears cannot therefore be interpreted

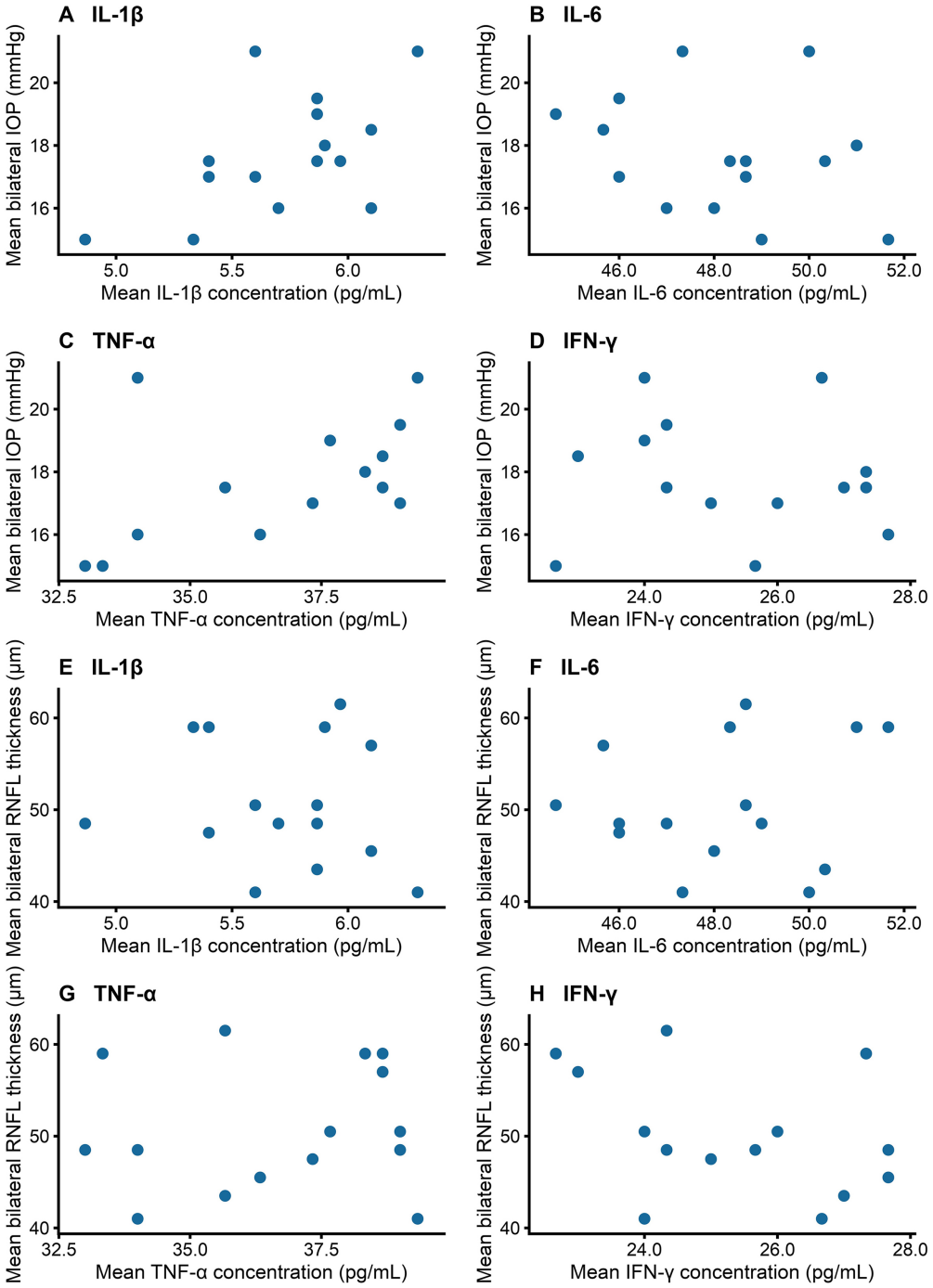


Fig. 2. The relationships between ocular measurements and tear cytokines in the glaucoma group were explored. (A–D) Intraocular pressure (IOP) plotted against IL-1β (A), IL-6 (B), TNF-α (C), and IFN-γ (D). (E–H) Retinal nerve fiber layer (RNFL) thickness plotted against IL-1β (E), IL-6 (F), TNF-α (G), and IFN-γ (H). The horizontal axes represent cytokine values and the vertical axes represent IOP or RNFL thickness. Each plotted observation represents the average of three samples taken from each subject. IL, interleukin; TNF, tumor necrosis factor; IFN, interferon. The displays are considered exploratory and do not establish adjusted clinical associations or predictive performance.

as a disease-specific inflammatory signature. The findings may have been influenced by chronic medication exposure, preservatives, mild or subclinical ocular surface inflammation, and their interactions [17,18]. The lack of detailed information prevented adjustment for medication burden, drug classes, preservative exposure, duration of treatment, and ocular surface findings. Neither age and sex matching nor adjustment for these variables resolves this confounding. Excluding moderate or severe dry eye does not exclude mild or subclinical ocular surface inflammation.

Standardized morning tear collection was performed from one eye per participant, with withdrawals pooled to obtain sufficient assay volume. Repeated sampling provided measurements across three visits. The primary analysis included a participant-specific random intercept and correction for multiplicity across the four primary comparisons. These design features support a structured exploratory comparison but do not establish the validity of model-based precision estimates, given the diagnostic concerns described above.

False discovery rate correction for the four main comparisons does not address unmeasured confounding or model misspecification and does not extend to the exploratory temporal analyses. In the same manner, concordant sensitivity estimates do not demonstrate biological stability, nor do they validate the confidence intervals reported. These results need to be confirmed in larger, clinically well-characterized cohorts.

Future research should investigate whether tear cytokine profiles track glaucoma progression, vary with IOP control, or predict treatment response. Longitudinal studies integrating tear biomarkers with OCT and visual-field measures could also help distinguish disease-related inflammation from the effects of chronic topical therapy and identify biologically distinct POAG phenotypes.

5. Limitations

Thirty individuals participated in this exploratory study. Through repeated sampling, the number of measurements was increased without increasing the number of independent participants. The limited size of the sample restricted assessment of clinical subgroups and potential confounders and provided limited power for interaction tests. Recruitment from ophthalmology and glaucoma services and the eligibility criteria may impact the generalizability of our findings to community populations, patients with untreated POAG, other glaucoma subtypes, and those with significant ocular surface disease. It is not possible to quantify the direction and magnitude of selection-related bias from these data.

All POAG participants were on topical therapy, while control subjects were unexposed to glaucoma medication. We were unable to adjust for detailed medication burden, drug class, preservative exposure, duration of treatment, and ocular surface information. Consequently, the differences noted cannot differentiate effects due to glaucoma from treatment or ocular surface effects. Inflammation induced by treatment could increase the observed between-group differences, but we cannot quantify its contribution or the overall direction of residual confounding. Excluding moderate or severe dry eye does not rule out mild or subclinical surface abnormalities.

The three visits were made over a period of approximately two weeks, preventing any conclusions about long-term biomarker dynamics, glaucoma progression, or treatment response. The assessment of variability between withdrawals was not possible as they were all pooled for analysis. Further, assay duplicates were not independent biological replicates but were technical replicates only. A nonsignificant group-by-visit interaction does not establish temporal equivalence or within-participant repeatability. Short-term reproducibility was not formally assessed using ICCs, and the reliability of cytokine measurements across visits remains unestablished.

There were singularity or boundary warnings from the mixed-effects analyses, with an essentially zero estimated random-intercept variance contribution for IL-6. Residual concerns included heteroscedasticity, non-normality, and extreme residuals for several analytes. The concurrence of the point estimates from the sensitivity analyses does not resolve uncertainty in variance estimation or confidence-interval coverage. The four main comparisons of cytokines were subjected to false discovery rate correction, whereas the exploratory temporal analyses were not. The IOP and RNFL plots are descriptive and do not establish clinical associations. Incomplete reporting of assay-specific analytical characteristics, including detection and quantification limits, calibration ranges, cross-reactivity, and precision estimates, represents an additional limitation of the study.

Tear measurements do not identify the anatomical source of the mediators, nor can they establish retinal or optic nerve inflammation. Concurrent intraocular measurements, cellular profiling, and an external validation cohort were not included in the study. Diagnostic accuracy, clinically meaningful thresholds, and predictive performance remain unestablished. The findings encourage more research into tear cytokines in treated POAG patients, but not the clinical use of a disease-specific biomarker.

The absence of a comprehensive baseline clinical characterization further limits assessment of the representativeness of the study population and the potential influence of disease severity and treatment-related confounding on the observed cytokine differences.

6. Conclusions

In this exploratory comparison, participants with treated POAG showed higher average concentrations of four tear cytokines than healthy controls, with reported adjusted GMRs ranging from 1.27 to 1.44. The study does not establish that these differences originate from glaucoma itself, because topical treatment, preservative exposure, and ocular surface characteristics could not be separated adequately from disease status. Model diagnostic findings also limit confidence in the reported precision. Larger studies incorporating detailed treatment and ocular surface assessments, appropriate comparison groups, and independently validated analyses are required before tear cytokine measurements can be considered for clinical application.

Availability of Data and Materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

LC, AA, MZ, CG, DR, RA, VMEB, and MV designed the research study. CG, GG, MC, FC, and FV performed the research and prepared the draft. LC, AA, CG, GG, MC, FC, FV, VMEB, DR, RA, MV, and MZ analyzed the data. LC and AA wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The study was conducted in compliance with the ethical principles of the Declaration of Helsinki and the International Council for Harmonisation (ICH) Good Clinical Practice (GCP) guidelines. Approval was received from the University of Enna "Kore" ethics committee. Written informed consent was obtained from patients and healthy controls before study entry. University of Enna "Kore" ethics committee approval n. protocol 21375.

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Conflicts of Interest

The authors declare no conflicts of interest. Dario Rusciano, Roberta Amato, Francesco Cappellani, and Caterina Gagliano have affiliations with Neurovisual Science Technology, Research Division (NEST-G.R.I.D.O.), Catania, Italy, or the Mediterranean Foundation "G.B. Morgagni", Catania, Italy. These affiliations did not influence data interpretation or manuscript preparation. Given his role as the Guest Editor and Editorial Board member, Dario Rusciano had no involvement in the peer review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to Sarah D. Atkinson.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/FBL57240.

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