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TIMP1, TIMP2, and TIMP4 are increased in aqueous humor from primary open angle glaucoma patients

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Purpose: Elevated intraocular pressure (IOP) is the only known modifiable risk factor for primary open angle glaucoma (POAG), and it can be caused by reduced aqueous humor outflow from the anterior chamber. Outflow is predominantly regulated by the trabecular meshwork, consisting of specialized cells within a complex extracellular matrix (ECM). An imbalance between ECM-degrading matrix metalloproteinases (MMPs) and the tissue inhibitors of MMPs (TIMPs) within the trabecular meshwork is thought to contribute to POAG. This study aimed to quantify levels of TIMPs and MMPs in aqueous humor samples from glaucomatous and non-glaucomatous eyes, analyze MMP/TIMP ratios, and correlate results with age, IOP, and Humphrey's visual field pattern standard deviation (PSD).

Methods: Aqueous humor samples were collected from 26 non-glaucomatous control subjects before cataract surgery and 23 POAG patients undergoing trabeculectomy or cataract surgery. Analyte concentrations were measured using multiplexed immunoassays. Statistical significance was assessed with Mann–Whitney U tests, and Spearman's method was used to assess correlations with age, IOP, and PSD.

Results: Concentrations of TIMP1 ($p = 0.0008$), TIMP2 ($p = 0.002$), TIMP4 ($p = 0.002$), and MMP2 ($p = 0.020$) were significantly increased in aqueous humor samples from POAG versus cataract samples. For the majority of MMP/TIMP molar ratios calculated for the cataract group, TIMPs outweighed MMPs. In POAG, molar ratios of MMP2/TIMP1 ($p = 0.007$) and MMP9/TIMP1 ($p = 0.005$) showed a significant decrease, corresponding to an elevated excess of TIMPs over MMPs in POAG compared to cataract samples. Conversely, MMP2/TIMP3 ($p = 0.045$) and MMP3/TIMP3 ($p = 0.032$) molar ratios increased. Several MMP/TIMP molar ratios correlated with IOP ($r = 0.476–0.609$, $p = 0.007–0.034$) and PSD ($r = -0.482$ to -0.655, $p = 0.005–0.046$) in POAG samples and with age in cataract control samples.

Conclusions: An imbalance among MMPs and TIMPs was found in glaucomatous aqueous humor samples, with a shift toward raised TIMP levels. This may result in the inhibition of MMP activity, leading to an altered ECM composition in the TM and thereby contributing to increased outflow resistance.

Glaucoma is a complex optic neuropathy involving loss of peripheral vision and ultimately leading to blindness if untreated [1]. Worldwide, over 60 million people are affected by glaucoma, the majority of which can be classified as having primary open angle glaucoma (POAG) [2,3]. Although the disease etiology is not completely understood, an elevated intraocular pressure (IOP) is a major risk factor and is currently the sole target of drug intervention [4,5].

Intraocular pressure is predominantly determined by the balance between aqueous humor production by the ciliary body and its outflow through the trabecular meshwork (TM) and uveoscleral pathways. The TM is a complex tissue located in the iridocorneal angle and consists of specialized cells embedded in an extracellular matrix (ECM) [6–8]. Regulation of aqueous humor outflow resistance is one of the TM's key roles, which is achieved by modification of the ECM composition [9], along with contraction of the ciliary muscle and alteration of the TM cell shape to modify the geometry and thus permeability of the TM [10,11]. TM composition is known to change with age, including a reduction in TM cellularity and an increase in ECM accumulation [12]. These alterations appear to occur largely in patients with glaucoma and they are considered to result in a reduced outflow facility and consequently increased IOP [12].

ECM composition is regulated by the continual specific degradation of ECM components and the selective deposition of new ECM material produced by TM cells including collagens, glycosaminoglycans, proteoglycans, fibronectin, and elastin-containing microfibrils [6,13,14]. Interestingly, fibronectin is also present in the aqueous humor, and significantly elevated concentrations have been reported in the aqueous humor of POAG patients compared to cataract controls [15]. The selective degradation of ECM components involves matrix metalloproteinases (MMPs), a family of secreted zinc-dependent proteinases with selective substrate specificity [16]. MMP synthesis and activity are tightly regulated, with MMPs secreted as pro-enzymes and activated extracellularly, where their activity is controlled by endogenous inhibitors

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[17]. Tissue inhibitors of metalloproteinases (TIMPs) are potent albeit non-selective inhibitors of active MMPs, inhibiting proteolytic activity by obstructing the active site in a tight, reversible interaction with a 1:1 stoichiometry [18].

It has been suggested that an imbalance in the MMP-to-TIMP ratio could be involved in the development of glaucoma due to reported changes in ECM composition and an increased deposition of ECM components in the TM of glaucoma patients [19]. Several groups have also described altered MMP and TIMP concentrations in glaucomatous aqueous humor samples in comparison to non-glaucomatous cataract aqueous samples [19-21]. However, the extent to which these imbalances correlate with any relevant clinical descriptors is not known. Thus, the aim of this study was to assess differences in TIMP and MMP concentrations in aqueous humor samples from POAG and non-glaucomatous cataract patients to evaluate changes in MMP-to-TIMP ratios by means of stoichiometric analyses. In addition, the study aimed to correlate these results with disease-associated parameters including age, IOP, optic cup-to-disc ratio (CDR), and degree of visual field loss, as quantified by Humphrey's visual field pattern standard deviation (PSD) score and disease duration.

METHODS

Patient eligibility and recruitment: This study was approved by the Health and Medical Human Research Ethics Committee Tasmania (EC00337) and executed in adherence to the tenets of the Declaration of Helsinki. All participants were recruited through the Launceston Eye Doctors and the Launceston Eye Institute and gave written consent with regards to the donation and use of aqueous humor samples.

Clinical descriptors including age, IOP, CDR, PSD, and disease duration were recorded for POAG patients: A diagnosis of POAG was made based on the characteristic optic disc cupping, corresponding visual fields loss, and thinning of the retinal nerve fiber layer (RNFL) recorded on the Ocular Coherence Tomography (OCT), irrespective of the presenting IOP. A gonioscopic examination was also performed to classify the anatomy of the drainage angle. IOP was measured in all patients using a calibrated Goldmann Applanation tonometer. For this study, only the latest treated IOP measurement taken during the consultation before the surgery was used. Vertical CDR was estimated by one observer (TYT) using a 60D lens during indirect slit lamp fundoscopy, and it was then confirmed with an optic disc profile scan on Ocular Coherence Tomography. PSD was obtained from the SITA Fast 24–2 Humphrey Visual Field analyzer, which was used under standard settings. PSD was chosen over mean deviation, as the latter did not permit a clear differentiation between any cataract effects on the field test and glaucoma-induced visual field loss, specifically in glaucoma patients undergoing cataract surgery or a combined procedure. The PSD score emphasizes a focal variation in the visual field that highlights typical glaucomatous field loss. A greater PSD score indicates a denser field defect that correlates with disease severity. Disease duration was recorded as number of years since diagnosis at the time the AH samples were collected. All POAG patients recruited were receiving IOP-lowering medication in the form of monotherapy or a combination of up to four of the following compounds: Timolol (β-blocker), Brimatoprost, Tafluprost, Latanoprost (prostaglandin derivatives), Brimonidine (**α**-2 agonist), Dorzolamide, and Brinzolamide (carbonic anhydrase inhibitors).

Aqueous humor collection: Aqueous humor samples (generally 50–100 µl) were obtained from 23 patients with POAG during either trabeculectomy or cataract surgery. POAG patients who had previously had a trabeculectomy or vitrectomy were excluded. To serve as a control, aqueous humor samples from 26 non-glaucomatous patients undergoing routine cataract surgery were collected. Case and control subjects were excluded if they had other retinal (such as diabetic retinopathy or age-related macular degeneration) or neurologic diseases. For all samples, aqueous humor samples were collected from the center of the anterior chamber by paracentesis at the beginning of surgery, immediately frozen at −20 °C, and transferred to −80 °C within 48 h, where they were stored for analysis.

Quantitation of MMPs and TIMPs: Aqueous humor samples were analyzed using the following magnetic bead-based multiplex assays: Magnetic Luminex Human TIMP multiplex kit (R&D Systems, Inc., Minneapolis, MN) and a custom Magnetic Luminex human premixed multi-analyte kit (R&D Systems). Using these kits, the concentrations of the following analytes were measured: TIMP1 (tissue inhibitor of metalloproteinase 1), TIMP2, TIMP3, TIMP4, MMP-1 (matrix metalloproteinase-1), MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-12, and MMP-13. The assays were performed in accordance with the manufacturers' instructions on a Bio-Plex 200 System (Bio-Rad Laboratories, Inc., Hercules, CA). Aqueous humor samples were diluted at 1:2.4 for the custom kit and at 1:6 for the TIMP kit using the assay diluents provided, and 50 µl of the diluted samples was added to each well. Fluorescence intensity (FI) was acquired and analyzed using Bio-Plex Manager 6.0. Concentrations out of range were generally below the measurable concentration, with the exception of TIMP1 and TIMP2 (n = 3 and 1 samples, respectively), which gave FI readings above the highest standard and thus were excluded from all analyses.

The number of samples in the range is given for each analyte concentration reported and median values were determined using only the measureable samples.

Statistical analyses: All statistical analyses were conducted with Prism 6 (GraphPad Software, San Diego, CA) using two-tailed tests with a p value <0.05,which was regarded as statistically significant unless otherwise noted. Differences with regards to age and gender were assessed using an unpaired *t* test and Fisher's exact test, respectively. The D'Agostino–Pearson omnibus normality test was used to assess the normality of analyte concentration distributions, and a subsequent comparison of analyte concentrations between POAG and cataract control samples was performed using the Mann–Whitney U test. A correction for multiple testing was performed using Bonferroni's method (adjusted p = 0.004).

Further analyses included a calculation of molar MMP/TIMP ratios to assess potential imbalances, followed by a correlation of analyte concentrations and MMP/TIMP ratios to clinical descriptors using Spearman's method. These analyses were performed only on analytes that were detected in at least 50% of cataract control and POAG samples.

Due to significant differences in size between MMPs (27.9–76.4 kDa) and TIMPs (20.7–22.4 kDa), the molecular weight was considered for a stoichiometric analysis of MMP-to-TIMP ratios. Thus, measured protein concentrations (pg/ml) were converted to mol/l using the amino acid sequence obtained from the UniProtKB database (access date 18 June 2014) [22]. The signal sequence, indicated in the database, was removed before the molecular weight calculation (Appendix 1) using the ExPASy ProtParam tool (access date 18 June 2014) [23]. Stoichiometric analyses combining each MMP with each TIMP were performed on individual samples and reported as median values with an interquartile range. Because TIMPs are known to be non-selective in their MMP-inhibitory activity, we calculated all possible molar ratios using only the TIMPs and MMPs that were measured in more than 50% of cataract and POAG samples, with the number of molar ratio analyses precluding the adjustment of resulting p values for multiple comparisons. Statistical significance was assessed using the Mann–Whitney U test (p<0.05 considered significant).

RESULTS

In this study, a total of 23 POAG and 26 cataract control samples were analyzed, measuring all four known TIMPs and several members of the MMP family. Clinical descriptors including age, IOP, PSD, CDR, and disease duration were collected for all POAG samples and they are presented in Table 1. No significant differences were found between the cataract control and the POAG group concerning age (p = 0.226) or gender (p = 0.245). However, a positive correlation was determined between PSD and CDR for the POAG group (r = 0.654, p = 0.0007). Correlations between all other clinical descriptors were non-significant. All POAG patients were treated with IOP-lowering medication; 95% were prescribed a prostaglandin derivative, of which 80% simultaneously received the β-blocker Timolol. Furthermore, 43% of POAG patients were treated with an α-2 agonist and 62% received a carbonic anhydrase inhibitor. The majority of POAG patients were treated with a combination of two or three compounds.

The levels of TIMP1, TIMP2, and TIMP4 are significantly increased in glaucomatous aqueous humor samples: In both POAG and cataract control aqueous humor samples, TIMP1, TIMP2, TIMP3, and MMP2 were present at the highest concentrations, ranging from 4 to 25 ng/ml (Table 2). Concentrations measured for MMP3, MMP7, MMP8, MMP9, and MMP13 were between 100 and 660 pg/ml, whereas TIMP4,

Table 1. Clinical data of non-glaucomatous cataract and POAG patients.

Parameters	Cataract	POAG	p-value
Age (Mean ± SD)	74.8±7.0	72.2±7.8	0.226
Sample number (M/F)	26 (13/13)	23 (16/7)	0.245
IOP (Median, IQR)	N/A	22.0, 19.0–23.0	N/A
PSD (Median, IQR)	N/A	3.47, 2.16–8.36	N/A
CDR (Median, IQR)	N/A	0.85, 0.7–0.9	N/A
DD (Mean ± SD)	N/A	5.78±3.0	N/A

POAG: Primary open angle glaucoma; SD: standard deviation; M: male; F: female; IOP: intraocular pressure in mmHg; PSD: pattern standard deviation; CDR: optic cup/disc ratio; DD: disease duration (years); IQR: interquartile range; N/A: not available. Statistical significance was assessed using unpaired *t* test (age) and Fischer's exact test (gender) and p<0.05 was considered significant. All POAG patients were receiving IOP-lowering medications and all IOPs reported correspond to treated IOPs.

Table 2. Aqueous humor analyte concentrations in cataract control versus POAG.

Analyte	Cataract			POAG			p-value
	Median	IQR	In Range	Median	IQR	In Range	
TIMP1	7,235	6,062–8,508	24/24	11,226	8,757–18,434	17/20	**0.0008**
TIMP2	15,298	13,200-17,767	24/24	20,735	16,167-30,406	19/20	**0.002**
TIMP3	3,967	2,941–5,380	24/24	4,610	2,941–6,647	20/20	0.396
TIMP4	43.9	43.9–50.3	15/24	57.6	47.2–97.6	16/20	**0.002**
MMP1	25.0	18.5–34.4	25/26	31.5	16.9–99.0	22/23	0.406
MMP2	20,641	16,730-24,127	26/26	24,965	20,458-36,854	22/23	0.020
MMP3	418.3	293.4–644.5	25/26	660.3	318.8–1272.0	23/23	0.149
MMP7	261.8	230.2–270.5	3/26	361	178–637	8/23	0.606
MMP8	208.8	53.9–293.2	7/26	108.4	57.8–181.8	6/23	0.311
MMP9	187.6	129.4–347.4	26/26	179.6	114.6–376.5	22/23	0.778
MMP12	36.2	28.9–39.7	6/26	38.3	33.6–45.9	5/23	0.307
MMP13	140.9	107.8–206.0	10/26	199.2	155.2–205.8	6/23	0.367

Median and interquartile range (IQR) calculated for values in range reported as pg/ml. Significance was tested using the Mann–Whitney U test and a p value <0.05 was considered significant. Correction for multiple testing was performed using Bonferroni's method (adjusted p value=0.004) and p values that remained significant are highlighted in bold.

MMP1, and MMP12 were present at concentrations below 100 pg/ml. The analyte concentrations obtained in this study are broadly consistent with those reported in the existing literature [19-21,24,25]. MMP7, MMP8, MMP12, and MMP13 could only be measured in a small number of samples (Table 2), with ≥50% of samples below the detection limits of the assay in both POAG and cataract control samples; consequently, these analytes were excluded from all subsequent analyses. Nevertheless, the proportion of samples in which MMP7, MMP8, MMP12, and MMP13 were out of range was not significantly different between the control and POAG groups (Fisher's exact test p = 0.184–1.0).

A comparison of POAG aqueous humor samples to cataract control samples revealed higher concentrations in POAG for the majority of analytes, but for many, this difference did not reach statistical significance. However, significant differences in concentration were seen for TIMP1 (p = 0.0008), TIMP2 (p = 0.002), and TIMP4 (p = 0.002), with all three analytes presenting an increased median concentration in POAG (Table 2 and Figure 1). The increases in TIMP1, TIMP2, and TIMP4 remained significant after correction for multiple testing (adjusted p value = 0.004). In addition, the median concentration of MMP2 was also increased in POAG (p = 0.020; Table 2 and Figure 1). No sample was consistently above the 95th percentile across all analytes measured, and with regards to the statistically significant analytes, the highest concentrations plotted in Figure 1 stem from different individuals. Furthermore, the highest concentrations measured for TIMP1 and TIMP2 were outside the range of the assay standard curve; thus, for TIMP1 and TIMP2, these samples were excluded before analysis.

Due to the known role of TIMPs as MMP inhibitors, we sought to determine the presence of an imbalance between specific MMP and TIMP proteins. Thus, MMP/TIMP stoichiometric ratios were calculated (Table 3 and Figure 2). With the exception of MMP2/TIMP3, MMP2/TIMP4, MMP3/TIMP4, and MMP9/TIMP4, the molar concentration of TIMP was higher than that of each MMP for all other median ratios presented in Table 3. When comparing the POAG group to cataract controls, the molar ratios of MMP2/TIMP1 (p = 0.007) and MMP9/TIMP1 (p = 0.005) showed a significant decrease in POAG samples, corresponding to an elevated excess of TIMPs over MMPs in POAG versus cataract samples. Conversely, the MMP2/TIMP3 (p = 0.045) and MMP3/TIMP3 (p = 0.032) molar ratios increased. These significant changes to MMP/TIMP molar ratios are likely due to the increase in TIMP1, TIMP2, and TIMP4 concentrations in POAG, as little difference was observed in MMP levels. Furthermore, the molar ratios demonstrate that in terms of total TIMP concentration, the increases in TIMP1, 2, and 4 outweigh the rise in the total MMP2 concentration observed.

Several MMP/TIMP molar ratios correlate with IOP and PSD in glaucomatous aqueous humor samples: Spearman's rank correlation method was used to assess the correlation of each analyte concentration and MMP/TIMP ratio with age for both POAG and cataract samples (Table 4). Significant positive correlations were determined between age and TIMP1

(r = 0.492, p = 0.015), TIMP3 (r = 0.413, p = 0.045), and MMP3 (r = 0.537, p = 0.006). Furthermore, MMP3/TIMP1 (r = 0.529, p = 0.010) and MMP3/TIMP2 (r = 0.492, p = 0.017) also presented positive correlations with age in cataract samples. Nevertheless, none of these analytes or molar ratios correlated with age for POAG samples.

Correlations with IOP, PSD, disease duration (Table 5), and CDR (Appendix 2) were determined for analyte concentrations and MMP/TIMP ratios for POAG samples. Molar ratios for MMP1 in combination with any of the four TIMPs, as well as MMP3/TIMP3 and MMP9/TIMP3, correlated positively with IOP (p = 0.007–0.034; Table 5). Several molar ratios also correlated negatively with PSD (p = 0.005–0.046), and MMP9/TIMP1 correlated positively with disease duration (p = 0.015), whereas no significant correlations were determined for CDR.

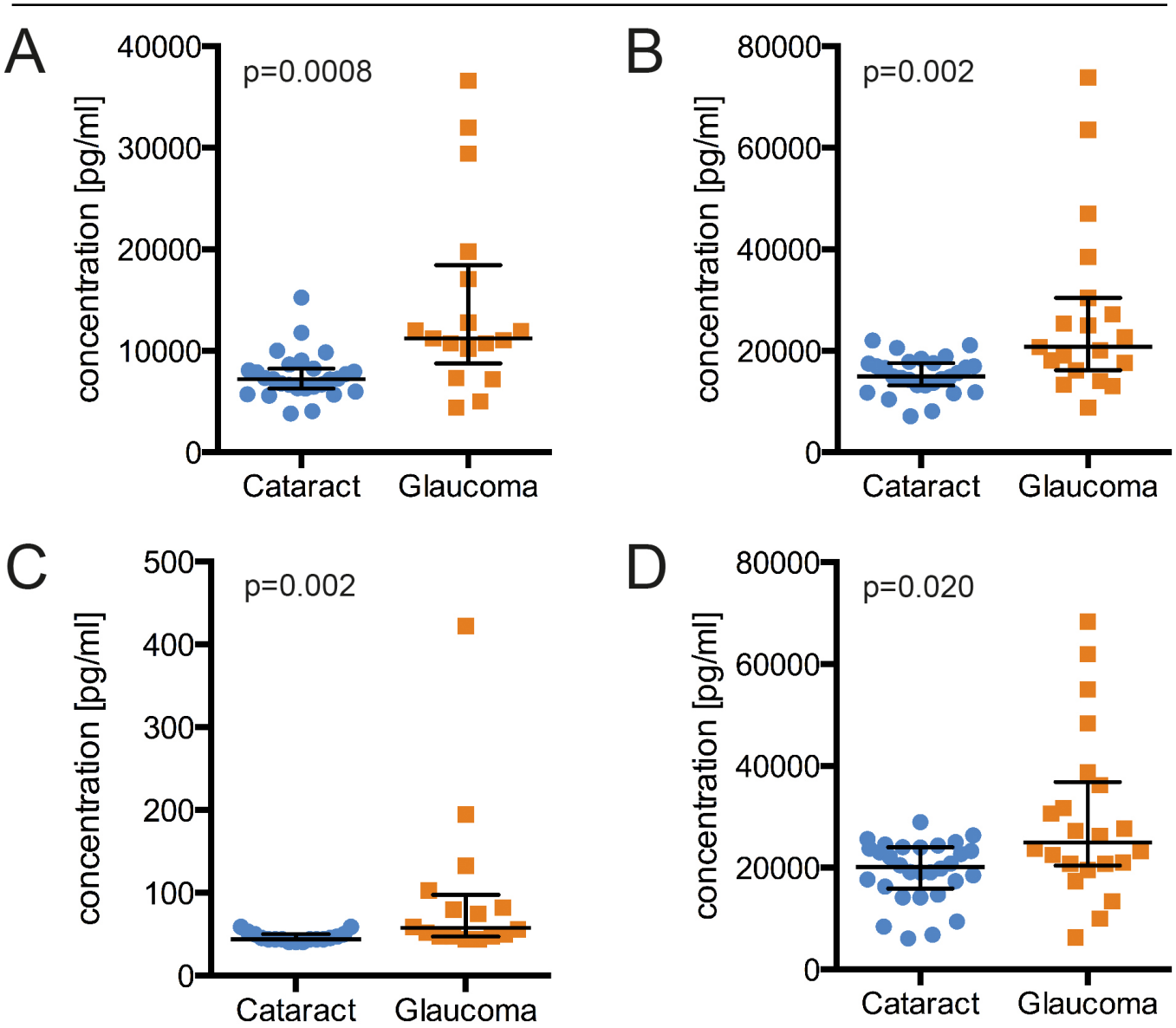

Figure 1. Distribution of TIMP1 (**A**), TIMP2 (**B**), TIMP4 (**C**), and MMP2 (**D**) concentrations in aqueous humor samples from non-glaucomatous cataract (blue; N=15-26) versus POAG (orange; N=16-22) patients. Levels of all four analytes shown were significantly increased in POAG aqueous humor samples, as determined using the Mann–Whitney U test (p<0.05). Medians and interquartile ranges are indicated. See Table 2 for a complete list of analyte concentrations measured.

Table 3. Stoichiometric analysis of MMP and TIMP ratios in cataract control versus POAG samples.

Ratio	Cataract			POAG			p-value
	Median	Interquartile range	N	Median	Interquartile range	N	
MMP1/TIMP1	1.28×10^{-3}	8.68×10^{-4}-2.07×10^{-3}	23	1.20×10^{-3}	6.17×10^{-4}-1.96×10^{-3}	16	0.353
MMP1/TIMP2	6.25×10^{-4}	4.65×10^{-4}-1.09×10^{-3}	23	5.88×10^{-4}	3.78×10^{-4}-1.22×10^{-3}	18	0.857
MMP1/TIMP3	2.46×10^{-3}	1.57×10^{-3}-3.40×10^{-3}	23	2.69×10^{-3}	1.58×10^{-3}-1.47×10^{-2}	19	0.193
MMP1/TIMP4	0.24	0.18–0.34	15	0.33	0.22–0.65	16	0.108
MMP2/TIMP1	0.74	0.69–1.04	24	0.64	0.46–0.77	16	**0.007**
MMP2/TIMP2	0.40	0.33–0.46	24	0.35	0.30–0.41	18	0.137
MMP2/TIMP3	1.32	1.06–1.88	24	2.01	1.48–2.29	19	**0.045**
MMP2/TIMP4	155.10	133.20–170.50	15	144.10	126.80–167.00	16	0.245
MMP3/TIMP1	2.40×10^{-2}	1.66×10^{-2}-3.97×10^{-2}	23	2.52×10^{-2}	1.27×10^{-2}-2.91×10^{-2}	17	0.492
MMP3/TIMP2	1.09×10^{-2}	9.16×10^{-3}-2.31×10^{-2}	23	1.53×10^{-2}	9.50×10^{-3}-2.56×10^{-2}	19	0.528
MMP3/TIMP3	4.26×10^{-2}	3.04×10^{-2}-8.22×10^{-2}	23	7.10×10^{-2}	4.29×10^{-2}-2.24×10^{-1}	20	**0.032**
MMP3/TIMP4	4.68	3.70–7.60	15	7.26	4.04–13.65	16	0.354
MMP9/TIMP1	8.55×10^{-3}	4.91×10^{-3}-1.25×10^{-2}	24	4.64×10^{-3}	2.20×10^{-3}-9.11×10^{-3}	16	**0.005**
MMP9/TIMP2	4.10×10^{-3}	2.46×10^{-3}-6.55×10^{-3}	24	2.70×10^{-3}	1.74×10^{-3}-4.70×10^{-3}	18	0.124
MMP9/TIMP3	1.45×10^{-2}	8.05×10^{-3}-2.88×10^{-2}	24	1.42×10^{-2}	7.96×10^{-3}-2.74×10^{-2}	19	0.631
MMP9/TIMP4	1.47	0.69–2.55	15	1.17	0.60–1.99	16	0.397

Values represent median stoichiometric ratio with interquartile range. Significance was tested by means of Mann–Whitney U and a p value <0.05 was considered significant, as highlighted in bold. N: number of ratios calculated.

DISCUSSION

An imbalance between MMPs and TIMPs in aqueous humor samples has been suggested to play a role in the development of glaucoma [19]. In this study, we quantified aqueous humor concentrations of TIMP 1–4 and several MMPs in POAG and cataract samples, assessed changes in MMP/TIMP molar ratios, and correlated these results with clinical descriptors. A significant increase in the concentrations of TIMP1, TIMP2, and TIMP4 was determined in POAG compared to cataract samples, which is broadly consistent with several reports in the literature [19-21,24,25]. Unlike most other studies of MMP and TIMP levels in aqueous humor samples, we have included clinical data in our analyses and found that several of the MMP/TIMP molar ratios calculated for POAG aqueous humor samples correlated strongly with IOP, PSD, or both.

Our results reveal an overall dominance of TIMPs over MMPs in cataract aqueous humor samples. However, the increased concentrations of TIMP1, TIMP2, and TIMP4 produce an MMP/TIMP molar ratio that is imbalanced in the aqueous humor samples of POAG patients compared to that of cataract patients and suggests that overall MMP activity may be decreased in POAG. All TIMPs are said to be able to inhibit all MMPs, with only a few exceptions. However, TIMP3 is more effective at inhibiting other enzymes, such as ADAMs and ADAMTs compared to MMPs [16]. Thus, in this study, all TIMPs relevant to MMP inhibition are upregulated in POAG aqueous humor samples, consistent with the reduction in active MMP levels reported for glaucomatous aqueous humor samples [19]. This observation may contribute to a reduced clearance of ECM components and is in agreement with the reported increase in ECM deposition within the glaucomatous TM [12].

Several significant correlations with age were found for TIMPs, MMPs, and MMP/TIMP molar ratios in cataract samples and which may contribute to the known age-associated increase in ECM deposition within the TM of healthy individuals [12]. In contrast, no such associations with age were observed in POAG samples, despite the larger extent to which this occurs in the glaucomatous TM. However, a correlation was determined between the MMP9/TIMP1 molar ratio and disease duration in POAG samples.

Glaucoma has been described as a disease of early and accelerated cell senescence [26-28], a state in which cells permanently exit the cell cycle, but remain metabolically active. A higher number of senescent cells in the TM of POAG patients compared to age-matched control individuals has been reported [26-28]; thus, changes in gene expression associated with senescence, which include increased secretions of TIMPs and MMPs [29], may contribute to the increased aqueous humor concentration of TIMPs reported here. It is

possible that senescence-associated increases of MMP and TIMP secretions occur to varying extents in different POAG patients, and this may reduce the extent of the correlation between these proteins and age. IOP may also modify the levels of MMPs and TIMPs by causing a mechanical stretch of the TM, which alters the secretion of MMPs and TIMPs [30]. This action has been suggested to involve TGFβ1 as a signaling intermediate [31]. Thus, it is plausible that TM cells are directly involved in the change of MMP and TIMP levels in the aqueous humor.

In POAG samples, MMP/TIMP imbalances were correlated with an elevated PSD score and therefore deterioration of the visual field (indicated by negative correlations between several MMP/TIMP molar ratios and PSD). An association between elevated IOP and the progression of visual field defects has been shown [32], and the hypothesis that decreased MMP activity within the TM leads to elevated IOP is well described [9]. Based on these findings, one may expect that the further imbalance of TIMPs over MMPs in POAG samples compared to cataract samples described here is likely to reduce ECM turnover, increase aqueous humor outflow

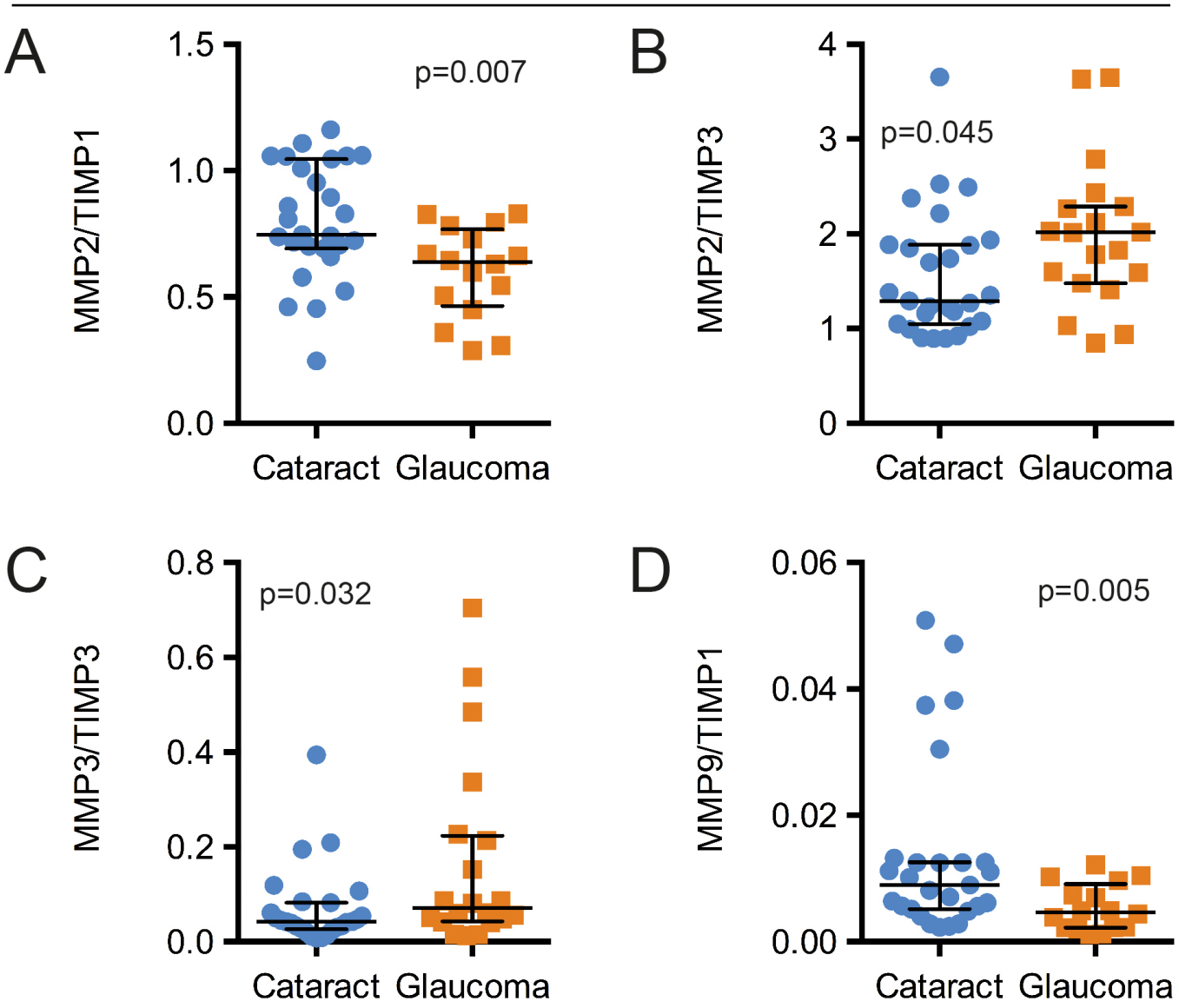

Figure 2. Distribution of MMP/TIMP ratios in cataract versus POAG patients. Stoichiometric ratios were calculated for individual aqueous humor samples from non-glaucomatous cataract (blue; N=23-24) and POAG (orange; N=16-20) patients. Median ratios and interquartile ranges are indicated. The ratios for MMP2/TIMP1 (**A**), MMP2/TIMP3 (**B**), MMP3/TIMP3 (**C**), and MMP9/TIMP1 (**D**) were significantly different between cataract and glaucoma ($p<0.05$) patients, as determined using the Mann–Whitney U test. See Table 3 for the full set of ratios calculated.

Table 4. Correlation of measured analytes and MMP/TIMP ratios to age for cataract control and POAG samples.

Analyte/ratio	Cataract			POAG		
	r-value	p-value	N	r-value	p-value	N
TIMP1	0.492	**0.015**	24	−0.012	0.949	17
TIMP3	0.413	**0.045**	24	0.011	0.962	20
MMP1	0.388	0.055	25	0.115	0.612	22
MMP3	0.537	**0.006**	25	0.052	0.814	23
MMP2/TIMP4	−0.104	0.686	15	0.443	0.087	16
MMP3/TIMP1	0.529	**0.010**	23	0.346	0.173	17
MMP3/TIMP2	0.492	**0.017**	23	−0.045	0.855	19
MMP9/TIMP4	0.509	0.055	17	0.074	0.785	16

Correlation of analyte concentrations and MMP/TIMP ratios to age were determined using Spearman's rank correlation method. A p value <0.05 was considered significant, as shown in bold. Only correlations with p<0.1 and their corresponding values for the other group are shown. r-value: Spearman correlation coefficient; N: number of correlation pairs.

resistance, and thereby elevate IOP. However, we found that increased concentrations of TIMPs altered the MMP/TIMP ratio, such that a more pronounced imbalance correlated with a lower IOP. This may be due to treatment with hypotensive medication, decreasing IOP to a variable extent between patients, or their impact on MMP and TIMP levels in the aqueous humor [33]. Several IOP-lowering drugs have been tested in Sprague-Dawley rats and in cell cultures (human fibroblasts and keratocytes) and have been shown to affect the expressions of some MMPs and TIMPs [34]. Broadly, the prostaglandin derivative latanoprost and the α-2 agonist brimonidine caused an increase in MMP3 and decreases in TIMP1 and TIMP3, whereas the β-blocker Timolol had the opposite effect. However, the effects of combinatorial treatments were not assessed in this study; therefore, such experiments do not fully reflect current treatment regimens, which

Table 5. Correlation of measured analytes and MMP/TIMP ratios to PSD, IOP and Disease Duration for POAG samples.

Analyte/ratio	IOP		PSD		Disease duration		N
	r-value	p-value	r-value	p-value	r-value	p-value	
MMP1	0.402	0.063	−0.147	0.516	0.134	0.553	22
MMP1/TIMP1	0.594	**0.017**	−0.538	**0.034**	0.368	0.161	16
MMP1/TIMP2	0.609	**0.007**	−0.482	**0.043**	0.240	0.338	18
MMP1/TIMP3	0.491	**0.033**	−0.407	0.084	0.215	0.378	19
MMP1/TIMP4	0.550	**0.029**	−0.556	**0.028**	0.005	0.976	16
MMP3/TIMP1	0.423	0.091	−0.655	**0.005**	0.316	0.216	17
MMP3/TIMP2	0.431	0.065	−0.492	**0.032**	0.050	0.838	19
MMP3/TIMP3	0.476	**0.034**	−0.348	0.133	0.166	0.484	20
MMP3/TIMP4	0.393	0.132	−0.509	**0.046**	−0.165	0.505	16
MMP9/TIMP1	0.247	0.353	−0.300	0.258	0.605	**0.015**	16
MMP9/TIMP2	0.334	0.175	−0.313	0.207	0.460	0.055	16
MMP9/TIMP3	0.527	**0.020**	−0.454	0.051	0.283	0.241	19
MMP9/TIMP4	0.467	0.070	−0.409	0.117	0.378	0.149	16

Correlation of analytes and MMP/TIMP ratios to IOP (intraocular pressure), PSD (Humphrey's visual field pattern standard deviation) and disease duration were determined using Spearman's rank correlation. A p value of <0.05 was considered significant, as shown in bold. Only correlations with p<0.1 and their corresponding values for the other clinical descriptors are shown. r-value: Spearman correlation coefficient; N: number of correlation pairs (applies to all analyses).

often involve a combination of two or more compounds. In this present study, most POAG patients were being treated with a prostaglandin analog (95%), of which 80% simultaneously received Timolol, and notably, no difference in MMP3 concentration between POAG and cataract samples was observed. Nevertheless, it is possible that the IOP-lowering treatments used in the care of the POAG study participants may have affected the MMP or TIMP concentrations reported here.

The positive correlations between MMP/TIMP ratios and IOP described in this study are also difficult to reconcile with the current knowledge of TIMPs as potent MMP inhibitors. However, TIMPs have recently been shown to possess additional activities independent of their roles as MMP inhibitors, including the regulation of cell growth, differentiation, migration, and apoptosis, as mediated through direct interactions between TIMPs and cell surface receptors [35,36]. Increased TIMP expressions in glaucomatous aqueous humor samples may therefore have as yet unrecognized MMP-independent effects, such as a direct inhibition of other TM cell functions, including contractibility and phagocytosis, which may affect aqueous humor outflow [10,11], or to the observed loss of cellularity within the TM of glaucomatous eyes, which may involve apoptotic signaling mechanisms [37-40]. Although the effect of TIMPs on apoptosis appears to be highly context-dependent [41], with several studies reporting pro- and anti-apoptotic responses to TIMP signaling [35], the positive correlations between MMP/TIMP ratios and IOP suggest that a higher IOP may be associated with a loss of anti-apoptotic (or pro-survival) TIMP signaling. This may lead to decreased cellularity within the TM and consequently insufficient drainage of the aqueous humor.

The strong correlations determined between MMP/TIMP ratios and the clinical determinants IOP and PSD indicate that the imbalance between MMPs and TIMPs is likely of importance in POAG. Nevertheless, it remains unknown whether the changes observed in POAG aqueous humor compositions are a cause or consequence of the disease and whether TIMP levels increase as a response to altered MMP secretion or vice versa or indeed as a response to other events occurring in the anterior chamber. In conclusion, this study suggests that further work should focus on TIMPs, with respect to not only changes in ECM composition, but also the cellularity of the TM in POAG.

APPENDIX 1. CALCULATION OF TIMP AND MMP MOLECULAR WEIGHTS FOR STOICHIOMETRIC ANALYSIS

Molecular weight (MW) given in Daltons (Da) was calculated using the amino acid sequence (AAs) stated (signal peptide sequence removed), retrieved from the UniProt database. To access the data, click or select the words "Appendix 1."

APPENDIX 2. CORRELATION OF MEASURED ANALYTES & MMP/TIMP RATIOS TO CDR FOR POAG SAMPLES

Correlation of analytes and MMP/TIMP ratios to CDR (optic cup-to-disc ratio) was determined using Spearman's rank correlation. A p value <0.05 was considered significant. Only correlations also shown in Table 5 are displayed. r-value: Spearman correlation coefficient; N: number of correlation pairs. To access the data, click or select the words "Appendix 2."

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