

# Analyzing cytokines as biomarkers to evaluate severity of glaucoma

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## Abstract

• **AIM:** To analyze cytokines as biomarkers for evaluation of severity of glaucoma.

• **METHODS:** This was a prospective case-control study including 29 eyes with glaucoma. Besides, 28 eyes with senile cataract were used as control. Patients were classified into four groups: acute angle closure glaucoma (AACG), chronic angle closure glaucoma (CACG), primary open angle glaucoma (POAG) and senile cataract. Undiluted vitreous samples were collected, then vitreous concentrations of 9 types of cytokines were determined by cytometric bead assay system:  $\gamma$ -interferon (IFN $\gamma$ ), interleukin (IL)-10, IL-2, IL-4, IL-5, interferon- $\gamma$ -inducible protein (IP)-10, monocyte chemoattractant protein (MCP)-1, tumor necrosis factor (TNF) - $\alpha$ , and vascular endothelial growth factor (VEGF). We also recorded the intraocular pressure (IOP) of patients in each group and Pearson correlated analysis was performed to analysis the correlation between each type of cytokine with IOP.

• **RESULTS:** Vitreous levels of IL-2, IL-5, MCP-1, TNF- $\alpha$  and IP-10 were significantly higher ( $P < 0.05$ ) in AACG group. Patients with AACG, CACG and POAG have higher IOP than senile cataract, but we didn't find any significant correlation between IOP with any type of the cytokines.

• **CONCLUSION:** Inflammation and immune reaction have a strong link with the pathology of glaucoma especially AACG. Some cytokines may act as biomarkers to evaluate the severity of glaucoma. Anti-inflammatory treatments and controlling of IOP are necessary for the therapy of glaucoma.

• **KEYWORDS:** glaucoma; cytokines; intraocular pressure

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## INTRODUCTION

Glaucoma is one of the main causes of blindness worldwide. The elevated intraocular pressure (IOP) is the main risk factor while it is also characterized by a progressive glaucomatous optic neuropathy and corresponding visual field loss<sup>[1-2]</sup>. Meanwhile, some studies have suggested that glaucoma actually involves multiple factors, including immune reactions<sup>[3]</sup>, inflammation<sup>[4]</sup>, ischemia<sup>[5]</sup>, hypoxia<sup>[6]</sup>, and oxidative stress<sup>[7]</sup>. Glaucoma can be categorized into acute angle closure glaucoma (AACG), chronic angle closure glaucoma (CACG), primary open angle glaucoma (POAG) depending on the different pathogenesis.

The role of immunological factors in glaucoma has become a major research topic recently and cytokines mediate immune and inflammatory responses may play an important role in the process of glaucomatous optic neuropathy<sup>[8]</sup>. Previous studies that measured cytokine concentrations in aqueous humor samples have detected elevated cytokine levels in eyes suffering from glaucoma, such as interleukin (IL)-9, 10, 12, interferon (IFN)- $\alpha$ ,  $\gamma$ , monokine induced by IFN- $\gamma$  (MIG or CXCL9), etc<sup>[9-13]</sup>. T-helper (Th) cells are the main source of cytokines, and some studies suggested that balance of Th1/Th2 cytokines plays an important role in the mechanism of glaucomatous optic neuropathy<sup>[14-15]</sup>. Vascular endothelial growth factor (VEGF) may also play an important role in the mechanism of neovascular glaucoma (NVG)<sup>[16]</sup>, which is a kind of cytokine that could promote neovascularization and has a strong link with inflammation and immunity<sup>[17]</sup>. The evidence from these previous studies indicates that the levels of cytokines in aqueous humor (AH) may be related to the

pathogenesis of glaucoma. Therefore, evaluation of those cytokines in AH may expand the understanding of glaucoma pathophysiology.

Cytometric bead assay has greater sensitivity than traditional enzyme-linked immunosorbent assay (ELISA) and spots enzyme immunoassay (elisspots), it allows simultaneous detection of multiple cytokines in a small volume clinical samples<sup>[18]</sup>. This technique has been successfully used to detect the levels of several cytokines in AH of patients with active panuveitis and anterior uveitis (AU) in other study<sup>[19]</sup>. In our study, we measured multiple cytokines in the AH of eyes with AACG, CACG, POAG and senile cataract using cytometric bead assay to investigate the possible roles of the intraocular cytokines in the pathogenesis of glaucoma.

**SUBJECTS AND METHODS**

This study was performed in accordance with the tenets of Declaration of Helsinki, and the produces were approved by the Institutional Review Board of Xinhua Hospital. All the patients signed a written informed consent after an explanation of nature and possible consequences of this study. All the samples were collected from April 2014 to January 2015.

**Subjects** Patient’s inclusion criteria: 1) patients have been diagnosed as any type of glaucoma and need filtering surgery therapy; 2) patients has no filtering operation therapy history before; 3) patients’ age and medical history were clear; 4) patients have signed the informed consent.

Patient’s exclusion criteria: 1) patients have other ophthalmic diseases; 2) patients have other systemic diseases; 3) patients have accepted systemic and local steroids therapy in one week; 4) patients have accepted ophthalmic surgery or intravitreous injections before.

Twenty-nine eyes of twenty-nine patients with glaucoma (8 patients with AACG, 15 patients with CACG and 6 patients with POAG) were studied. Besides, twenty-eight eyes of twenty-eight patients with senile cataract and have no history of IOP exceeding 21 mm Hg were used as control.

At last, patients were classified into four groups: AACG group, CACG group, POAG group and senile cataract group.

**Sample Collection** All the patients with glaucoma underwent filtering surgery at Xinhua Hospital while the patients with cataract underwent phaco+IOL surgery. IOP was recorded for patients in each group. After anesthesia, 0.1 mL AH in anterior chamber was collected through a lateral hyal-corneal incision by using a 30-gauge needle connected with 1 mL syringe before the surgery. The needle was not contact with iris, lens or corneal endothelium. The collected vitreous samples were stored at -80°C until the assay validation.

**Cytometric Bead Assay** The vitreous levels of 9 types of cytokines were determined simultaneously by a commercially available cytometric bead assay (Becton, Dickinson and

**Table 1 Demographics of cases in each groups**

Parameters	n	Age (a)
AACG	8	66.25 (47-94)
CACG	15	70.77 (50-95)
POAG	6	63.33 (50-82)
Senile cataract	28	72.75 (50-84)

AACG: Acute angle closure glaucoma; CACG: Chronic angle closure glaucoma; POAG: Primary open angle glaucoma.

Company). Analyses were performed for  $\gamma$ -interferon (IFN $\gamma$ ), IL-10, IL-2, IL-4, IL-5, interferon- $\gamma$ -inducible protein (IP)-10, monocyte chemoattractant protein (MCP)-1, tumor necrosis factor (TNF) - $\alpha$ , and VEGF.

The samples were thawed in room temperature, then centrifuged at 15 000 rpm for 10min at 4°C . The 50  $\mu$ L of each sample and different concentrations of each cytokine standard were added to 50  $\mu$ L antibody-conjugated beads in a 96-well filter plate. After 30min incubation, the plate was washed and after another 30min of incubation, 25  $\mu$ L biotinylated antibody solution was added to each well. Then washed the plates, and 50  $\mu$ L streptavidin-conjugated phycoerythrin was added to each well and incubated for 10min. After a final wash, the contents of each well were resuspended in 125  $\mu$ L assay buffer and were analyzed using a BD Bead Array Reader. The concentrations of the cytokines were calculated from a standard curve for each cytokine.

**Statistical Analysis** Statistical analysis was performed using SPSS 13.0 software. Data was presented as average and range. If *P*-value of the homogeneity of variance >0.05, a one way-AVONA analysis was used to detect the differences of the vitreous concentrations of each cytokine among AACG; CACG; POAG and senile cataract group, followed by Bonferroni test to detect the differences between each two groups. If *P*-value of the homogeneity of variance <0.05, a Kruskal-Wallis 1-way analysis was performed to test the differences for the vitreous concentrations of each cytokine among those groups, followed by Tamhane’s T2 to detect the differences between each two groups. The correlation between two parameters was determined by Pearson correlation. A *P*-value <0.05 was considered to be statistically significant.

**RESULTS**

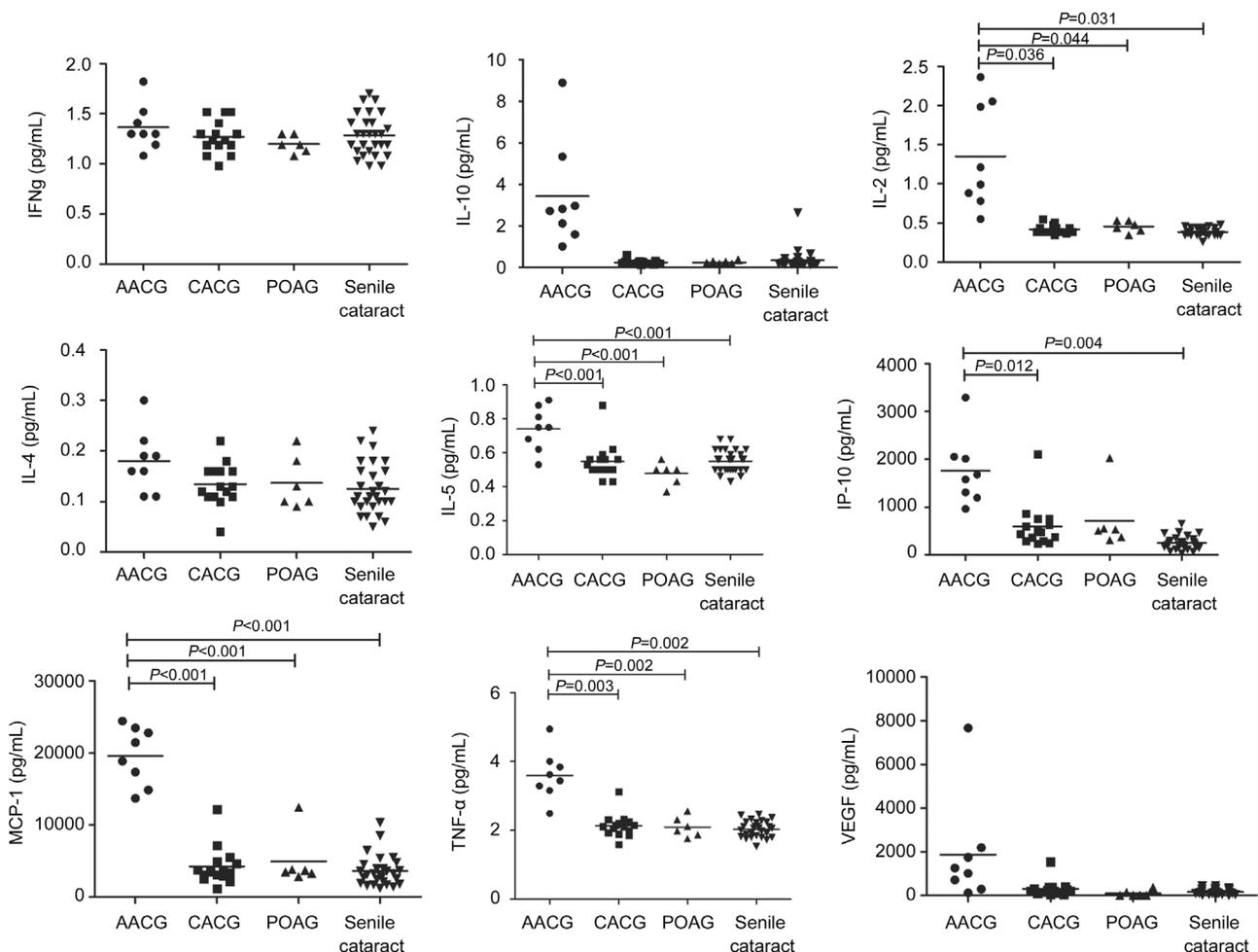
The sample sizes and average ages of each group are shown in Table 1. The average age is 66.25 in AACG group; 70.77 in CACG group; 63.33 in POAG group and 72.75 in senile cataract group.

**Vitreous Levels of 9 Types of Cytokines** The vitreous levels of IL-10, IL-2, IL-5, IP-10, MCP-1, TNF- $\alpha$  and VEGF were significantly different (all *P*<0.001) among AACG, CACG, POAG and senile cataract group. We also did comparison between each two groups. There is no significant difference of IL-10 or VEGF between each two groups. The vitreous level

**Table 2 Vitreous levels of 9 types of cytokines in eyes with AACG, CACG, POAG, senile cataract**

Parameters	AACG (n=8)	CACG (n=15)	POAG (n=6)	Senile cataract (n=28)	P
IFNg	1.36 (1.08-1.82)	1.27 (0.98-1.52)	1.20 (1.08-1.30)	1.29 (0.98-1.70)	0.434 <sup>a</sup>
IL-10	3.43 (1.01-8.90)	0.25 (0.14-0.60)	0.25 (0.18-0.37)	0.35 (0.10-2.63)	<0.001 <sup>b</sup>
IL-2	1.35 (0.55-2.36) <sup>d</sup>	0.42 (0.35-0.55) <sup>d</sup>	0.46 (0.35-0.53) <sup>d</sup>	0.39 (0.26-0.48) <sup>d</sup>	<0.001 <sup>b</sup>
IL-4	0.18 (0.11-0.30)	0.13 (0.04-0.22)	0.14 (0.09-0.22)	0.13 (0.05-0.24)	0.069 <sup>a</sup>
IL-5	0.74 (0.53-0.91) <sup>c</sup>	0.55 (0.43-0.88) <sup>c</sup>	0.48 (0.37-0.56) <sup>c</sup>	0.55 (0.43-0.68) <sup>c</sup>	<0.001 <sup>a</sup>
IP-10	1758.88 (965-3292) <sup>d</sup>	594.20 (239-2105) <sup>d</sup>	713.33 (307-2025)	251.72 (30-653) <sup>d</sup>	<0.001 <sup>b</sup>
MCP-1	19611 (13695-24413) <sup>c</sup>	4255.87 (1150-12169) <sup>c</sup>	4930.17 (2848-12436) <sup>c</sup>	3608.32 (1209-10330) <sup>c</sup>	<0.001 <sup>a</sup>
TNF- $\alpha$	3.60 (2.49-4.94) <sup>d</sup>	2.13 (1.59-3.12) <sup>d</sup>	2.10 (1.76-2.56) <sup>d</sup>	2.04 (1.53-2.47) <sup>d</sup>	<0.001 <sup>b</sup>
VEGF	1872.25 (117-7659)	300.17 (35.78-1536)	86.40 (1.79-362)	158.34 (1.79-448)	<0.001 <sup>b</sup>

AACG: Acute angle closure glaucoma; CACG: Chronic angle closure glaucoma; POAG: Primary open angle glaucoma; IFNg:  $\gamma$ -interferon; IL: Interleukin; IP: Interferon- $\gamma$ -inducible protein; MCP: Monocyte chemoattractant protein; TNF: Tumor necrosis factor; VEGF: Vascular endothelial growth factor. Levels are expressed as the average (range) pg/mL. <sup>a</sup>One way-AVONA analysis was performed to compare the four groups; <sup>b</sup>Kruskal-Wallis 1-way analysis was performed to compare the four groups; Significant ( $P<0.05$ ) difference for comparison versus control: <sup>c</sup>Bonferroni test and <sup>d</sup>Tamhane's T2.



**Figure 1 Scatter plots: distribution levels of 9 types of cytokines in eyes with AACG, CACG, POAG and senile cataract.**

of IL-2 was significantly higher in AACG group than CACG group ( $P=0.036$ ), POAG group ( $P=0.044$ ) and senile cataract group ( $P=0.031$ ). The vitreous level of IL-5 was significantly higher in AACG group than CACG group ( $P<0.001$ ), POAG group ( $P<0.001$ ) and senile cataract group ( $P<0.001$ ). The vitreous level of IP-10 was significantly higher in AACG group than CACG group ( $P=0.012$ ) and senile cataract group

( $P=0.004$ ). The vitreous level of MCP-1 was significantly higher in AACG group than CACG group ( $P<0.001$ ), POAG group ( $P<0.001$ ) and senile cataract group ( $P<0.001$ ). The vitreous level of TNF- $\alpha$  was significantly higher in AACG group than CACG group ( $P=0.003$ ), POAG group ( $P=0.002$ ) and senile cataract group ( $P=0.002$ ) (Table 2). Figure 1 shows the scatter plots of each type of cytokine in each group.

**Table 3 IOP of eyes with AACG, CACG, POAG and senile cataract**

Parameters	AACG	CACG	POAG	Senile cataract	<i>P</i>
<i>n</i>	5	15	6	27	
IOP (mm Hg)	29.74 (18.9-39.3)	27.33 (9-52) <sup>b</sup>	28.55 (16-42.3)	14.63 (9.8-19.5) <sup>b</sup>	<0.001 <sup>a</sup>

AACG: Acute angle closure glaucoma; CACG: Chronic angle closure glaucoma; POAG: Primary open angle glaucoma; IOP: Intraocular pressure. Levels are expressed as the average (range) mm Hg. <sup>a</sup>Kruskal-Wallis 1-way analysis was performed to compare the four groups; Significant (*P*<0.05) difference for comparison versus control: <sup>b</sup>Tamhane's T2.

**Correlation Analysis** We recorded IOP for patients in each group (Table 3) and Pearson correlated analysis was performed to check the correlation between each type of cytokine with IOP (Table 4). Patients with AACG, CACG and POAG have higher IOP than senile cataract, but because of the small sample size, only the difference between CACG group and senile cataract group is significant. Meanwhile, we didn't find any significant correlation between any type of cytokine with IOP in those group.

**DISCUSSION**

Levels of 9 different cytokines were analyzed simultaneously in 0.1 mL AH. Although the sample volume is very small, cytometric bead assay has great sensitivity and also allows for simultaneous detection of multiple cytokines in small volume clinical samples.

We included patients with AACG, CACG, POAG and detected cytokines for each group to see if different pathogenic mechanisms could impact the vitreous levels of cytokines. The result shows that IL-2, IL-5, MCP-1, TNF- $\alpha$  and IP-10 were significantly higher in AACG group. However, there is no significant difference was found among any other groups. IL-2 induces T-cell proliferation and affects the levels and function of cytotoxic and regulatory T cells as well as the production of antibodies. Hou *et al*<sup>[20]</sup> found significant mRNA elevation for IL-2 on the iris of patients with neovascular glaucoma. TNF- $\alpha$  is a kind of pleiotropic cytokine which has many physiological functions. A study showed that TNF- $\alpha$  which produced by retinal glial cells is one of the risk factors for glaucoma<sup>[21]</sup>. Upregulation of expression of TNF- $\alpha$  and its receptor TNF-R1 can induce apoptosis of retinal ganglion cells. In the rat model of high IOP induced by laser photocoagulation, the expression of TNF-R1 gene was 8 times higher than the normal rat, the level of TNF- $\alpha$  was also significantly increased<sup>[22]</sup>. Another study proved that adding anti TNF- $\alpha$  antibodies or TNF-R1 conditioned medium of ischemia, retinal ganglion cell death are greatly reduced<sup>[23]</sup>. Tezel and Wax<sup>[24]</sup> found that anti TNF- $\alpha$  antibodies can lead to decreased rate of retinal ganglion cells apoptosis by about 66%. Xin *et al*'s<sup>[25]</sup> study showed that patients with open angle glaucoma have higher TNF- $\alpha$  levels in AH compared with the control subjects. IP-10 belongs to the CXC chemokine family which can induce chemotaxis, cell growth, apoptosis, angiogenesis, and inflammation

**Table 4 Correlations between cytokines and IOP**

Parameters	AACG		CACG		POAG	
	$\rho$	<i>P</i>	$\rho$	<i>P</i>	$\rho$	<i>P</i>
IFNg	0.638	0.246	-0.008	0.979	-0.759	0.08
IL-10	0.431	0.469	0.158	0.573	0.346	0.502
IL-2	-0.122	0.845	0.299	0.279	-0.133	0.801
IL-4	0.226	0.715	0.042	0.882	0.523	0.287
IL-5	0.196	0.752	-0.365	0.18	0.64	0.171
IP-10	0.761	0.135	0.208	0.456	-0.001	0.999
MCP-1	-0.4	0.505	0.194	0.488	0.014	0.978
TNF- $\alpha$	-0.141	0.822	0.334	0.223	0.457	0.362
VEGF	-0.146	0.814	0.29	0.295	-0.054	0.919

AACG: Acute angle closure glaucoma; CACG: Chronic angle closure glaucoma; POAG: Primary open angle glaucoma; Pearson correlation ( $\rho$ ) and *P* value were calculated by pearson correlation and a significant difference was accepted when *P*<0.05.

mediated by combining with the CXC chemokine receptor 3 (CXCR3) and also involved in the formation of inflammatory and immune responses to infection and tumor. Studies have shown that IP-10 is related to diabetic macular edema, neovascular macular degeneration and polypoidal choroidal vasculopathy<sup>[26-27]</sup>, but the relationship between IP-10 and glaucoma is still unreported. IL-5 and MCP-1 are also involved in the inflammatory and immune responses in many situations and higher level of MCP-1 in eyes with glaucoma was proved by Huang *et al*<sup>[28]</sup>.

There are other previous studies test the concentrations of different cytokines in eyes with glaucoma. Huang *et al*<sup>[28]</sup> found elevated concentrations of IL-6, IL-8, granulocyte colony-stimulating factor (G-CSF), MCP-1, MCP-3, and VEGF in eyes with AACG. Chua *et al*'s<sup>[9]</sup> study certificated that concentration of IL-9, IL-12, IFN- $\alpha$ , IFN- $\gamma$ , CXCL9 and IL-10 are higher in eyes with glaucoma and especially eyes with POAG have higher IL-12, IFN- $\gamma$  and CXCL9 levels, while eyes with PACG had higher interleukin-8 (CXCL8) and CXCL9 levels. In Takai *et al*'s<sup>[29]</sup> study, the concentrations of IL-8, transforming growth factor (TGF)-1, and serum amyloid A (SAA) were significantly higher and IL-6 was significantly lower in the eyes with POAG. Borkenstein *et al*<sup>[10]</sup> also found a lower level of IL-6 in eyes with POAG. The previous studies together with our study all show that immune and

inflammatory responses may have a close relationship with the pathology of glaucoma. IL-2, IL-5, IL-10, MCP-1, IP-10 and TNF- $\alpha$  can mediate immune and inflammatory responses in lots of situations. Among those 6 cytokines, IL-2, TNF- $\alpha$  are regulated by Th1 cells while IL-5, IL-10 are regulated by Th2 cells. Some studies considered that imbalance of Th1/Th2 are associated with many diseases, such as allergies, tumor progression, graft rejection and so on<sup>[30-33]</sup>. What we found in our study suggesting that imbalance of Th1/Th2 cytokines could also play an important role in the mechanism of glaucomatous optic neuropathy.

However, the specific reason and mechanism of the relationship between high levels of cytokines and glaucoma are still uncertain. The elevated levels of cytokines may caused by the development of glaucoma and are the results of the acute crisis. Different pathogenesis may lead to different levels of cytokines. The concentrations of the cytokines are also possibly influenced by the use of medicine since our study did not detect the relationship between the cytokines and medicine which used by the patients to control the symptoms of glaucoma. Thus, more researches should be performed to study the basic mechanisms and the reasons of the high levels of cytokines in glaucoma eyes.

We didn't detect any significant correlation between those cytokines with IOP, the reason is probably because our sample size is small. However, Freedman and Iserovich's<sup>[33]</sup> study shows that levels of intraocular cytokines increase with an increase in IOP. Huang *et al*<sup>[28]</sup> also found that IOP may be responsible for the production of cytokines in eyes with AACG and elevated cytokine levels may in turn influence AH dynamics and thus lead to IOP elevation. Takai *et al*'s<sup>[29]</sup> study showed that cytokine networks including TGF-1, IL-8, and SAA in AH may have critical roles in IOP elevations in patients with POAG. The results of these studies suggest that anti-inflammatory treatments are necessary for controlling IOP in eyes suffering from glaucoma.

Another limitation of our study is the sample size. It is too small to get significant results for some groups. Besides, during the sample collection, any possible contamination may affect the result even if we tried to avoid it. Therefore, more precise further studies with a larger sample size are still needed to detect the relationship between inflammation and immune with different kinds of glaucoma and the relationship between different cytokines with IOP.

In conclusion, the significant results of our study suggest that inflammation and immune reaction have a strong link with the pathogenesis of glaucoma especially AACG. Some cytokines may act as biomarkers to evaluate the severity of glaucoma. Anti-inflammatory treatments and controlling of IOP are very necessary for the therapy of glaucoma.

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**Authors' Contributions:** Wang ZY and Zheng Y conceived and designed the study. Tong Y, Zhou YL, Wang ZY, and Zheng Y collected the samples. Tong Y and Zhou YL performed the data statics and analysis. Tong Y wrote the paper. Biswal M and Zhao PQ reviewed and edited the manuscript.

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