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New perspectives in glaucoma pathophysiology, diagnosis, and treatment

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Published in
Frontiers in Medicine

ISSN 1664-8714
ISBN 978-2-8325-2436-7
DOI 10.3389/978-2-8325-2436-7

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New perspectives in glaucoma pathophysiology, diagnosis, and treatment

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Citation

Martucci, A., Nucci, C., Pinazo-Duran, M. D., eds. (2023). *New perspectives in glaucoma pathophysiology, diagnosis, and treatment*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-2436-7

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frontiers | Frontiers in Medicine

TYPE Editorial
PUBLISHED 03 May 2023
DOI 10.3389/fmed.2023.1200427

OPEN ACCESS

EDITED AND REVIEWED BY
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RECEIVED 04 April 2023
ACCEPTED 14 April 2023
PUBLISHED 03 May 2023

CITATION
Martucci A, Nucci C and Pinazo-Duran MD (2023) Editorial: New perspectives in glaucoma pathophysiology, diagnosis, and treatment. *Front. Med.* 10:1200427. doi: 10.3389/fmed.2023.1200427

Editorial: New perspectives in glaucoma pathophysiology, diagnosis, and treatment

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KEYWORDS

glaucoma, retinal ganglion cells (RGCS), visual field, quality of life, glaucoma diagnosis, glaucoma pathophysiology, glaucoma treatment

Editorial on the Research Topic
New perspectives in glaucoma pathophysiology, diagnosis, and treatment

Glaucoma is the most common cause of legal blindness worldwide. This progressive optic neuropathy is characterized by the degeneration of retinal ganglion cells and consequent changes in the optic nerve head anatomy.

The prevalence of the different forms of glaucoma varies on basis of age, gender, ethnicity, and economical levels.

People with glaucoma, initially experience peripheral vision loss, and if not treated promptly, vision loss may get worse, possibly leading to blindness over time. The disease is extremely complex, and its pathogenesis is still not fully understood. Although intraocular pressure is considered the major cause of retinal ganglion cell death, glaucoma can even occur if intraocular pressure is within the normal range. In these cases, other risk factors such as abnormally low cerebrospinal fluid pressure, impaired microcirculation, mitochondrial deficits, altered immunity, excitotoxicity, and oxidative stress or coexisting ocular or systemic diseases may also concur to the development and the progression of the disease (1).

Genetics may also play a role in the disease. An increasing body of evidence suggests the presence of susceptibility loci in the DNA. However, the mechanism by which these genes might contribute is not clear.

Due to the disease's complexity, early glaucoma diagnosis can be challenging for clinicians. Visual field examination provides late information as damage is expected when at least 30% of retinal ganglion cells have been damaged. Hence the need for new diagnostic tools that allow an easier and earlier diagnosis of the disease (2).

Once diagnosed, glaucoma treatment may be challenging for clinicians. By now, intraocular pressure reduction is the primary strategy to treat glaucoma. However, some patients may not respond to the treatment hence the need for new intraocular pressure lowering molecules or different therapeutic approaches that may comprehend neuroprotection, systemic intervention, para surgical or surgical intervention (3).

In this Research Topic, 14 original research articles examined several topics including the prevalence of glaucoma, new pathophysiological insight, including psychometric aspects and impact on quality of life, as well as new options for diagnosis, treatment, and overall management of this complex disease.

One paper described the characteristics, epidemiology, management, and outcomes of glaucoma in pediatric patients in central China. Liu Q. et al. reported that primary congenital glaucoma, juvenile open-angle glaucoma, and traumatic glaucoma turned out the most prevalent subtypes of pediatric glaucoma in central China.

Six papers evaluated novel biomarkers or diagnostics tools useful for the early diagnosis of glaucoma. Lillo et al. showed that glaucoma, even when the ocular pressure is pharmacologically kept under control, causes significant variation in the level of the compounds of the aqueous humor which are also essential for mitochondrial function. In this regard, Pinazo-Duran et al. reported that the development of composite biomarkers from tears, aqueous humor and blood, seems to be an appropriate solution in ophthalmological practice for early diagnosis and to predict therapeutic response in glaucoma patients.

Yuan et al. analyzed the role of anthropometric aspects in glaucoma. Herein, the Mendelian randomization method revealed that increased body mass index and waist circumference are potential risk factors for glaucoma.

Yang et al. evaluated the usefulness of optic disc photographs for morphologic features and glaucoma likelihood, showing that the stereoscopic method was superior compared to the monoscopic method for general ophthalmologists.

In this context, Seo et al. highlighted that deep learning models may be the future of diagnostic tools. Convolutional neural network models have been shown to be able to discriminate early normal-tension glaucoma from glaucoma suspect eyes using Bruch's membrane opening-based optic disc photography.

This new disc photography of Bruch's membrane opening overview can aid in the diagnosis of early glaucoma.

Besides, increasing evidence suggests that glaucoma is a neurodegenerative disease that originates in the brain but manifests as an eye disease (4–7). Liu K. et al. showed the usefulness of the Mendelian randomization tool to understand the causal effect of brain alterations on glaucoma. Thus, supporting the rising evidence of the existence of the brain-eye axis.

Three articles dealt with the therapeutic aspect of glaucoma. Martucci et al. proposed the combined use of neuroprotectors, such as citicoline and Coenzyme Q10, due to their putative synergistic effect and combined action on the different pathogenetic targets causing the onset and progression of glaucoma. Using combined treatment may downregulate more pro-apoptotic pathways and boost the effect on one or more pathways on which the different molecules act.

Besides, some patients need special care. In the case of moderate glaucoma, Wang et al. proposed ultrasound cyclo-plasty as a safe and effective method for reducing IOP. While, according to Li et al., patients who have undergone keratoplasty, are more likely to develop glaucoma before surgery. In these cases, surgical treatment should be selected according to the ocular surface condition to reduce the occurrence of complications.

Psychological factors may also interfere with the treatment. Two papers on this topic focused on this aspect. A case-control study by Chu et al. documented that IOP was significantly reduced in patients with open-angle glaucoma after "planning" selective laser trabeculoplasty treatment, even without actually performing it.

Peng et al. underlined the importance of resilience as a positive factor, which could improve patients' quality of life with glaucoma. However, other factors such as sleep disturbance may reduce this positive impact. Thus, supporting the importance of interventions that enhance the levels of resilience and promote healthy sleep in glaucoma patients.

As seen, studies on primary open-angle glaucoma extend well beyond ophthalmology to biochemistry molecular biology, general internal medicine, pharmacology, pharmacy, science technology, and other areas. Two papers focused on the importance of the literature analysis also using the emerging techniques of artificial intelligence. Zhao et al. conducted a bibliometric analysis of publications on glaucoma from 2000 to 2021 in the Web of Science database. Thus, suggesting that the analysis of co-occurrence networks provides researchers with information about potential collaboration opportunities with other institutions and researchers. Bibliometric analyses provide valuable guidance for researchers in the selection of Research Topics. In this contest, AlRyalat et al. proposed that with the emergence of artificial intelligence and machine learning techniques that seek to decipher new learnings from these large datasets, a deep and nuanced understanding of the designs and methodologies used may be key to unlocking even more data to enhance patient care.

In summary, this Research Topic focuses on new insights into the disease prevalence and pathophysiology, on the development of a new generation of diagnostic tools and biomarkers, and on new treatments scheme that may assist ophthalmologists in the early care of their patients, preserving their quality of life.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Acknowledgments

We appreciate all the significant works and thank the contributors to this Research Topic.

Conflict of interest

AM is medical consultant for Visufarma SpA.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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frontiers in Medicine

OPINION
published: 26 January 2022
doi: 10.3389/fmed.2022.818568

Designs and Methodologies Used in Landmark Clinical Trials of Glaucoma: Implications for Future Big Data Mining and Actionable Disease Treatment

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Keywords: glaucoma, open angle, clinical trials, diagnosis, optic nerve

INTRODUCTION

A widely agreed upon definition of glaucoma with clear diagnostic criteria to classify disease presence and status remains elusive in both clinical and research settings. For decades, the diagnosis of glaucoma was primarily based on documenting visual field changes through static or kinetic perimetry and correlating these findings with structural changes at the optic nerve head (1, 2). Recently, the 10th World Glaucoma Association (WGA) Consensus Meeting supported the use of optic nerve structural endpoints alone to provide sufficient information for a diagnosis of glaucoma, even in the absence of visual field changes, a state termed pre-perimetric glaucoma (3). The National Institute for Health and Care Excellence (NICE), the national body tasked with issuing clinical guidelines for England and Wales, does not provide strict criteria to diagnose glaucoma (4). There is scarce guidance on criteria to definitively diagnose glaucoma which leads to difficulties in research efforts focused on phenotyping ocular imaging/diagnostic data as a first step toward identifying and predicting disease to enhance clinical care.

LANDMARK TRIALS IN GLAUCOMA

The field of glaucoma has the second largest number of published randomized controlled trials in all of ophthalmology, the majority of which evaluate glaucoma treatments (5, 6). Landmark randomized controlled trials have shaped the practice of glaucoma care and are commonly used to teach medical learners the basics of treating patients with suspected or diagnosed glaucomatous optic neuropathy (7–10). While it is clear that these trials have provided a wealth of information for practical patient care, the added benefit of these data sets is the ability to mine information that can guide the planning of future studies. This can take the form of feeding large labeled data sets into novel machine learning algorithms which potentially can produce new findings to inform patient care (11, 12). The large data sets from previous landmark trials are often published in top tier journals and receive a great deal of attention. However, the building blocks of these studies; including methodologies, patient selection criteria and diagnostic operating procedures, are usually published in a separate document and receive less rigorous attention (1, 2, 13). The aim of this brief report is to clarify the diagnostic criteria used by landmark glaucoma trials with a focus on design and methodology.

We reviewed diagnostic criteria used by landmark glaucoma clinical trials commonly cited in ophthalmic textbooks and review articles (7–10). We also supplemented the mentioned trials by

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Ophthalmology, a section of the journal Frontiers in Medicine

Received: *19 November 2021*
Accepted: *04 January 2022*
Published: *26 January 2022*

Citation:
AlRyalat SA, Ertel MK, Seibold LK and Kahook MY (2022) Designs and Methodologies Used in Landmark Clinical Trials of Glaucoma: Implications for Future Big Data Mining and Actionable Disease Treatment. Front. Med. 9:818568. doi: 10.3389/fmed.2022.818568

TABLE 1 | Landmark glaucoma clinical trials and each of the functional, structural, and intraocular pressure criteria used in glaucoma diagnosis or enrollment.

Trial	Year	IOP (mmHg) criteria	Visual field criteria	Structural criteria
The Glaucoma Laser Trial (GLT) (1)	1991	>21	Glaucomatous visual field defect/deterioration on Program 32 (1)	None
		≥27	-	cup/disc ratio disparity ≥0.3
		≥31	-	cup/disc ratio ≥0.8
The Advanced Glaucoma Intervention Study (AGIS) (2)	1994	>21	Visual field defect score of at least (25)	-
		17–21	Visual field deterioration	Disc rim deterioration
Collaborative Normal-Tension Glaucoma Study (CNTGS) (19)	1998	≤24*	Glaucomatous visual field defect/deterioration on Program 32 (19)	Glaucomatous disc judged by physicians
The Collaborative Initial Glaucoma Treatment Study (CIGTS) (13)	1999	≥20	At least three contiguous points on the total deviation probability plot at the <2% level and a Glaucoma Hemifield Test result that is "outside normal limits," (25)	Glaucomatous optic disc
		20–26	At least two contiguous points in the same hemifield on the total deviation probability plot at the <2% level (25)	Glaucomatous optic disc
		≥27	-	Glaucomatous optic disc
Ocular Hypertension Treatment Study (OHTS) (20)	1999	≥24 and ≤32*	Corrected pattern standard deviation <0.05 OR glaucoma hemifield test outside normal limits (26)	-
			-	Stereoscopic optic disc photographs showing a change in the position of vessels (greater than expected by eye movement), development of notch, pit, or development of thinning or pallor in the neural rim.
Early Manifest Glaucoma Trial (EMGT) (21)	1999	<30*	Glaucoma hemifield test outside normal limits (26)	-
			Glaucoma hemifield test borderline (26)	Glaucomatous optic disc features correspond to visual field
The European Glaucoma Prevention Study (EGPS) (22)	2002	>21 to ≤29*	Deterioration from baseline (22)	-
			-	Deterioration from baseline
Low-Pressure Glaucoma Treatment Study (LoGTS) (23)	2005	≤21*	At least 3 contiguous points depressed more than 8 decibels or 2 contiguous points depressed more than 10 decibels (23)	Glaucomatous optic disc consistent with visual field
UK Glaucoma Treatment Study (UKGTS) (18)	2013	<30*	Reduction in sensitivity at 2 or more contiguous points with $P < 0.01$ loss or more, 3 or more contiguous points with $P < 0.05$ loss or more, or a 10-dB difference across the nasal horizontal midline at 2 or more adjacent points in the total deviation plot (27).	Cup-to-disc ratio of ≥0.7, focal narrowing of the neural rim, or both

*These intraocular pressure thresholds were used for enrollment, rather than for establishing the diagnosis.

an advanced PubMed search for design and methodology articles published for glaucoma clinical trials where we used the following search strategy:

((Design[Title]) OR (Method[Title])) AND (Glaucoma [Title])

We included trials that were concerned mainly with open angle glaucoma, as this represents the majority of the effort in this space. Studies that had design protocols published in a separate "design and methodology" article were of particular interest.

Fourteen trials were identified in our assessment, however, five of them were surgical trials focused on outcome comparisons and used criteria previously utilized in larger randomized clinical trials (14–18). As a result, we focused on nine clinical trials (1, 2, 13, 19–24). Glaucoma diagnosis was based on one or more of the following criteria: functional criteria in terms of visual field performance, structural criteria in terms of optic disc features, and/or intraocular pressure (IOP). **Table 1** details the specific glaucoma diagnostic criteria adopted by each of the landmark clinical trials. Visual field criterion was a pre-requisite diagnostic criterion for most clinical trials, although they varied in their definition for what qualified as a glaucomatous visual field (1, 13, 19, 22, 23, 25, 26). The Glaucoma Laser Trial (GLT) and the Collaborative Initial Glaucoma Treatment Study (CIGTS) did not require visual fields for patients with an IOP of 27 or higher, where only structural evidence of glaucomatous optic disc damage was required (1, 13). More recent trials, including the European Glaucoma Prevention Study (EGPS) (22), Low-Pressure Glaucoma Treatment Study (LoGTS) (23), and UK

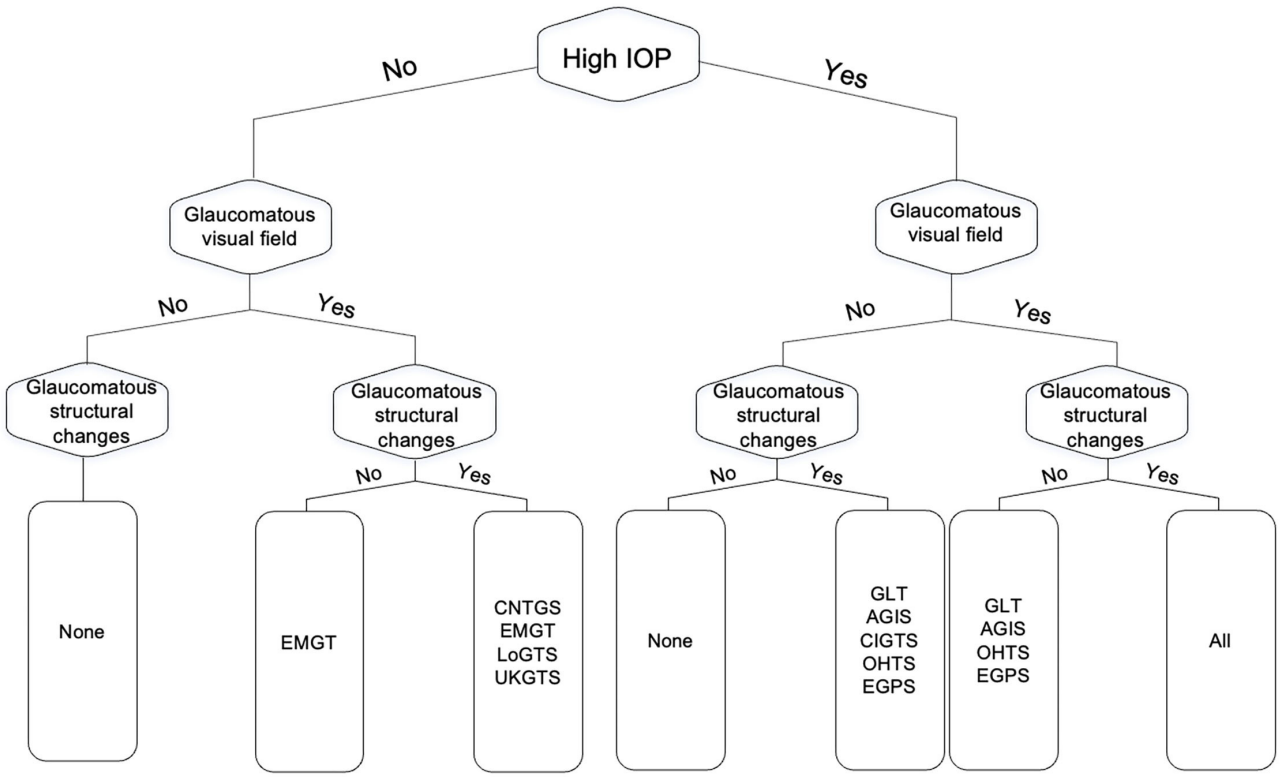

FIGURE 1 | Flow chart of the different combinations from the three glaucoma diagnostic facets (functional, structural, and intraocular pressure) as they relate to the major clinical trials in glaucoma. GLT, The Glaucoma Laser Trial; AGIS, The Advanced Glaucoma Intervention Study; CNTGS, Collaborative Normal-Tension Glaucoma Study; OHTS, Ocular Hypertension Treatment Study; EMGT, Early Manifest Glaucoma Trial; EGPS, The European Glaucoma Prevention Study; LoGTS, Low-Pressure Glaucoma Treatment Study; UKGTS, UK Glaucoma Treatment Study.

Glaucoma Treatment Study (UKGTS) (18), required structural glaucomatous features and/or visual field glaucomatous features. **Figure 1** shows how the different combinations of functional, structural and IOP criteria were utilized across these studies.

DISCUSSION

Detailing the presence and/or progression of glaucomatous optic neuropathy is based on the functional and structural characteristics of the optic nerve, relying on the combination of both subjective and objective data obtained from clinical examination and output from various diagnostic modalities. While large randomized clinical trials have provided insights into the treatment of glaucoma, the criteria used for enrollment and interventions in each trial are disparate, making application of findings in the clinical setting difficult at best. With the emergence of artificial intelligence and machine learning techniques that seek to decipher new learnings from these large datasets, a deep and nuanced understanding of the designs and methodologies used is key to unlocking even more data to enhance patient care. We have provided an overview of diagnostic criteria used in landmark randomized controlled trials of open angle glaucoma. These criteria differ in the diagnostic weight placed on subjective visual field studies, objective structural changes of the optic nerve, as well as the use of IOP metrics. Outlining these criteria in a single resource may act as a starting point for discussions on proper methods of mining past data sets while also reaching some consensus for implementing a commonly agreed upon set of diagnostic criteria in future studies to facilitate broader analyses. The ultimate goal is to make findings from large randomized clinical trials more actionable in a real-world clinical setting by leveraging big data sets toward predictive output and guidance for when to observe and when to intervene with escalating care.

AUTHOR CONTRIBUTIONS

SA, ME, LS, and MK contributed in research conception, literature review, and manuscript writing. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers | Frontiers in Medicine

ORIGINAL RESEARCH
published: 01 April 2022
doi: 10.3389/fmed.2022.874369

Clinical Analysis of Pediatric Glaucoma in Central China

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Purpose: We aimed to describe the characteristics, epidemiology, management, and outcomes of glaucoma in pediatric patients in central China.

Methods: This study retrospectively analyzed inpatients with pediatric glaucoma at Henan Provincial People's Hospital, Henan Eye Institute, and Henan Eye Hospital between 2017 and 2020.

Results: Overall, 239 cases (276 eyes) of pediatric glaucoma in patients, comprising 87 girls (36.40%) and 152 boys (63.60%) were analyzed. The mean age was 6.65 ± 4.46, and 2.93% of the patients had a family history of glaucoma. Primary congenital glaucoma (PCG) was the most common type of glaucoma, followed by traumatic glaucoma in 8.33% of the patients, which was considered secondary glaucoma. The most common signs and symptoms were elevated intraocular pressure (IOP) and eye pain. Trabeculotomy (Trab) and microcatheter-assisted 360° trabeculotomy (MAT) combined with Trab were the most commonly performed surgeries. The IOP of patients with PCG, juvenile open-angle glaucoma (JOAG), and secondary glaucoma were 15.27 ± 7.48 mmHg, 17.16 ± 10.05, and 18.65 ± 8.55, respectively, at the final follow up. The rate of re-operations in patients with PCG, JOAG, and secondary glaucoma were 9.15%, 6.78%, and 4.69%, respectively. The mean visual acuity of the eyes with PCG, JOAG, and secondary glaucoma was 0.79 ± 0.68, 0.51 ± 0.48, and 0.53 ± 0.50, respectively.

Conclusion: PCG, JOAG, and traumatic glaucoma were the most prevalent subtypes in patients with pediatric glaucoma in central China. Trab and MAT combined with Trab were the most common interventions used in this study. Pediatric amblyopia might require full attention during the entire treatment, especially after glaucoma surgery. Effective preventive measures and more public education on glaucoma prevention and the importance of early diagnosis and treatment is necessary.

Keywords: pediatric glaucoma, trabeculotomy, juvenile open-angle glaucoma, primary congenital glaucoma, intraocular pressure

OPEN ACCESS

Edited by:
Alessandro Meduri, University of Messina, Italy

Reviewed by:
Giovanni William Oliverio, University of Messina, Italy
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Specialty section:
This article was submitted to Ophthalmology, a section of the journal Frontiers in Medicine

Received: *12 February 2022*
Accepted: *14 March 2022*
Published: *01 April 2022*

Citation:
Liu Q, Liu C, Li H, Yang X, Dong Y, Feng X and Cheng W (2022) Clinical Analysis of Pediatric Glaucoma in Central China. Front. Med. 9:874369. doi: 10.3389/fmed.2022.874369

INTRODUCTION

Pediatric glaucoma is a group of eye diseases that causes irreversible blindness and affects infants and children. Congenital glaucoma is an important risk factor for visual impairment in children (1). Hence, the burden of pediatric glaucoma may substantially diminish the quality of life in affected individuals (2).

The primary goal of glaucoma treatment is intraocular pressure (IOP) reduction, for which medical treatment is often the first-line treatment. For long-term treatment, surgery may be the definitive option for IOP reduction especially in cases of pediatric glaucoma (2). Various types of new glaucoma surgeries have emerged in recent years and may benefit pediatric glaucoma patients (3, 4). Henan Province is in the middle of China and is a traditional agricultural province. Many patients attend and receive treatment at Henan Eye Hospital, which is the leading domestic eye hospital. Therefore, many patients with glaucoma from remote areas are already in an advanced stage of disease at the time of the first visit and are commonly lost to follow-up because of economic or reasons related to long-distance travel. These circumstances may indicate that the possibility of childhood glaucoma resulting in blindness is higher in this province than in economically developed or coastal areas in China.

Pediatric glaucoma, resulting in blindness, is a serious public health problem in Henan. To date, no large studies have examined the characteristics and outcomes of pediatric glaucoma in central China. Therefore, we analyzed the characteristics of pediatric glaucoma in patients at the Department of Ophthalmology, Henan Eye Hospital, Henan Eye Institute, and Henan Provincial People's Hospital.

MATERIALS AND METHODS

We conducted a retrospective analysis of all patients with pediatric glaucoma diagnosed and surgically treated at the Department of Ophthalmology of Henan Eye Hospital, Henan Eye Institute, Henan Provincial People's Hospital between 2017 and 2021. Patients with pediatric glaucoma aged <18 years were included. Age, sex, IOP combined with glaucomatous history, visual acuity (VA), abnormal signs under a slit lamp, medications, surgical options, and outcomes were analyzed. This retrospective study was approved by the Ethics Committee of Henan Eye Hospital and Henan Eye Institute and the Henan Provincial People's Hospital Human Research Ethics Committee [approval number HNEECKY-2022(08)]. This study adhered to the tenets of the Declaration of Helsinki. Informed consent was obtained from all guardians or patients.

The patients with pediatric glaucoma in this study were identified by a qualified glaucoma specialist. Surgical treatments were performed if the patient demonstrated progression of glaucomatous damage or, in the surgeon's opinion, the IOP was at a level that would cause additional damage or primary disease requiring treatment. Here, the IOP was measured using a Tono-Pen tonometer and Goldmann applanation tonometer. Pediatric glaucoma is classified as primary or secondary (5, 6). Primary pediatric glaucoma includes primary congenital glaucoma (PCG) and juvenile open-angle glaucoma (JOAG) (7, 8).

Statistical Analysis

Data were analyzed using SPSS software (version 19.0.0; IBM Corp., Armonk, NY, USA). Continuous and categorical variables are presented as means ± standard deviations and percentages, respectively.

TABLE 1 | Pediatric glaucoma patient and ocular characteristics.

Characteristics	Total ($N = 239$ patients and 276 eyes)
Age (at time of surgery)*	
Mean (standard deviation)	6.65 (4.46)
Gender	
Male	63.60% (152)
Female	36.40% (87)
Number of eyes in study	
1	202
2	37
Prior surgery	
Glaucoma surgery	**40**
Trabeculectomy (Trab)	19
Trabeculotomy	2
Non-penetrating trabeculectomy	2
Endoscopic cyclophotocoagulation (ECP)	2
Gonioscopy-assisted transluminal trabeculotomy (GATT)	1
Microcatheter-assisted 360° trabeculotomy (MAT)	8
Ahmed glaucoma valve implantation	5
Xen glaucoma implant	1
Non-glaucoma surgery	**38**
Phacoemulsification	6
Penetrating Keratoplasty	2
Suture of ocular trauma	5
Vitrectomy	15
Others	10
Prior numbers of glaucoma medications	
1	66
2	61
3	31
4	7
Uncertain	39
Glaucoma family history	**7**
Congenital cataract	5

For the 37 patients with both eyes included in the study, their ages at surgery can differ from 0 to 17 years.

Abbreviations: AGV, Ahmed glaucoma valve; IOL, intraocular lens; IOP, intraocular pressure; JOAG, juvenile open-angle glaucoma; MAT, microcatheter-assisted 360° trabeculotomy; PCG, primary congenital glaucoma; Trab, trabeculotomy; VA, visual acuity.

TABLE 2 | Diagnostic findings in patients with surgical pediatric glaucoma*.

Characteristic	N (eyes)	(%)
Primary		
Primary congenital glaucoma	153	(55.43%)
Male:Female	103:50	
Juvenile open-angle glaucoma	59	(21.38%)
Male:Female	36:23	
Secondary		
Lens-related		
Pseudophakia	3	(1.09%)
Subluxation of lens	2	(0.72%)
Aphakia	1	(0.36%)
Phacomatoses		
Sturge-Weber Syndrome	1	(0.36%)
Uveitis		
Uveitis (intermediate/anterior)	4	(1.45%)
Anterior segment dysgenesis		
Aniridia	3	(1.09%)
Peters anomaly	1	(0.36%)
Others		
Traumatic	23	(8.33%)
Silicone oil-related	13	(4.71%)
Steroid-induced	4	(1.45%)
Intraocular tumor	4	(1.45%)
Neovascular Glaucoma	3	(1.09%)
Pigment dispersion syndrome	2	(0.72%)

Data are shown as NO. (%).

TABLE 3 | Different main symptoms and signs of the pediatric glaucoma patients*.

Characteristic	N (number of eyes)	(%)
Signs		
Elevated IOP	91	(32.97%)
Impaired vision	85	(30.80%)
Buphthalmos	19	(6.88%)
Leukocoria	15	(5.43%)
Enlargement of C/D ratio	8	(2.89%)
Exophthalmos	3	(1.09%)
Strabismus	2	(0.72%)
Nystagmus	1	(0.37%)
Hyphema	1	(0.37%)
Symptoms		
Eye pain	11	(3.99%)
Red eye	10	(3.62%)
Photophobia	9	(3.26%)
Lacrimation	7	(2.54%)
Foreign body sensation	1	(0.37%)
Systemic association		
Headache	5	(1.81%)
Uncertain	8	(2.89%)

Intraocular pressure: IOP; C/D: cup/disc ratio.
Data are show as NO. (%).

RESULTS

Pediatric Glaucoma Characteristics and Epidemiological Findings

In the 5-year study period, 239 glaucoma cases (276 eyes) comprising 87 girls (36.40%) and 152 boys (63.60%) were analyzed. Their demographic characteristics are listed in **Table 1**. The mean age was 6.65 ± 4.46 years. Overall, 40 eyes received glaucoma surgery, and 38 eyes received non-glaucoma surgery. Six eyes had previously received cataract extraction. A total of 198 eyes had no history of glaucoma surgery, seven patients had a family history of glaucoma, and five patients had a family history of congenital cataracts (**Table 1**). Of the 276 eyes, 204 eyes (73.91%) were treated with glaucomatous medications before visiting Henan Eye Hospital. Overall, 48.53% eyes received more than one medication for lowering IOP.

Different Types of Pediatric Glaucoma

A total of 212 eyes had primary glaucoma, and 64 eyes had secondary glaucoma (**Table 2**). Among the pediatric eyes, PCG accounted for 55.43%. The most common type of the secondary glaucoma in pediatric patients was trauma-related glaucoma (23 eyes; 8.33% of all the involved eyes). Other common types of secondary glaucoma were silicone oil-related (eyes 13, 4.71%) and lens-related (six eyes, 2.17%). Four eyes had uveitis, anterior segment dysgenesis, steroid-induced glaucoma, and retinoblastomas (1.45%).

Different Signs and Symptoms of Pediatric Glaucoma

The main signs and symptoms observed in this study are summarized in **Table 3**. Parental concerns were included in the presentation of signs. The common clinical signs observed by an ophthalmologist included elevated IOP (91 eyes, 32.97%), impaired vision (85 eyes, 30.80%), buphthalmos (19 eyes, 6.88%), leukocoria (15 eyes, 5.43%), and enlargement of the C/D ratio (eight eyes, 2.89%). The most common symptoms included eye pain (11 eyes, 3.99%), red eyes (10 eyes, 3.62%), photophobia (nine eyes, 3.26%), lacrimation (seven eyes, 2.54%).

Different Interventions for Pediatric Glaucoma

After evaluation by a glaucoma specialist in our center, the patients received surgery as follows: 23.55% underwent trabeculotomy (Trab), 19.93% underwent microcatheter-assisted 360° trabeculotomy (MAT) combined with Trab, and 14.86% underwent Ahmed glaucoma valve (AGV) implantation (**Table 4**). Eyes with lens-related glaucoma received intraocular lens (IOL) repositioning, IOL suspension fixation, and IOL extraction. Four retinoblastoma eyes received ophthalmectomy.

TABLE 4 | Management of pediatric glaucoma patients*.

Characteristic	N (numbers of eyes)	(%)
Surgical treatment		
Trabeculectomy (Trab)	65	(23.55%)
Microcatheter-assisted 360°trabeculotomy (MAT) combined Trab	55	(19.93%)
Ahmed glaucoma valve implantation	41	(14.86%)
Non-penetrating trabeculectomy	31	(11.23%)
Phacoemulsification	16	(5.80%)
Trabeculotomy combined Trab	15	(5.43%)
MAT	12	(4.35%)
Trabeculotomy	7	(2.54%)
Phacoemulsification combined Ahmed	6	(2.17%)
Ophthalmectomy	4	(1.45%)
Paracentesis and irrigation for hyphema	5	(1.81%)
Removal of silicone	5	(1.81%)
Xen glaucoma implant	2	(0.73%)
Intraocular lens reposition	2	(0.73%)
Evisceration of the eye	2	(0.73%)
Suspension fixation of intraocular lens	1	(0.36%)
Intraocular lens extraction	1	(0.36%)
Ahmed valve reposition	1	(0.36%)
Others		
Endoscopic cyclophotocoagulation (ECP)	5	(1.81%)

Data are shown as NO. (%).

Follow-Up Findings of Patients With Pediatric Glaucoma

The clinical parameters of the different types of glaucoma are described in **Table 5**. In all patients, the IOP decreased to 16.33 ± 8.28 mmHg at the final follow-up. PCG manifests at an earlier age than JOAG and secondary pediatric glaucoma. Among 153 eyes with PCG, the IOP decreased from 33.56 ± 10.05 to 15.27 ± 7.48 mmHg at the final follow-up. The IOP of patients with JOAG and secondary glaucoma were 17.16 ± 10.05 and 18.65 ± 8.55, respectively, at the final follow up. The VA of pediatric patients with PCG, JOAG, and secondary glaucoma was determined respectively: 8 (5.23%), 4 (6.78%), and 3 (4.69%) eyes had no light perception at the final follow up; 10 (6.54%), 2 (3.39%), and 2 (3.13%) eyes had light perception; 18 (11.76%), 6 (10.17%), and 11 (17.19%) eyes had perceived hand movement; and 9 (5.88%), 7 (11.86%), and 2 (3.13%) eyes had perceived finger counting. The mean VA of the eyes with PCG, JOAG, and secondary glaucoma was 0.79 ± 0.68, 0.51 ± 0.48, and 0.53 ± 0.50, respectively. The rate of reoperation interventions in patients with PCG was 9.15% (14 eyes), which was higher than those of other pediatric glaucoma types. Among eyes with PCG, one eye received three surgeries, and one eye received four surgeries. The number of glaucoma medications used was similar among the different types of glaucoma.

DISCUSSION

Glaucoma is the leading cause of irreversible blindness worldwide. Pediatric glaucoma is characterized by impairment of aqueous outflow, resulting in elevated IOP, which further results in glaucomatous optic neuropathy and eventual blindness (9–11). Severe visual impairment in pediatric glaucoma is commonly observed in underdeveloped and developing countries. It is a major and preventable cause of irreversible visual loss in pediatric patients.

Despite recent advances in surgical and medical treatments, pediatric glaucoma has resulted in irreversibly impaired visual function. In underdeveloped and developing countries and districts, the level of medical care is uneven, especially in remote areas. Henan is an agricultural province with limited economic and medical development compared to the coastal and relatively developed areas in China. Pediatric glaucoma may substantially diminish the quality of life over their entire lifetime, which can be devastating to patients and their families, and even to society. To date, epidemiological investigations on pediatric glaucoma in Henan and its surrounding regions have been insufficient. In this study, we included patients with confirmed pediatric glaucoma who underwent surgery in the ophthalmology department of the Henan Eye Institute.

The average age of the patients in the study by Baig et al. was similar to that in another study conducted in Hong Kong, and patients with JOAG were older (2). Surukrattanaskul et al. reported an earlier presentation age in their study (12). This could be related to the different medical referral systems. The number of male pediatric patients was higher than that of female patients (63.60% vs. 36.40%; 1.75:1), which is similar to previously published results (12–16). Moreover, 8.79% of patients in this study had a family history of glaucoma, which was slightly higher than in studies conducted in eastern China and parts of Asia, including Thailand (12, 13). Pediatric glaucoma is commonly treated surgically, and medical treatment is assisted by therapy (17). Here, 16.74% of the patients had previously received at least once glaucoma surgery, 27.62% patients had a history of medical treatment, and 2.93% of the 239 patients with an available family history were identified in this study. A higher rate of glaucoma history was observed in the Dallas Glaucoma Registry (18). The economic development and awareness of prevention could be attributed to this high rate. There was a tendency to check the eyes because of a family history of glaucoma. By contrast, family history was lower in this study than in those reported in Beijing, Shanghai, and Hong Kong districts of China.

PCG was the most prevalent type of glaucoma in this study population, and it was present in 55.43% of the patients. Many studies have reported varied distributions of subtypes of pediatric glaucoma (5, 7, 8, 13, 16, 19). Similar results were observed in mainland reports from Chinese populations of Beijing Tongren Hospital and Shanghai Eye, Ear, Nose and Throat Hospital (13, 14). In the United States, Canada, and Brazil, PCG was the most common diagnosis (7, 15). PCG also comprised the majority of the cases in Britain and the Republic of Ireland (5). Conversely, many studies have reported a higher prevalence of secondary

TABLE 5 | Final clinical outcomes among different types of pediatric glaucoma*.

	Primary congenital glaucoma	Juvenile open-angle glaucoma	Secondary glaucoma
Presentation			
AGE	4.59 ± 3.59	9.86 ± 3.67	8.60 ± 4.39
SEX (Male:Female)	82:49	24:20	46:18
IOP (mmHg)	33.56 ± 10.05	32.59 ± 14.99	33.30 ± 11.56
LogMAR visual acuity	NLP∼0, (0.89 ± 0.41)#	NLP∼0, (0.76 ± 0.50) #	NLP∼-0.08 (0.83 ± 0.49)#
Final follow-ups			
IOP (mmHg)	15.27 ± 7.48	17.16 ± 10.05	18.65 ± 8.55
LogMAR visual acuity	NLP∼ 0, (0.79 ± 0.68) #	NLP∼0, (0.51 ± 0.48) #	NLP∼-0.08 (0.53 ± 0.50)#
Re-operation interventions/eye	14 (9.15%)	4 (6.78%)	3 (4.69%)
No. of glaucoma medications	2.05 ± 0.98	2.00 ± 1.95	2.07 ± 0.99

Data are shown as mean ± standard deviation or range.
#*mean ± standard is from LogMAR visual acuity more than 1.0 in primary congenital glaucoma.*

glaucoma than PCG. The prevalence of PCG in Hong Kong and Thailand was similar to the 17% reported in the USA and was much lower than in our studies (12, 16). The number of boys was more than twice that of girls in this study, which is consistent with reports from northern China, the United States, and Europe (20, 21). Papadopoulos et al. reported that the incidence of PCG was significantly greater in pediatric patients of Pakistani origin than in Caucasian children (5). The varied results among different countries and districts suggest that glaucoma subtypes may be related to ethnicity. Although consanguine marriages are not allowed in China, the phenomenon of consanguine marriages might not disappear.

Traumatic glaucoma is the most common type of secondary pediatric glaucoma in Henan Province. Eye trauma is an important cause of ocular morbidity and a leading cause of non-congenital unilateral blindness in children (22). The rate of pediatric traumatic patients was relatively lower in the United States and the United Kingdom (22, 23). In developing countries, the incidence of ocular trauma is higher. Henan Province is an agricultural province and a major source of migrant peasant workers in China. Many young parents go to work without their children. The low level of economic development and child neglect might be some of the reasons for the higher incidence observed in this study compared with previous studies conducted in the Hong Kong district (22, 23). The Tongren Hospital Eye Center has the highest level of specialized care in treating patients with eye trauma, which might lead to a higher incidence of traumatic glaucoma in pediatric patients (14). Therefore, IOP should be measured periodically in pediatric patients after ocular trauma.

Beck et al. have reported that congenital angle anomalies and postoperative inflammation leading to angle dysfunction or synechia closure, and some unknown influences of aphakic state or vitreous interaction may cause reduced aqueous humor outflow (24). Current lens-related glaucoma was less common in this study than that in previous studies. Baig et al. have reported lens-related glaucoma as the most prevalent type of secondary glaucoma (18.0% of all involved eyes) (2). A similar incidence rate was observed (1.6%) on the mainland at Beijing Tongren Hospital. Patients undergoing cataract surgery at an early age are at a high risk of developing glaucoma with or without IOL implantation (25).

Elevated IOP was observed in 32.97% of the patients and was the most common reason for referral and visiting our center. In Hong Kong, only 12.2% of pediatric glaucomatous eyes were identified by high IOP (2). The relatively concentrated medical resources in provincial capital cities in mainland China could be the reason for this. Impaired vision was also an important reason for visiting our center. Medical resources and ophthalmological specialties are quite disparate in mainland China compared with other developed areas worldwide. Hence, most patients with pediatric glaucoma present with an advanced stage of disease and impaired vision at our center. Therefore, early diagnosis and treatment are crucial to prevent vision loss. Moreover, eye pain and redness were the most common symptoms reported by patients or their parents. The results suggest that pediatricians might be familiar with the common symptoms and refer to an ophthalmologist if necessary.

Trab has been effective as the primary approach for pediatric glaucoma (26, 27). Here, the most common initial glaucoma intervention for each eye was Trab. The most commonly performed surgery was Trab and MAT combined with Trab. Thus, Trab remains the preferred surgical treatment for pediatric glaucoma in central China. The most common surgical procedure in North America and the United Kingdom is goniotomy (7, 28, 29). However, Trab and MAT combined with Trab have been adopted rather than goniotomy for anterior angle surgery in China (14). Previous reports have shown that angle surgeries (regardless of goniotomy or Trab) were effective for PCG, JOAG, and uveitic glaucoma (30–36). Non-penetrating trabeculectomy was used to treat the patients with PCG and JOAG. AGV was treated for multiple surgical failures or secondary pediatric glaucoma in this study. In Hong Kong, the most common surgical intervention is AGV (16). Similar to the Hong Kong report, we observed that patients with aniridia accepted AGV as the primary treatment in central China (16). Differences in economic levels and healthcare systems could be one of the influencing factors in the different conditions of pediatric glaucoma treatments.

Here, in most eyes that were analyzed, stable IOP reduction was observed in different types of pediatric glaucoma. The average LogMAR VA improved at the final observation in all groups of patients with pediatric glaucoma. Chan et al. reported that over 73% of affected eyes experienced VA worsening (16). Long-term visual functional outcomes of infants were better than those of newborns with PCG (37). Moreover, the improvement in the average VA was more significant in the JOAG group than in the PCG group at the final follow-up in this study. The results suggest that an effective reduction in IOP might not save VA for all patients. Therefore, amblyopia should be considered after glaucoma surgery in children. The 9.15% rate of reoperation was highest in patients with PCG in this study, which was lower than the 18% rate in a Würzburg clinical study (28). This may be due to the development of anti-glaucomatous medicines and surgeries. Effective treatment of pediatric glaucoma, preserving vision, and improving amblyopia remain challenging for all pediatric glaucoma therapeutics.

This study had several limitations. First, the retrospective nature of this study may have led to bias. Second, no widely established or standardized management protocol guidelines currently exist. Last, this study was a hospital-based data analysis of patients with severe pediatric glaucoma who underwent surgical treatment. Therefore, population-based epidemiological studies are needed.

In summary, the present study summarizes the characteristics, epidemiology, management, and outcomes of pediatric glaucoma patients. PCG is the most common type in central China. This finding may be related to ethnicity and possibly consanguine marriages. Traumatic glaucoma was the most common type of secondary glaucoma in this study and may be caused by low levels of economic development and child neglect in rural areas. Elevated IOP, poor vision, and eye pain were the most common signs and symptoms. Some pediatricians are familiar with the common symptoms and refer patients to an ophthalmologist when necessary. Trab and MAT combined with Trab were the most common interventions used in this study, and AGV was administered for multiple surgical failures or secondary pediatric glaucoma. Effective IOP control was observed in the PCG, JOAG, and secondary pediatric glaucoma groups; however, the improvement in average VA was larger in the JOAG group than in the PCG group at the final follow-up. Pediatric amblyopia might require full attention during the entire treatment, especially after anti-glaucoma surgery. Effective preventive measures and more public education on glaucoma prevention and the importance of early diagnosis and treatment are necessary. Expanded medical services and education in glaucoma screening in rural places and in at-risk populations are required.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

This retrospective study was approved by the Ethics Committee of Henan Eye Hospital, Henan Eye Institute, and Henan Provincial People's Hospital Human Research Ethics Committee [ethics number is HNEECKY-2022(08)]. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

QL, CL, XF, and WC performed the initial clinical database searches. QL, HL, XY, and YD performed the statistical analyses. QL produced the first draft of the manuscript, tables, and supervised the study. All authors contributed to study revision, edited the manuscript, reviewed the manuscript, and approved the submitted version.

FUNDING

QL received support from Henan Medical Science Foundation of China (SBGJ2018083) and the Basic Research Project of Henan Provincial Eye Hospital (No. 21JCQN005).

ACKNOWLEDGMENTS

The authors thank the editor and reviewers for their valuable comments and suggestions.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 01 July 2022
doi: 10.3389/fmed.2022.956339

Implicating Causal Brain Magnetic Resonance Imaging in Glaucoma Using Mendelian Randomization

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Edited by:
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Specialty section:
This article was submitted to Ophthalmology, a section of the journal Frontiers in Medicine

Received: 30 May 2022
Accepted: 13 June 2022
Published: 01 July 2022

Citation:
Liu K, Wu P, Chen B, Cai Y, Yuan R and Zou J (2022) Implicating Causal Brain Magnetic Resonance Imaging in Glaucoma Using Mendelian Randomization. Front. Med. 9:956339. doi: 10.3389/fmed.2022.956339

Background: Glaucoma is hypothesized to originate in the brain but manifests as an eye disease as it possesses the common features of neurodegeneration diseases. But there is no evidence to demonstrate the primary brain changes in glaucoma patients. In the present study, we have used Mendelian randomization (MR) to understand the causal effect of brain alterations on glaucoma.

Methods: Our MR study was carried out using summary statistics from genome-wide associations for 110 diffusion tensor imaging (DTI) measurements of white matter (WM) tracts (17,706 individuals), 101 brain region-of-interest (ROI) volumes (19,629 individuals), and glaucoma (8,591 cases, 210,201 control subjects). The causal relationship was evaluated by multiplicative random effects inverse variance weighted (IVW) method and verified by two other MR methods, including MR Egger, weighted median, and extensive sensitivity analyses.

Results: Genetic liability to fornix fractional anisotropy (FX.FA) (OR = 0.71, 95%CI = 0.56–0.88, $P = 2.44 \times 10^{-3}$), and uncinate fasciculus UNC.FA (OR = 0.65, 95%CI = 0.48–0.88, $P = 5.57 \times 10^{-3}$) was associated with a low risk of glaucoma. Besides, the right ventral diencephalon (OR = 1.72, 95%CI = 1.17–2.52, $P = 5.64 \times 10^{-3}$) and brain stem (OR = 1.35, 95%CI = 1.08–1.69, $P = 8.94 \times 10^{-3}$) were associated with the increased risk of glaucoma. No heterogeneity and pleiotropy were detected.

Conclusion: Our study suggests that the fornix and uncinate fasciculus degenerations and injures of the right ventral diencephalon and brain stem potentially increase the occurrence of glaucoma and reveal the existence of the brain-eye axis.

Keywords: diffusion tensor imaging, white matter, region-of-interest, glaucoma, Mendelian randomization

INTRODUCTION

Glaucoma is one of the common blinding disorders characterized by progressive damage to the retinal ganglion cells (RGCs) along with visual field loss (1). Many elements are considered major risk factors, including intraocular pressure (IOP), genes, family factors, vascular factors, and high myopia (2, 3). However, elevated IOP is the only modifiable risk factor; thus, IOP reduction is considered a helpful treatment until now (4, 5). However, whereas many normal IOP individuals suffer from the disorder, IOP reduction could not completely stem the glaucoma progression (6). This suggests that it is necessary to find other effective ways to alleviate glaucomatous neurodegeneration.

The eye is the extension of the brain and has many embryological, functional, and developmental similarities with the central nervous system (7). As the hallmark of glaucoma, the primary cells to die in glaucoma are RGCs, which are typical neurons (8). Although the cell bodies of RGCs lie in the retina, most portions of axons lie outside the ocular area, which forms the optic nerve, optic chiasm, and optic tract (8). Besides, many observational studies have shown that glaucoma patients had cognition and memory decline (9, 10). In addition, motor coordinating impairment and psychological disorders such as depression and anxiety were prevalent among the glaucoma patients (11, 12). Based on these theories, various scholars hypothesize that brain tissues are associated with glaucoma pathogenesis (13, 14).

Magnetic resonance imaging (MRI) studies are an important tool as a non-invasive method to evaluate the structure, function, and neurochemistry of the brain (15). Some researchers investigated the structural and functional brain changes in patients with glaucoma using MRI. They reported white matter structural abnormalities in different parts of the visual pathway and altered brain connectivity beyond the visual system (16, 17). These observations led to the question of whether brain changes precede or follow the RGCs degeneration. Although an anterograde tans-synaptic degeneration might be responsible for some visual pathway damage, the brain structures outside the visual pathway generally reflect a primary neuropathological process. Besides, due to the high cost and limited accessibility to MRI facilities, it is hard to complete randomized, controlled trials to explore the primary brain changes in glaucoma patients.

Mendelian randomization (MR) uses a genetic variation to evaluate the inter-causality of disease risk factors, which is not affected by most acquired confounding factors (such as environment) (18). Thus far, MR has been used to derive the causal relationship between kidney damage and brain cortical structure, brain structures, and neuropsychiatric disorders, including Alzheimer's disease (AD) (19, 20). Recently, a GWAS study reported an association of single nucleotide polymorphisms (SNPs) of brain structural measurements with glaucoma (21–24). These studies bought insight into finding the causal relationship between glaucoma and brain structures. Thus, we aimed to identify the causal relationship between white matter (WM) structures [diffusion tensor imaging (DTI), brain subregion volumes region of interest (ROI)], and glaucoma by using the two-sample MR analysis. Our results indicate the potential risks of glaucoma in the brain and provide new insight into the possible existence of the brain-eye axis.

MATERIALS AND METHODS

Assessment of Assumptions

The valid genetic instrumental variables (IVs) in the MR study must fulfill the 3 assumptions (**Figure 1**): (1) The IVs must be associated with DTI parameters of WM tracts and ROI volumes. (2) The IVs must not be associated with factors that confound the relationship between DTI parameters of WM tracts and glaucoma, ROI volumes, and glaucoma. (3) The IVs are only associated with glaucoma through DTI parameters of WM tracts and ROI volumes.

The Data Source for Diffusion Tensor Imaging Measurements

The genetic variants of DTI parameters of WM traces were taken from the genome-wide association studies (GWAS) of Zhao et al. (21). This GWAS study consisted of a total of 110 DTI parameters from 17,706 subjects of European ancestry, including fractional anisotropy (FA), mean diffusivity (MD), axial diffusivity (AD), radial diffusivity (RD), mode of anisotropy (MO), and the average values of 21 WM tracts. The 21 WM tracts were labeled through the Enhancing NeuroImaging Genetics through Meta-Analysis (ENIGMA) DTI pipeline (22, 23), and the full names of the tracts are shown in **Supplementary Table 1**.

The Data Source for the Region-of-Interest Volumes

The genetic variants of the ROI volumes came from the genome-wide association studies (GWAS) study of Zhao et al. (24). This GWAS study analyzed MRI data from 19,629 European participants through consistent procedures *via* normalization tools (ANTs)[1] and used Mindboggle-101 atlas for labeling.

Selection of Genetic Instruments

The single nucleotide polymorphisms (SNPs) related to DTI and ROI were screened to obtain the required IVs. First, the SNPs associated with DTI or ROI exhibited genome-wide significance ($P < 5 \times 10^{-8}$). Also, SNPs with linkage disequilibrium (LD) were removed ($R^2 < 0.001$) in the LD data from the 1,000 Genomes Project (25). In addition, we removed the SNPs with the presence of palindromes. Finally, to clarify the direction of causality, all SNPs were examined using MR Steiger (26), and the SNPs that could lead to causality were removed.

The Data Source for Glaucoma

To avoid the overlapping between DTI and ROI-related and glaucoma-related data sources as much as possible, the association of glaucoma relative SNPs was obtained from the FinnGen consortium (Release 5, May 11, 2021, comprising 218,792 individuals). This study defines glaucoma by H40-H42 of the International Classification of Disease-10 (ICD-10).

[1] http://stnava.github.io/ANTs/

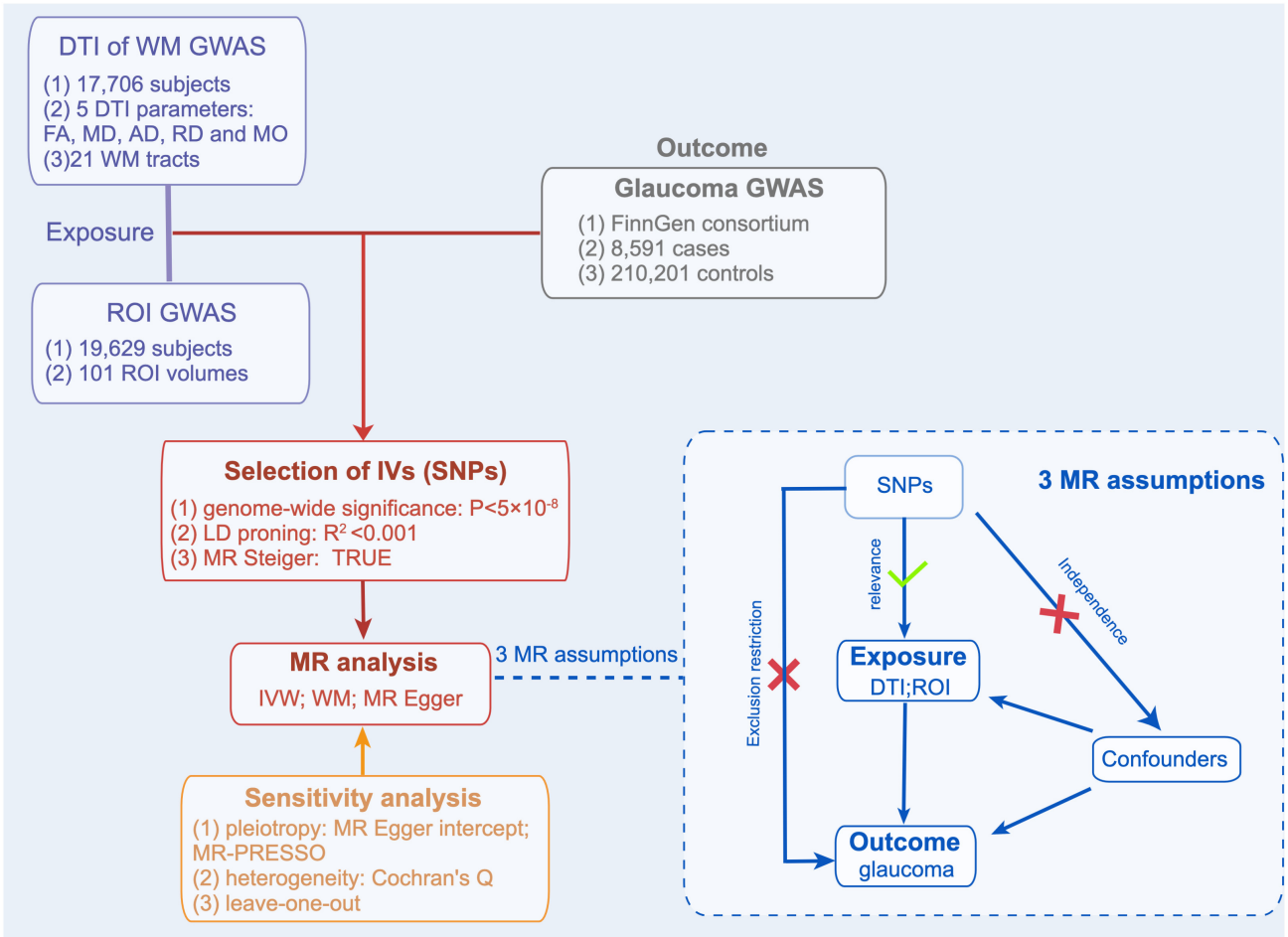

FIGURE 1 | Mendelian Randomization Analysis Overview. Overview of Mendelian randomization analyses and major assumptions. DTI, indicates diffusion tensor imaging; WM, white matter; ROI, region of interest; FA, fractional anisotropy; MD, mean diffusivity; AD, axial diffusivity; RD, radial diffusivity; MO, mode of anisotropy.

Genotype data from the FinnGen research project included 8,591 glaucoma patients of Finnish origin and 210,201 control subjects.

Sensitivity Analysis

To test the robustness of the MR estimates, we applied sensitivity analysis. First, MR Egger's intercept values were used to assess the multiplicity of SNPs. The closer the intercept to 0, the lower the multiplicity was considered. Meanwhile, MR-Pleiotropy RESidual Sum and Outlier (PRESSO) (27) was used to evaluate further the SNPs pleiotropy that has the potential causality, and the SNPs with abnormalities were removed. Meanwhile, Cochran's Q test was used for the assessment of heterogeneity. Finally, to ensure the robustness of the results, the analysis was carried out using the leave-one-out test.

Statistical Method

"Two Sample MR" packages were used to estimate the MR results using the R software (version 1.4.1717). The Wald ratio was used to assess the causal effect of individual SNPs. Provided that sensitivity analysis was passed, inverse variance weighting (IVW) was used as the main method to assess MR effects. The weighted median (28) and MR-Egger regression (27) were used as additional methods to determine the MR results validity further. $P < 0.05$ was considered to have potential causality.

RESULTS

Instrumental Variables Selection

A total of 394 SNPs were selected to predict 81 DTI parameters of WM traces genetically, and 389 SNPs were used to predict 61 ROI volumes (**Supplementary Tables 2**, **3**). An overview of the flow charts is shown in **Figure 1**.

Causal Associations Between Diffusion Tensor Imaging and Glaucoma

We evaluated the DTI-Glaucoma association by Mendelian randomization analysis, and the specific statistical results are shown in **Figure 2** and **Supplementary Table 4**. There were two FA parameters of the white matter that reached statistical

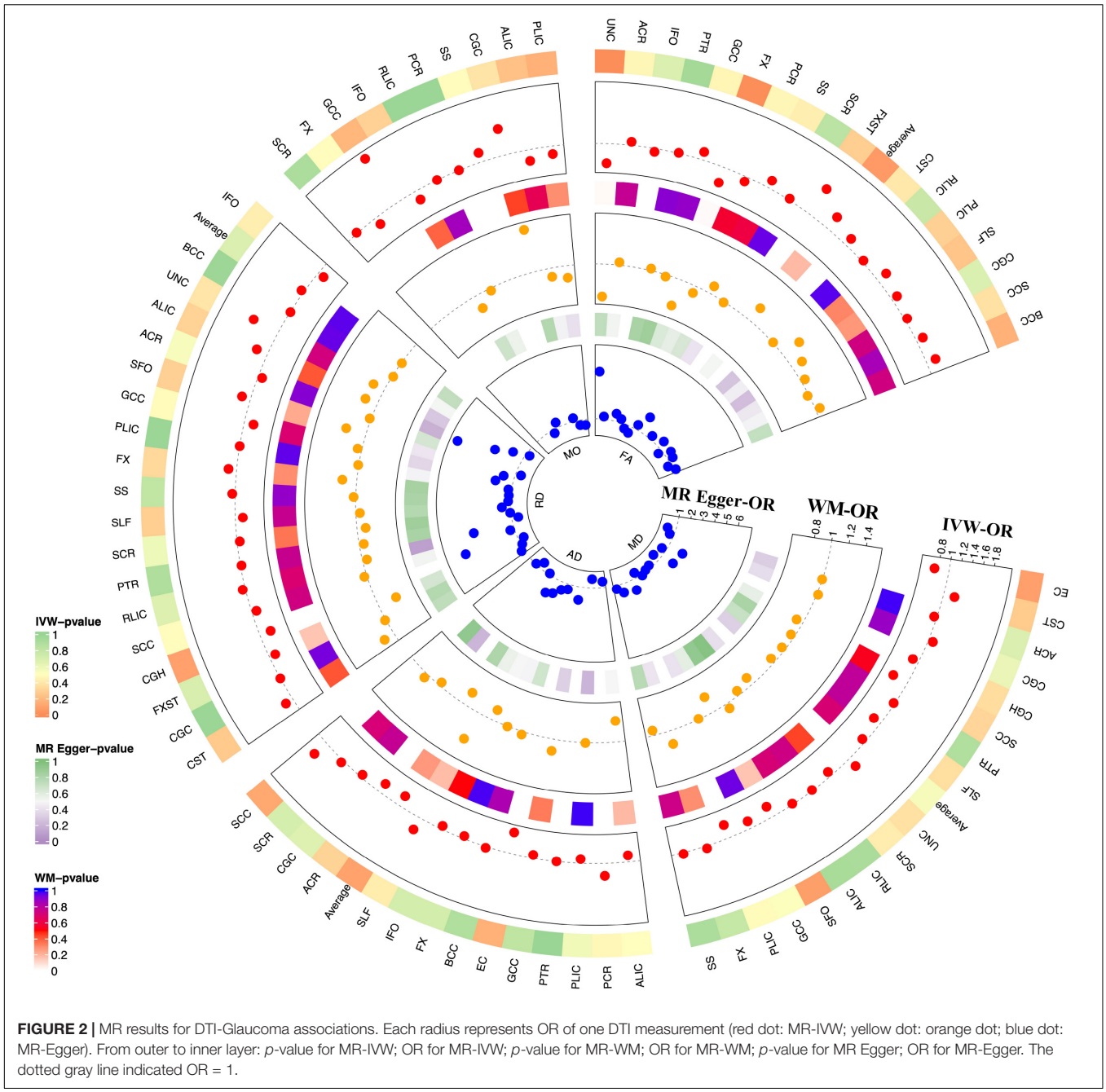

FIGURE 2 | MR results for DTI-Glaucoma associations. Each radius represents OR of one DTI measurement (red dot: MR-IVW; yellow dot: orange dot; blue dot: MR-Egger). From outer to inner layer: *p*-value for MR-IVW; OR for MR-IVW; *p*-value for MR-WM; OR for MR-WM; *p*-value for MR Egger; OR for MR-Egger. The dotted gray line indicated OR = 1.

significance: fornix fractional anisotropy (FX.FA) and uncinate fasciculus fractional anisotropy (UNC.FA) (**Table 1**). Then, IVW was used to confirm that FX.FA (OR = 0.71, 95%CI = 0.56–0.88, $P = 2.44 \times 10^{-3}$), UNC.FA (OR = 0.65, 95%CI = 0.48–0.88, $P = 5.57 \times 10^{-3}$) was associated with a low risk of glaucoma. Additionally, we further validated the results of IVW by weighted median (FX.FA: $P = 1.03 \times 10^{-2}$; UNC.FA: $P = 2.42 \times 10^{-2}$) and MR-egger (FX.FA: $P = 0.614$, UNC.FA: $P = 0.735$) (**Table 1**).

MR genetic instruments should only impact the outcome *via* exposure and not any other pathway (horizontal pleiotropy and heterogeneity). Therefore, we confirmed our results by applying sensitivity tests (**Supplementary Tables 5**, **6**). The result showed little pleiotropy (FX.FA: $P = 0.91$ and UNC.FA: $P = 0.67$) and heterogeneity [FX.FA (IVW: $P = 0.65$; MR Egger: $P = 0.76$) and UNC.FA (IVW: $P = 0.53$; MR Egger: $P = 0.70$)] (**Table 2**). Further, MR-PRESSO demonstrated no significant horizontal pleiotropy of FX.FA ($P = 0.762$). The MR-PRESSO test could not be performed due to insufficient SNPs in UNC.FA. Additionally, the leave-one-out method confirmed the robustness of the causal association in FX.FA-Glaucoma and UNC.FA-Glaucoma pairs (**Supplementary Figures 1A,B**). Considering no

TABLE 1 | MR results between WM and glaucoma.

WM	Method	SNP	OR	95%CI	*P*-value
FX.FA	IVW	7	0.71	0.56–0.88	2.44×10^{-3}
FX.FA	WM	7	0.69	0.52–0.92	1.03×10^{-2}
FX.FA	MR egger	7	0.64	0.13–3.23	0.614
UNC.FA	IVW	3	0.65	0.48–0.88	5.57×10^{-3}
UNC.FA	WM	3	0.63	0.42–0.94	2.42×10^{-2}
UNC.FA	MR egger	3	4.93	0.004–5815.19	0.735

MR, Mendelian randomization; IVW, inverse variance weighted; WM, white matter; FX, fornix (column and body of fornix); UNC, uncinate fasciculus; FA, fractional anisotropy.

TABLE 2 | Sensitivity analysis between WM and glaucoma.

	Method	Q	*P*-value	Intercept	*P*-value
FX.FA	IVW	3.35	0.76	0.006	0.91
FX.FA	MR egger	3.33	0.65		
UNC.FA	IVW	0.71	0.70	−0.130	0.67
UNC.FA	MR egger	0.39	0.53		

MR, Mendelian randomization; IVW, inverse variance weighted; WM, white matter; FX, fornix (column and body of fornix); UNC, uncinate fasciculus; FA, fractional anisotropy.

TABLE 3 | MR results between ROI and glaucoma.

ROI	Method	SNP	OR	95%CI	*P*
Right.ventral.DC	IVW	5	1.72	1.17–2.52	5.64×10^{-3}
Right.ventral.DC	WM	5	2.07	1.34–3.21	1.10×10^{-3}
Right.ventral.DC	MR egger	5	1.63	0.35–7.51	0.578
Brain.stem	IVW	13	1.35	1.08–1.69	8.94×10^{-3}
Brain.stem	WM	13	1.48	1.13–1.95	5.06×10^{-3}
Brain.stem	MR egger	13	1.00	0.31–3.20	0.994

MR, Mendelian randomization; IVW, inverse variance weighted; WM, weighted median. Right.ventral.DC, right ventral diencephalon; brain.stem, Brain stem.

heterogeneity or horizontal pleiotropy was present in the selected SNPs after sensitivity testing, the results of IVW were more reliable. Therefore, these results demonstrated that reducing fornix and uncinate fasciculus fractional anisotropy made a causal contribution to glaucoma.

Causal Associations Between Region-of-Interest Volume and Glaucoma

By Mendelian randomization analysis, we found that the volumes of the right ventral diencephalon (right.Ventral.DC) and brain stem (Brain.stem) had a significant causal relationship with glaucoma (**Table 3**). The statistical results for the other regions are shown in **Figure 3** and **Supplementary Table 7**. The results of IVW confirmed the volume of the right.ventral.DC (OR = 1.72, 95%CI = 1.17–2.52, $P = 5.64 \times 10^{-3}$) and Brain.stem (OR = 1.35, 95%CI = 1.08–1.69, $P = 8.94 \times 10^{-3}$) were positively associated with glaucoma risk. Meanwhile, weighted median (right.ventral.DC: $P = 1.10 \times 10^{-3}$; Brain.stem: $P = 5.06 \times 10^{-3}$) and MR-Egger (right.ventral.DC: $P = 0.578$; Brain.stem: $P = 0.994$). were used to verify the findings further.

We performed pleiotropic analysis (**Supplementary Table 8**) and heterogeneity tests (**Supplementary Table 9**). By calculating the MR-Egger intercept, no significant horizontal pleiotropy was observed in the right.ventral.DC (intercept = 0.003, $P = 0.95$) and Brain.stem (intercept = 0.018, $P = 0.62$) (**Table 4**). MR-PRESSO further validated the absence of significant horizontal pleiotropy (right.ventral.DC: $P = 0.430$; Brain.stem: $P = 0.126$). Besides, the results of heterogeneity tests showed that the right.ventral.DC (IVW: Q = 5.67, $P = 0.22$; MR-Egger: Q = 5.66, $P = 0.13$) and Brain.stem (IVW:Q = 17.92, $P = 0.12$); MR-Egger: Q = 17.51, $P = 0.09$) associated SNPs that were not significantly heterogeneous (**Table 4**). Meanwhile, the leave-one-out test confirmed the causal association in the right.ventral.DC-Glaucoma and Brain.stem-Glaucoma pairs (**Supplementary Figures 1C,D**). Therefore, the results of IVW were more reliable as no heterogeneity or horizontal pleiotropy was present in the selected SNPs after sensitivity testing. Thus the inferred causality between right.ventral.DC-glaucoma and Brain.stem-Glaucoma pairs were plausible.

DISCUSSION

To the best of our knowledge, this is the first MR analysis that leveraged population-scale human genetics to understand the causal relationship between neuroimaging polymorphisms and glaucoma in an unbiased manner. The imaging changes in the brain could not be directly associated as a cause of glaucoma in many current neuroimaging studies. However, our results show that the changes in the microstructures of the white matter and the volume of specific brain regions can affect the onset of glaucoma and reveal the existence of the brain-eye axis.

Large cohort studies have confirmed that patients with glaucoma have shown progressive vision loss even with a significant reduction in IOP (6). This finding indicates that IOP-independent mechanisms contribute to the etiopathogenesis of this disorder. Recently, there has been growing attention about the brain changes in glaucoma. In particular, numerous MRI studies performed on glaucoma patients have shown brain structural differences. Hernowo et al. (29) found decreased volume along the full length of the visual pathway for both gray and white matter in a follow-up study of glaucoma patients. Moreover, changes in the brain beyond the primary visual pathways, including the middle occipital gyrus, inferior temporal gyrus, the anterior thalamic radiation, corticospinal tract, superior temporal gyrus, and occipital lobe white matter, were also reported (30–32). However, the distinction between these brain changes as cause or consequence remains difficult as nearly all current literatures are cross-sectional with limited population and testing methods. Thus, understanding the brain-eye interactions across disease severity could provide valuable insights into the pathogenesis of glaucoma and lead to new treatment modalities.

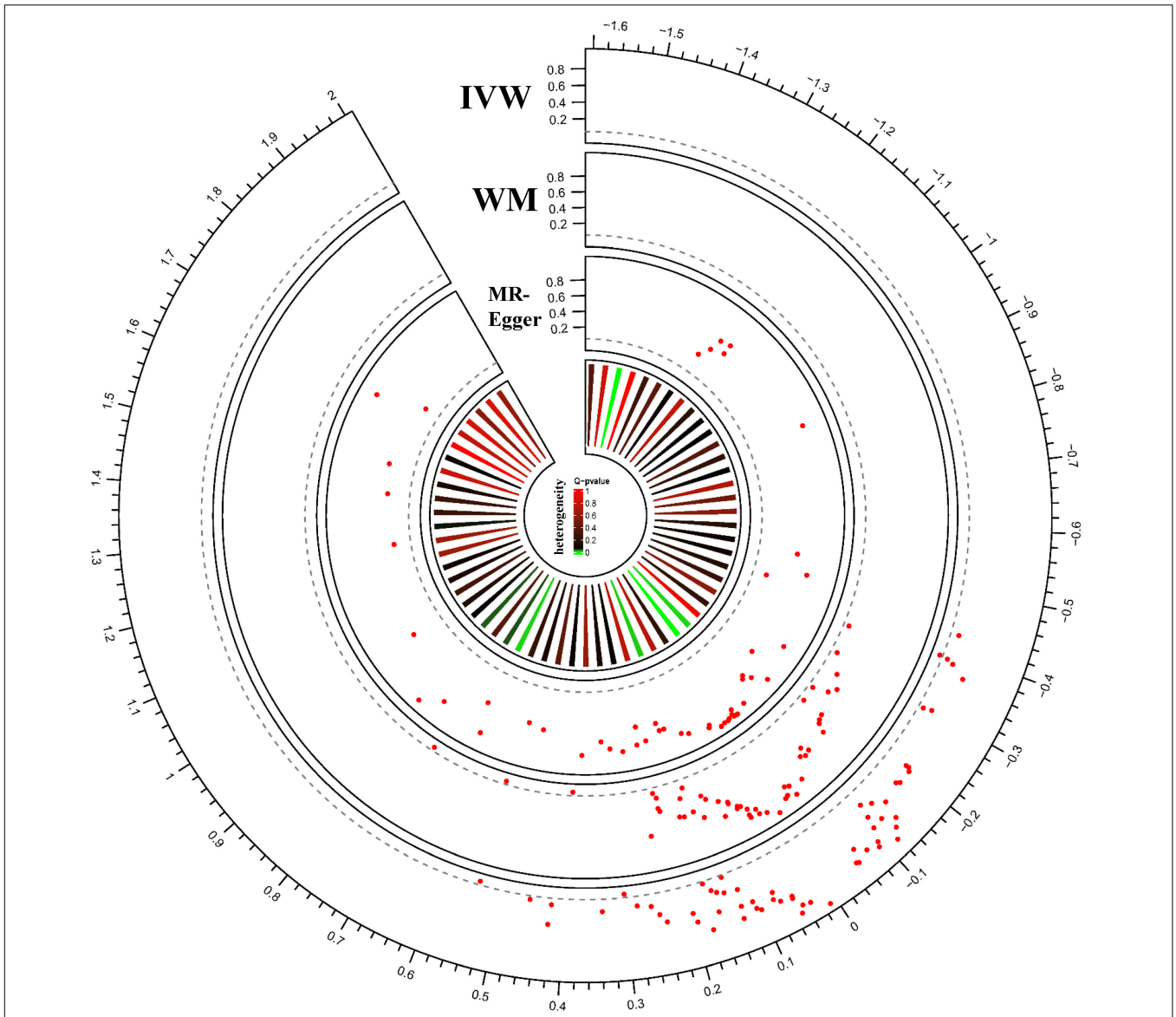

FIGURE 3 | MR results for ROI-Glaucoma associations. Each radius represents one ROI measurement. From outer to inner layer: MR results analyzed with IVW; MR results analyzed with WM; MR results analyzed with MR-Egger; heterogeneity of each ROI measure. The red dot represents the ROI-Glaucoma relation. The X-axis shows the beta value, and Y-axis shows the *p*-value. The dotted gray line indicated the nominal p threshold (0.05).

TABLE 4 | Sensitivity analysis between ROI and glaucoma.

	Method	Q	*P*	Intercept	*P*
Right.ventral.DC	IVW	5.67	0.22	0.003	0.95
Right.ventral.DC	MR egger	5.66	0.13		
Brain.stem	IVW	17.92	0.12	0.018	0.62
Brain.stem	MR egger	17.51	0.09		

MR, Mendelian randomization; IVW, inverse variance weighted; Right.ventral.DC, right ventral diencephalon; Brain.stem, brain stem.

MRI provides a non-invasive method to evaluate structural, metabolic, and functional changes in the brain. Diffusion-weighted imaging measures the 3-dimensional displacement of water molecules and provides important information about changes in the microstructures of the white matter (33). Many studies have used DTI to describe the white matter integrity in glaucoma. Hui et al. (34) reported decreased FA and increased MD at the optic nerve for the first time in a rat model of glaucoma. Decreased FA at the proximal portion of the optic nerve and chiasm were also detected in glaucoma patients (35, 36). Meanwhile, recent DTI studies demonstrated that glaucoma patients had a lower FA within the visual white matter pathway, negatively correlated with glaucoma severity (37). Additionally, the decreased FA in the inferior fronto-occipital fasciculus, the longitudinal and inferior frontal fasciculi, the putamen, the caudate nucleus, the

anterior and posterior thalamic radiations, and the anterior and posterior limbs of the internal capsule were reported in glaucoma (38). In our results, decreased FX.FA and UNC.FA was a novel risk factor for glaucoma. Lower FA values indicate the presence of axonal disruption and reduced structural integrity. The fornix carries the axons projecting from the hippocampus, and fornix FA reduces in subjects with hippocampal atrophy, correlating with memory function and emotion regulation (39). Uncinate fascicles are the connecting fibers of the medial and lateral orbitofrontal gyrus, anterior cingulate gyrus, and anterior temporal lobe, including the amygdala and hippocampus. Lower FA in the uncinate fasciculus is involved in emotional regulation, including bipolar disorder and late-life depression (40, 41). Of note, glaucoma patients were associated with a reduction in cognition, memory, and psychological disorders such as depression and anxiety (12). Moreover, the hippocampal shrinks in glaucoma patients were associated with disease severity (42). Given this evidence, we speculate that the FA reduction in the fornix and uncinate fasciculus may precede glaucoma's hippocampal atrophy and clinical symptoms.

Using a similar methodology, we detected the increased volumes of the right.ventral.DC and Brain. stem are both risk factors for glaucoma. Brain stem is involved in many of the basic functions of the encephalon, such as motor control and sensory analysis. Consistent with our results, Williams et al. (31). reported the volume of brain stem was larger in the early stage of glaucoma group and the volume decline later stage of the disease. The ventral diencephalon (VDC) is a group of structures including the hypothalamus, papillary body, subthalamic nucleus, substantia nigra, red nucleus, lateral geniculate nucleus (LGN), and medial geniculate nucleus (MGN) that cannot be normally distinguished by standard MRI images. Several primate studies have reported LGN input with additional modulatory input from various brain regions (including the striate cortex, the brain stem, and the thalamic reticular nucleus) (43). Moreover, recent observations with experimentally induced glaucoma have confirmed the loss of LGN neurons in the layers associated with the eye (44). Increased volume is likely a result of inflammatory processes and neuronal injuries, such as cellular swelling or microglial activation (31). Therefore, the injuries of right ventral diencephalon and stem brain may induce irreversible damage to the optic nerve in glaucoma.

Many research hypotheses that glaucoma originates in the brain but manifest as an eye disease. One of these studies indicated that glaucoma should be seen as part of a neurological disorder. They found that trans-lamina cribrosa pressure difference (TLCPD), calculated as the IOP minus the cerebrospinal fluid pressure, had a better association with glaucoma presence than IOP alone (45). Other studies have revealed that a higher proportion of AD patients had a probable diagnosis of glaucoma than controls, while the primary open-angle glaucoma (POAG) patients had a higher risk of developing AD (46, 47). RGCs are located in the retinal ganglion cell layer, and the axons of RGCs constitute the optic nerve and reach up to the LGN. Retrograde trans-synaptic degeneration is currently responsible for RGC loss in various neurodegeneration diseases, including Alzheimer's (AD), and Parkinson's (PD) disease (48). Meanwhile, various neuroimaging research converges evidence that glaucoma is associated with central nervous system changes and shares commonality with neurodegeneration diseases, including loss of RGCs and the deposition of abnormal proteins in specific anatomical areas like the hippocampus. The primary brain alterations could induce the RGCs death through retrograde trans-synaptic degeneration. Besides, recent studies also considered that glaucoma shares the common pathogenic mechanisms (oxidative stress, mitochondrial dysfunction, inflammation, and apoptosis) with neurodegeneration diseases (49). Our study conducted hypothesis-free, data-driven MR analyses and identified FA reduction in the fornix and uncinate fasciculus and increased volume in right.ventral.DC and brain stem are the primary causes of glaucoma. Therefore, combined with previous results, our data suggest that brain damage is an important factor in the onset of glaucoma. Future molecular and clinical research is required to improve our understanding of the underlying mechanisms. These potential pathogenic mechanisms linking brain alterations to retinal physiology will probably lead to early diagnosis of glaucoma with more appropriate and accurate approaches for its treatment.

Although more reliable conclusions can be obtained using large-scale GWAS studies, some limitations should be considered for the present study. First, data used in the current study were derived from the European population; therefore, the causal relationship between brain structural alteration and glaucoma in other populations such as Asia is unknown. Therefore, further research on different ethnicities should be conducted to explore the variation. Second, although we assessed the associations between brain alteration and glaucoma, it is expected that more MRI metrics of larger sample sizes can be used in future MR studies to find out the precise lesions in the brain of glaucoma patients. Third, the brain alteration in different types of glaucoma might be different, and further studies considering the glaucoma group are required. So forth, it was difficult to achieve a full non-overlap between exposure (DIT and ROI) and outcome (Glaucoma), even though we chose the data from publicly available summary data.

In summary, FA reduction in the fornix and uncinate fasciculus and increased volume of the right ventral diencephalon and brain stem might be the primary causes of glaucoma. Furthermore, our study illustrates that the fornix and uncinate fasciculus degenerations and injuries of the right ventral diencephalon and stem brain potentially increase the risk of glaucoma onset. These novel findings support the hypothesis that brain alteration can trigger glaucoma, and can be utilized for the advancement of diagnostics and therapies for glaucoma in the future.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories

and accession number(s) can be found in the article/ **Supplementary Material**.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

JZ: conceptualization and writing—review and editing. PW: methodology. KL: investigation and writing—original draft. BC: resources. RY and YC: supervision. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by a grant from the National Natural Science Foundation of China (No. 81600713) to JZ.

ACKNOWLEDGMENTS

We thank the participants and investigators of the FinnGen study for their GWAS data.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.956339/full#supplementary-material

Supplementary Figure 1 | Leave-one-out permutation analysis for the fornix fractional anisotropy **(A)**, uncinate fasciculus fractional anisotropy **(B)**, and the volume of right ventral diencephalon **(C)** and brain stem **(D)**.

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frontiers | Frontiers in Medicine

REVIEW
published: 05 July 2022
doi: 10.3389/fmed.2022.922527

Depicting Developing Trend and Core Knowledge of Primary Open-Angle Glaucoma: A Bibliometric and Visualized Analysis

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Objective: The prevalence of glaucoma is rising due to an increasing aging population. Because of its insidious and irreversible nature, glaucoma has gradually become the focus of attention. We assessed primary open angle glaucoma, the most common type of glaucoma, to study its present status, global trend, and state of clinical research.

Methods: Publications from 2000 to 2021 in Web of Science database were retrieved and analyzed by bibliometrics. VOSviewer and Citespace were used for analysis.

Results: A total of 6,401 publications were included in this review, and we found that the number of publications increased from 139 in 2000 to 563 in 2021. American researchers have published the most papers and had the highest h-index and the most citations, while the *Journal of Glaucoma* has published the most papers on this topic. Some key researchers, contributing institutions, their partnerships, and scientific masterpieces were identified. The publications we reviewed fall into seven categories: publications on intraocular pressure, normal tension glaucoma, risk factors, the trabecular meshwork, optical coherence tomography, surgery, and mutation. Clear study hotspots were described, which began with epidemiology and transitioned to pathogenesis and diagnosis and then to treatment.

Conclusion: Studies on primary open angle glaucoma extend well beyond ophthalmology to biochemistry molecular biology, general internal medicine, pharmacology, pharmacy, science technology, and other areas. Interest, research and publications on primary open angle glaucoma are on the rise.

Keywords: primary open angle glaucoma, intraocular pressure, bibliometrics analysis, citespace, VOSviewer

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Ophthalmology, a section of the journal Frontiers in Medicine

Received: *18 April 2022*
Accepted: *13 June 2022*
Published: *05 July 2022*

Citation:
Zhao L, Li J, Feng L, Zhang C, Zhang W, Wang C, He Y, Wen D and Song W (2022) Depicting Developing Trend and Core Knowledge of Primary Open-Angle Glaucoma: A Bibliometric and Visualized Analysis. Front. Med. 9:922527. doi: 10.3389/fmed.2022.922527

INTRODUCTION

Glaucoma affects nearly 80 million people and is the leading cause of irreversible blindness globally (1, 2). It is classified as primary glaucoma and secondary glaucoma based on the etiological mechanism. Primary glaucoma can be categorized into primary angle-closure glaucoma and primary open angle glaucoma (POAG) according to the anterior chamber angle morphology (3). The prevalence of the same type of glaucoma varies by region (4). The most common type of

glaucoma is POAG—which is a complex genetic disease characterized by the loss of retinal ganglion cells and damage to the optic nerve, leading to progressive visual field loss (3, 5). The global prevalence of POAG among people aged 40–80 years was estimated to be 3.05% (1.69–5.27%) and 5.9 million people were blind due to this disease in 2020 (1). However, the severity of the problem may be greater than these figures indicate because epidemiological studies have indicated that approximately 50% of POAG cases are not diagnosed because glaucoma is an insidious disease and with population aging, the severity of the problem will be further aggravated (6). Even so, the global trend of POAG has not been well analyzed. Bibliometric analysis is a feasible strategy to qualitatively and quantitatively summarize and predict research trends by evaluating the research of main authors, journals, research institutes, and countries (7–9). In addition, bibliometric analysis has contributed to clinical decision-making and the development of guidelines (10–12). The purpose of this study is to analyze the current state and the trend of clinical research regarding POAG.

METHOD

Data Source

Although several databases can meet the needs of global-level analyses (13), we chose Science Citation Index Expanded (SCI-Expanded, 1999-present) of the WoS Core Collection (WoSCC) database for our evaluation. The WoSCC database covers more than 12,000 international scientific journals with great impact and quality and is the most commonly applied database for bibliometric analysis (14, 15)[1]. Apart from the general literature search, it also possesses an important function of citation index searching, which is helpful for assessing the academic performance of literature in a specific field (16).

Search Strategy

The topic was "primary open angle glaucoma," and the "topic" field contained the title, abstract, author keywords, and keyword plus. We focused on publications between 2000 and 2021, and the search date was April 7, 2022. We selected "articleORreview" as the article type, excluded other language than English and retrieved and analyzed 532 reviews and 5,869 articles. All searches were performed in one day to avoid database update bias. The detailed data retrieval strategies and inclusion criteria for this study are summarized in **Figure 1A**.

Data Extraction

We downloaded the data extraction information of all identified publications, including the title, author(s), year of publication, country of submission, affiliated institutions, journals, keywords, and abstracts. Two authors independently browsed and extracted data from eligible publications.

Bibliometric Analysis

The basic feature of publications is the intrinsic function of WOS. The h-index is estimated for a scholar or scientist who has published h papers and each paper has been cited no less than h times by other studies (17). Therefore, the h-index determines the number of papers published by each researcher and all related citations to assess the productivity of the authors and the impact of the published research (18). Moreover, although H-index was initially developed to evaluate individual academic achievement, it could be extended to describe the publication output of a nation or region, an institution, or a journal (19).

Visual Analysis

VOSviewer (Leiden University, Leiden, Netherlands) is a program for creating and visualizing bibliometric networks (20). In this study, VOSviewer was used for co-authoring, co-citation, and co-occurrence analyses. In the network map developed by VOSviewer, different nodes represent different elements, including authors, countries, institutions, and keywords, and the size of the node reflects the number or frequency of releases (21). The links between nodes represent associations, including co-authorship or co-occurrence, while the colors of nodes/lines reflect different clusters or years (22). The strength of the link is expressed in terms of total link strength (TLS). Co-authorship analysis illustrates the links between projects based on the number of co-authored papers. This is an effective tool for evaluating cooperation trends and identifying leading researchers, countries, and institutions (23). Co-occurrence analysis illustrates the relationship between keywords based on the number of publications where the keywords are found together (24). This analysis explores popular subjects and research directions. Therefore, it is an important indicator of the development of a particular research field. Similar to VOSviewer, CiteSpace is often used for literature analysis and visualization. It is used to capture keywords associated with strong citation bursts and explore keywords' time co-occurrence to predict research frontiers and explore keywords' co-evolutionary pathways (25). In this study, we used CiteSpace to make up for the gaps of VOSviewer. Microsoft Excel 2016 was used to predict the future trends of POAG publications. The equation of the prediction model was as follows: $f_{(x)} = a^x$, in which x represented the publication year, and $f_{(x)}$ represented the cumulative number of publications. In this way, we effectively captured the current status, emerging trends, and recent developments in the research of POAG. **Figure 1B** summarizes the entire process of bibliometric analysis.

RESULT

Ophthalmic literature has been subjected to scientometrics in the past for glaucoma and specific journals to add insight to the evolving trends (26, 27). However, glaucoma is a general term which contains a large number of subspecies and the POAG is the most common type of glaucoma. The pathogenesis and epidemiology of different types of glaucoma are different, thus it is necessary to classify and discuss them separately in bibliometric analysis. Our research is mainly focused on POAG and is the first time to analysis the research of POAG specifically.

[1]https://Images.Webofknowledge.Com/Wokrs533jr18/Help/Wos/Hp_Database.Html

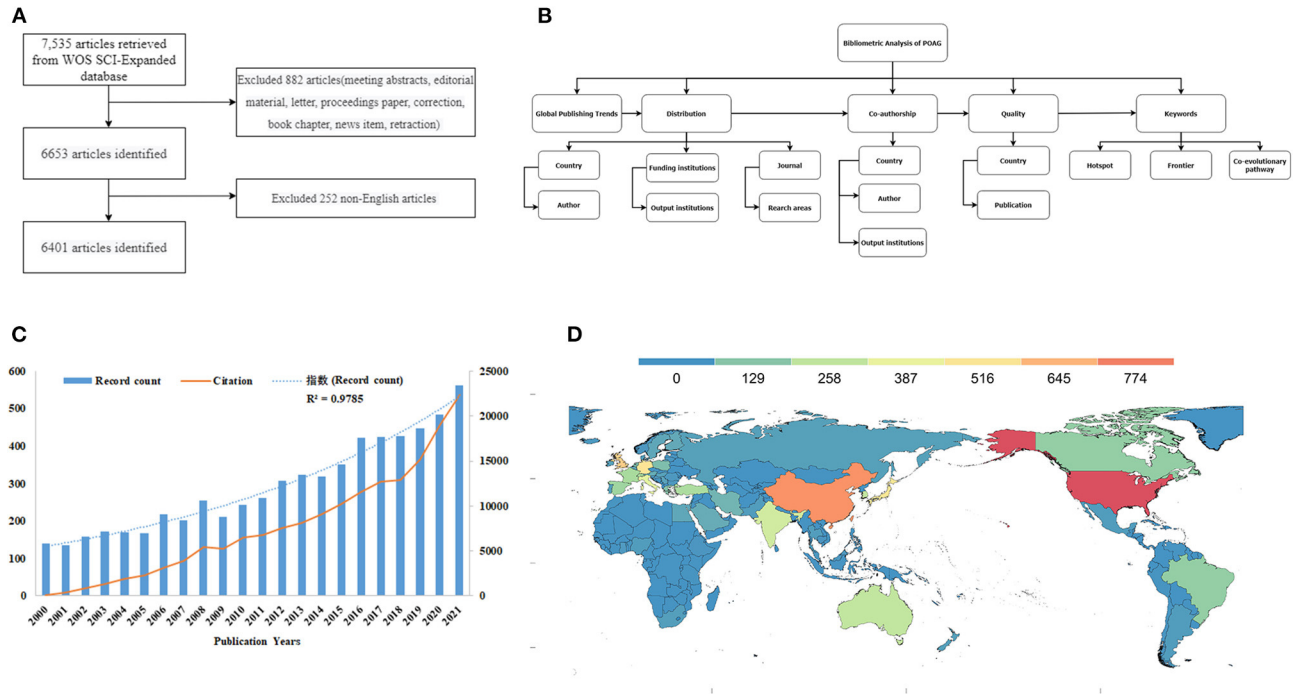


FIGURE 1 | The methods of bibliometric analysis and the trends of POAG. **(A)** A flowchart representing retrieval strategies for POAG articles from the WOS SCI-Expanded database and the inclusion criteria for the study; **(B)** The whole flow chart of bibliometric analysis for POAG in this study; **(C)** Trends in the growth of publications and the number of cited articles worldwide from 2000 to 2021; **(D)** The distribution world map of POAG.

Publication Output and Development Trend

The WOS database contains a total of 6,401 publications (532 reviews and 5,869 articles) related to POAG between 2000 and 2021. The distribution of articles was analyzed according to the year of publication (**Supplementary Table 1**). The report showed that from 2000 to 2021, the total number of publications increased from 139 to 563. The number of papers published in 2021 was the highest (563, 8.796%). The annual increase in publication and citations is shown in **Figure 1C**, which indicates that citation changes are roughly synchronized with the number of publications.

The Distribution and Co-authorship Analysis of Countries, Authors, Funding Institutions, and Output Institutions

Distribution

Country

The countries that made the greatest contributions are presented in **Supplementary Table 2**. A distribution world map of POAG research is shown in **Figure 1D**. The US had the largest number of publications (1,898), with a centrality of 31%.

Author

The top 10 authors with the highest number of publications are listed in **Table 1**. Over the past 22 years, these authors have published a total of 874 publications, accounting for 13.65% of the global total publications. It is important to note that we included all the authors in our analysis regardless of their relative contributions (first author, corresponding author, or co-author).

Funding Institutions

A total of 3,109 institutions funded the publication of POAG related works. **Table 2** lists the top 10 funding institutions from 2000 to 2021. Seven of the top 10 are from the US, with one each from Japan, China and Europe.

Output Institutions

The top 10 output institutions are also shown in **Table 2**. A total of 4,306 institutions have participated in POAG research. Four of the top 10 institutions are from the US, while the other top output institutions are from the UK, Singapore, and Germany.

Co-authorship Analysis

We used VOSviewer to map the network, visually showing the links between countries, authors, and institutions that have contributed to POAG research.

Country

A total of 69 countries with at least ten publications were identified. As shown in **Figure 2A**, the top five countries for TLS are as follows: the US (TLS = 1,399 times), the UK (TLS = 694 times), China (TLS = 448 times), Germany (TLS = 441 times), and Singapore (TLS = 416 times). The US has conducted extensive research on POAG and cooperated closely with other countries around the world in this research field to jointly

TABLE 1 | The top 10 authors in the study of POAG.

Rank	Author	Country	Affiliate	Publications	TLS
1	Weinreb RN	USA	University of California San Diego	127	280
2	Aung T	SINGAPORE	Singapore National Eye Center	127	258
3	Pasquale LR	USA	Icahn School of Medicine at Mount Sinai	104	122
4	Wiggs JL	USA	Harvard Medical School	95	139
5	Mackey DA	Australia	University of Western Australia	75	173
6	Ritch R	USA	New York Eye & Ear Infirmary of Mount Sinai	73	65
7	Allingham RR	USA	Duke University	70	111
8	Craig JE	Australia	Flinders University South Australia	70	192
9	Park KH	Korea	Seoul National University College of Medicine	69	155
10	Wang NL	China	Capital Medical University	64	64

TLS, Total link strength.

TABLE 2 | The top 10 funding institutions and output institutions in the study of POAG.

Rank	Funding institutions	Country/Region	Publications	Output institutions	Country	Publications	TLS
1	United States Department of Health Human Services	USA	939	University of California System	USA	324	486
2	National Institutes of Health NIH USA	USA	925	University of London	UK	267	359
3	Nih National Eye Institute Nei	USA	782	University College London	UK	223	347
4	Research to Prevent Blindness Rpb	USA	367	Harvard University	USA	212	317
5	National Natural Science Foundation of China Nsfc	China	248	League of European Research Universities Leru	Europe	206	528
6	Ministry of Education Culture Sports Science and Technology Japan Mext	Japan	153	National University of Singapore	Singapore	202	523
7	European Commission	Europe	125	Singapore National Eye Center	Singapore	194	550
8	Pfizer	USA	115	Duke University	USA	191	542
9	Abbvie	USA	107	Moorfields Eye Hospital Nhs Foundation Trust	Germany	183	116
10	Allergan	USA	103	Massachusetts Eye Ear Infirmary	USA	168	110

TLS, Total link strength.

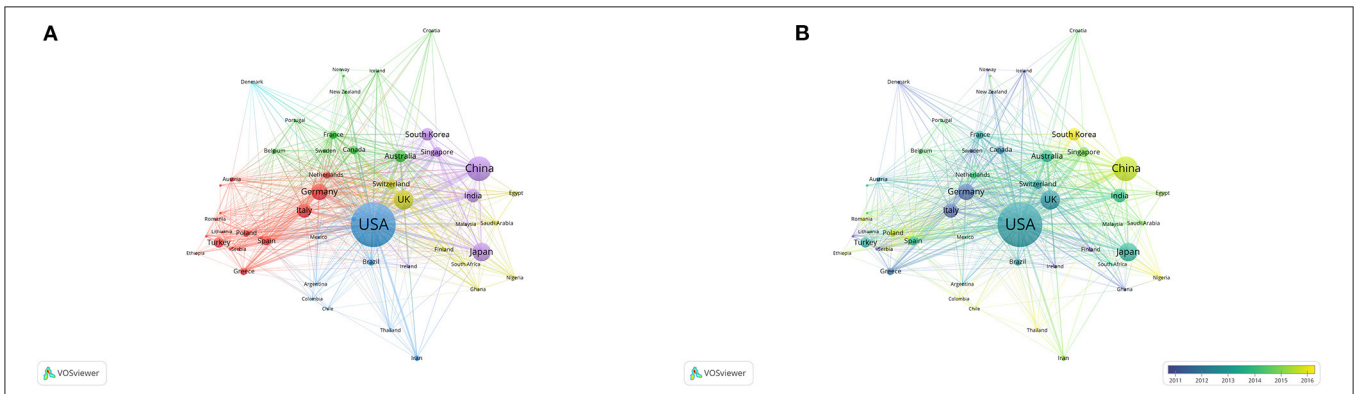


FIGURE 2 | Cooperation map of countries in the studies of POAG. **(A)** Mapping of the co-authorship analysis amongst 69 identified countries. Each node represents an individual country, and the node size is proportional to the number of publications. Line thickness between nodes indicates link strength of a collaboration relationship (weighted by a quantitative evaluation indicator of TLS). **(B)** Country overlay. The color of each node represents the average year of POAG publications in a country. Blue represents the earlier published countries, while yellow represents the recently published countries.

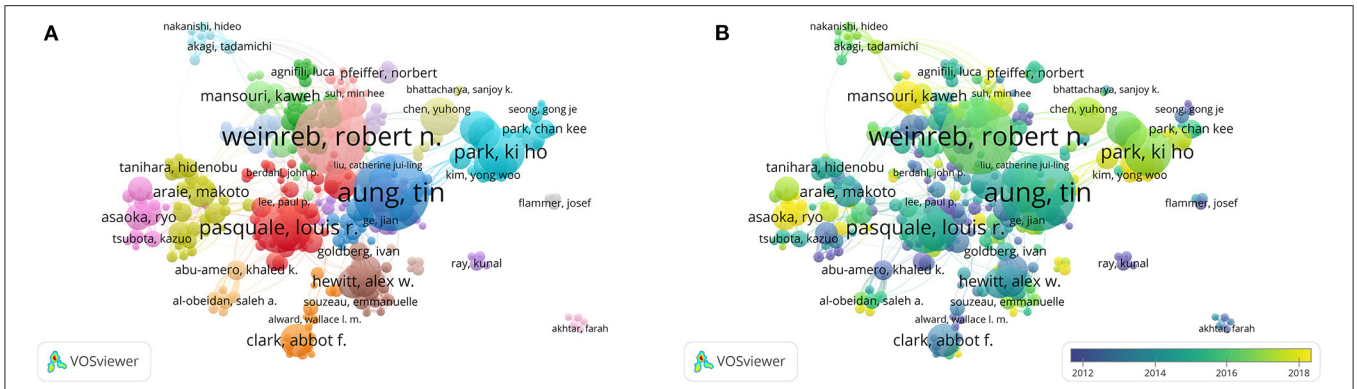


FIGURE 3 | Cooperation map of authors in the studies of POAG. **(A)** Author network; **(B)** Author overlay. Explanatory figure legend is the same as for **Figure 2**.

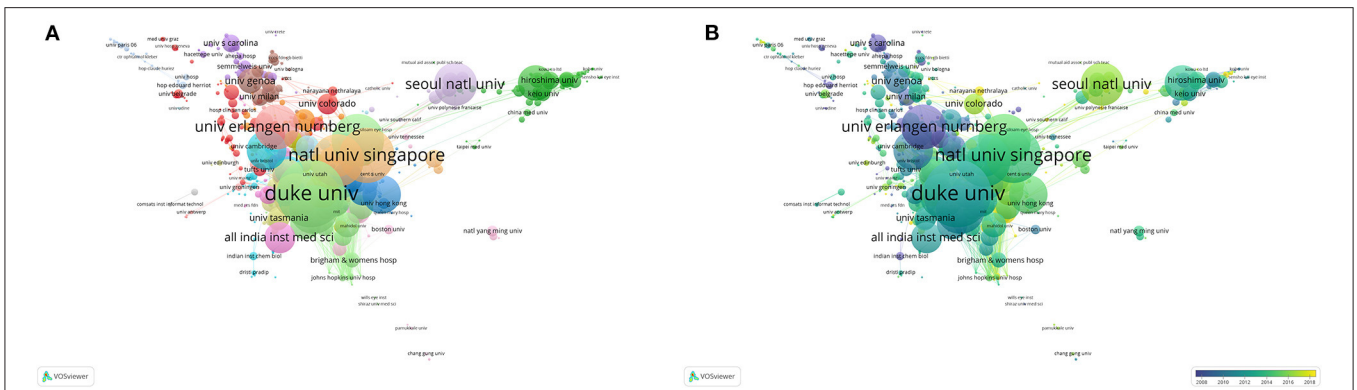


FIGURE 4 | Cooperation map of output institution in the studies of POAG. **(A)** Institution network; **(B)** Institution overlay. Explanatory figure legend is the same as that of **Figure 2**.

TABLE 3 | The top 10 journals and research areas in the study of POAG.

Rank	Journal	Country	Publications	IF(2020)	Research areas	Publications
1	Journal of glaucoma	USA	644	2.503	Ophthalmology	4601
2	Investigative ophthalmology visual science	USA	483	4.799	Biochemistry molecular biology	399
3	Ophthalmology	Netherlands	281	12.079	General internal medicine	364
4	American journal of ophthalmology	Netherlands	237	5.258	Science technology other topics	331
5	British journal of ophthalmology	UK	227	4.638	Pharmacology pharmacy	290
6	Molecular vision	USA	205	2.367	Research experimental medicine	265
7	Plos one	USA	201	3.240	Genetics heredity	238
8	Graefes archive for clinical and experimental ophthalmology	Germany	178	3.117	Neurosciences neurology	131
9	European journal of ophthalmology	Italy	168	2.597	Surgery	83
10	Eye	UK	155	3.775	Cell biology	79

IF, Impact factor.

promote the development of POAG research. The superimposed visualization map (**Figure 2B**) of co-authorship analysis shows that China, South Korea, Poland and other countries have also made some progress in the research field of POAG in recent years.

Author

Figure 3A shows the degree of collaboration among authors, with a total of 803 authors who have at least 10 publications. The top five authors with the highest TLS were Weinreb RN (TLS = 280), Aung T (TLS = 259), Craig JE (TLS = 192), Hewitt AW (TLS = 188), and Mackey DA (TLS = 173). The superimposed visualization map of co-authorship analysis was also performed in **Figure 3B**.

Output Institution

As shown in **Figure 4A**, Duke University (542 TLS); the National University of Singapore (523 TLS); the Singapore National Eye Center (500 TLS); the University of California, San Diego (486

TLS); and the University of Melbourne (378 TLS) were the top five universities for TLS. According to the average output time, the superimposed visual map of co-authorship analysis was performed, as shown in **Figure 4B**.

Journal Distribution and Research Areas

Journal Distribution

A total of 206 academic journals have published articles on POAG. **Table 3** lists the top 10 most published journals and their impact factors.

Research Areas

The top 10 research areas among the major journals are also shown in **Table 3**.

Analysis of the Quality of the Publications

The total number of citations and h-index reflect the quality of publications and the academic impact of one country (28). In addition to analyzing the number of publications in different countries around the world, we also analyzed the quality of publications in **Table 4**.

TABLE 4 | The top five countries with the largest citation and highest H-index.

Rank	Citation frequency	Average citation frequency.	H-index
1	USA (81339)	Iceland (51.27)	USA (116)
2	UK (20577)	Singapore (40.62)	Germany (66)
3	Germany (16392)	Portugal (36.78)	UK (64)
4	China (13397)	USA (35.19)	China (54)
5	Japan (13064)	France (34.35)	Italy (53)

The Top 10 Most Cited Publications

The average number of citations per paper on POAG was 26.55. **Table 5** shows the 10 most cited articles about POAG.

Keywords Analysis

Research Hotspot

We identified and analyzed keywords that have been used more than 10 times in publications through VOSviewer. As shown in **Figure 5A**, 1,000 keywords can be grouped into approximately seven research clusters by color. **Figure 5B** shows an overlay visualization of co-occurrence analysis, where items are colored according to the average time in which the keywords appear. The most common keywords in each group are listed in **Figure 5C**.

Research Frontier

Burst keywords are frequently cited words over a period, indicating a sharp increase in the frequency of keywords, which can last for years (29). CiteSpace is used to detect burst keywords that are considered indicators of cutting-edge topics of research over time. As can be seen from the keywords listed in **Table 6**, "localization" showed the strongest outbreak intensity, followed by "timolol" and "locus," etc.

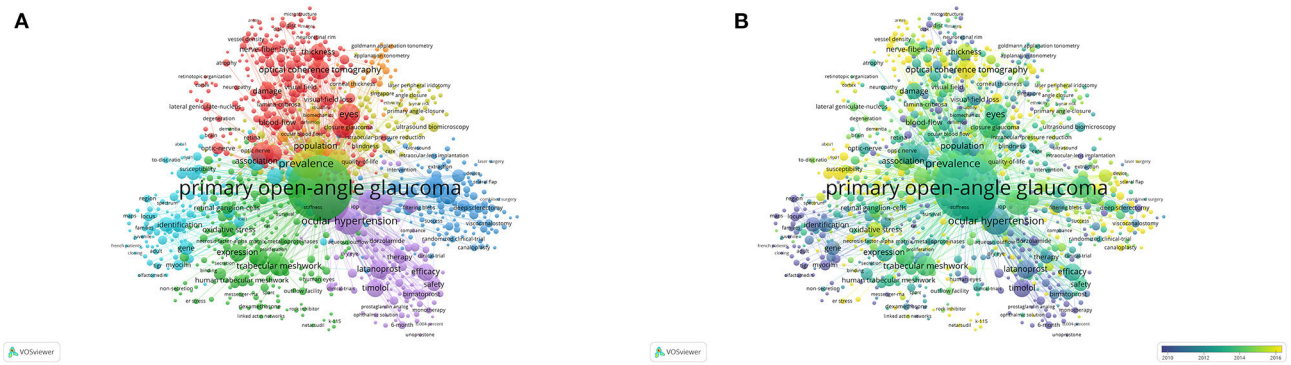
Co-evolutionary Pathway

Figure 6 shows the change in the keywords over time. Citespace is used to make co-evolutionary pathway and

TABLE 5 | The top 10 most cited publications in the study of POAG.

Rank	Title	TC	Author	Source Title	Published
1	The ocular hypertension treatment study - a randomized trial determines that topical ocular hypotensive medication delays or prevents the onset of primary open-angle glaucoma	2,458	Kass MA, HeuerDK, Higginbotham EJ et al.	Archives of ophthalmology	Jun 2002
2	Global prevalence of glaucoma and projections of glaucoma burden through 2040 a systematic review and meta-analysis	2,325	Tham YC, Li X, Wong TY et al.	Ophthalmology	Nov 2014
3	The Ocular Hypertension Treatment Study - Baseline factors that predict the onset of primary open-angle glaucoma	1,791	Gordon MO, Beiser JA, Brandt JD et al.	Archives of ophthalmology	Jun 2002
4	The pathophysiology and treatment of glaucoma a review	1,404	Weinreb Robert N, Aung T, Medeiros FA et al.	Jama-journal of the american medical association	May 2014
5	Development of the 25-item national eye institute visual function questionnaire	1,371	Mangione CM, Lee PP, Gutierrez PR et al.	Archives of ophthalmology	Jul 2001
6	The ubiquitin kinase PINK1 recruits autophagy receptors to induce mitophagy	1,292	Lazarou M, Sliter DA, Kane LA et al.	Nature	Aug 2015
7	Primary open-angle glaucoma	1,238	Weinreb RN, Khaw PT	Lancet	May 2004
8	The impact of ocular blood flow in glaucoma	1,127	Flammer J, Orgul S, Costa VP et al.	Progress in retinal and eye research	JUL 2002
9	Mutations of optineurin in amyotrophic lateral sclerosis	861	Maruyama H, Morino H, Ito H et al.	Nature	May 2010
10	Adult-onset primary open-angle glaucoma caused by mutations in optineurin	788	Rezaie T, Child A, Hitchings R et al.	Science	Feb 2002

TC, Total citation.



C

Groups	Keywords
intraocular pressure	Latanoprost, timolol, travoprost, dozolamine, bumoprost
normal tension glaucoma	Optic nerve papilla, central corneal thickness, intraocular blood flow, hypertension, ocular hyperperfusion
risk factors	Population, management, ultrasonic biological microscopy, quality of life, Baltimore Ophthalmic Survey
trabecular mesh	Aqueous humor, oxidative stress, retinal ganglion cells, extracellular matrix, gene expression
optical coherence tomography	Thickness, nerve fiber layer, visual field defect, optic nerve and age
surgery	Lens phacoemulsification, mitomycin C, deep sclerectomy, pseudoexudation syndrome and complications
mutation	Genome-wide association, trabecular meshwork cells, myofibrin, epidermal detachment syndrome, optic nerve protein

FIGURE 5 | Co-occurrence analysis on POAG research. **(A)** Network visualization map of keyword co-occurrence analysis using VOS viewer. All keywords are labeled. The size of the node reflects the occurrence frequency of a certain keyword. The larger the size of the node is, the more frequently the keyword co-occurs. VOS viewer marks keywords with different colors, and the color of the nodes and labels indicates the cluster in which they belong to. Closely related keywords are grouped into one cluster with the same color. The higher the quantity of co-occurrences of two keywords, the closer will they be located in the network; **(B)** Overlay visualization map of keyword co-occurrence analysis. The meanings of the node and label in this map are the same as in **Table 6**. However, the color of each node in this map indicates the average year of the keyword in the article according to the color gradient in the lower right.

TABLE 6 | The top keywords with the strongest citation bursts.

Keywords	Strength	Begin	End	2000-2020
Localization	22.46	2000	2009	
Timolol	21.28	2000	2006	
Region	15.61	2000	2009	
Tiger gene	14.44	2000	2004	
Baltimore eye survey	13.36	2000	2006	
Mutation	12.36	2000	2007	
Prostaglandin analogs	10.83	2000	2006	
Locus	17.81	2002	2010	
Elevated intraocular pressure	16.19	2002	2012	
Fixed combination	12.72	2005	2010	
Genome wide scan	10.75	2005	2011	
Filtering surgery	11.33	2006	2011	
Optical coherence tomography	20.9	2016	2018	
Vessel density	14.89	2017	2021	
Coherence tomography angiography	14.99	2018	2021	

some significant keywords such as "normal tension glaucoma" (NTG), "trabecular mesh," "surgery," "intraocular pressure," "risk factors" and "optical coherence tomography" are determined.

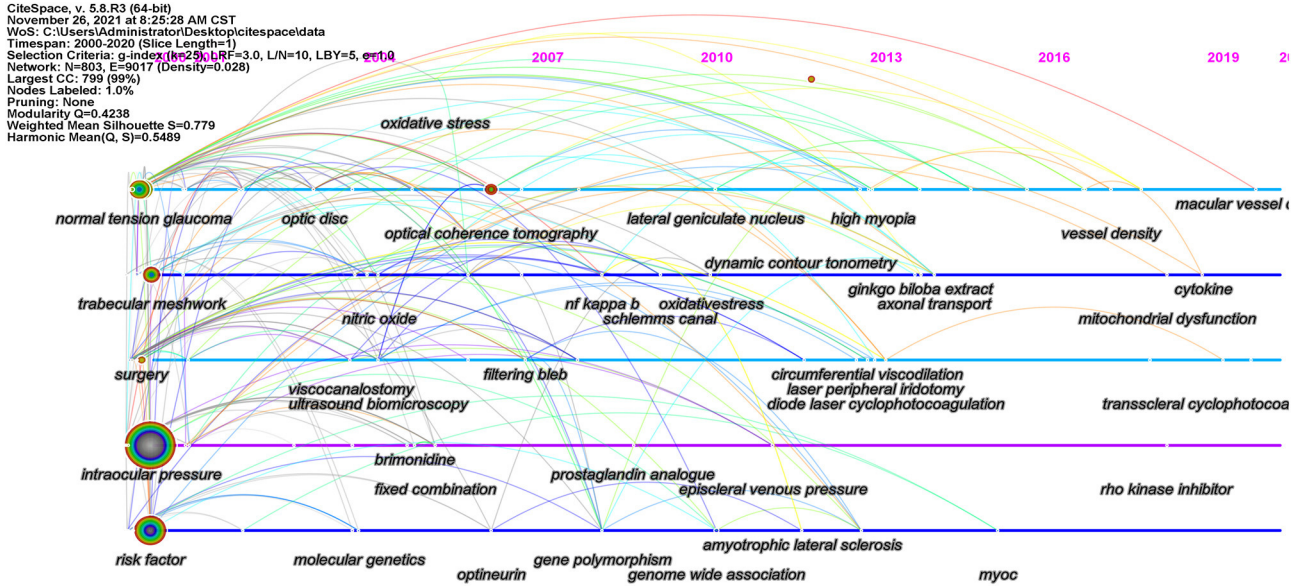


FIGURE 6 | Evolutionary pathway in the study of POAG: The position of each node represents when it first appeared, and the lines between nodes represent relationships between keywords. The node colors represent different years, from cold to warm means period from 2000 to 2021. Bluish purple indicates the previous keyword, and red indicates the latest keyword. Longer colored segments indicate a larger reference time span. The flow of knowledge between clusters from cool to warm colors can also be observed over time.

DISCUSSION

Analysis of Research Results

In this analysis, 6,401 POAG related publications were found on WOS from 2000 to 2021. We observed that from 2000 to 2021, the number of POAG publications showed a wavy increase within 3–5 years. The search time for our survey was April 2022, so we believe all the publications in 2021 were included. During this 22 year period, the US, China, the UK, Germany, and Japan became the top five countries with the highest number of POAG related publications. This is roughly consistent with the distribution of the funding institutions. Singapore is not among the top 10 countries with publications, but two of the top 10 output institutions are in Singapore. In terms of global publication quality (specifically citation frequency and h-index), the US, the UK, and Germany are the top three countries; however, the average citation frequency of Iceland (51.27) is much higher than that of other countries. In addition, some other countries, such as Singapore, Portugal, USA, and France, also have good publication quality. The Journal of Glaucoma has the highest number of publications associated with POAG (644), but the journal with the highest impact factor is Ophthalmology (IF2020 = 12.079), the only one among the top 10 journals with an IF score > 10. The analysis of research hotspots showed that research fields of POAG go far beyond ophthalmology and that there will be some progress in biochemistry, internal medicine, pharmacology, research experimental medicine, genetics, neuroscience, cell biology, and other areas, as shown in **Table 3**.

The top 10 authors with the most publications were from the US, Singapore, and Australia. Dr. Weinreb RN and Dr. Aung T have published the same 127 articles over the past 22 years, making themself the most prolific author of the period. The most cited article of Dr. Aung T is "Global Prevalence of Glaucoma and Projections of Glaucoma Burden through 2040: A Systematic Review and Meta-analysis," which has been cited up to 2,325 times and is the second most cited article among studies on POAG. His review suggests that the number of patients with glaucoma worldwide will increase to 111.8 million by 2040, with a higher proportion in Asia and Africa. These have important implications for glaucoma screening and treatment and the design of relevant public health strategies (2). Dr. Weinreb RN's most cited article is "The Pathophysiology and Treatment of Glaucoma: A Review," which ranks fourth in terms of citations in this search area. This review further discussed the pathophysiological mechanism and treatment of POAG (3). Prof. Pasquale LR and Prof. Wiggs JL's conducted research on central corneal thickness in the US (30). The most widely cited publication in this field is "The Ocular Hypertension Treatment Study: A Randomized Trial Determines that Topical Ocular Hypotensive Medication Delays or Prevents the Onset of Primary Open-Angle Glaucoma," which was published by Prof. Kass MA in 2002 with 2,458 citations in WOS. This paper suggests that topical ocular antihypertensive agents can effectively delay or prevent POAG in people with elevated intraocular pressure (IOP). While this does not mean that all patients with elevated IOP should be treated with medications, clinicians should consider initiating treatment for patients with elevated IOP who are at moderate or high risk of POAG (31).

The co-authorship map was constructed to visualize authors, countries and regions, and institutes that published at least 10

publications between 2000 and 2021. Not surprisingly, the top five authors who collaborated most closely were grouped among the top 10 authors who published the most. The top five most collaborative countries and territories (except for Singapore) and the top five institutions (except for Australia's University of Melbourne) were also in the top 10. We used co-occurrence cluster analysis to produce the network graph of co-occurrence relationship by analyzing the keywords found in the studies on POAG. A total of seven potential research directions were identified. Nodules of different colors (ranging from blue to red) showed considerable density in all seven clusters, suggesting a balanced pattern of development in all seven directions. In addition, research hotspots in each direction are changing, indicating that research is diversifying. The frequency of global cooperation has become closer, reflecting the growing trend of international and global cooperation among researchers who have interest in POAG.

Research Trend Change Analysis

Overview of Research Trends Change

In keywords co-occurrence analysis, blue coded keywords, including "timolol," "mutation," "myofibrin," "bromonidine," "dozolamine," "olfaction," and "adjuvant therapy," appeared earlier. After 2010, as more detailed studies were carried out, keywords such as "risk factors," "normal intraocular pressure glaucoma," "optic nerve fibers," and "oxidative stress" began to appear. In the burst keywords analysis of **Table 6**, drugs such as "timolol" and "prostaglandin analogs" and keywords related to gene research, such as "localization," "region," "*TIGR* gene," and "mutation," appeared earlier. In addition, "scanning laser polarimetry" and "Baltimore Eye Survey" also emerged early. From 2000 to 2021, other keywords such as "locus," "elevated intraocular pressure," "fixed combination," "genome-wide scan," and "filtering surgery" broke out successively. Coherence tomography angiography has been developed based on OCT and vessel density in recent years. The keywords "vessel density" and "coherence tomography angiography" showed persistence after explosion in recent years, indicating that this field is the next research frontier. To further verify the research trend, co-evolutionary pathway is made: keywords such as "normal tension glaucoma" (NTG), "trabecular mesh," "surgery," "intraocular pressure," and "risk factors" appeared frequently and early, which had a broad relationship with other clusters and represented the main research content and direction in the studies on POAG. OCT is an emerging hotspot for studies on POAG due to its late appearance and extensive connections with other clusters.

Diversity of Glaucoma Etiology

Because of the choice of database, our research focused only on publications from the 21st century, but the research content of the last century is the foundation. In the 1990s, James et al. (32) conducted detailed ophthalmic screening of residents of 16 clusters in East Baltimore to study the association of POAG with risk factors such as IOP, systemic hypertension, perfusion pressure, ethnic differences, family history, age, and optic disc structure. These findings are gradually being confirmed by studies in other communities or countries. In addition, other related risk factors have been identified in recent years. The Ocular Hypertension Treatment Study showed that the thinner the central cornea is, the greater the risk of developing POAG (33). In the Bayesian meta-regression model, men were more likely to develop POAG than women, and urban residents were more likely to develop POAG than rural residents (2).

In the 1950s, elevated IOP was thought to be a determinant factor for glaucoma. In patients with POAG, the increase in IOP is mainly due to increased resistance to outflow of aqueous humor through the trabecular meshwork (32). With the discovery of NTG and physiologic high IOP in recent years, we have realized that the increase in IOP alone cannot explain the whole pathogenesis of glaucoma development (34, 35). Berdahl et al. (36) put forward in 2008 that in patients with POAG and NTG, displacement of the ethmoid plate is mainly due to an increased pressure gradient of the ethmoid plate caused by a decrease in cerebrospinal fluid (CSF) pressure. Compression, deformation, and remodeling of the ethmoid plate lead to mechanical axon damage and disruption of axon transport, which in turn leads to damage to retinal ganglion cells (4). Therefore, the concept of transethmoid pressure difference was proposed to describe the difference between the preethmoid pressure (i.e., IOP) and the retroethmoid pressure (i.e., CSF pressure) (37). Studies have shown that in addition to lowering IOP, CSF pressure can be regulated through systemic therapy to rebalance the pressure gradient across the ethmoid plate in patients with NTG (38). Furthermore, studies have shown that high IOP can also change the environment of other retinal neurons and cells in the central visual pathway by causing microcirculation disorders, immune changes, excitatory toxicity, and oxidative stress or by increasing their sensitivity to injury (39). Genetic factors have long been implicated in the pathophysiology of POAG. *MYOC/TIGR* mutations are associated with some forms of adolescent open-angle glaucoma (40). With the development of genome-wide association analysis in recent years, several genes associated with POAG have been highlighted in previous reviews. The most mature genes include *CAV1, TMCO1, CDKN2B-AS1, Six6, ABCA1, GMDS, AFAP1, Gas7, TGFBR3, TXNRD2, ATXN2,* and *FOXC1*, and many of the associations in these genes have been repeated in multiple studies and in different ethnic populations (41). The mechanism by which these genes lead to POAG is unclear, but they may interact with transforming growth factor beta, a molecule that regulates cell growth and survival throughout the body to cause trabecular fibrosis and prevent aqueous outflow, which in turn leads to IOP and death of retinal ganglion cells (42).

Earlier Diagnosis of Glaucoma Is Associated With Better Prognosis

Patients do not show any symptoms until the glaucoma progresses, leading to extensive nerve damage. When symptoms do occur, the disease causes irreversible vision loss and reduced visual field, so early diagnosis and intervention are crucial to slow the progression of the disease. Although IOP measurement alone is no longer the sole basis for the diagnosis of POAG, IOP remains a consistent risk factor for glaucoma. With the

death of retinal ganglion cells and loss of optic nerve fibers in glaucoma, the appearance of the optic nerve head and the retinal nerve fiber layer changes characteristically (43). Examination of the optic nerve head with an ophthalmoscope can reveal signs of neuronal loss, but its appearance in healthy people is highly variable, making it challenging to identify early damage (44). OCT, which has been developing rapidly in recent years, is a hot research keyword lately. It can provide more objective and quantitative information about the loss of optic nerve fibers (retinal ganglion cell axons) (45). However, by the time OCT will show some morphological changes, the retina would have been permanently damaged. Therefore, we need technologies that can be used for early diagnosis and effective treatment. Doppler optical coherence tomography, also known as OCT angiography, is a technology that combines the Doppler effect principle with OCT. It can observe the capillary morphology and blood flow in the optic disc area of patients with glaucoma, which can be used as a sensitive index for early diagnosis of POAG (46).

The Only Effective Treatment to Slow Down the Progress of POAG Is to Reduce IOP With Eye Drops, Laser or Surgery

Most patients with glaucoma prefer prostaglandins, as they reduce IOP by increasing aqueous drainage through the uveal sclera pathway (47). Other second-line drugs, such as β-adrenergic blockers, α-adrenergic agonists, and carbonic anhydrase inhibitors, are not as effective as prostaglandin analogs in reducing IOP and may have some side effects (48). When medication does not achieve sufficient IOP reduction with acceptable adverse reactions, laser or surgical procedures are recommended. In recent years, the continuous improvement of surgical methods for glaucoma, the advancement in technology, the research of postoperative antiscar formation drugs, and the invention and implication of new implants have brought the surgical treatment of POAG to a new field. While a substantial reduction in IOP can be achieved in most patients, the effect diminishes over time, with an annual failure rate of about 10% (49). Some patients still have impaired visual function after normal IOP; hence, the development of neuroprotective glaucoma treatment has become a hot research topic at home and abroad (50). In addition, there are systemic therapeutics that can rebalance the pressure gradient of the ethmoid plate by modulating the CSF pressure in patients with POAG who have low CSF pressure and patients with normal intraocular glaucoma. For patients with advanced glaucoma, since most retinal ganglion cells have been apoptotic, stem cell replacement therapy has become the most valuable potential means and needs to become a new hotspot in the treatment of POAG (51).

LIMITATIONS

This bibliometric analysis study inevitably has some limitations. First, it is regrettable that our analysis of global POAG research is somewhat flawed due to the fact that the majority of published articles are in English and there are a large number of journals in other languages that are also worth studying. Second, there are intrinsic differences between the results of bibliometric analysis and real-world studies. For instance, some comparatively new publications of high quality may not attach sufficient attention due to lower citation frequency, whilst older articles have a tendency to accumulate more citations. Thirdly, since the version we used was based on a customized subset (SCI-Expanded, 1999-present) of the whole WoS Core Collection, some excellent articles that are not included in this subset are ignored in this process, which makes the articles lose some luster. Since different databases have different properties including citation frequency counting, document type marking, and export formats for documents, merging of the databases may not optimal choice (52).

CONCLUSIONS

A total of 6,401 articles on POAG research published between 2000 and 2021 were retrieved from the WoSCC SCI-Expanded database. The number of publications, key institutions and countries, published journals, primary authors, and cooperative networks were systematically analyzed using hybrid analysis and visualization technologies (CiteSpace and VOSviewer). The analysis of co-occurrence networks provides researchers with information about potential collaboration opportunities with other institutions and researchers. Bibliometric analyses also reveal the current research hotspots and research frontiers in an objective and comprehensive manner, thus indicating the retrospective view of POAG and providing valuable guidance for researchers in the selection of research topics.

AUTHOR CONTRIBUTIONS

Conception and design: LZ, WS, and CW. Data analysis: LZ and JL. Writing: LZ, LF, and CZ. Data collection: WZ, YH, and DW. All authors contributed to the article and approved the submitted version.

FUNDING

The study was supported by National Nature Science Fund of China (81974132), National Nature Science Fund of China (81770927), National Key R&D Program of China (2021YFA1101202), Hunan Provincial Health Commission (20220702839).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.922527/full#supplementary-material

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frontiers | Frontiers in Medicine

ORIGINAL RESEARCH
published: 22 July 2022
doi: 10.3389/fmed.2022.935084

Targeted Metabolomics Shows That the Level of Glutamine, Kynurenine, Acyl-Carnitines and Lysophosphatidylcholines Is Significantly Increased in the Aqueous Humor of Glaucoma Patients

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The composition of the aqueous humor of patients with glaucoma is relevant to understand the underlying causes of the pathology. Information on the concentration of metabolites and small molecules in the aqueous humor of healthy subjects is limited. Among the causes of the limitations is the lack of healthy controls since, until recently, they were not surgically intervened; therefore, the aqueous humor of patients operated for cataract was used as a reference. Sixteen aqueous humor samples from healthy subjects undergoing refractive surgery and eight samples from glaucoma patients were used to assess the concentration of 188 compounds using chromatography and mass spectrometry. The concentration of 80 of the 188 was found to be reliable, allowing comparison of data from the two groups (glaucoma and control). The pattern found in the controls is similar to, but not the same as, that reported using samples from "controls" undergoing cataract surgery. Comparing data from glaucoma patients and healthy subjects, 57 of the 80 compounds were significantly ($p < 0.05$) altered in the aqueous humor. Kynurenine and glutamine, but not glutamate, were significantly increased in the glaucoma samples. Furthermore, 10 compounds were selected considering a statistical score of $p < 0.0001$ and the degree of change of more than double or less than half. The level of C10 (decanoyl)-carnitine decreased, while the concentration of spermidine and various acyl-carnitines and lysophosphatidylcholines increased in glaucoma. Principal component analysis showed complete segregation of controls and cases using the data for the 10 selected compounds. The receiver operating characteristic curve these 10

OPEN ACCESS

Edited by:
Vito Romano,
University of Brescia, Italy

Reviewed by:
Neeru Vallabh,
University of Liverpool, United Kingdom
Hanlin Zhang,
University of California, Berkeley, United States

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Specialty section:
This article was submitted to Ophthalmology, a section of the journal Frontiers in Medicine

Received: *03 May 2022*
Accepted: *23 June 2022*
Published: *22 July 2022*

Citation:
Lillo A, Marin S, Serrano-Marín J, Binetti N, Navarro G, Cascante M, Sánchez-Navés J and Franco R (2022) Targeted Metabolomics Shows That the Level of Glutamine, Kynurenine, Acyl-Carnitines and Lysophosphatidylcholines Is Significantly Increased in the Aqueous Humor of Glaucoma Patients. Front. Med. 9:935084. doi: 10.3389/fmed.2022.935084

compounds and for glutamine allowed finding cut-off values and significant sensitivity and specificity scores. The concentration of small metabolites in the aqueous humor of glaucoma patients is altered even when they take medication and are well controlled. The imbalance affects membrane components, especially those of the mitochondria, suggesting that mitochondrial abnormalities are a cause or consequence of glaucoma. The increase in glutamine in glaucoma is also relevant because it could be a means of keeping the concentration of glutamate under control, thus avoiding its potential to induce the death of neurons and retinal cells. Equally notable was the increase in kynurenine, which is essential in the metabolism of nicotine adenine dinucleotides.

Keywords: lipidomics, glutamine, eye disease, mass spectrometry, biomarker, glaucoma, kynurenine, mitochondria

INTRODUCTION

The aqueous humor (AH) is necessary for correct visual perception. Among others, the aqueous humor maintains the three-dimensional structure of the mammalian eye and facilitates the focusing of images on the retina. Consequently, it must be transparent and have an appropriate refractive index. The first report on the physiology of AH formation and chemical composition was made about a century ago by Yudkin (1). Although it was supposed to have few components to achieve transparency, various studies have found molecules as diverse as vitamins, amino acids, intermediate metabolites, and lipids (2–4). Unfortunately, AH is a bodily fluid that requires surgery to obtain a sample, which limits its use in the diagnosis and/or prevention of eye diseases. The composition of the AH is likely to be altered in diseases of the eye.

One of the most prevalent eye diseases, glaucoma, is the second leading cause of permanent blindness worldwide. Although there are several therapeutic approaches, including pharmacological options, trabeculoplasty (application of a laser beam to the trabecular meshwork to increase AH outflow), and surgical options, the etiology and pathophysiology remain unclear (5). In poor/developing countries where medication is scarce and/or unaffordable, glaucoma becomes a more serious problem (6). The main alteration is the accumulation of AH inside the eye, thus causing an increase in intraocular pressure (IOP) that leads to retinal damage and, in the long run, alterations in the optic nerve (7). AH, which comes mainly from the stromal fluid of the ciliary processes, is secreted in the posterior chamber of the anterior cavity of the eye (8). The abnormal accumulation of fluid in glaucoma is mainly due to poor drainage through the trabecular meshwork and/or increased AH production (7). Outflow can be affected for a number of reasons, including disturbances that increase drain resistance. For example, it has been described that endothelial cells that have a greater rigidity in the cytoskeleton can inhibit the formation of pores that facilitate the exit of AH (9). Another factor is the decrease in the volume of Schlemm's canal and/or the surface of the collecting canals (10). In addition, the activation of retinal microglia directly affects the physiology of the trabecular meshwork (11), which leads to a deregulation of the outflow of AH. It is hypothesized that the composition of AH may help understand the causes of glaucoma and ultimately improve therapeutic interventions.

Glaucoma, as well as other diseases that course with inflammation and neurodegeneration, e.g., Alzheimer's disease and Parkinson's disease, have been linked to mitochondrial deterioration (12, 13). On the one hand, one of the most common mechanisms of neuronal death is excitotoxicity caused by the increase in extracellular glutamate (14–16); therefore, it is expected that altered glutamate metabolism and/or glutamate-related events may be affecting the risk of glaucoma. In this sense, it would be relevant to know the level of glutamate and glutamine in AH in healthy controls and in glaucoma patients. On the other hand, inflammatory events in the retina (11) are accompanied by altered metabolic dysfunction, oxidative stress, and altered lipid metabolism. These processes that occur in the mitochondria affect the composition of the surrounding extracellular medium. Until recently, the quantification of some metabolites likely to be altered in inflammation has been difficult for technical reasons. An example is the acyl-carnitines whose variation in the retina can be translated into AH through microfluidic circulation (8). Therefore, finding changes in AH composition related to mitochondrial function could be a key step in advancing our understanding of the pathophysiological mechanisms underlying glaucoma. The opinion that the inflammatory component is important for the development of glaucoma is growing (12, 13).

The composition of the AH of patients with open-angle glaucoma, compared with that of patients with a variety of eye diseases other than glaucoma, shows increased levels of creatinine and decreased levels of taurine and spermine. The same metabolomics study led to the identification of a further 12 compounds when *p*-values were thresholded at 0.05 (3). In a more recent conceptually similar study using a different methodological platform, 22 metabolites were found whose levels were altered in AH samples from patients with open-angle glaucoma (17); control samples in this study were obtained from people undergoing cataract surgery. The compounds with higher score were cyclic AMP (cAMP), 2-methylbenzoic acid, 3'-sialyllactose, lysophosphatidylcholines 18:0 and 15:0 and hypoxanthine. The fold change for these compounds was moderate and more marked (2/3-fold) for uric acid,

hydroxyphenyllactic acid N^6-succinyl adenosine. Using healthy controls a lipidomic-based showed that 37 of 110 lipids were significantly altered in samples from glaucoma patients, the majority of the 37 were increased in glaucoma with the exception of some lysophosphatidylcholines (18). In the present paper we compared the composition of AH from open-angle glaucoma patients and healthy controls using a targeted metabolomics platform that determines the concentration of 188 metabolites, amino acids, biogenic amines, lysophosphatidylcholines, acyl-carnitines, sphingomyelins and total sugars. The controls in this work did not suffer from cataracts or any other eye disease.

METHODS

Subjects

A total of 23 samples were collected, 16 were from healthy controls; 5 were men and 9 women, mean age was 36 (range 25–56), and 8 were from open angle glaucoma patients; 4 were men and 4 women, mean age was 67.5 (range 51–81). None of the individuals had previously undergone eye surgery. Comorbidities in the patient's group were hypertension ($n = 5$), hyperlipidemia ($n = 3$) and altered thyroid function ($n = 1$). Participants were informed and, in terms of ethical standards, this study adhered to the tenets of the declaration of Helsinki. The study has been evaluated by the *Comitè d'Ètica de la Investigació de les Illes Balears (CEI-IB)* and approved under the conditions of good practices of the *Illes Balears* Health Service in the use of information systems and in the treatment of personal data (document available at: (https://www.ibsalut.es/docs/docs/CA/cbp_cat.pdf; accessed on June 3, 2022).

All glaucoma patients were well controlled with topical drop therapy. AH was collected at the beginning of the intervention; both in the glaucoma group and in the control group there were individuals with myopia or hyperopia but without cataracts or ocular pathologies. The ocular pressure of controls and patients was within the reference interval. The mean of controls and patients was −4.5 diopters (range −2.5 to −7 diopters). The controls were not diagnosed with any pathology. No previous surgery is reported. Individuals underwent refractive lensectomy or collamer lens (ICL) implantation (without removing the natural eye lens). All surgeries were performed by the same surgeon (J.S.N.) on an empty stomach, and after pupil dilation and disinfection with 5% ophthalmic povidone-iodine, under topical anesthesia and identical preoperative and operative protocol. The first side port was made about 1 mm width under the microscope in the operating room, and aspirate 100-150 μl with a 27G needle to avoid deepening into the anterior chamber of the eye. Each sample was transferred to a 0.5 mL Eppendorf tube and stored at −80 C until analysis.

Metabolomics

The approach in the paper was selected to determine as many metabolites as possible also aiming at the determination of compounds whose level is difficult to assess due to technical issues, for instance the measurement of carnitines having acyl chains of different number of carbons. Accordingly, targeted metabolomics using the AbsoluteIDQ™ p180 Kit (Biocrates Life Sciences, Innsbruck, Austria), was performed. This approach allows the absolute quantitation of up to 188 metabolites, including amino acids, biogenic amines, hexoses, acyl-carnitines, phospho- and sphingolipids. Individual metabolites may be found in www.biocrates.com/products/research-products/absoluteidq-p180-kit. Up to 30 μl of AH was plated in each well. Standards, quality controls and internal standards were placed in the plate, which was processed according to manufacturer instructions. Derivatized samples were analyzed in the AB Sciex 6500 QTRAP MS/MS mass spectrometer (AB Sciex LLC, Framinghan, MA, USA) coupled to an Agilent 1290 Infinity UHPLC system (Agilent, Santa Clara, CA, USA), following the instructions provided with the kit. Spermine could not be detected in our hands, neither in standards nor in samples (values were below detection limit and/or too low in comparison to the lowest value of the standard). Analyst and the MetIDQ™ software packages were used to analyze the obtained data and calculate metabolite concentrations. Final concentrations were calculated taking into account the volume that was placed in each well.

Statistical Analysis

Univariate analysis of data was performed using one-way ANOVA comparing one by one the 80 compounds whose concentration was reliably determined by MS. Significant differences were considered when $p < 0.05$. We selected those metabolites that meet both criteria: $\log_2$ fold change (FC) $> |1|$ and $p < 0.0001$. The receiver operating curve (ROC) was constructed using IBM SPSS software. The Principal Component Analysis (PCA) was done using the R software.

RESULTS

Summary of Findings in Samples From Healthy Controls Without a Diagnosis of Eye Disease

Analysis of data from healthy controls and patients diagnosed with glaucoma led to finding reliable concentrations for 80 compounds (see values of compounds in all samples in **Supplementary Table S1**). Metabolites with concentration values below the detection limit or whose values are not within the standard curve were not included in the analysis. We then compared our control data with the control data in the Buisset et al. (3) report, in which the same kit was used (3). Unlike the controls in our study, the controls in Buisset et al. study had cataracts. **Table 1** shows the comparison of concentrations of the 22 compounds selected in the study by Buisset et al. (3) report [see **Table 2** in (3)]. The concentrations of 23 compounds vary by more than two-fold or less than one-half (i.e., $\log_2$ fold change between −1 and 1). In our study, the concentration of taurine, butyrylcarnitine and glycine was lower by, respectively, 2.2, 2.3 and 3.3 times. Although the trend was to find lower values in our study, this was not always the case, for example, for acetylornithine and leucine among the compounds listed in **Table 1**.

TABLE 1 | Comparison of concentration of metabolites (median) in controls with those in the Buisset et al. (3) report.

Metabolite	Median in (3) (μM)	Median in the present report (μM)
Creatinine	38.0	24.0
Propionylcarnitine (C3)	0.33	0.19
PC aa C34:1	0.10	0.10
Glutamine	580.0	563.3
Acetylcarnitine (C2)	3.05	1.81
Taurine	41.20	18.33
PC aa C36:2	0.045	0.034
PC aa C36:4	0.030	0.019
PC aa C38:4	0.037	0.019
SM C18:1	0.009	0.0065
Carnitine (C0)	14.8	9.32
PC aa C32:1	0.016	0.014
PC aa C34:2	0.056	0.056
Trans-4-OH-Proline	3.46	2.08
Isoleucine	74.6	73.7
PC aa C30:2	0.002	0.002
Alanine	251.0	159.3
Acetylornithine	0.16	0.34
Butyrylcarnitine (C4)	0.15	0.054
Lyso PC a C28:1	0.018	0.0098
Leucine	96.9	134.7
Glycine	21.95	6.60

Only the data for compounds selected in the Buisset et al. (3) report are shown. Compounds for which the ratio between both values is higher than 2 or lower than a half are in red.

Concentration of Total Sugars, Amino Acids and Biogenic Amines in the Aqueous Humor of Control Individuals

The methodology used only allows the determination of total sugars and the results show that it is high (>3 mM) and similar in control samples and in glaucoma samples (details in **Supplementary Table S1**).

The concentration of the 20 L-amino acids that can be present in proteins varies by 2 orders of magnitude. The lowest concentration was found for aspartic acid (1.5 μM) while the highest was for glutamine (550 μM). The glutamic acid (glutamate) level was (6.6 μM). In addition to glutamine, the amino acids whose concentrations exceeded 100 μM were: alanine, leucine, lysine, threonine and valine. In summary, the level of amino acids in AH was very heterogeneous and did not present any specific pattern (details in **Supplementary Table S1**).

Regarding biogenic amines, the highest levels were found for creatine (26.7 μM), taurine (17.3 μM) and methionine sulfoxide (14.5 μM), and spermidine was among the lowest (0.3 μM). Interestingly, 3,4-dihydroxyphenylalanine (DOPA), the precursor of catecholamines was detectable (0.14 μM). Kynurenine, which was significantly increased in glaucoma samples (see below), was also detectable (0.49 μM) (details in **Supplementary Table S1**).

TABLE 2 | Values of sensitivity, specificity and area under the curve obtained from the ROC plots in **Figure 2**.

Metabolite	Sensitivity	Specificity	Cut-off (μM)	AUC[a]
C2	1	0.937	2.58	0.992
C3	1	0.875	0.270	0.969
C3-DC (C4-OH)	1	0.937	0.012	0.945
C4	0.875	0.937	0.0943	0.945
C5	0.75	1	0.102	0.941
C10	0.875	1	0.084	0.984
PC aa C42:6	1	1	0.0582	1
PC ae C30:1	0.875	0.937	0.0093	0.914
PC ae C36:3	0.75	1	0.0112	0.938
PC ae C40:2	0.75	1	0.0058	0.914
Glutamine	0.875	0.812	623	0.805

[a]*AUC, Area under the curve.*

Concentration of Acyl-Carnitines in the Aqueous Humor of Control Individuals

The concentration of up to 13 carnitines in the AH of both control individuals and glaucoma patients was reliably determined. Information on these compounds is relevant since acyl-carnitines are involved in lipid transport across membranes. By far, carnitines C0, C2 and C3 were the most abundant with concentration values of 9.8, 1.7 and 0.2 μM, respectively. Other acyl-carnitines were at concentrations one to two orders of magnitude lower (**Supplementary Table S1**). Virtually all acyl-carnitine levels were altered in the glaucoma samples (see below).

Concentration of Glycerophospholipids and Sphingomyelins in the Aqueous Humor of Control Individuals

Consistent with the hydrophobicity of glycerophospholipids and sphingomyelins and the high-water content of AH, the concentration of these compounds in this body fluid was relatively low. However, it was possible to reliably determine the concentration of 26 glycerophospholipids and 6 sphingomyelins. Sphingomyelin levels ranged from 0.04 μM (sphingomyelin C16:0) to 0.0021 μM (sphingomyelin C22:3) (**Supplementary Table S1**). Regarding glycerophospholipids, their levels ranged between 0.14 μM (C34:1 phosphatidylcholine) and 0.0025 μM (C40:2 phosphatidylcholine). Surprisingly, except for glycerophospholipid C36:6 and sphingomyelin SM C22:3, the levels of all detectable glycerophospholipids and sphingomyelins were significantly altered in glaucoma samples (**Supplementary Table S1**).

Compounds Whose Concentration Is Altered in the AH of Glaucoma Patients

When comparing data in samples from glaucoma patients and healthy controls, the concentration of 57 of 80 compounds was significantly different (indicated in red columns in **Supplementary Table S1**. A volcano plot (**Figure 1**) led to selecting the compounds whose range of variation between

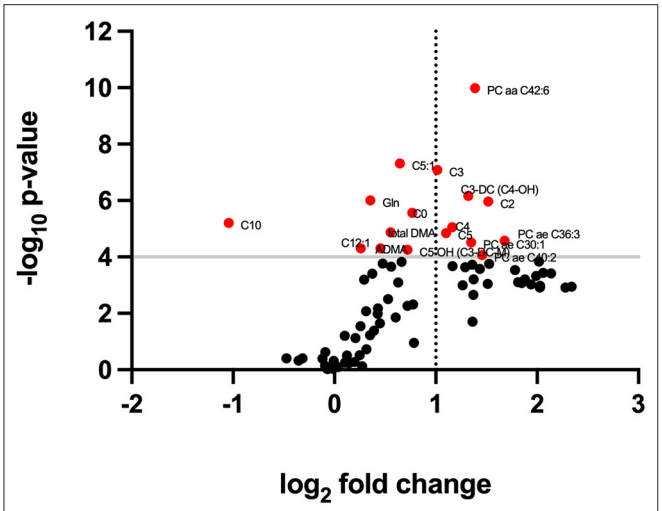

FIGURE 1 | Volcano plot relating significance levels and fold change in data from glaucoma and control samples. Compounds for which differences have a $p < 0.0001$ score are shown in red.

individuals with glaucoma and control was greater and with greater reliability (lower p value). A receiver operating characteristic (ROC) curve was constructed for the 10 compounds selected considering $p < 0.0001$ and $\log_2$ fold change (FC) $> |1|$; ROC for glutamine was also included because, in absolute values, the increase in glaucoma patients was marked (**Figure 2**). The area under the ROC (AUC) for all 11 compounds was close to 1 and the sensitivity and specificity were also close to one. Consequently, the cut-off values in **Table 2** seem very reliable. Consistent with the modest fold change of glutamine concentration in glaucoma vs. control, the scores were the lowest among the 11 selected compounds; Sensitivity, specificity, and AUC values for glutamine were 0.875, 0.812, and 0.805, respectively. Glutamine concentration of AH is relatively high and the cut off value of this compound in AH from glaucoma patients is 623 μM (**Table 2**). Significant differences between controls and glaucoma patients are shown in **Figure 3** for glutamine and the other 10 compounds. Principal component analysis shows good separation between controls and cases; **Figure 4** shows a three-dimensional view of the Cartesian space where 75% of the individuals of each population would be located. The principal component (PC) 1, the PC2, and the PC3 explain, respectively, the 71.9%, the 7.9%, and the 5.3% of the variance between groups. To understand the level of general affectation within a biochemical framework, **Figure 5** shows the compounds that are differentially expressed; the red color highlights the compounds whose concentration in glaucoma is significantly different from that in glaucoma. Marked alterations were found in amino acid metabolism, in amino acid-mediated production of secondary metabolites, including secondary amino acid metabolism, in the composition of various membrane lipids, and in the handling/transport of lipids across the mitochondrial membrane. It should be noted that, with the exception of C10 acyl-carnitine, the glaucoma samples showed an increase in the concentration of metabolites whose levels were significantly altered, from amino acids and biogenic amines to lipids and acyl-carnitines. Finally, it was noticed a significant increase ($p < 0.001$) in kynurenine, 0.49 μM in controls to 0.76 μM in glaucoma.

DISCUSSION

The composition of the AH is important for understanding the physiology of the eye and the homeostatic mechanisms that support the sense of sight. In addition, it is likely that alterations in the concentration of the compounds in the AH provide information on the pathophysiology of diseases that affect the eye and/or the optic nerve.

Here we present novel information, namely the composition of AH sampled from healthy individuals with no known eye disease. Comparing our data with other studies using control samples from controls with cataracts, there are many similarities but also important differences. A limitation of our study is that controls and patients were not matched for age. The reason is that glaucoma appears late in life and therefore the age of the patients was relatively high. In contrast, controls were selected for not having cataracts and this led to the selection of younger individuals.

Table 1 shows that there are 4 of 22 compounds whose concentration is markedly different when comparing our results with those of Buisset et al. (3). Three of them were taurine, butyrylcarnitine, and acetylornithine, all of which we found increased in the glaucoma samples. Although the levels of isoleucine and glutamine were similar in the two studies, the concentration of glycine and alanine was lower in our study and that of leucine was higher in our study. We confirmed the reported increases in lysine and arginine in glaucoma samples compared to controls who underwent cataract surgery (4, 19), but not in glycine as the difference between cases and controls was not significant (**Supplementary Table S1**). The reasons for these discrepancies do not seem to be due to a different surgical procedure but, probably, to a similar but not equal composition of compounds in the AH of healthy controls and individuals with cataracts.

The composition of samples from glaucoma patients differs from that of controls; in fact, 57 of 80 compounds were increased in the glaucoma samples. 10 compounds had fold changes >2 or <1/2. The level differences for other compounds were neither doubled nor halved, but in some cases the changes in concentration were marked. Apart from glutamine, whose median was 563 in the control vs. 697 μM in the glaucoma individuals, the concentration of kynurenine, lysoPC a 24:0, C12:1 acyl-carnitine and asymmetric dimethylarginine was substantially higher in the glaucoma samples. As noted above, the only compound whose concentration decreased in the glaucoma samples was C10 acyl-carnitine. Regarding spermine, we are aware of the report on variations in glaucoma (3) our study has not provided reliable results for spermine and, therefore, we cannot say whether or not spermine levels vary in glaucoma; on the contrary, it was

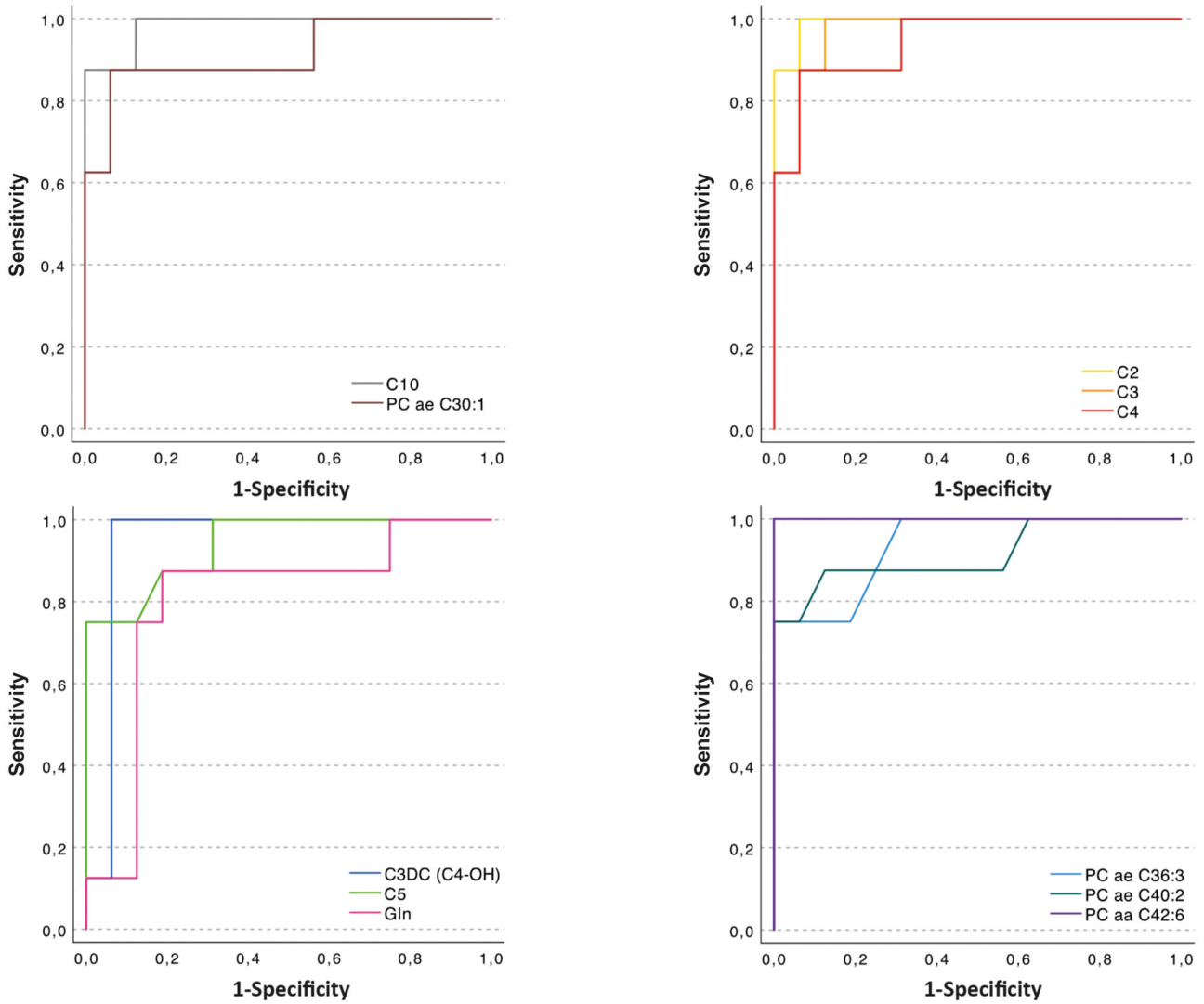

FIGURE 2 | Sensitivity vs. specificity ROC curve of glutamine (Gln) and of the 10 compounds that have a $p < 0.0001$ score and whose $\log_2$ FC > |1|.

possible to reliably determine the level of spermidine, which is higher in glaucoma samples. We did not find the decreases in taurine reported elsewhere (3) but we did find increases. The level of taurine in the healthy controls used here is lower than in the study by Buisset et al. (3) and this may be the reason why we detected significant increases in this biogenic amine in glaucoma samples. The reasons for the discrepancies between studies are not readily apparent as the two studies found, in the glaucoma samples, similar increases in the concentration of other compounds. Comparing controls (cataract) and glaucoma samples, the study by Tang et al. (17) did not look for spermine or taurine concentration and its metabolomic profile led to the identification of compounds that were not determined in our study, e.g., purine metabolism intermediates, and of 3-(4-hydroxyphenyl)-propionic acid, N-lactoyl-phenylalanine, D-mannitol, guanidinoethyl sulfonate, hydroxyacetone, 2-aminoadipic acid, cAMP, 3′-sialyllactose, 2-methylbenzoic acid, dulcitol, lysoPC18:0 and lysoPC 15:0.

Principal component analysis of our data clearly separates controls from patients using the 10 selected compounds (**Figure 3**). The sensitivity and specificity for the 10 selected compounds and for glutamine stand out (**Figure 2**, **Table 2**). It should be noted that the increase in the levels of many compounds occurs in the AH of patients who are under therapy to control glaucoma, so the therapy, being useful to prevent optic nerve damage, does not restore the "standard" composition of the humor. Our study confirms the alteration in the level of acyl-carnitines and phosphatidylcholines reported in the Buisset et al. (3) and Tang et al. (17) studies, but increasing the number of compounds whose concentration is altered and better defining the cut-off values for all of them (see **Figure 1** and **Supplementary Table S1**). Of the 5 compounds that were

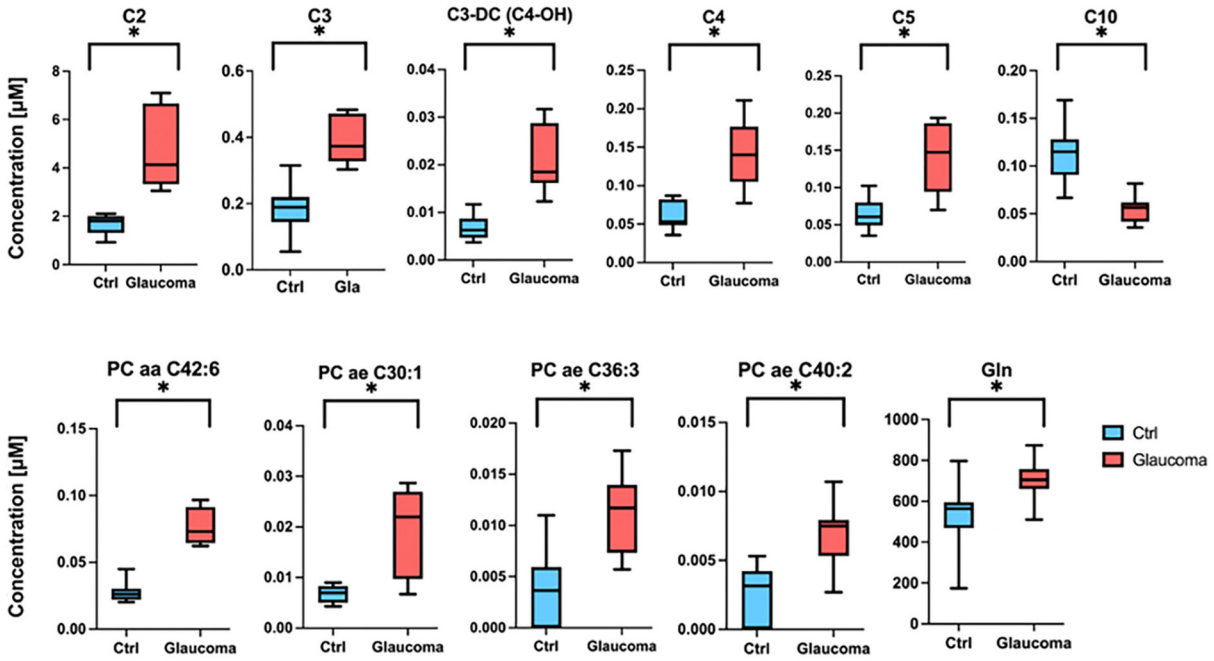

FIGURE 3 | Concentration of glutamine (Gln) and of acyl-carnitines and lysophosphatidylcholines in aqueous humor (AH) from healthy individuals and glaucoma patients. Data are shown as median in box-and-whisker plots (whiskers denote the highest and lowest value determined for each compound in each group, control or glaucoma). *$p < 0.05$.

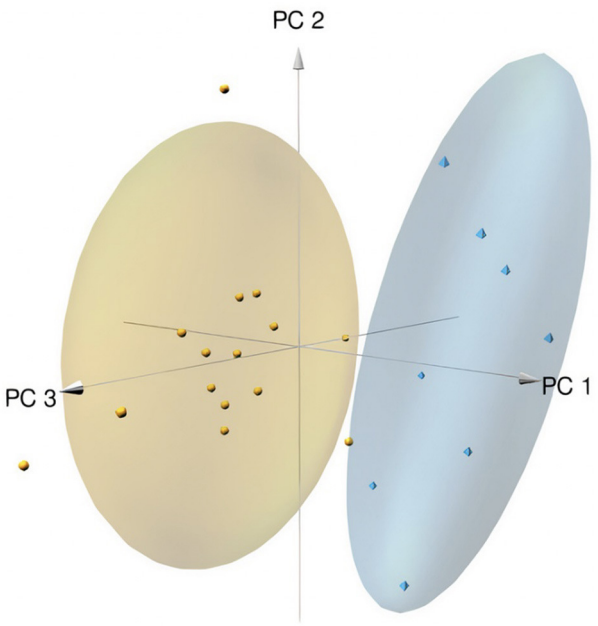

FIGURE 4 | Principal component analysis graphic. The space and data for controls are in yellow and the space and data for glaucoma individuals are in blue. The principal component (PC) 1, the PC2, and the PC3 explains the 62.61%, the 20.31%, and the 11.20% of the variance between groups, respectively. See **Supplementary Tables S2, S3** for details.

determined in both Tang et al. (17) and in our study, the fold change (glaucoma vs. control) of 4 of them (16:0, 16:1, 18:0 and 18:1 lysophosphatidylcholines) was similar but for 2-aminoadipic acid Tang et al. reports a decrease ($\log_2$ FC = −0.73) and we report a small increase ($\log_2$ FC = 0.27). The reason may be the different methodology and/or the fact that in one case the controls had cataracts while in the other only samples from healthy individuals were used.

It is of interest to speculate on the reason for the changes in glutamine, lysophosphatidylcholines, and acyl-carnitines in the AH of glaucoma patients. Increased glutamine levels in glaucoma may be a consequence of trying to keep glutamate (Glu) concentration unchanged. As glutamine is considered generally safe and is used for cryopreservation of tissues/cells (20, 21), an increase in [glutamine] to keep glutamate concentration low (and constant) could be a mechanism to prevent excitotoxic damage caused by accumulation of the amino acid. In paired values in a non-human primate glaucoma model, the concentration of glutamate in the AH before and after the onset of glaucoma was not significantly different. In such a model there was a marked degree of variability (ranges: 2.95–87.3 μM before intervention and 2.77–87.4 after establishment of glaucoma) that we do not find in human samples. In contrast, several years ago, it was reported that elevated intraocular pressure and increased glutamate can lead to the death of retinal ganglion cells. In the study it is interesting the information about glutamate concentration: "*elevated level of glutamate in the vitreous humor*

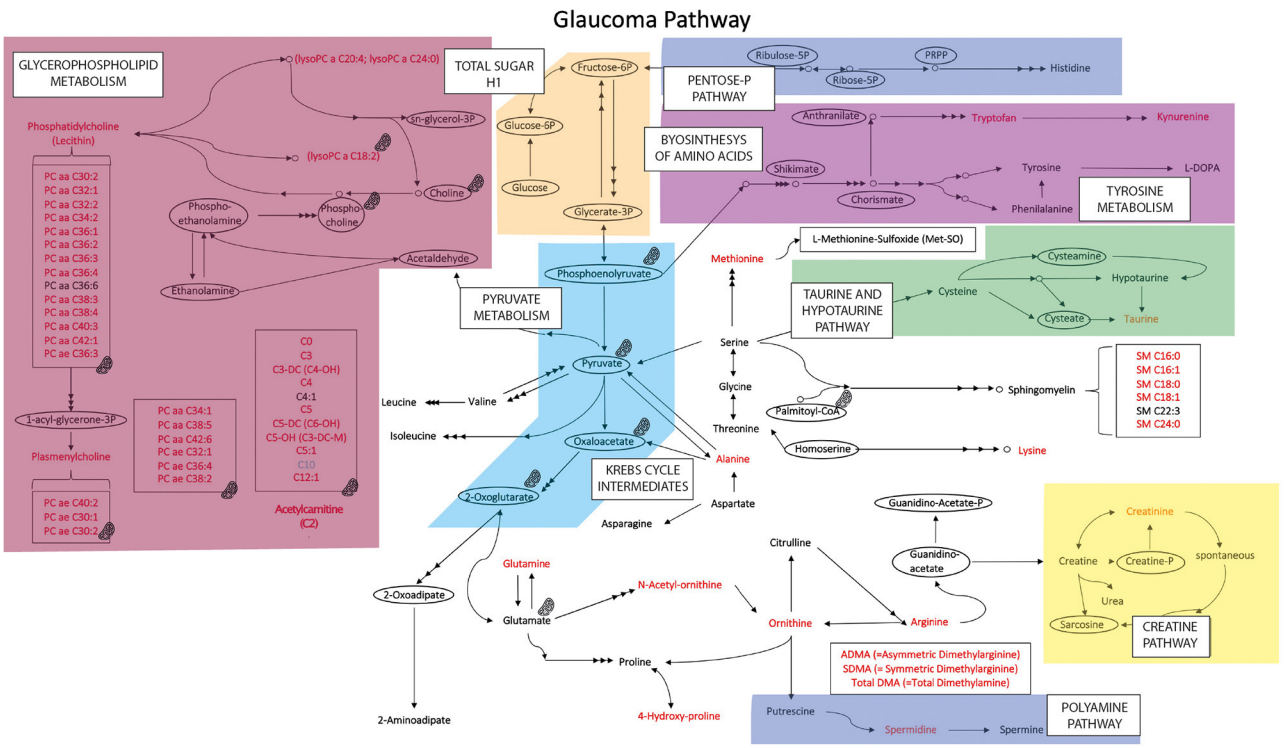

FIGURE 5 | Metabolic pathway map summarizing the results obtained in glaucoma samples. In red the metabolites whose concentration is increased in the glaucoma samples (compared with values in samples from healthy controls), in light blue the metabolite whose concentration is reduced in glaucoma samples. Compounds within ellipses were not determined in this study; they are included as nodes in metabolism interconnections. Areas in color represent main metabolic pathways. Icon indicates compound/group of compounds that may be in mitochondria or are directly/indirectly related to mitochondria.

of glaucoma patients (27 microM as compared to 11 microM in the control population)" (22). We wonder if such a high concentration in the vitreous humor of the patients was due to the lack of treatment to normalize ocular pressure; our study shows AH concentrations of 6.55 μM in controls and 7.40 μM in patients with glaucoma.

The altered levels of acyl-carnitines and key membrane phospholipids, suggest that in glaucoma, even if corrected with medication, there are alterations in the membranes, mainly of mitochondrial origin. In fact, carnitines are necessary for the transport of lipids across the mitochondrial membrane. On the one hand, the content of lysophosphatidylcholines is higher in the mitochondrial membranes than in the plasma and endoplasmic reticulum membranes. On the other hand, it is tempting to speculate that the complex phospholipid transport machinery of mitochondrial membranes (23) is altered in glaucoma. Evidence from lymphoblasts from glaucoma patients points to mitochondrial alterations leading to impaired ATP production through defective function of Complex I of the electron transport chain (24, 25); such changes could eventually modify the serum levels of mitochondrial markers that may end up modifying their concentration in the AH. There is also a correlation in increases in 34:2, 34:4 and 36:4 phosphatidylcholines in plasma and AH samples from glaucoma patients (26). However, the involvement of local events affecting the variety of structures that make up the eye cannot be ruled out. A mitochondrial implication may be behind the significant increase in kynurenine in glaucoma samples, since the kynurenine pathway is key in the metabolism of nicotine and adenine dinucleotides, which are essential, among others, for the production of ATP in mitochondria. Targeting the kynurenine pathway has been proposed to combat neurodegeneration caused acutely by hypoxia/ischemia, or chronically due to expression of mutant proteins leading to Huntington's disease or Parkinson's disease (27–29).

Previous studies have shown increases in AH of glaucoma samples of C0, C2, C3, and C4, but to our knowledge, no one has tested (in this body fluid) the other acyl-carnitines that have been determined here. Those studies also show that C4 (butyryl)-carnitine) is found in both the AH and plasma of glaucoma patients (3, 26). In our study, all acyl-carnitines whose concentration is above the detection limit, 13 in total, increased in glaucoma, with two exceptions i) C4:1 and C14:2OH, whose concentrations were similar, and ii) C10, which decreased in the glaucoma samples. These results confirm the mitochondrial alterations and the need to

evaluate the role of (decanoyl)-carnitine C10 in the functionality of the mitochondria. This compound showed interest in the past because exogenous administration led to impaired mitochondrial handling and fatty acid oxidation and inhibited ketogenesis (30). Apparently, acyl-carnitines have the potential to better understand the pathophysiological mechanisms of ocular diseases, in the same way that the analysis of the profile of acyl-carnitines has served to better understand monogenic diseases that affect the metabolism of organic acids and of fatty acids (31).

Taken together, the data herein serve to better understand the composition of AH in healthy controls. Due to the power of the approach, which is based on mass spectrometry, amino acids, biogenic amines but also lipophilic molecules such as glycerophospholipids, sphingomyelins and acyl-carnitines could be measured. Glaucoma, even when the ocular pressure is kept under control by pharmacological means, causes a variation in the level of 71% of the measured compounds. The imbalance in the glaucoma samples was substantial for glutamine, probably due to the need to keep the concentration of glutamate low, which is toxic to retinal cells if it accumulates in the AH. The comparison of glaucoma and control samples confirmed that mitochondria play a role in the disease as acyl-carnitines, which are essential for mitochondrial function, were significantly altered.

DATA AVAILABILITY STATEMENT

Virtually all data obtained from metabolomics analysis are in the **Supplementary Material**. Any data allegedly missing from the **Supplementary Material** may be obtained from the corresponding author upon reasonable request.

ETHICS STATEMENT

The study has been evaluated by the Comitè d'Ètica de la Investigació de les Illes Balears (CEI-IB) and deemed not to require ethics approval samples are considered waste and no data on patient identification (neither name, address nor ID numbers) are available to experimenters.

AUTHOR CONTRIBUTIONS

RF and JS-N designed the project. JS-N was the surgeon that took the samples. SM and AL did, in single blinded conditions, all experimental and *in silico* work related to processing samples until obtaining raw data. Data analysis and construction of tables and figures was done by JS-M and NB. RF, GN, and MC supervised all the project and validated the final results. MC, JS-N, and RF wrote the first draft of the manuscript. The manuscript was further edited by all authors who approved the submitted version.

ACKNOWLEDGMENTS

We thank the CCiTUB (Scientific and Technological Centers of the UB) of the Universitat de Barcelona (Barcelona, Spain), for the expert and technical help provided in the use of the LC-MS/MS equipment.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.935084/full#supplementary-material

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frontiers | Frontiers in Medicine

TYPE Original Research
PUBLISHED 23 September 2022
DOI 10.3389/fmed.2022.999974

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Ophthalmology, a section of the journal Frontiers in Medicine

RECEIVED 21 July 2022
ACCEPTED 29 August 2022
PUBLISHED 23 September 2022

CITATION
Yuan R, Liu K, Cai Y, He F, Xiao X and Zou J (2022) Body shape and risk of glaucoma: A Mendelian randomization. *Front. Med.* 9:999974. doi: 10.3389/fmed.2022.999974

Body shape and risk of glaucoma: A Mendelian randomization

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Background: Body size (BS) is one of the risk factors for the development of many clinical diseases, but the relationship between BS and glaucoma is controversial. Herein, we try to use Mendelian randomization (MR) method to study BS causal association with glaucoma risk from the genetic level.

Methods: The Body Size was determined through anthropometric traits (ATs), such as body mass index (BMI), waist-to-hip ratio adjusted by body mass index (WHRadjBMI), waist-to-hip ratio (WHR), and waist circumference (WC). Association of single nucleotide polymorphisms (SNPs) with each AT and glaucoma were determined individually from the aggregated data of the Genetic Investigation of Anthropometric Traits (GIANT) consortium and the FinnGen study summary data (8,591 cases with glaucoma and 210,201 controls). To explore the role of BS and glaucoma, a two-sample MR analysis was performed on genome-wide association study (GWAS) data. Besides, three MR methods [inverse variance weighted (IVW), Weighted median, and MR-Egger regression] were used to get the whole causal estimate for multiple instrumental SNPs.

Results: BMI (OR = 1.20; 95% CI = 1.02–1.41; $P = 0.03$) and WC (OR = 1.32; 95% CI =1.04–1.69; $P = 0.03$) were associated with a risk of glaucoma. Besides, genetically predicted WHRadjBMI (OR = 1.10; 95% CI = 0.88–1.35; $P = 0.43$) and WHR (OR = 1.22; 95% CI = 0.93–1,572; $P = 0.14$) were not associated with glaucoma. No heterogeneity and directional pleiotropy were detected.

Conclusion: The data of this study revealed that increased BMI and WC are potential risk factors for glaucoma, and WHRadjBMI and WHR are not associated with the occurrence of glaucoma.

KEYWORDS

body shape, glaucoma, body mass index, waist-to-hip ratio adjusted by body mass index, waist-to-hip ratio, waist circumference, Mendelian randomization

Introduction

Glaucoma is a chronic condition of progressive optic neuropathy associated with characteristic damage to the optic nerve, loss of visual field, and lead irreversible blindness. The cases of glaucoma are expected to observe an increment of 111.8 million by 2040 (1). The raised intraocular pressure (IOP) on the optic nerve is the sole modifiable risk factor in glaucoma; however, it does not help in all cases (2, 3). Thus, we need to start looking at factors other than intraocular pressure that may be associated with glaucoma to find a new method of prevention and treatment. Glaucoma is considered a multifactorial disease, and family history of glaucoma (4) and age (5) are considered the chief risk factors for glaucoma. At present, a large number of clinical studies have shown that immune components are also involved in the neurodegenerative process of glaucoma (6). In addition, other factors, including hemodynamic factors, metabolic syndrome, and obesity, were associated with glaucoma (7).

Body size (BS) is generally measured through anthropometric characteristics (ATs), like body mass index (BMI), waist to hip ratio adjusted according to body mass index (WHRadjBMI), waist to hip ratio (WHR), and waist circumference (WC) (8). Among them, BMI is a common tool to assess obesity. The World Health Organization (WHO) defines obesity as a body mass index $\geq$30 kg/m^2, overweight as a body mass index between 25 to 29.9 kg/m^2 (9). Obesity is a growing problem worldwide and has an impact on eye diseases including age-related cataracts, age-related macular degeneration and diabetic retinopathy (10). However, whether there is a direct link between obesity and intraocular pressure remains elusive. On the one hand, BMI had a positive linear correlation with IOP (11). And on the other hand, BMI was inversely associated with the risk of open-angle glaucoma (12). Whereas, no significant differences were found in BMI when comparing patients with and without glaucoma in a case-control study (13). Recently, many scholars studied the impact of anthropometric parameters on the incidence of glaucoma. In a national health and nutrition survey from South Korea, fat mass/weight ratio and fat mass/muscle mass ratio were found to be negatively associated with glaucoma. On the contrary, muscle mass parameter/BMI ratio was observed to be significantly positively related with glaucoma ($P < 0.05$) in males. In contrast, height and fat mass/BMI showed a serious relationship with onset of glaucoma ($P < 0.05$) in females (14). Furthermore, a positive correlation was found for BMI with IOP in the Chinese and Singaporeans population. (15). Conversely, lower BMI led to higher prevalence of glaucoma in the Indian population (16). Therefore, whether these parameters are positively or negatively correlated with the disorder is debatable because of ethnic differences and confounding factors found in different studies and different research methods. Although previous studies had suggested that some of the relevant items in BS may be associated with the occurrence of glaucoma, limited follow-up and research methods have made it difficult to complete randomized controlled trials to explore the specific relationship between patients with glaucoma and BS.

The Mendelian randomization (MR) analysis is a type of instrumental variable (IV)-based study, which is extensively utilized to assess potential causal relationships among exposure and outcome (17). Moreover, the gene distribution of human genetics follows Mendelian genetic law and does not get affected by most acquired confounding factors. Recently, Robert Carreras-Torres used the MR method to verify a strong causal relationship between BMI, as one of BS, and the risk of pancreatic cancer [ODDS Ratio (OR) = 1.34, 95% Confidence Interval (CI) = 1.09–1.65, for Each Standard Deviation Increase in BMI (4.6 kg/m^2)] (18). However, as we know, there were no studies conducted using genetic data to explore the causal relationship between BS and glaucoma. Therefore, we have conducted an extensive MR analysis to understand the influence of Body Shape on glaucoma incidence by simulating the possible effects of BMI, WHRadjBMI, WHR and WC on glaucoma risk. Our results indicate the potential risks of glaucoma in BMI and WC in BS and provides new diagnostic ideas and preventive measures for glaucoma.

Materials and methods

Study design

When using a large sample size genetic database, the two sample MR analyses can assess the causal effect of BMI, WHR, WHRadjBMI, and WC on glaucoma by using SNPs as instrumental variables (IVs) to avoid accidental influence (19, 20). Our MR study was conducted on the following assumptions: First, the IVs are associated with BMI, WHR, WHRadjBMI, and WC; Second, the IVs affects glaucoma only through its effect on BMI, WHR, WHRadjBMI, and WC; Third, the IVs was not associated with any factors that confound the relationship between the exposure and outcome (Figure 1) (21). In addition, the data used by these MR analyses were processed from the data provided.

Ethics declarations

The study used publicly available aggregate-level data without additional participant consent and ethical approval. All original and GWAS studies are approved by the ethics committees of their respective institutions. The ethical approval for each study can be found in the original publication. All methods and procedures in this study were performed in accordance with the Declaration of Helsinki. All methods

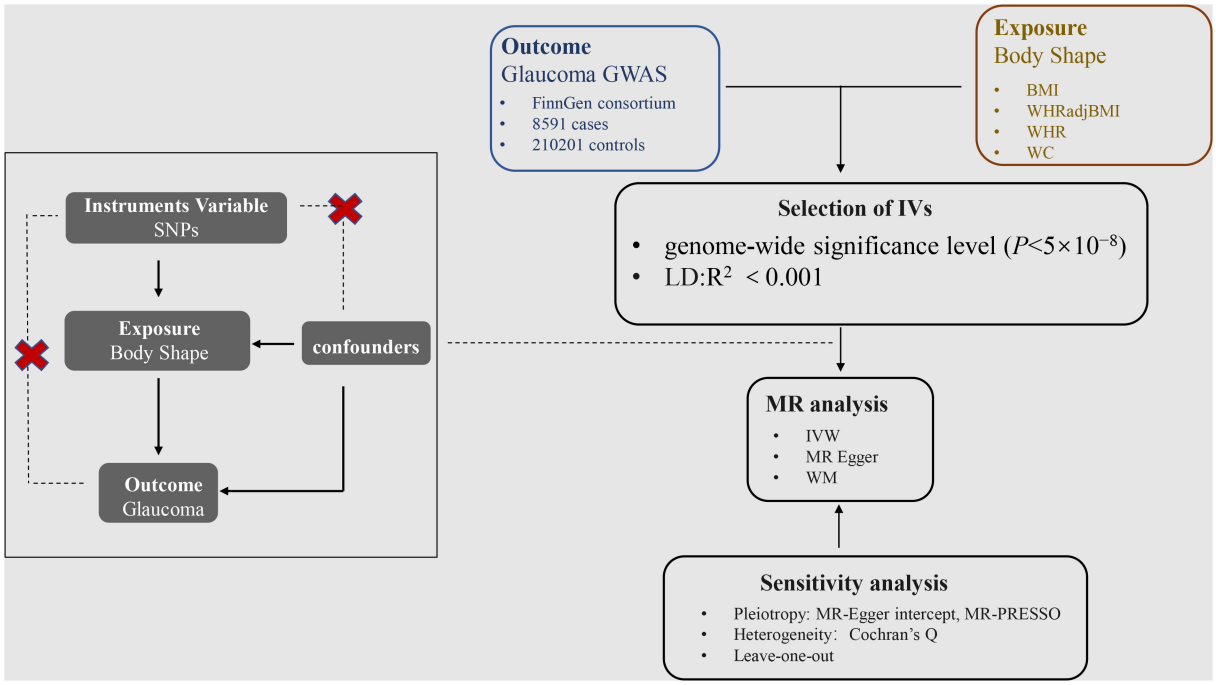

FIGURE 1
Acyclic graph interpretation of Mendelian randomization analysis.

in our study of MR were performed by the STROBE-MR statement (22).

Data sources for MR analyses

The two sample MR analysis was completed utilizing summary data from genome-wide association studies (GWAS) (23). In our study, the whole subjects have homogeneous characteristics which were both from Europe. The relationships for SNPs with BMI, WHRadjBMI, WHR, and WC were derived from data aggregated from the Genetics of Anthropometric Traits (GIANT) Consortium in the year of 2015 (24).

In addition, the association of exposure relative SNPs with glaucoma was obtained from the FinnGen study (https://r5.finngen.fi/) (25). The database contains 8,591 glaucoma patients and 210,201 health participants in the European population. The demographic information of participants, such as age and gender, is provided in the FinnGen study. The data set can be accessed as required.

The selection and validation of instrumental variables

To prove the independence of IVs, we evaluated linkage disequilibrium (LD) by testing the clustering test. Because LD can introduce bias and it was required that the instrumental variables of exposure selection should be independent of each other. This was ensured by clustering the majority of variants into a series of indexed SNPs (8). Then the SNPs were gathered by aggregating all SNPs according to LD ($R^2 = 0.001$). The significant association between the SNPs with each SNP reaching the genome-wide significance level ($P < 5 \times 10^{-8}$) was also determined. Palindrome and ambiguous SNPs were discarded.

Sensitivity analysis

To test and correct the robustness of the MR estimates, we applied sensitivity analysis. Firstly, MR-Egger's intercept values were used to assess the multiplicity of SNPs. The closer the intercept to 0, the lower the multiplicity was considered. Secondly, MR Pleiotropy RESidual Sum and Outlier (MR-PRESSO) (26) was used to evaluate further the SNPs pleiotropy that has the potential causality, and the SNPs with abnormalities were removed. When the number of variables is >50%, MR-PRESSO can still get accurate results. Meanwhile, Cochran's *Q*-test was used for the assessment of heterogeneity. Finally, to ensure the robustness of the results, the analysis was carried out using the Leave-one-out test.

TABLE 1 Associations between BS and risk of glaucoma using Mendelian randomization.

Exposure	Method	OR	95% CI	*P*-value
BMI	IVW	1.20	1.02–1.41	0.03
BMI	Weighted median	1.26	0.95–1.66	0.11
BMI	MR Egger	1.37	0.93–2.02	0.12
WHRadjBMI	IVW	1.09	0.88–1.35	0.43
WHRadjBMI	Weighted median	1.01	0.74–1.40	0.93
WHRadjBMI	MR Egger	0.87	0.26–2.93	0.82
WHR	IVW	1.21	0.93–1.57	0.14
WHR	Weighted median	1.27	0.87–1.85	0.22
WHR	MR Egger	1.82	0.53–6.24	0.35
WC	IVW (random effects model)	1.32	1.04–1.69	003
WC	Weighted median	1.32	0.95–1.84	0.09
WC	MR egger	1.41	0.75–2.67	0.29

SNP, Single nucleotide polymorphism; BMI, including body mass index; WHRadjBMI, waist-to-hip ratio adjusted by body mass index; WHR, waist-to-hip ratio; WC, waist circumference; IVW, Inverse variance weighted; OR, The effect of the effect allele; CI, Confidence interval; *P* *P*-value from the GWAS.

Statistical methods

All analyses were completed using R software (version 1.4.1717) and the R packages "Two Sample MR." The Wald ratio is often accustomed to deriving causal estimates for a single SNP (8). Three MR methods, namely Inverse variance weighted (IVW) linear regression, the MR-Egger regression (27), and the Weighted median method (28), were used for overall causal estimation for multiple SNPs (29, 30). The IVW was transformed into a weighted regression of the outcome effects of instrumental variables on exposure effects to obtain an overall estimate of the impact of BMI, WHRadjBMI, WHR and WC on glaucoma risk. Meanwhile, the fixed effect model or random effect model were selected according to the heterogeneity (31, 32). The MR-Egger method can still provide unbiased estimators even if pleiotropy exists for all selected instrumental variables. Moreover, even if more than half of the instrumental variables are invalid, the Weighted median still provides a consistent estimate of the causal effect. $P < 0.05$ is considered to have potential causality.

Results

The causal effect of BMI on glaucoma

There were 64 SNPs associated with BMI and glaucoma (Supplementary Table 1). In MR analysis between BMI and glaucoma, overall causal estimation of the IVW method showed that BMI had a significant relationship with glaucoma (OR = 1.20; 95% CI = 1.02–1.41. $P = 0.03$) (Table 1). The Weighted median (OR = 1.26, 95% CI = 0.95–1.66; $P = 0.11$) and MR-Egger (OR = 1.37, 95% CI = 0.93–2.02, $P = 0.12$) did not show a correlation between BMI and glaucoma (Table 1; Supplementary Figure 1A). A Leave-one-out test validated the impact of each SNP on the results to verify the robustness of the data (Figure 2A). The pleiotropy were performed by MR-Egger intercept ($P = 0.45$) and MR-PRESSO ($P = 0.63$) (Table 2). At the same time, the funnel plot results confirm that there was no horizontal pleiotropy of the selected tool variables (Figure 2B), so as the results of Cochran's *Q*-test (Table 2, $P = 0.62$). As the sensitivity analysis confirms the robustness of the data and the results of the IVW were more reliable, there was a clear causal relationship between BMI and glaucoma.

The causal effect of WHRadjBMI on glaucoma

Thirty-six SNPs were associated with WHRadjBMI and glaucoma (Supplementary Table 2). In the MR analysis between WHRadjBMI and glaucoma, the whole causal estimation of the IVW method showed that each SD change had no impressive impact on the risk of glaucoma in WHRadjBMI (OR = 1.10; 95% CI = 0.88–1.35; $P = 0.43$) (Table 1). The results of the weighted median (OR = 1.01, 95% CI = 0.74–1.40; $P = 0.93$) and MR egger (OR = 0.87, 95% CI = 0.26–2.93, $P = 0.81$). Did not show the correlation between WHRadjBMI and glaucoma (Table 1; Supplementary Figure 1B). The impact of each SNP on the results verified the robustness of the data by the leave-one-out of the test (Figure 3A). The pleiotropy was tested by MR egger intercept ($P = 0.71$) and MR PRESSO ($P = 0.08$) (Table 2). At the same time, the results of the funnel chart (Figure 3B) and Cochran's *Q*-test (Table 2, $P = 0.07$). Also confirmed no horizontal pleiotropy for the selected instrumental variables. Because there is no pleiotropy of the data and the results of

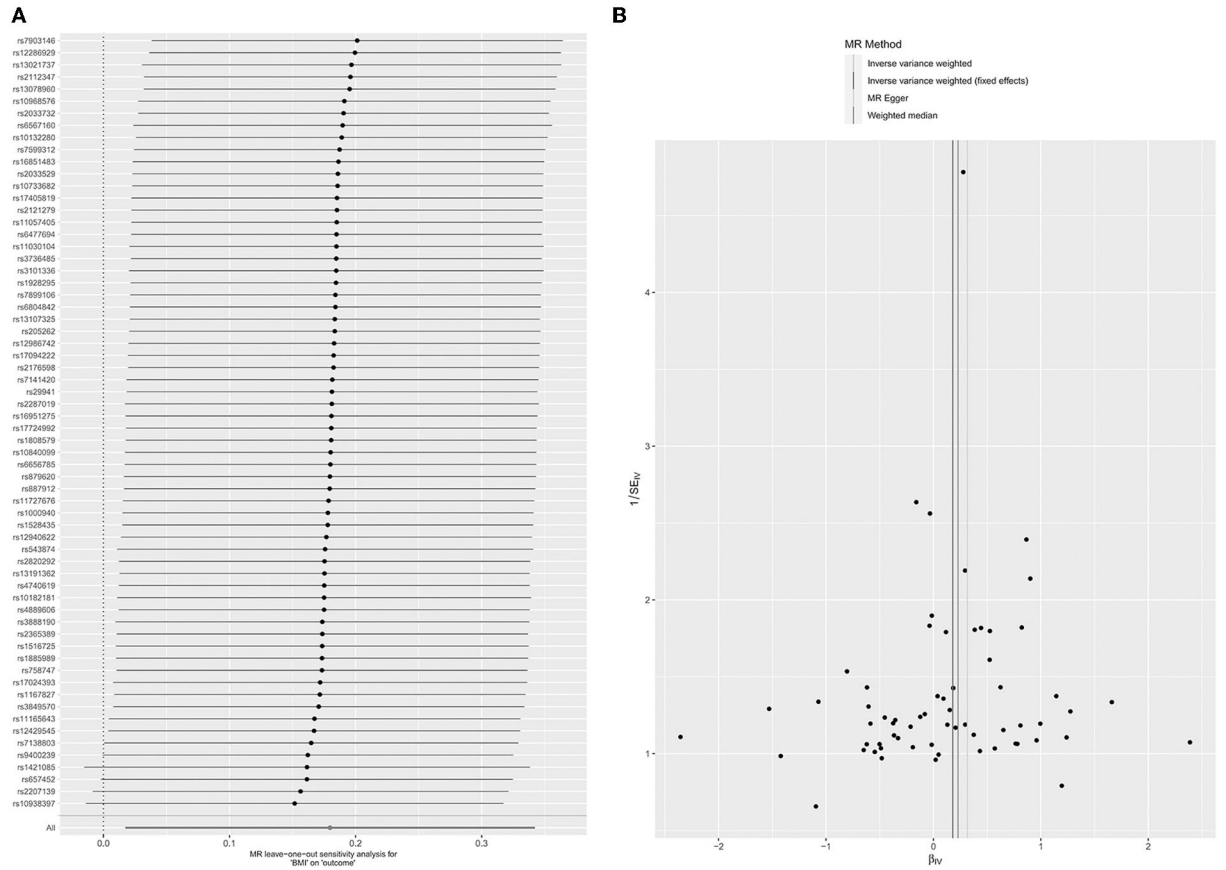

FIGURE 2
Leave-one-out permutation analysis plot **(A)** and Funnel plot **(B)** of BMI.

TABLE 2 Sensitivity analysis for the associations between BS and risk of glaucoma.

Exposure	*P* (Cochran's Q)	Intercept (MR-egger)	*P* (MR-egger)	*P* (MR- PRESSO)
BMI	0.62	-4.32×10^{-3}	0.45	0.63
WHRadjBMI	0.07	6.70×10^{-3}	0.71	0.08
WHR	0.20	-1.10×10^{-2}	0.51	0.22
WC	0.04	-2.23×10^{-3}	0.82	0.06

MR, Mendelian randomization; BMI, including body mass index; WHRadjBMI, waist-to-hip ratio adjusted by body mass index; WHR, waist-to-hip ratio; WC, waist circumference; SE, Standard error, *P* *P*-value from the GWAS.

the IVW test were more reliable, there was no obvious causal relationship between WHRadjBMI and glaucoma.

The causal effect of WHR on glaucoma

There were 29 SNPs associated with WHR and glaucoma (Supplementary Table 3). From the MR analyses between WHR and glaucoma, the overall causality estimated by the IVW method showed that each SD change had no significant effect on the risk of glaucoma in WHR (OR = 1.22; 95% CI = 0.94–1.58; *P*= 0.14) (Table 1). The Weighted median (OR = 1.27, 95% CI = 0.87–1.85; *P* = 0.22) and MR-Egger (OR = 1.82, 95% CI = 0.53–6.24, *P* = 0.35) did not show a correlation between WHR and glaucoma (Table 1; Supplementary Figure 1C). The impact of each SNP on the results was verified by the Leave-one-out test (Figure 4A). Pleiotropic tests were performed on MR-egger Intercept (*P* = 0.51) and MR-PRESSO (*P* = 0.22, Table 2). At the same time, the funnel plot results also confirmed that the selected instrumental variables did not have the horizontal pleiotropic effect (Figure 4B). There is no heterogeneity by

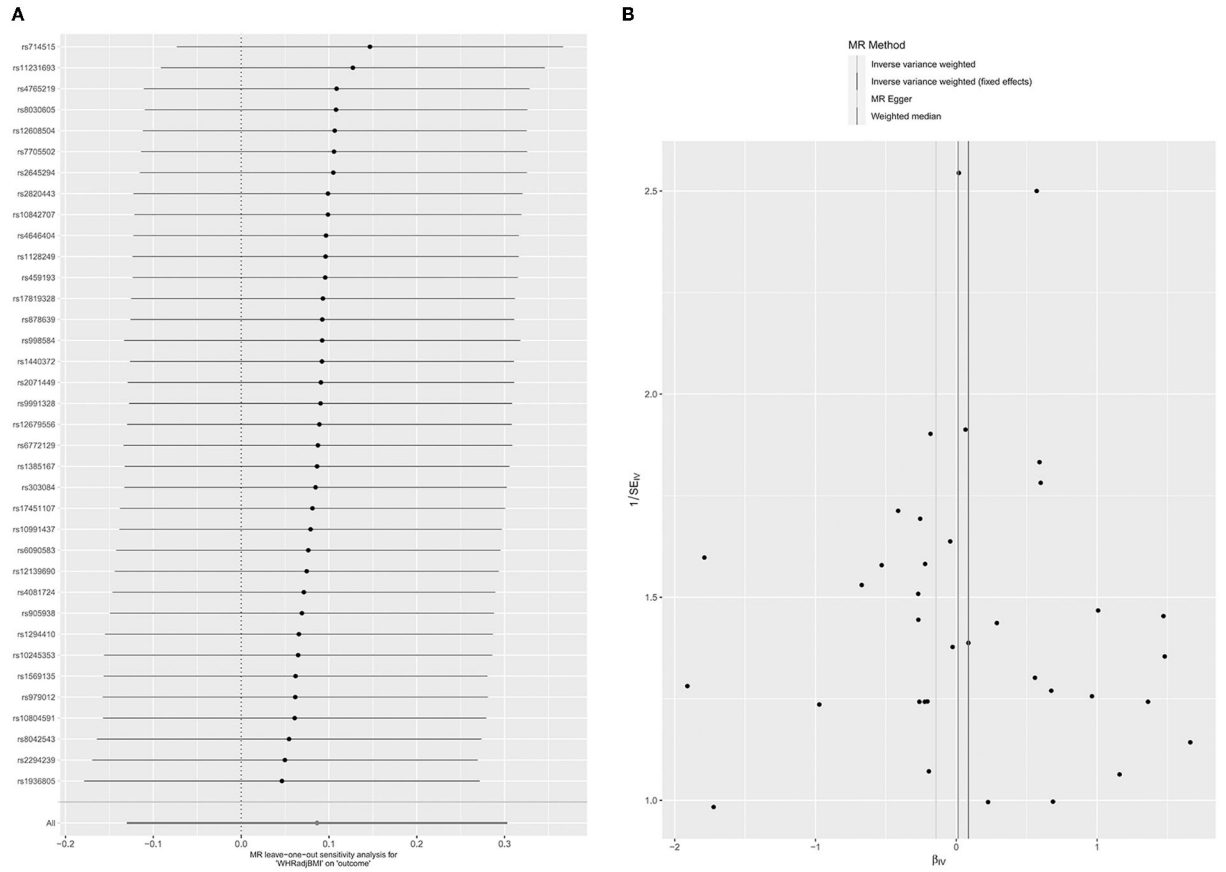

FIGURE 3
Leave-one-out permutation analysis plot **(A)** and Funnel plot **(B)** of WHRadjBMI.

using Cochran's *Q*-test (Table 2, $P = 0.20$). Since there is no pleiotropy in the data, the IVW test results were more reliable. Therefore, there was no clear causal relationship between WHR and glaucoma.

The causal effect of WC on glaucoma

There were 36 SNPs associated with WC and glaucoma (Supplementary Table 4). In the MR analysis between WC and glaucoma, the overall causal estimates from the IVW method indicated that $P = 0.01$ (OR = 1.32; 95% CI = 1.08–1.62) in WC. The result of MR-Egger's sensitivity analysis (OR = 1.41, 95% CI = 0.75–2.67, $P = 0.29$) and Weighted median (OR = 1.32, 95% CI = 0.95–1.84; $P = 0.09$) in Table 1 and Supplementary Figure 1D. Pleiotropy tests were performed by MR-Egger intercept ($P = 0.82$) and MR PRESSO ($P = 0.06$, Table 2). The results of MR Egger and MR PRESSO in the pleiotropy test were >0.05 which means there was no pleiotropy. The funnel plot results also confirmed the absence of pleiotropy (Figure 5B). There is heterogeneity by using Cochran's *Q*-test (Table 2, $P = 0.04$). Considering the existence of heterogeneity, we used a random effects model (31) to IVW and we still observed a significant association between WC and glaucoma (OR = 1.32, 95 CI% = 1.34–1.69, $P = 0.03$ <0.05, Table 1). The impact of each SNP on the results was validated by a Leave-one-out test (Figure 5A). Therefore, there was an obvious causal relationship between WC and glaucoma.

Discussion

To the best of our knowledge, this is the first MR analysis that detect the causal relationship between glaucoma and Body Size. In our study, two-sample MR analyses with a genetic instrument were selected from a wide-ranging GWAS to evaluate the body size role in the risk of glaucoma based on the genetic data obtained from European databases. MR approach is more likely to avoid confounding bias than the literature-reported risk in observational epidemiological studies (33). After adjusting for genetic linkage using the three different estimation methods (Weighted median, IVW, and MR-Egger regression), our results

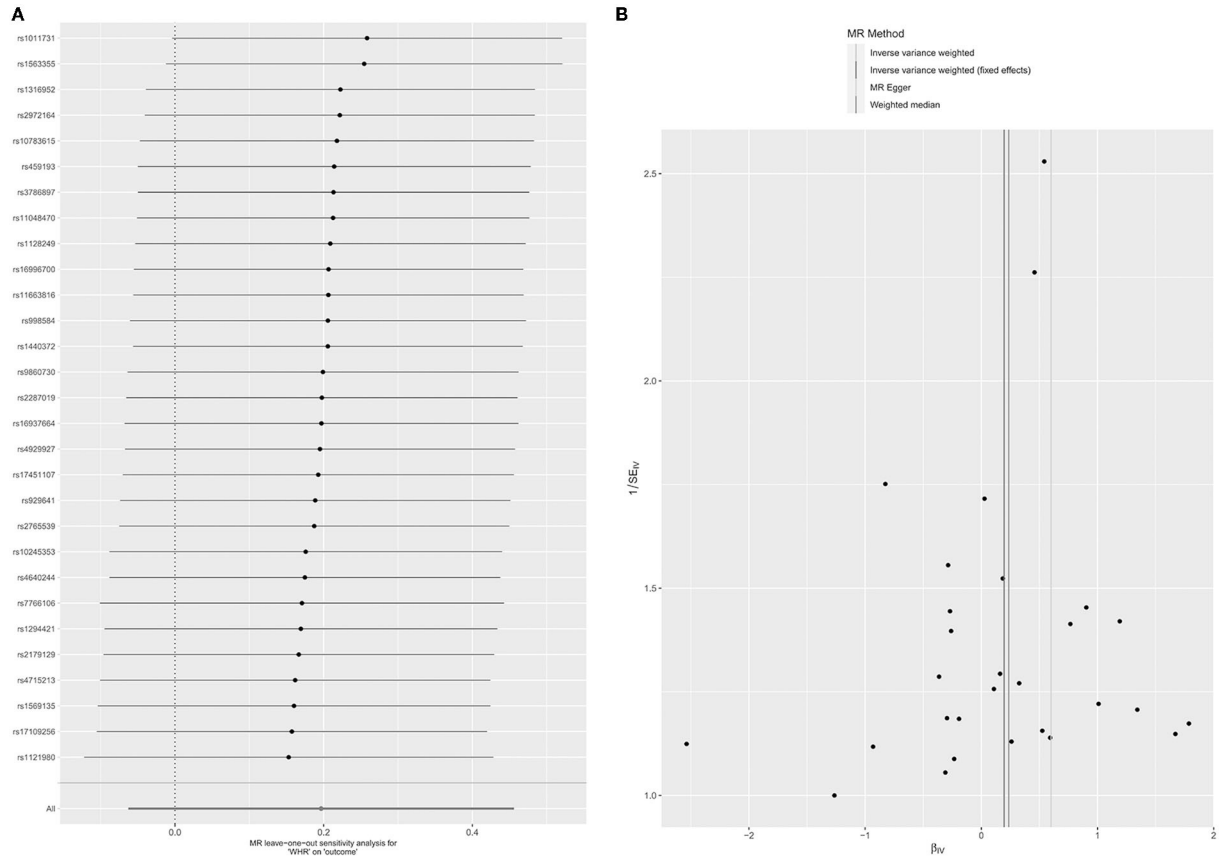

FIGURE 4
Leave-one-out permutation analysis plot **(A)** and Funnel plot **(B)** of WHR.

suggest that BMI and WC might associate with the glaucoma risk except WHRadjBMI and WHR.

In previous studies, BMI, WHRadjBMI, WHR, and WC were reported as important BS indicators. These indicators were shown to be closely related to many diseases. For instance, a cross-sectional study involving 1,891 subjects (59.1% Chinese, Malays 22.2%, Indians 18.7%) from Singapore believed that the combination of BMI and WHtR might have clinical importance for segregating patients using cardiovascular disease risk factors (34). Moreover, patients with high BMI, WC, and WHtR often represent obesity, which is considered a higher risk factor for cardiovascular disease due to abnormal blood pressure, blood lipids, blood sugar, and even blood volume (35). A study in China showed that WC and WHtR were more closely related to diabetes, especially in female patients older than 40 years old (36). This study suggested that WC and WHtR reflect the abdominal or ectopic fat in diabetes, a more important risk factor for metabolic disease than general obesity indicators. Previous studies had reported that combinations such as BMI and WHtR (Waist-to-Height Ratio) play an important role in understanding risk factors for cardiovascular disease in adults (36). Besides, somatic symptoms of depression have been considered positively correlated with anthropometric findings (38). Recently, several meta-analyses showed that different types of anthropometric data might be potential causes of gastric and esophageal cancers (39) and demonstrated the importance of anthropometric indices in disease risk factors and even disease prevention. These studies emphasized the important role of BS indicators in measuring disease risk factors.

Recently, several studies have reported BMI relationship with glaucoma. A prospective study from the Korean National Health Insurance System classified people according to metabolic syndrome and obesity, expressed the presence, severity, and concluded that metabolically healthy and obesity were had a significant risk of glaucoma. For instance, someone with a BMI of 30 kg/m^2 or more was prone to develop glaucoma than those with a BMI of 18.5–22.9 kg/m^2 (40). In a population-based study, Ko et al. reported that people with a BMI of 30 kg/m^2 or higher were more likely to be associated with glaucoma incidence (41). Consistently, many studies performed on the Chinese and Singaporeans population have reported a correlation between IOP, body shapes, and BMI

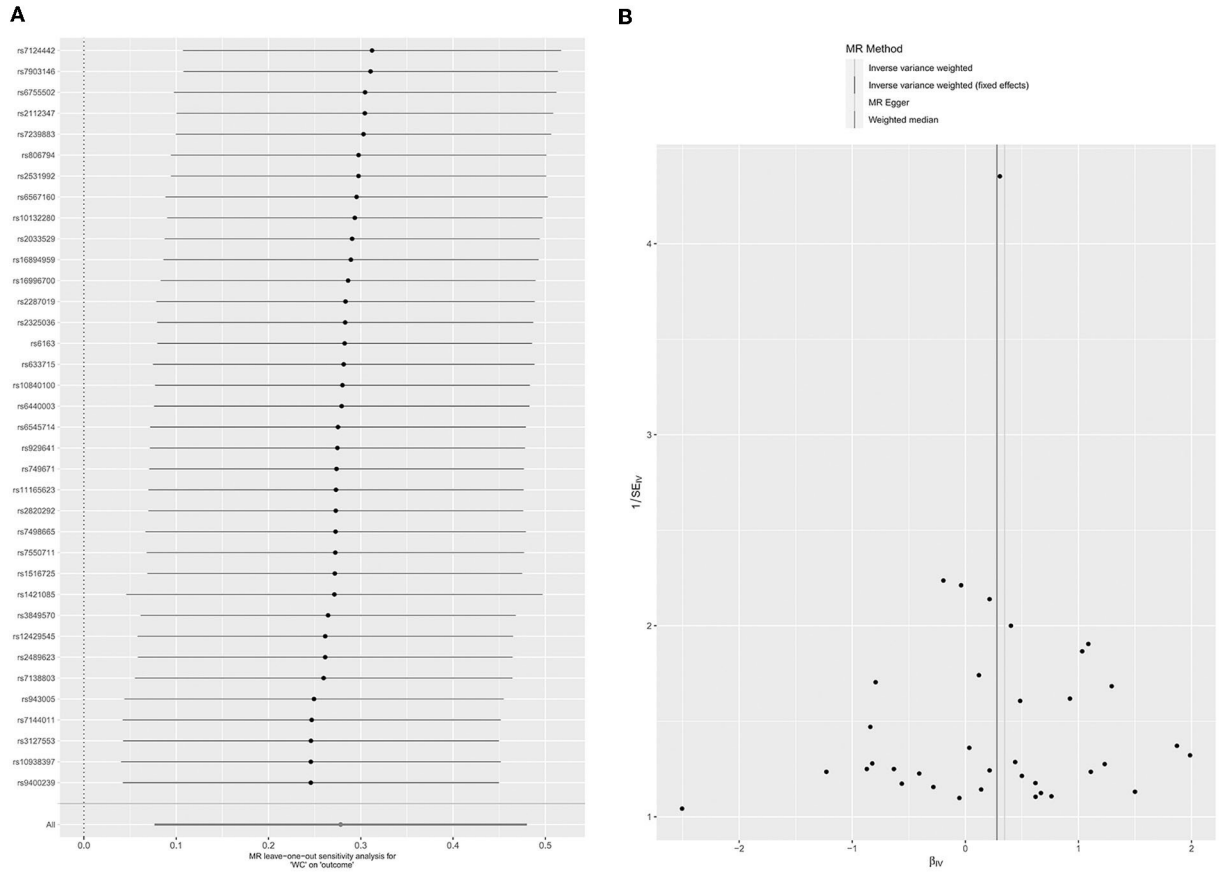

FIGURE 5
Leave-one-out permutation analysis plot **(A)** and Funnel plot of **(B)** of WC.

(15). In contrast, people with a low BMI were associated with a higher risk of glaucoma (16). These results might be influenced by the different sociodemographic groups, with differences in race, age, and population composition ratio. As another two key indicators of BS, WHR and waist circumference were documented by very few researchers (16). A study from India showed that increasing age and elevated WHR were the risk factors for elevated intraocular pressure (42). However, these cross-section studies have not completely demonstrated the relationship between BS and glaucoma (43). In our study, BMI and WC was a novel risk factor for glaucoma which is consistent with the results of previous studies (37). Hemodynamics factors have been reported to involve in the pathogenesis of POAG and metabolic health along with obesity might affect the overall primary open angle glaucoma development (7). Despite similar levels of obesity, BMI might show differential clinical outcomes, based on the body's metabolic state (44). However, BMI cannot distinguish between fat and lean body mass, while WC, WHC, etc., can reflect visceral obesity. Different BS indexes complement each other, and the synergistic effect reflects the overall body's metabolism. Jung Y pointed out that diabetes, hypertension, and hypercholesterolemia can be hypermetabolic. The metabolic syndrome may increase intraocular pressure (7) as the high blood pressure may lead to excessive aqueous humor production by facilitating more blood flow to the ciliary arteries (45). In addition, diabetes may also contribute to an increase in intraocular pressure by influencing the osmotic gradient, allowing more aqueous humor to enter the anterior chamber (46). Hyperglycemia leads to the dysfunctional trabecular meshwork, accumulation of fibronectin, disruption of water outflow facilities, changes in osmotic gradients associated with the dysregulated autonomic system, or microvascular damage to the optic nerve or peripheral retina (47). This effect might also arise due to the use of systemic drugs. However, if systemic drugs are associated with glaucoma risk, it might have implications for glaucoma prevention and treatment (31).

Few studies have explored the specific relationship between WC and glaucoma, with an Asian study of 5,255 participants from the KNHANES V database finding no relationship between WC and POAG (17), and another finding that higher WC was associated with high intraocular pressure (48). These studies have some limitations, in addition to the use of cross-sectional

studies so they can't speculate on causation, they also simply studied one type of POAG in glaucoma or only studied the direct relationship between high intraocular pressure and these data. We know that although intraocular pressure is the most important factor in glaucoma, the severity of it is not directly related to intraocular pressure. Therefore, we need to find other direct factors to explain the onset of glaucoma. Our study suggests there is the potential relationship between WC and glaucoma in the Western population, and it's worth noting that people in developed countries have a propensity toward more abdominal fat (49), but it may not apply to the Chinese and most of the Asian population. So we also need to expand the database in the future. Changes in BMI and WC often represent a relative increase in fat and a relative decrease in skeletal muscle content, and according to previous reports, oxidative stress can affect glaucoma optic neuropathy (50), so we can boldly assume that it may be due to changes in body composition that cause differences in the patient's oxidative stress response and thus lead to the occurrence of glaucoma. In the life cycle, BMI is believed to increase with age (51), based on this, we can also speculate that it may be due to the influence of age on systemic metabolism, which is the phenomenon of aging and thus to indirect glaucoma, so in the future, we can refine age and even gender to the group, and further study the degree of correlation between BC and glaucoma in these different groups.

The current study had some limitations, we did not conduct a classification analysis of glaucoma, and the correlation between BS and different types of glaucoma in different regions can be further clarified in future studies. Since causality may be related to race, MR studies are required in other ethnic groups.

Conclusion

To conclude, we performed a two-sample MR analysis. The size-related SNPs were used to study the effect of BS on the risk of glaucoma. Current research suggests increase of BMI and WC in BS might be risk factors of glaucoma. No causality was observed between WHRadjBMI and WHR and glaucoma risk. These novel findings can trigger glaucoma and can be utilized for the advancement and therapies for glaucoma in the future.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

JZ and XX: conceptualization and writing—review and editing. RY: investigation and writing—original draft. KL: methodology. FH and YC: resources and supervision. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the National Natural Science Foundation of China (No. 81600713) and Natural Science Foundation of Hunan Province (No. 2022JJ40855) to JZ and Natural Science Foundation General Program of Hunan Province (No. 2022JJ40830) and Natural Science Foundation General Program of Changsha City (No. kq2014290) to XX.

Acknowledgments

We want to acknowledge the participants and investigators of GIANT consortium and FinnGen study.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.999974/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Scatter plots for the genetic associations of BMI **(A)**, WHRadjBMI **(B)**, WHR **(C)**, and WC **(D)**.

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frontiers | Frontiers in Medicine

TYPE Original Research
PUBLISHED 29 September 2022
DOI 10.3389/fmed.2022.986176

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Ophthalmology, a section of the journal Frontiers in Medicine

RECEIVED 04 July 2022
ACCEPTED 09 September 2022
PUBLISHED 29 September 2022

CITATION
Li Z, Wang Q, Zhang S-F, Huang Y-F and Wang L-Q (2022) Timing of glaucoma treatment in patients with MICOF: A retrospective clinical study. *Front. Med.* 9:986176. doi: 10.3389/fmed.2022.986176

Timing of glaucoma treatment in patients with MICOF: A retrospective clinical study

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Purpose: To summarize and discuss the treatment and timing of glaucoma in patients with MICOF keratoprosthesis implantation to guide follow-up clinical treatment.

Methods: The data of 39 eyes (39 patients) with the Moscow Eye Microsurgery Complex in Russia (MICOF) keratoprosthesis implantation in our hospital from 1 January 2002 to 31 December 2017 were collected, including patients with preexisting glaucoma and those who developed glaucoma *de novo* after MICOF. The sex, age, preoperative diagnosis, glaucoma surgery, keratoplasty, times of keratoplasty, best corrected visual acuity (BCVA) and final follow-up corrected visual acuity, visual field (VF) defect, and cup-to-disk ratio (CDR) were statistically analyzed.

Results: Among 16 eyes with preexisting glaucoma, eight eyes underwent glaucoma surgery before MICOF, 4 eyes underwent glaucoma surgery combined with MICOF, and four eyes were managed medically. Among 23 eyes with *de novo* glaucoma, seven eyes were treated with surgery and 16 eyes were treated with medication only. A total of 9 (56.3%) eyes had corneal transplants with preexisting glaucoma, which was a higher percentage than that in the patients with *de novo* glaucoma ($n = 5$, 21.7%, $P = 0.043$). In both the preexisting glaucoma group and the *de novo* glaucoma group, the most common causes were alkali burns (56.3% of preexisting glaucoma and 43.5% of *de novo* glaucoma). There was no significant difference between the operation and initial visual acuity, postoperative visual acuity, BCVA, CDR, or VF defect. In the *de novo* glaucoma group, the final follow-up visual acuity of the glaucoma surgery group (1.56 ± 1.07) was worse than that of the mediation group (0.44 ± 0.53) ($P < 0.017$). Among the complications, the incidence of cornea melting in the patients treated with medications only (n=10) was significantly higher than that in the patients treated with glaucoma surgery ($n = 0$, $P = 0.007$), but there was no significant difference in the other complications.

Conclusion: Among patients with MICOF, those patients who have undergone keratoplasty are more likely to develop glaucoma before surgery and glaucoma needs to be prevented. Surgical treatment can be selected according to the ocular surface condition in the patients with *de novo* glaucoma to reduce the occurrence of complications.

KEYWORDS

MICOF, KPro, glaucoma, treatment, retrospective clinical study

Introduction

In some patients with severe corneal pathologies, such as chemical burns, autoimmune disease, Steven–Johnsons syndrome, and severe dry eye, amniotic membrane transplantation or corneal transplantation was mostly used in the past, but the curative effect was not ideal because of poor ocular surface conditions or inflammatory reactions caused by the sutures (1). Although it has been reported that the use of sutureless amniotic membrane transplantation improves the efficacy, large studies are still needed (2).

At present, keratoprostheses are developing rapidly, which provide more opportunities and choices for the abovementioned patients with severe disease to have their eyesight restored. There are three common types of keratoprosthesis: the Boston keratoprosthesis (Boston KPro), osteo-odonto keratoprosthesis (OOKP), and MICOF keratoprosthesis (developed by the Moscow Eye Microsurgery Complex in Russia thus, called MICOF) (3, 4). OOKP uses mucous membranes and alveolar bone to fix the optical cylinder (5), and the scope of operation is relatively large. The Boston KPro surgery is less invasive, but requires a donor cornea for support. However, MICOF does not require a donor cornea, and the surgical invasiveness is mild (6). MICOF may have greater application prospects. The gratifying thing is that MICOF has been clinically tested in our hospital and has achieved good results.

However, patients with all the types of keratoprostheses are at risk of glaucoma affecting vision. Preoperative assessment of glaucoma is affected by the transparency of the cornea, and the state of the ocular surface after keratoprosthesis implantation makes intraocular pressure (IOP) monitoring extremely difficult. Glaucoma is a blinding disease with a high incidence, so it is important to monitor the patients' relevant examination data to assess glaucoma progression and determine the appropriate glaucoma timing (7, 8).

Currently, the most research on the treatment of keratoprosthesis glaucoma is with the use of Boston KPro. Because the structures of keratoprosthesis are different, the treatment of MICOF glaucoma cannot be simply applied, and there are few studies on MICOF glaucoma. Our study analyzes the impact of preoperative diagnosis, and glaucoma surgery on the prognosis of glaucoma by evaluating the clinical data related to preexisting and *de novo* glaucoma in patients with MICOF, to draw experience for clinical treatment.

Materials and methods

Patients

Thirty-nine eyes of 39 patients were included in this retrospective study. The data of inpatients with MICOF in the Chinese PLA General Hospital from 1 January 2002 to 31 December 2017 were collected. All the patients underwent MICOF implantation, a two-stage surgery, which includes inserting a titanium frame and screwing a polymethyl methacrylate (PMMA) optical cylinder. All the surgeries were performed by two experienced surgeons. When there were no complications, the patients had the same medication regimen after the operation. All the patients had glaucoma. The studies involving human participants were reviewed and approved by. The study adhered to the tenets of the Declaration of Helsinki and was approved by the Chinese PLA General Hospital.

Definitions and data collection

For statistical research, the glaucoma patients with MICOF were divided into two groups: preexisting glaucoma and *de novo* glaucoma. The diagnosis of preexisting glaucoma was based on IOP and a history of glaucoma before MICOF. *De novo* glaucoma was diagnosed by an experienced physician according to IOP, VF defect, and CDR after MICOF.

The sex, age, preoperative diagnosis, glaucoma, glaucoma surgery, keratoplasty, times of keratoplasty, preoperative BCVA, postoperative BCVA and final follow-up corrected visual acuity, VF progress, and CDR were statistically analyzed.

Intraocular pressure can be measured by iCare before MICOF. Due to the existence of an optical cylinder, the IOP can only be estimated by experienced physicians pressing the eyeball with their fingers, which was objective. All the patients had poor visual acuity before MICOF and could not be examined by visual field examination. If the postoperative BCVA was higher than 1.0 logarithm of the minimum angle of resolution (logMAR), it was feasible to detect VF. Significant widening of the VF defect or a decrease in the visual field index (VFI) was defined as VF progression. Due to opaque optical media presence in those patients before MICOF, optical coherence tomography (OCT) examination of CDR could not be performed, so only the postoperative OCT data were counted.

According to the data, the preoperative diagnoses included acid burn, alkali burn, thermal burn, explosion injury, autoimmune disease, glaucoma, and keratoconus. Glaucoma surgery is divided into: cyclocryotherapy, endoscopic cyclophotocoagulation, transscleral cyclophotocoagulation, trabeculectomy, and glaucoma drainage device implantation. According to the literature, BCVA was converted into logMAR: 0.1 = 1.0 logMAR, no light perception = 3.0 logMAR, light perception = 2.3 logMAR, hand motion = 2.0 logMAR, and counting fingers = 1.7 logMAR (9).

Statistical analysis

Data were analyzed using the SPSS software version 22.0. The *t*-test or the Mann–Whitney *U*-test was used to compare

the differences in continuous variables between the two groups, and Pearson's chi-square tests or Fisher's exact tests were used to evaluate the differences in the classified variables between the groups. GraphPad Prism version 8 was used to analyze the statistical results.

Results

General data of patients

The data showed that 39 eyes (39 patients) had glaucoma, including 33 eyes (84.6%) of males and six eyes (15.4%). of females. The average age of the patients was 48.21 ± 13.33 years old. The follow-up time was 69.38 ± 50.22 months, and the median follow-up time was 62 months.

Comparison of related data between preexisting glaucoma and *de novo* glaucoma

Table 1 shows the comparison of sex, eye type, age, preoperative diagnosis, keratoplasty, and postoperative complications between the preexisting glaucoma ($n = 16$) and *de novo* glaucoma after MICOF ($n = 23$) (Table 1). The related data of the patients in the two groups were similar. The most common preoperative diagnosis was alkali burns (56.3% of preexisting glaucoma and 43.5% of *de novo* glaucoma). The proportion of the eyes with preexisting glaucoma treated with keratoplasty (56.3%) was higher than that of the eye with *de novo* glaucoma (21.7%, $P < 0.05$).

Comparison of glaucoma surgery

According to the data, the patients were divided into the preexisting glaucoma group and the *de novo* glaucoma group. Then, the patients with preexisting glaucoma were divided into the preoperative glaucoma operation group ($n = 8$), the intraoperative glaucoma surgery group ($n = 4$), and the medication group ($n = 4$). The patients with *de novo* glaucoma were divided into the glaucoma surgery group ($n = 7$) and the medication group ($n = 16$). Among the preexisting glaucoma group, eight eyes underwent glaucoma surgery before the operation, including cyclocryotherapy ($n = 3$, 37.5%), endoscopic cyclophotocoagulation ($n = 1$, 12.5%), transscleral cyclophotocoagulation ($n = 1$, 12.5%), trabeculectomy ($n = 1$, 12.5%), glaucoma drainage device implantation ($n = 1$, 12.5%), and trabeculectomy combined with glaucoma drainage device implantation ($n = 1$, 12.5%). A total of 4 eyes underwent glaucoma surgery during MICOF surgery, cyclocryotherapy in 2 eyes (50%), endoscopic cyclophotocoagulation in 1 eye (25%), and cyclocryotherapy combined with glaucoma drainage device implantation in 1 eye (25%). A total of four eyes were treated with medication only. Among the patients with *de novo* glaucoma, seven eyes underwent glaucoma surgery, including endoscopic cyclophotocoagulation (n=5, 71.4%), transscleral cyclophotocoagulation ($n = 1$, 14.2%), and cyclocryotherapy combined with glaucoma drainage device implantation ($n = 1$, 14.2%). A total of 16 eyes were treated with medication only (Table 2).

Comparison of the glaucoma surgery group and the medication group

The study compared the effects of glaucoma surgery and medication on visual acuity (initial BCVA, postoperative BCVA, best BCVA, and final BCVA), CDR (per year), visual field loss, and complications. There was little difference in the initial BCVA, postoperative BCVA, and best BCVA, but in the *de novo* glaucoma group, the final BCVA in the glaucoma surgery group (1.56 ± 1.07) was worse than that in the medication group (0.44 ± 0.53) ($P = 0.017$). There was no significant difference in CDR or visual field loss. Regarding complications, the incidence of cornea melting in the eyes with *de novo* glaucoma treated with medication only ($n = 10$) was significantly higher than that in the eyes with glaucoma surgery ($n = 0$) ($P = 0.007$). There was no significant difference in the incidence of other complications (Table 3).

Effect of glaucoma surgery on visual field loss

The Kaplan–Meier survival curve was used to analyze the loss of the VF in the glaucoma surgery pre, during, or after MICOF, and the medication only group (Figure 1).

Discussion

In recent years, the research and clinical application of keratoprosthesis have become increasingly widespread, which brings hope for the recovery of vision for patients with severe ocular surface diseases. However, glaucoma, like an invisible killer, quietly affects the vision of patients. Studies have shown that before keratoprosthesis implantation, the prevalence of glaucoma is 36–76%, and 8–75% of eyes develop new glaucoma after surgery (10, 11). In the patients with severe ocular surface diseases, such as chemical burns, autoimmune keratopathy, severe dry eyes, and repeated keratoplasty failures, the transparency of the corneal conjunctiva is reduced, which makes the evaluation of glaucoma difficult. After keratoprosthesis in these patients, IOP monitoring becomes very difficult because of

TABLE 1 Comparison of related data between preexisting glaucoma and *de novo* glaucoma.

	Preexisting glaucoma	*De novo* glaucoma	*P*-value
	***n* = 16**	***n* = 23**	
Male gender	14 (87.5%)	19 (82.6%)	1
Right operated eye	8 (50%)	7 (30.4%)	0.182
Age, years	45.75 ± 12.13	49.91 ± 14.12	0.344
Preoperative diagnosis			
Alkali burn	9 (56.3%)	10 (43.5%)	0.523
Acid burn	2 (12.5%)	2 (8.7%)	1
Thermal burn	1 (6.3%)	4 (17.4%)	0.631
Explosion burn	1 (6.3%)	3 (13.0%)	0.638
Autoimmune disease	1 (6.3%)	4 (17.4%)	0.631
Glaucoma	1 (6.3%)	0	0.41
Keratoconus	1 (6.3%)	0	0.41
Keratoplasty	9 (56.3%)	5 (21.7%)	***0.043***
Number of keratoplasty	0.81 ± 0.911	0.45 ± 0.963	0.255
Initial BCVA	2.23 ± 0.13	2.14 ± 0.18	0.139
Postoperative BCVA	0.76 ± 0.61	0.63 ± 0.52	0.412
Best BCVA	0.43 ± 0.58	0.17 ± 0.20	0.107
Final BCVA	1.03 ± 1.00	0.78 ± 0.88	0.374
Complications			
Cornea melting	2	10	0.076
Overgrowth of the surface mucosa	3	5	1
Retroprosthetic membrane	1	1	1
Infective endophthalmitis	2	2	1
Aseptic endophthalmitis	1	1	1
Macular edema	0	3	0.255
Retinal detachment	0	1	1

The meaning of the bold + italic values is that the difference is statistically significant.

the uniqueness of the optical cylinder. Therefore, the prevention and treatment of glaucoma are particularly important to maintain the vision of patients with keratoprosthesis. According to our previous study (12), 17 of 91 patients with MICOF had preexisting glaucoma. Among them, 7 patients developed glaucoma after MICOF, and 16 patients had *de novo* glaucoma after MICOF, which threatened their vision. However, most of the studies are Boston KPro currently. There are few studies on glaucoma in patients with MICOF. Due to the different types and structures of corneal prostheses, the results of other types of KPro cannot be simply applied to patients with MICOF. Therefore, this study used the statistics of patients with MICOF, to explore the timing of glaucoma treatment.

In this study, alkali burns were the most common preoperative diagnosis of glaucoma. Chemical burns, especially alkali burns, are well indications for keratoprosthesis. After keratoprosthesis implantation, patients with alkali burns can obtain better vision in a short time, but the maintenance of vision will be a challenge. Because of its strong penetration ability, alkaline substances can not only damage the ocular surface, but also cause the destruction of intraocular structure and inflammation, which increase the incidence of glaucoma, even if the retina and optic nerve look normal temporarily. However, the retina ganglion cell layer may have been destroyed (13, 14), making it extremely sensitive to IOP, and the loss of vision can be caused without excessive IOP (15). In addition, this study found that the proportion of patients with preexisting glaucoma with previous keratoplasty was higher than that in the patients with *de novo* glaucoma after MICOF, and the difference was statistically significant. Abnormalities in the intraocular structure may be aggravated during keratoplasty (16), and the use of dexamethasone after keratoplasty is also a risk factor for glaucoma (17). Therefore, attention should be given to the evaluation of glaucoma in patients with alkali burns and previous keratoplasty.

To accurately evaluate the treatment and timing of glaucoma, the patients with preexisting glaucoma were divided into three groups: the glaucoma surgery pre-MICOF group, the glaucoma surgery during MICOF group, and the medication only group. The patients with *de novo* glaucoma were divided into the glaucoma surgery after MICOF group and the medication only group. In this study, the patients with

TABLE 2 Treatment of the preexisting glaucoma and *de novo* glaucoma.

	Preexisting glaucoma ($n = 16$)			*De novo* glaucoma ($n = 23$)	
	Glaucoma surgery pre-MICOF ($n = 8$)	Glaucoma surgery during MICOF ($n = 4$)	Medication only ($n = 4$)	Glaucoma surgery after MICOF ($n = 7$)	Medication only ($n = 16$)
Glaucoma surgery					
Cyclocryotherapy	3 (37.5%)	2 (50%)			
Endoscopic cyclophotocoagulation	1 (12.5%)	1 (25%)		5 (71.4%)	
Transscleral cyclophotocoagulation	1 (12.5%)			1 (14.2%)	
Trabeculectomy	1 (12.5%)				
Glaucoma drainage devices implantation	1 (12.5%)				
Trabeculectomy combined with glaucoma Drainage devices implantation	1 (12.5%)				
Cyclocryotherapy combined with glaucoma drainage devices implantation	1 (25%)		1 (14.2%)		

preexisting glaucoma were not treated with glaucoma surgery after MICOF. Among the patients with preexisting glaucoma, there was no significant difference in the postoperative BCVA, best BCVA, or final BCVA between the operation group and the medication group, which may indicate that there was previous nerve loss in the patients with glaucoma. As long as IOP can be maintained, surgery has no effect on glaucoma. In the patients with *de novo* glaucoma, the final BCVA in the surgery group was worse than that in the medication group, which may be because the degree of glaucoma in the patients requiring surgical treatment was already more severe than that in the patients treated with drugs. In the study of Boston KPro by Dominique (18), it was found that the eyes with glaucoma surgery after KPro progressed faster than the eyes with glaucoma surgery pre-KPro or medication in the patients with preexisting glaucoma, and the complications did not increase. Therefore, it is recommended to combine glaucoma drainage device implantation during KPro. In this study, regardless of preexisting or *de novo* glaucoma, ciliary body destruction was often used because the eye surface state of patients with MICOF is generally very poor, and the conjunctival condition is not good. Shunt surgery may not have a good effect (19).

Because of the difficulty of IOP measurement in patients with keratoprosthesis, it can only be measured artificially, and there is certain subjectivity. When the refractive stroma is transparent, glaucoma progression can be evaluated by optic disk OCT or visual field progression monitoring. In this study, there was no significant difference in the change in CDR or the progression of the visual field between the preexisting glaucoma group and the *de novo* glaucoma group, the glaucoma surgery group and the medication group. This is consistent with the study of Boston KPro by Dominique (18). Silva (20) found that the narrowing of the temporal chamber angle has diagnostic significance for the progression of Boston KPro glaucoma through anterior segment OCT, but this is based on the premise that the chamber angle imaging is clear.

Regarding complications, the incidence of cornea melting in the medication treatment group was significantly higher than that in the glaucoma surgery group. In terms of the process of operation, the aim for the implantation of MICOF is to make a lamellar cornea pocket to place the titanium frame, and a PMMA optical cylinder was screwed into the frame, the donor cornea which is thin compared to the cornea covered by Boston KPro (21). When the IOP is high, the autologous tissue nutrition is poor, and the pressure of the optical cylinder and frame on the outer corneal tissue increases, which increases the incidence of cornea melting. In addition, persistent inflammation of the ocular surface can also lead to cornea melting (22). Although the follow-up visual acuity in the glaucoma surgery group was worse than that in the medication

TABLE 3 Comparison of the glaucoma surgery group and the medication group.

	Preexisting glaucoma			*De novo* glaucoma		
	Glaucoma surgery pre or during MICOF ($n = 12$)	Medication only ($n = 4$)	*P*-value	Glaucoma surgery after MICOF ($n = 7$)	Medication only ($n = 16$)	*P*-value
Initial BCVA	2.23 ± 0.14	2.23 ± 0.15	1	2.13 ± 0.24	2.15 ± 0.15	1
Postoperative BCVA	0.85 ± 0.66	0.50 ± 0.43	0.299	0.53 ± 0.45	0.67 ± 0.55	0.437
Best BCVA	0.49 ± 0.64	0.25 ± 0.38	0.389	0.27 ± 0.23	0.13 ± 0.17	0.1
Final BCVA	1.09 ± 0.87	0.83 ± 1.46	0.161	1.56 ± 1.07	0.44 ± 0.53	***0.017***
CDR change/year	0.119 ± 0.103	0.069 ± 0.089	0.18	0.150 ± 0.135	0.096 ± 0.111	0.151
Visual field loss	4 (33.3%)	1 (25%)	1	5 (71.4%)	4 (25%)	0.066
Complications						
Cornea melting	1	1	0.45	0	10	***0.007***
Overgrowth of the surface mucosa	3	0	0.529	2	3	0.621
Retroprosthetic membrane	0	1	0.25	0	1	1
Infective endophthalmitis	1	1	0.45	0	2	1
Aseptic endophthalmitis	1	0	1	0	1	1
Macular edema	0	0		1	2	1
Retinal detachment	0	0		1	0	0.304

The meaning of the bold + italic values is that the difference is statistically significant.

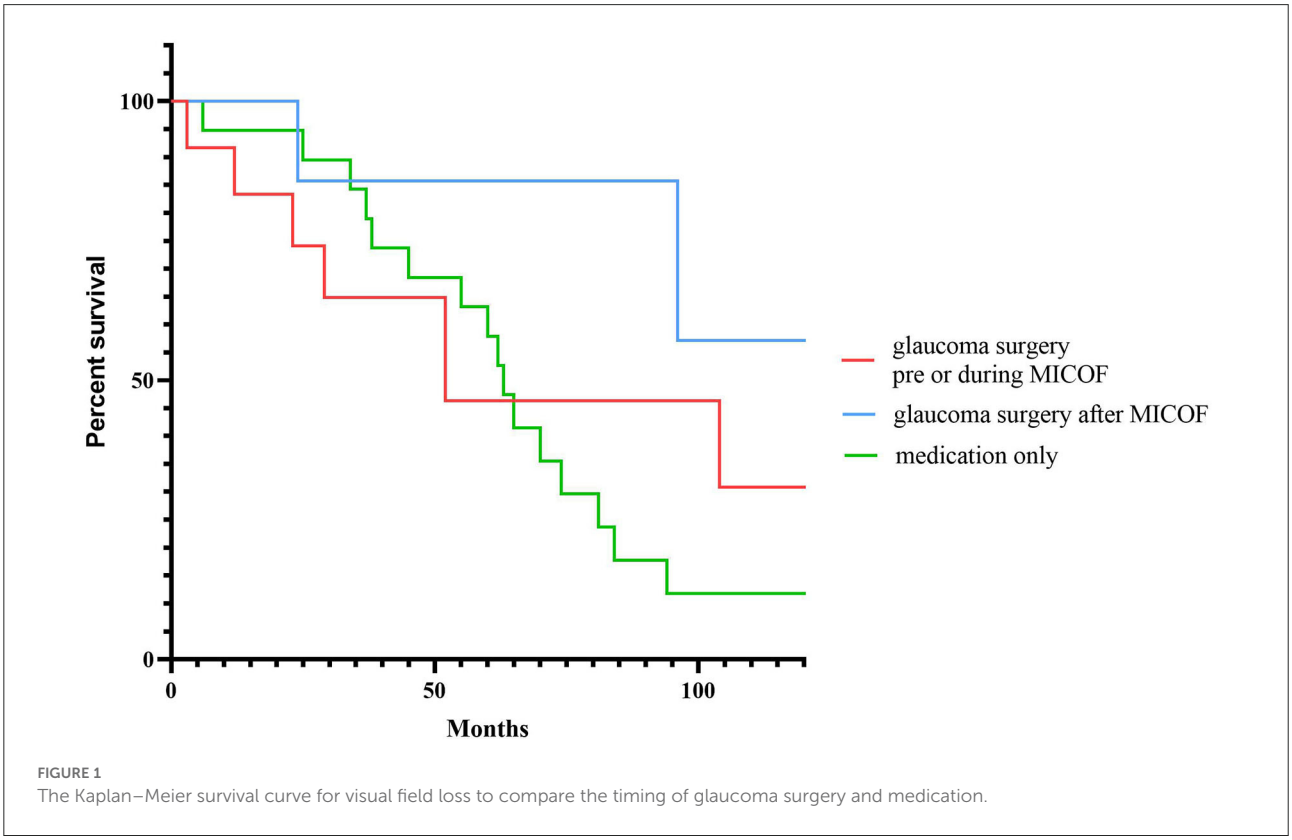

FIGURE 1
The Kaplan–Meier survival curve for visual field loss to compare the timing of glaucoma surgery and medication.

group, it was observed, by generating the Kaplan–Meier survival curve, that the visual field of early drug treatment and glaucoma surgery after MICOF was slow, according to whether the VF progressed. However, in the long run, there was persistent visual field loss in the medication only group, and the drugs that are ocularly applied may not be absorbed well due to ocular surface scarring.

Conclusion

According to our study, patients with MICOF who have undergone keratoplasty are more likely to develop glaucoma, and the focus should be on the prevention and treatment. *De novo* glaucoma can be treated by surgery according to the ocular surface condition to reduce the incidence of complications. However, the number of patients included in this study was small, and more clinical data need to be collected for research.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

Ethics statement

The study adhered to the tenets of the Declaration of Helsinki and was approved by the Chinese PLA General Hospital, informed consent was obtained from all subjects and/or their legal guardian(s). The patients/participants provided their written informed consent to participate in this study.

Author contributions

ZL: collected data, analyzed data, and wrote articles. QW: critical correction of the manuscript. S-FZ: collected data. Y-FH and L-QW: critical revision and approval of the article. All authors contributed to the article and approved the submitted version.

Funding

This study has been funded by the Fund Project of China Joint Logistic Support Force (LB20201A010024).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers | Frontiers in Medicine

TYPE Original Research
PUBLISHED 06 October 2022
DOI 10.3389/fmed.2022.1013260

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Ophthalmology, a section of the journal Frontiers in Medicine

RECEIVED 06 August 2022
ACCEPTED 20 September 2022
PUBLISHED 06 October 2022

CITATION
Chu Y-C, Chang P-Y, Wang J-K, Huang T-L and Hsu Y-R (2022) The IOP lowering effects of "planning" selective laser trabeculoplasty in open angle glaucoma. *Front. Med.* 9:1013260. doi: 10.3389/fmed.2022.1013260

The IOP lowering effects of "planning" selective laser trabeculoplasty in open angle glaucoma

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Purpose: To investigate whether the planning of selective laser trabeculoplasty (SLT) influences the intraocular pressure (IOP) in patients with open angle glaucoma (OAG).

Methods: In this retrospective case-control study conducted on patients with OAG who planned to undergo SLT treatment (SLT group) or a visual field examination (VF group), we collected the demographic data, IOP on the planning day and on the scheduled day of the SLT treatment or VF examination. ΔIOP was defined as the IOP change between the planning day and the scheduled day. We used multivariable regression analyses and linear mixed model to evaluate the association between the abovementioned factors and ΔIOP in the VF group and the treatment eye (SLT_t) and fellow eye (SLT_f) of the SLT group.

Results: One hundred and fifty-three eyes of 102 patients with OAG were included, of which 51 patients in the SLT group and 51 patients in the VF group. The ΔIOP was -1.92 ± 2.77 mmHg in the SLT_t, -0.65 ± 2.47 mmHg in the SLT_f and -0.08 ± 1.73 mmHg in the VF group ($P < 0.05$). Both multivariable regression analysis between the VF and SLT_t group and linear mixed model in the SLT group showed significant negative association between the ΔIOP and SLT arrangement ($P < 0.05$). There was no significant association between ΔIOP and age, gender, baseline IOP, IOP fluctuation, nor SE.

Conclusions: The IOP was significantly reduced in patients with OAG after "planning" of SLT treatment, even without actual performing the laser treatment in our retrospective case-control study.

KEYWORDS

selective laser trabeculoplasty, intraocular pressure, IOP fluctuation, adherence, open angle glaucoma

Background

Intraocular pressure (IOP), a major risk factor for glaucoma progression, was well known that it was not fixed but a fluctuated value over time. The IOP fluctuation could be categorized according to the period 1. Studies on IOP fluctuation defined the IOP variation occurring within a 24-h period as diurnal or short-term IOP fluctuation (1–5). Short-term IOP fluctuation also referred to IOP variation that occurred within a day or over days to weeks (1–5) while long-term was that through months to years (1, 5–7).

Selective laser trabeculoplasty (SLT) has selective effect on melanotic elements within the trabecular meshwork, facilitating flow into Schlemm's canal and then subsequent reduction in IOP (8). The IOP lowering effect of SLT was about 11–40% reduction in OAG (9, 10) and angle closure glaucoma (ACG) (11–14). In eyes with OAG or ACG, in terms of IOP control, primary treatment with SLT was comparable or even better than traditional treatment with topical prostaglandin analog medications (15, 16). Most studies regarding the SLT treatment were focused on the effect on IOP reduction, but there were limited studies designed to find the association of IOP fluctuation and the arrangement of SLT.

The IOP fluctuation presented higher in patients with glaucoma and abundant studies reported that IOP fluctuation may be related to the risk of glaucoma development and progression (6, 7, 17). The Los Angeles Eye Study revealed that IOP fluctuations were associated with OAG risk when IOP<15 mmHg (7). Asrani et al. reported that diurnal IOP fluctuation in well controlled glaucoma patients was a risk factor for disease progression (18). A *post-hoc* study conducted by Nouri using the patients in the Advanced Glaucoma Intervention study (AGIS) showed that IOP fluctuation between visits was an independent risk factor for glaucoma progression (19).

Numerous studies aimed to evaluate the factors associated with IOP fluctuation, such as baseline IOP (20), blood pressure (21), medications use (22), postural change (23), or exercise (24). Few studies were designed to evaluate the association of IOP fluctuation with the arrangement of an examination or intervention. Therefore, we performed a retrospective study to investigate whether the planning of selective laser trabeculoplasty influences the IOP in OAG patients.

Materials and methods

The protocol of the study, which followed the principles of the Declaration of Helsinki, was approved by the Institutional Review Board of Far Eastern Memorial Hospital (FEMH) in Taiwan (109108-E).

The SLT group included the patients with OAG who were scheduled to receive SLT treatment between January 2018 and March 2019. Bilateral eyes of the patients in the SLT group were divided into two groups, the treatment eye (SLT_t) and the fellow eye (SLT_f). The treatment eye was the eye scheduled to receive the SLT treatment. If the patient received SLT treatment of both eyes during this period, only the first eye received SLT treatment was included as the treatment eye. The VF group included the patients who visited the glaucoma clinic in FEMH between January and Dec 2019, and the worse eye was chosen for the statistical analysis. Patients were 20 years or older, visual acuity of 20/40 or better, and no previous intraocular surgery, except uncomplicated cataract surgery 6 months before entering the trial.

The exclusion criteria were eyes with ocular trauma, macular disease, or other optic nerve disease; those whose corneal pathology or anterior chamber pathology which obscured gonioscopic view to the angle and fundoscopic exam. Additionally, patients who used steroid eye drops within 3 months of the SLT treatment or VF examination were also excluded.

Age, gender, mean deviation (MD) of the visual field examination, central corneal thickness (CCT), spherical equivalent (SE), IOP, and the types and bottles of anti-glaucoma medications were collected in both groups. Noncontact pneumotonometry (Tonopachy^{TM} NT-530P, NIDEK, Japan) was used for IOP measurement. We collected the IOP on the scheduled day of the SLT treatment or VF examination on the planning day and the two previous visits. Baseline IOP and IOP fluctuation was defined as the mean and standard deviation of the three IOP measurements before the scheduled day. The change of IOP (ΔIOP) was defined as the IOP of the scheduled day minus the IOP of the planning day.

Statistical analysis

Statistical analysis was performed using SPSS ver. 22.0 (SPSS Inc., Chicago, IL, USA). To evaluate the difference between the SLT group and the VF group, we compared the treatment eyes of the SLT group (SLT_t) and the VF group. Student *t*-test and the Mann-Whitney test were used to compare the differences of the following continuous variables between the two groups: age, CCT, SE, MD, baseline IOP, IOP fluctuation, and ΔIOP based on the distribution of the variables. Simple linear regression analysis was used to evaluate the association between the above factors and ΔIOP. If the factor had significant correlation with ΔIOP or significant difference between two groups, multivariable linear regression would be performed for further analysis. $P < 0.05$ was considered statistically significant.

To evaluate the difference between each eye in the SLT group, paired *t*-test and Wilcoxon sign-rank test were performed to compare the differences between the treatment eye and the fellow eyes in the SLT group. Linear mixed model was used to compare IOP changes in the SLT group between both eyes.

Results

One hundred and fifty-three eyes of 102 patients with OAG were included in this study, of which 51 people were in the SLT group and 51 people were in the VF group.

The mean age of the patients was 56.21 ± 14.05 in the SLT group and 45.08 ± 12.40 the VF group ($P < 0.05$). The visual field defect was significantly worse in the treatment eye of the SLT group (SLT_t) than the VF group (−13.68 ± 8.94 dB and −4.78 ± 6.15 dB, $P < 0.05$).

In the SLT_t group, the IOP on the planning day was 15.88 ± 3.05 mmHg and the IOP on the scheduled day was 17.80 ± 2.56 mmHg. In the VF group, the IOP on the planning day was 16.68 ± 3.08 mmHg and the IOP on the scheduled day was 16.76 ± 3.31 mmHg. The baseline IOP was 17.27 ± 3.21 mmHg in the SLT_t and 16.98 ± 3.09 mmHg in the VF group. IOP fluctuation was 2.53 ± 1.79 mmHg in the SLT_t and 1.61 ± 1.12 mmHg in the VF group ($P < 0.001$). The ΔIOP was −1.92 ± 2.77 mmHg in the SLT_t group and −0.08 ± 1.73 mmHg in the VF group ($P < 0.001$). The patients in the SLT group received significantly more amounts of glaucoma medications (1.86 ± 0.60 mmHg in the SLT_t, and 1.31 ± 0.55 mmHg in the VF group, $P < 0.001$), more carbonic anhydrase inhibitors and prostaglandin analogs for treatments (Table 1).

Univariable analysis through simple linear regression model revealed that the following factors had significant association with ΔIOP: SE ($b = -0.293$, $P = 0.003$), IOP fluctuation ($b = -0.387$, $P < 0.001$), baseline IOP ($b = -0.221$, $P = 0.026$), SLT arrangement ($b = -0.373$, $P < 0.001$). Multivariable linear regression model showed only SLT arrangement ($b = -0.268$, $P = 0.009$) has significant negative association with ΔIOP between the scheduled day and the planning day. There was no significant association between ΔIOP and age, SE, baseline IOP, nor IOP fluctuation was found through multivariable analysis (Table 2).

Within the two eyes of the patient receiving SLT, the visual field test revealed significantly worse VF in the treatment eye than the fellow eye (MD = −13.68 ± 8.94 dB and −7.84 ± 7.88, $P < 0.001$). The baseline IOP and IOP fluctuation was 15.59 ± 2.45 and 1.83 ± 0.94 in the SLT_f group, which was significantly lower than those of the SLT_t group ($p < 0.05$). The ΔIOP in the SLT_f, was significantly different from that of SLT_t (−0.65 ± 2.47 mmHg and −1.92 ± 2.77 mmHg, $p < 0.001$). Linear mixed models with age, gender, baseline IOP, IOP fluctuation, MD and SLT arrangement revealed that SLT arrangement had significant negative influence on ΔIOP in the SLT group (Table 3).

Discussion

We compared the IOP change between the SLT_t group and the VF group to find the difference between the subjects who received SLT treatment and who did not. The both eyes of the SLT group (SLT_t and SLT_f) were compared to search for the influence of planning SLT on IOP change. We found that the IOP was significantly reduced after planning of the SLT treatment. To the best of our knowledge, the current study was the first study that investigated the influence of planning SLT on IOP change in patients with OAG.

In the SLT_t group and VF group, the IOP change from the planning day to the scheduled day of intervention (ΔIOP) had significant association with age, SE, IOP fluctuation, baseline IOP and arrangement of SLT using univariable simple linear regression analysis. Multivariable linear regression model showed that only the arrangement of SLT had significant negative association with ΔIOP, which meant the IOP was significantly reduced in patients with OAG after "planning" of SLT treatment, even without actual performing the laser treatment. We assumed that compliance or adherence issues might be the most possible reason to account for this finding. Poor adherence to antiglaucoma medication has been reported to be related to higher IOP and IOP fluctuation (25–27). A randomized control study investigating refill adherence showed that newly prescribed medications in OAG patients with good adherence had less IOP fluctuation in 24 months follow-up (25). A cross-sectional study using Morisky medication adherence scale revealed a trend of increase in IOP with increase in the score of nonadherences (26). In a study evaluating the reasons for medication prescription, the physicians commented that persistent IOP elevation was often a sign of nonadherence (27). Nonadherence has been reported to be related to patients with multiple medications. Adherence reported by patient interviewing and chart review decreased from 81 to 50% with the amounts of medication increased (28). In a large retrospective study including more than 37,000 glaucoma patients, the persistence of medication through 1 year was decreased from the one-bottle group (35.3%) to the three-bottle group (23.9%) (29). In our study, the average amount of anti-glaucoma agents was 1.78 bottles in the SLT group, which was significant more than 1.31 bottles in the VF group. Because of more IOP lowering bottles, the adherence of medication may be less in the SLT group than in the VF group. We speculated that the arrangement of SLT, which is an invasive intervention for IOP control clearly delivered the message of progression of disease and poor control of the IOP to the patients. Therefore, the arrangement of SLT may increase the patients' adherence for antiglaucoma medication compared to those who were only arranged for a routine follow-up examination like VF examination.

Nakakura conducted a prospective study using Goldmann applanation tonometry revealed that the office IOP fluctuation in 6 months was 2.75 ± 1.68 mmHg in POAG patients using three kinds of antiglaucoma agents (30). Tojo et al. using Triggerfish® contact lens sensor measured the 24-h IOP fluctuation and found positive correlation of short term IOP fluctuation (24-h fluctuation) and long term IOP fluctuation (5). It was believed that IOP fluctuation had significant correlation

TABLE 1 Patients' characteristics in the SLT and VF group.

	SLT group ($n = 102$)			VF group ($n = 51$)	P_2
	Treatment eye ($n = 51$)	Fellow eye ($n = 51$)	P_1		
Age (years)	56.21 ± 14.05			45.08 ± 12.40	<0.001
Gender (male, %)	37 (72.5%)			30 (58.8%)	0.144
SE (D)	−4.66 ± 3.98	−4.03 ± 5.76	0.31	−5.94 ± 3.13	0.08
CCT (mm)	552.47 ± 30.08	551.88 ± 28.98	0.74	561.49 ± 37.05	0.18
MD (dB)	−13.68 ± 8.94	−7.84 ± 7.88*	<0.001	−4.78 ± 6.15*	<0.001
Baseline IOP (mmHg)	17.27 ± 3.21	15.59 ± 2.45*	<0.001	16.98 ± 3.09	0.64
IOP fluctuation (mmHg)	2.53 ± 1.79	1.83 ± 0.94*	<0.001	1.61 ± 1.12*	<0.001
ΔIOP (mmHg)	−1.92 ± 2.77	−0.65 ± 2.47*	<0.001	−0.08 ± 1.73*	< 0.001
Average medication bottle amount	1.86 ± 0.60	1.67 ± 0.74	0.171	1.31 ± 0.55*	<0.001
Medication type					
α-agonist (*n*, %)	19 (37.3)	15 (29.4)	0.264	13 (27.7)	0.143
β-blocker (*n*, %)	43 (84.3)	38 (74.5)	0.164	34 (66.7)	0.038
CAI (*n*, %)	28 (54.9)	23 (45.1)	0.214	13 (25.5)	0.002
PG (*n*, %)	39 (76.5)	36 (70.6)	0.327	26 (51.0)	0.01

CAI, carbonic anhydrase inhibitor; CCT, central corneal thickness; MD, mean deviation; IOP, intraocular pressure; ΔIOP, IOP changes; PG, prostaglandin analogs; SE, Spherical equivalent; SLT, Selective laser trabeculoplasty; VF, Visual Field test.
P_1 = *P* value between treatment eye and fellow eye of the SLT group.
P_2 = *P* value between treatment eye of the SLT group and the VF group.
*$P < 0.05$ significance.

TABLE 2 Correlation between selected variables and ΔIOP (In SLT vs. VF group).

	Univariable linear regression analysis		Multivariable linear regression analysis	
	Coefficient (β)	*P*-value	Coefficient (β)	*P*-value
Age	−0.278	0.005*	−0.029	0.818
Gender	0.042	0.676		
SE	−0.293	0.003*	−0.193	0.095
CCT	0.109	0.273		
MD	−0.112	0.26		
SLT	−0.373*	<0.001	−0.268	0.009*
Baseline IOP	−0.221*	0.026	−0.162	0.121
IOP fluctuation	−0.387*	<0.001	−0.186	0.090
Med bottles	−0.049	0.625		

CCT, central corneal thickness; IOP, intraocular pressure; ΔIOP, IOP changes; MD, mean deviation; Med bottle, the total bottles of glaucoma medications; SE, Spherical equivalent; SLT, Selective laser trabeculoplasty; V, Visual Field test.
*$P < 0.05$ significance.

with mean IOP (6, 20, 28, 29), which means patients with higher baseline IOP usually had larger IOP fluctuation. In our study, we also found significant positive correlation between IOP fluctuation and the mean IOP of the three visits before the scheduled SLT treatment. Comparing bilateral eyes of the patients in the SLT group, the ΔIOP were negative values which means the IOP decreased at the scheduled day from the planning day of SLT treatment in bilateral eyes. The value of ΔIOP was more in the SLT_t than in the SLT_f, and it may be related to the higher baseline IOP in the SLT_t group. On the other hand, the mean IOP and IOP fluctuation had significant correlation with the ΔIOP in univariable analysis. However, no significant correlation was found when the mean IOP and IOP fluctuation were put simultaneously in the multivariable analysis. It may be caused by the confounding effect of the significant positive correlation between baseline IOP and IOP fluctuation.

The average age of patients in the current study was 45.08 years old in the VF group and 56.21 years old in the SLT group. Numeral studies investigated the relation between age

TABLE 3 The influence of planning SLT treatment on ΔIOP in the SLT group.

Covariate	ΔIOP		
	Coefficient (β)	95% CI	*p*-value
Age	−0.05	−0.10, 0.01	0.08
Male gender	−0.19	−1.38, 1.30	0.8
Baseline IOP	−0.15	−0.37, 0.07	0.18
IOP fluctuation	−0.22	−0.64, 0.20	0.30
MD	−0.02	−0.09, 0.04	0.51
SLT	−0.98	−1.83, −0.13	0.02*

MD, mean deviation; IOP, intraocular pressure; ΔIOP, IOP changes from the planning day to the scheduled day of SLT; SLT, Selective laser trabeculoplasty.
*$P < 0.05$ significance.

and adherence, but the results were controversial. In newly treated individuals with diagnosed OAG, greater adherence to medications was noted with increasing age (31). A retrospective chart review in the United Kingdom showed that glaucoma treatment adherence, which was defined as average difference in the actual number of prescriptions collected compared to 12 prescriptions required annually, improved with increasing age but may be related to the drop wastage in elderly patients (32). However, there was no significant correlations between age and adherence were found in a multicenter observational study in Korea (33) and in Taiwan (34). In our study, no significant correlation between the age and ΔIOP was noted in multivariable analysis. Although the mean age of the two groups were significant different in the current study, they were relatively younger and usually counted in the same age groups in other studies (32–34). Besides, the adherence of medication in age 40–49 was similar to that of age 50–59 in Nordstrom's study (31). There was no significant correlation in the age and ΔIOP may be related to the relatively younger age in our patients.

There were some limitations in our study. First, our study was a retrospective study, there was a significant difference in the age and glaucoma severity between the two groups. However, we found age and visual field defect had no significant correlation with the IOP change (ΔIOP). Second, the IOP had diurnal change which may influence the value of IOP change. In our study, the patients came to our clinics regularly in the morning or in the afternoon for IOP measurement, which may decrease the influence of the diurnal IOP fluctuation. Third, we did not directly evaluate the adherence of our patients due to our retrospective design. We could not provide direct evidence of the relationship of the IOP and the adherence.

In summary, we found that the arrangement of SLT had significant negative association with the change of the IOP in this retrospective case-control study. Additional prospective studies investigating whether the arrangement of intervention such as laser treatment or even filtering surgery influence the IOP and the adherence to glaucoma medications in the long-term follow-up are needed.

Value statement

What was known

Poor adherence to topical antiglaucoma medication was associated with higher IOP fluctuation.

What this study adds

The arrangement of selective laser trabeculoplasty (SLT) even without performing it lowered the IOP in the patients with open angle glaucoma (OAG).

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The protocol of the study was approved by the Institutional Review Board of Far Eastern Memorial Hospital (FEMH) in Taiwan (109108-E). Informed consent was waived by the IRB due to retrospective design of the study.

Author contributions

Y-CC and P-YC designed the study, collected the clinical data, performed the statistics, wrote the main manuscript text, and prepared tables. J-KW, T-LH, Y-RH, and P-YC reviewed, corrected, and approved the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This project was supported by a grant obtained from the Far Eastern Memorial Hospital (FEMH-2022-C-060).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers | Frontiers in Medicine

TYPE Original Research
PUBLISHED 13 October 2022
DOI 10.3389/fmed.2022.990611

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Ophthalmology, a section of the journal Frontiers in Medicine

RECEIVED 10 July 2022
ACCEPTED 08 September 2022
PUBLISHED 13 October 2022

CITATION
Yang J, Qu Y, Zhao J, Cong J, Sun Z, Du Y, Yang G, Ding D, Chen Y and Cheng G (2022) Stereoscopic vs. monoscopic photographs on optic disc evaluation and glaucoma diagnosis among general ophthalmologists: A cloud-based real-world multicenter study. *Front. Med.* 9:990611. doi: 10.3389/fmed.2022.990611

Stereoscopic vs. monoscopic photographs on optic disc evaluation and glaucoma diagnosis among general ophthalmologists: A cloud-based real-world multicenter study

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Purpose: To investigate whether stereoscopic vs. monoscopic viewing condition influences the evaluation of optic disc photographs for morphologic features and glaucoma likelihood in a general ophthalmologist population from multicenters on a cloud-based platform.

Methods: A cross-sectional study of 519 pairs of stereoscopic and monoscopic photographs of optic discs with adequate quality were selected and presented using a cloud-based platform. A total of 21 general ophthalmologists from 14 centers assessed 15 morphologic features based on 5R's rules and estimated glaucoma likelihood for each assigned photograph. There were 93 pairs of stereoscopic and monoscopic photographs evaluated by a panel of glaucoma specialists and set as ground truth. The main outcome measures were the agreement between estimates and ground truth and the inter-grader agreements.

Results: There were good agreements between ground truth and both monoscopic and stereoscopic estimates (stereo κ 0.532 and mono κ 0.494). There was also a substantial intra-grader agreement between monoscopic and stereoscopic evaluation of glaucoma likelihood (κ 0.636). In eyes with probable glaucoma, the accuracy of the stereo method was greater than that of the mono method (stereo 0.238 vs. mono 0.118) When compared with ground truth, stereoscopic photographs had a better agreement for disc size (stereo κ 0.447 vs. mono κ 0.183), disc color (stereo κ 0.612 vs. mono κ 0.549), neuroretinal rim shape (stereo κ 0.356 vs. mono κ 0.274) on the whole. The stereoscopic method also had a better inter-grade agreement for disc size,

disc color, neuroretinal rim shape, and glaucoma likelihood (stereo κ 0.402 vs. mono κ 0.359) on the whole.

Conclusions: In the evaluation of optic disc photographs for morphologic features and glaucoma likelihood, the stereoscopic method showed superiority compared to the monoscopic method for general ophthalmologists. The stereoscopic method is more likely to identify glaucomatous eyes which need medical intervention.

KEYWORDS

diagnosis, glaucoma, optic disc, photograph, stereoscopic

Introduction

Accurate and reproducible assessment of the optic disc and adjacent retinal structures using images of the optic disc has a key role in the evaluation of the condition of the optic disc and in the diagnosis and management of glaucoma (1–3). Taking photographs is currently the main method for optic disc documentation, and both monoscopic and stereoscopic methods have been widely used in clinical practice. Monoscopic optic disc photographs have relative advantages in convenience and cost, while stereoscopic ones provide more topographic information, which has been one of the gold standards for detecting glaucomatous optic disc (4, 5).

However, previous studies showed mixed results when comparing stereoscopic and monoscopic photographs in evaluating optic disc conditions, and the performance of the stereoscopic method has not been evaluated among general ophthalmologists. Several studies found that stereoscopic photographs had a similar inter-grader agreement among glaucoma specialists or experts to that of their monoscopic counterparts (6, 7), while some studies reported significant variability in non-stereo and stereo photographs among glaucoma specialists when evaluating optic disc (8–10). The differences between stereo and mono methods might come from the fact that stereoscopic photos provide a better understanding of the three-dimensional structure of the optic disc theoretically. In order to find a better method for accurate analysis of optic disc and optimum management of glaucomatous patients, a comparison between the stereoscopic method and its monoscopic counterpart is necessary.

For a good consistency of evaluations for the optic disc photographs, a standardized method to obtain stereoscopic images is necessary when comparing various photographic methods as well as taking photos in clinical practice. However, previous methodological studies mostly used sequential stereoscopic images (6–10), rather than simultaneous stereoscopic images, of which the sequential technique usually requires changing the position and angle of the camera manually to produce a horizontal offset and thus introduces bias due to lack of standardization. In contrast, a camera that allows a simultaneous record of side-by-side images with a synchronous fixed angle and the same condition of exposure could provide standardized images and theoretically more consistent with the real appearance of the optic disc.

Moreover, the assessing procedure of optic discs is usually subjective, which highly relies on extensive experience to achieve high diagnostic precision (11). However, the precision may not be applicable to general healthcare providers in real-world clinical practice (9). Considering that a great proportion of patients who visit clinics for glaucoma screening were examined by ophthalmologists with experience that might not equal to that of glaucoma experts, systematic and strategic observation of every feature of the optic disc on photographs by general ophthalmologists is necessary.

This study employs a cloud-based standardized assessment system for simultaneous stereoscopic photos that were taken in a real-world clinical setting. This study was designed to compare accuracy and agreement for a series of optic disc parameters and glaucoma likelihood of stereoscopic optic disc photographs with those of monoscopic photographs by general ophthalmologists in a real-world multicenter clinical setting.

Materials and methods

Approval from Peking Union Medical College Hospital's institutional review board was obtained for this project (approval number S-K2061), and the research was conducted in accordance with the Declaration of Helsinki. All data collected from the institutions were analyzed anonymously. As this study involved an anonymous medical record review with no more than minimal risk to participants, it met all requirements for a waiver of informed consent per institutional policy.

Abbreviations: CDR, cup-to-disc ratio; CI, confidence interval; ICC, intraclass correlation coefficient; ISNT, I for inferior, S for superior, N for nasal, T for temporal; RNFLD, retinal nerve fiber layer defect.

Data collection

Six hundred pairs of monoscopic and stereoscopic photographs from 600 eyes were recruited consecutively from the Department of Ophthalmology, Peking Union Medical College Hospital. These clinic-based photographs were captured in our clinical practice and selected for further evaluation. None had a history of coexisting ocular diseases, a history of intraocular surgery, or systemic diseases with possible ocular involvement. Photographs of inadequate quality were excluded because they might not exhibit the differences between monoscopic and stereoscopic photographs. Exclusive criteria of image quality included poor illumination of the disc, poor quality image, lens opacity, poor stereoimage, and optic discs of anomalous configuration (those which were totally tilted, congenital abnormal, or high myopic). A total of 519 pairs of images were determined to be suitable for further evaluation by a masked glaucoma specialist.

Each pair of photographs included a monoscopic photograph at a 45-degree field of view and stereoscopic photographs at a 20 × 27 degree field of view. The photographs were taken using a Kowa nonmydriatic retinal camera WX 3D (Kowa, Tokyo, Japan) (examples in Supplementary Figure 1). Photographs were saved as TIFF files (monoscopic) and JPEG files (stereoscopic). Images were uploaded to an interactive platform (https://anno.vistel.cn, one example webpage on this website is shown in Supplementary Figure 1) for further annotating, diagnosing, and grading.

Optic disc assessment

Five R's Rules were followed when assessing optic discs to establish a standardized system for comprehensive evaluation of morphologic features of optic discs without omission (12). The morphologic features included disc size, disc color, disc shape, disc contour, neuroretinal rim shape, ISNT rule consistency, cup-to-disc ratio, retinal nerve fiber layer, beta zone, hemorrhage, and small vessels (Table 1). Furthermore, we set several quantitative thresholds for metrics instead of subjective evaluation. For the range of retinal nerve fiber layer defect (RNFLD), quantitative analysis was performed in the superotemporal (10 to 12 clock h for right eyes, and 12 to 2 clock h for left eyes) and inferotemporal (6 to 8 clock h for right eyes, and 4 to 6 clock h for left eyes) quadrants, while the range of hemorrhages was evaluated all circle around the disc. For the range of RNFLD, a non-overlapping range of more than 1 clock h in each evaluated quadrant between two graders was regarded as inconsistency. Similarly, for the range of hemorrhages, inconsistency was defined as a non-overlapping range of more than 0.5 clock h.

Glaucoma likelihood was classified into four subcategories based on optic disc appearance: definite, probable, suspect, and none glaucoma (Table 1) (4, 13, 14). This detailed classification system could help distinguish subtle differences between monoscopic and stereoscopic photos, which might contribute to corresponding therapy according to gradings of risks or severities of glaucoma.

In order to set a gold standard for training and assessment, 93 pairs of stereoscopic and monoscopic photographs from 93 eyes were selected randomly. These photos were evaluated based on 5R's rule and 4-scale glauoma likelihood classification. These results were further discussed and assessed by an expert panel of five glaucoma professors *via* video meeting. In cases of disagreement, the leading glaucoma specialist (GW.C) made the final decision. These estimates assessed by glaucoma experts were set as ground truth.

There were 21 volunteer national certificated general ophthalmologists of various seniority of clinical practice, from 14 various hospitals, who participated in analyzing and grading the whole photograph set. Of these, 18 ophthalmologists had worked for more than 3 years, and at least seven ophthalmologists were fellows or attendings while the other ophthalmologists worked as residents. All of them were fully trained with 5 R's Rules and 4-scale glaucoma likelihood classification in an offline workshop until they could estimate the stereoscopic and monoscopic photos based on the same standard in tests. They could discuss with a glaucoma specialist (GW.C) if they had any question about the process of grading in training. They were grouped randomly into four groups. There were four rounds of annotation throughout the process, with about 75 pairs of photographs per round (Supplementary Table 1). Mandatory evaluation of the photographs from the abovementioned 93 eyes was required, and their estimates were used to evaluate their agreement with the ground truth. The inter-grader agreement of stereoscopic and monoscopic photos was evaluated, respectively. For each grader, about 50 monoscopic photographs whose corresponding stereoscopic ones have been evaluated by themselves were selected randomly and estimated for intra-grader agreement (Figure 1). Each photo was evaluated by at least two graders. In order to wash out the memory effect, the monoscopic and stereoscopic photos were dispatched to ophthalmologists in various batches over at least a 1-month interval. In order to provide a standardized environment during evaluation, all of these graders were assigned uniform stereo glasses, and the brightness, contrast, size, and resolution of images were adjusted automatically on the cloud-based platform. Glasses for correcting refractive error were recommended, but the ambient lightning were not standardized forcibly because the environment in which ophthalmologists view the photos in clinical practice were not same all across the world.

TABLE 1 Evaluation scale of morphologic characteristics and glaucoma diagnosis based on the Five R's Rules and 4-subcategory glaucoma likelihood classification.

Morphologic characteristics based on Five R's Rule	Scale
R1	
Disc size	Normal | Abnormal (including small, large, and not sure)
Disc color	Normal | Abnormal (including rosy, pallor, pale, and not sure)
Disc shape	Normal | Abnormal/Tilt
Disc contour	Clear | Unclear (including blurring, and not sure)
R2	
Neuroretinal rim shape	Normal | Abnormal (including suspected and abnormal)
Abnormal rim shape	Localized thinning | Notching | Diffuse thinning
ISNT rule consistency	Yes | No
CDR	Quantitative measurements, including vertical CDR, and area CDR, by identifying manually
R3	
Retinal nerve fiber layer (RNFL)	Normal | Abnormal
Abnormal RNFL	Quantitative measurements for RNFL defect range
R4	
Zone beta	Presence | Absence
Contour of zone beta	Clear | Unclear
R5	
Retinal and optic disc hemorrhages	Presence | Absence
Presence of hemorrhages	Quantitative measurements for hemorrhagic range
Small vessels	Normal | Abnormal
Abnormal small vessels	Narrowing | Tortuosity or distortion | Bridging
Diagnosis	
Glaucoma likelihood	None | Possible | Probable | Definite

CDR, cup-to-disc ratio; ISNT, I for inferior, S for superior, N for nasal, T for temporal.

Statistics

The levels of agreement for each morphologic characteristic were calculated. Agreement for nominal variables was calculated using weighted kappa (κ). The kappa is a numerical value that ranges from −1 (complete disagreement) to +1 (total agreement). For the continuous variables, the agreement was assessed using the intraclass correlation coefficient (ICC). The degree of agreement was classified according to the value of kappa or ICC as follows: slight (0–0.2), fair (0.2–0.4), moderate (0.4–0.6), substantial (0.6–0.8), and almost perfect (0.8–1) (15). Differences between various kappa or ICC values were considered statistically if the mean value for one viewing method lay outside two standard deviations of the mean value for the other viewing method (7, 16). When further and direct comparisons were available, paired *t*-test was performed.

Intra-grader agreement was computed by comparing the evaluating results of each morphologic characteristic using stereoscopic photographs and their matching monoscopic counterparts. Inter-grader agreements of the stereoscopic method and monoscopic method were calculated by comparing each grader's answers to variables, respectively. Agreement for each feature between estimates and ground truth was also calculated.

All statistical analysis, other than kappa statistics, was performed using SPSS 25.0 (IBM, Chicago, IL, USA). Kappa statistics were performed using a custom algorithm with Python.

Results

A total of 600 simultaneous stereoscopic photographs and their monoscopic counterparts were used for the evaluation of optic discs and glaucoma likelihood. Although both stereoscopic and monoscopic photos showed good agreement with the ground truth of glaucoma likelihood, the stereoscopic photos had better accuracy than monoscopic did for probable glaucoma (stereo κ 0.238 vs. mono κ 0.118). The stereoscopic method had better agreement with the ground truth than the monoscopic did when evaluating disc size, disc color, and neuroretinal rim shape. On the contrary, the monoscopic method misled graders more easily on disc size in probable glaucoma with significance, and RNFLD in definite and probable glaucoma with borderline significance. The overall levels of agreements

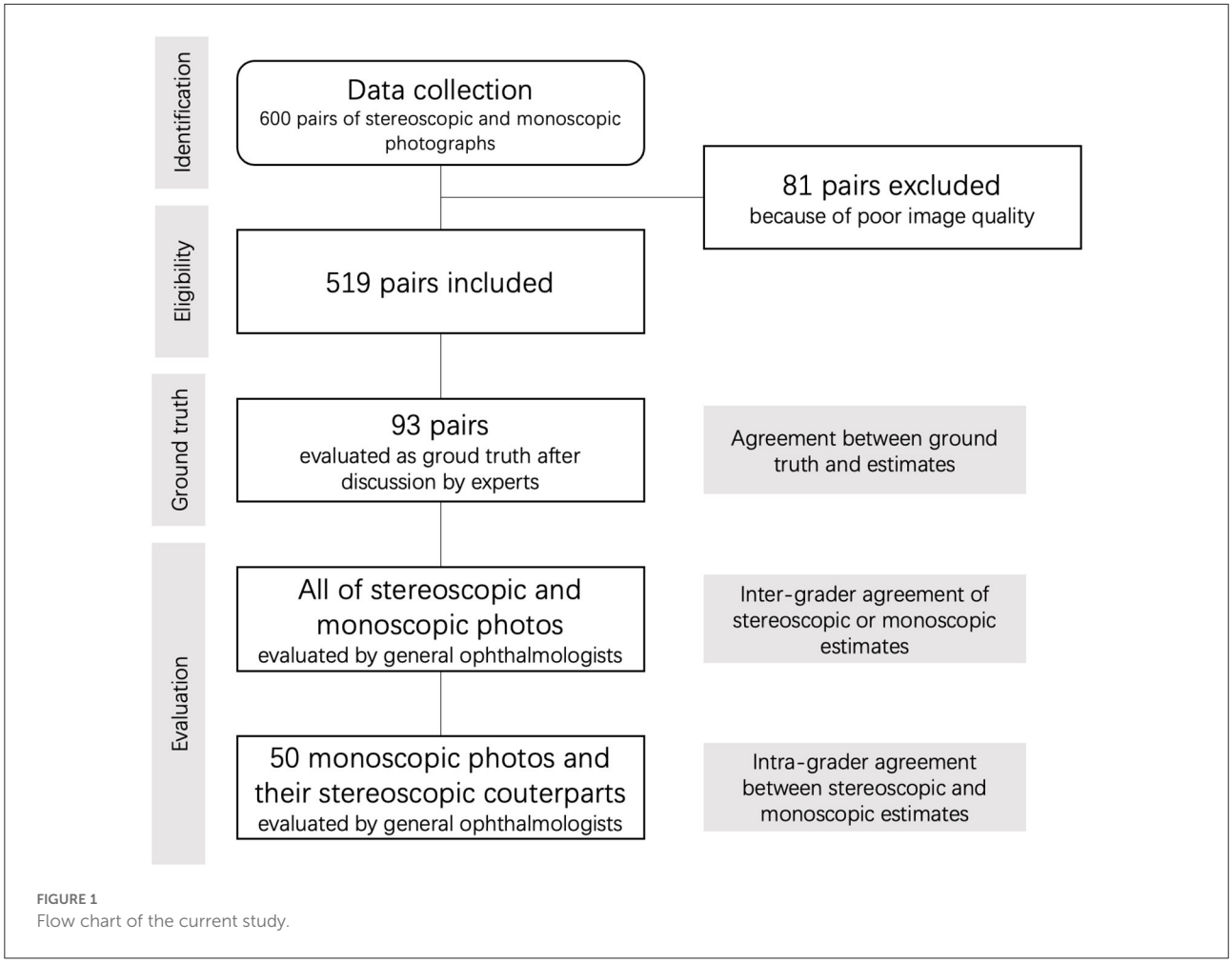

FIGURE 1
Flow chart of the current study.

for each morphologic feature and glaucoma likelihood between ground truth and viewing method were shown in Figure 2. The overall levels of inter-grader agreement of various viewing methods for each morphologic feature and glaucoma likelihood were shown in Figure 3. Detailed values were listed in Supplementary Tables 2–13.

R1: Scleral ring for optic disc and its size

The agreement between ground truth and stereoscopic estimates was superior or comparable to that between ground truth and monoscopic estimates for disc size [stereo κ 0.447, confidence interval (CI) 0.356–0.539 vs. mono κ 0.183, CI 0.121–0.244], disc color (stereo κ 0.612, CI 0.565–0.659 vs. mono κ 0.549, CI 0.490–0.608), disc shape (stereo κ 0.409, CI 0.334–0.483, and mono κ 0.339, CI 0.259–0.419), and disc contour (stereo κ 0.063, CI −0.013–0.139 and mono κ 0.088, CI −0.025–0.202) (Supplementary Table 2).

The inter-grader agreement using the stereoscopic method was also significantly greater than or similar to that using the monoscopic method for disc size (stereo κ 0.347, CI 0.323–0.370 vs. mono κ 0.276, CI 0.256–0.296), disc color (stereo κ 0.531, CI 0.516–0.546 vs. mono κ 0.505, CI 0.492–0.519), disc shape (stereo κ 0.355, CI 0.327–0.383 vs. mono κ 0.344, CI 0.322–0.365), and disc contour (stereo κ 0.172, CI 0.125–0.219 vs. mono κ 0.144, CI 0.110–0.179), as well as in each glaucoma likelihood subcategories, except for disc size and disc color in eyes with probable glaucoma (Supplementary Tables 3–5).

These two viewing methods had a substantial intra-grader agreement for disc color (κ 0.619) and disc shape (κ 0.568), and fair intra-grader agreement for disc size (κ 0.252) and disc contour (κ 0.278).

R2: Optic disc rim

For neuroretinal rim shape, the agreement between ground truth and stereoscopic estimates was greater than that between ground truth and monoscopic estimates (stereo κ 0.356, CI 0.287–0.426 vs. mono κ 0.274, CI 0.199–0.350), and the inter-grader agreement using the stereo method was better than that

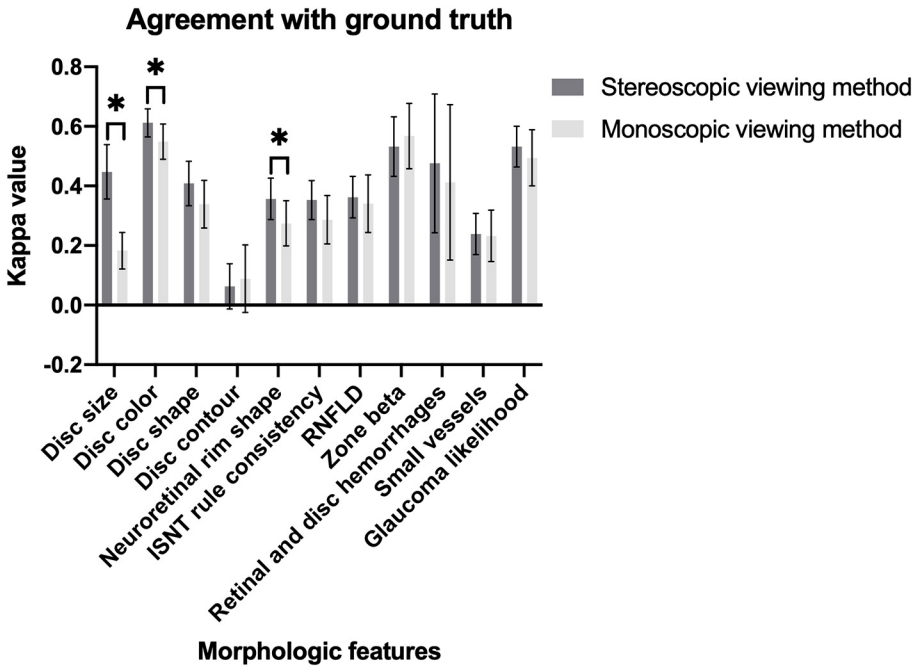

FIGURE 2
Magnitude of agreement between two viewing methods and ground truth for morphologic features of optic disc and glaucoma likelihood (*$P <$ 0.05).

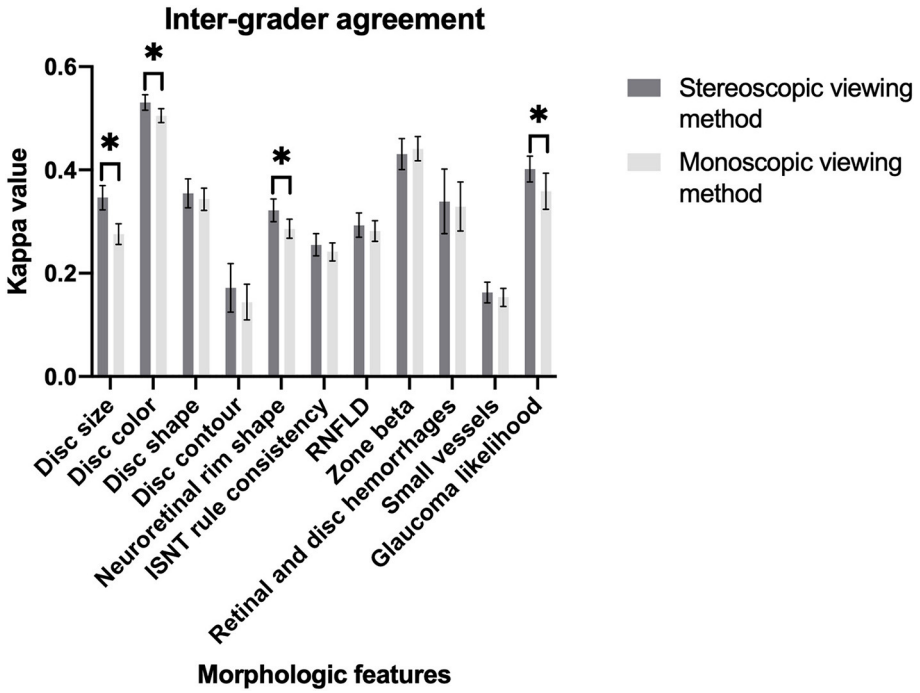

FIGURE 3
Magnitude of inter-grader agreement of different viewing methods for morphologic features of optic disc and glaucoma likelihood (*$P < 0.05$).

using mono method (stereo κ 0.322, CI 0.300–0.344 vs. mono κ 0.286, CI 0.268–0.305) (Supplementary Table 6).

The agreements of ISNT rule consistency between ground truth and both monoscopic and stereoscopic estimates were fair (stereo κ 0.353, CI 0.287–0.418, and mono κ 0.286, CI 0.405–0.368). The levels of inter-grader agreement for stereoscopic and monoscopic assessments showed no significant differences (stereo κ 0.255, CI 0.234–0.277 vs. mono κ 0.242, CI 0.224–0.259). Compared with the mono method, the stereo method showed better inter-grader agreement in probable glaucoma and unsatisfying inter-grader agreement in suspect glaucoma (Supplementary Table 7).

These viewing methods had moderate intra-agreement for neuroretinal rim shape (κ 0.487) and ISNT rule consistency (κ 0.427).

When assessing vertical CDR, stereoscopic assessments showed slightly greater value than monoscopic assessments (stereo 0.690 ± 0.111 vs. mono 0.684 ± 0.106, *P* 0.003). In subcategory evaluation, the vertical CDRs in stereoscopic photos of suspect glaucoma were slightly greater than those in monoscopic photos (stereo 0.686 ± 0.101 vs. mono 0.677 ± 0.099, *P* 0.011), while other subcategories of glaucoma likelihood showed no significant differences of vertical CDR between monoscopic and stereoscopic estimates. The area CDR in stereoscopic photos was also greater than that in monoscopic photos (stereo 0.449 ± 0.133 vs. mono 0.443 ± 0.125, *P* 0.001), and this phenomenon was noticed in definite and suspect glaucoma (Supplementary Table 8).

R3: Retinal nerve fiber layer

When assessing RNFLD, the agreements between ground truth and both monoscopic and stereoscopic photos were both moderate and similar (stereo κ 0.36, CI 0.29–0.43 vs. mono κ 0.34, CI 0.24–0.44), and the levels of inter-grader agreement for monoscopic and stereoscopic estimates were similar as well (stereo κ 0.29, CI 0.27–0.32 vs. mono κ 0.28, CI 0.26–0.30). But the inter-grader agreement of the mono method was greater than that of the stereo method in eyes with definite glaucoma and probable glaucoma. For the eyes with RNFLD, graders had a slightly higher inter-grader agreement in stereoscopic estimates than that in monoscopic estimates when detecting RNFLD (stereo 51.6 vs. mono 47.3%). The κ value of intra-grader agreement between these two methods was 0.538 (Supplementary Table 9).

R4: Region of parapapillary atrophy

The level of agreement between monoscopic estimates and ground truth for the beta zone was moderate (κ 0.568, CI 0.458–0.677), which was similar to the level between stereoscopic estimates and ground truth (κ 0.532, CI 0.432–0.632). The inter-grader agreements for the beta zone in stereoscopic and monoscopic estimates, on the whole, were relatively fair, and no significant difference between these viewings was found (stereo κ 0.431, CI 0.401–0.461 vs. mono κ 0.441, CI 0.418–0.465). Except in eyes with definite and suspect glaucoma, monoscopic photos showed better inter-grader agreements (Supplementary Table 10).

Both stereoscopic viewing and monoscopic viewing showed no significant agreement with ground truth (stereo κ −0.005, CI −0.055–0.044 vs. mono κ 0.109, CI −0.027–0.246), and these viewing methods showed no significant difference. Although stereoscopic and monoscopic methods had similar inter-grader agreements on the whole (stereo κ 0.234, CI 0.176–0.291 vs. mono κ 0.214, CI 0.170–0.257), but the stereoscopic method had better inter-grader agreements in eyes with definite, suspect and none glaucoma (Supplementary Table 11).

The levels of intra-grader agreement for the beta zone and its contour were substantial (κ 0.623 and 0.455, respectively).

R5: Retinal and optic disc hemorrhages and small vessels

For retinal and optic disc hemorrhages, stereoscopic viewing and monoscopic viewing had similar agreements with ground truth (stereo κ 0.476, CI 0.243–0.709 vs. mono κ 0.412, CI 0.151–0.673). They also had similar inter-grader agreements on the whole (stereo κ 0.339, CI 0.277–0.402 vs. mono κ 0.329, CI 0.282–0.377) (Supplementary Table 12).

When evaluating small vessels, the levels of agreements between ground truth and stereo estimates and between ground truth and mono estimates showed no significant difference (stereo κ 0.239, CI 0.170–0.308 vs. mono κ 0.232, CI 0.146–0.319). Although stereoscopic and monoscopic methods had similar inter-grader agreements on the whole (stereo κ 0.163, CI 0.143–0.183 vs. mono κ 0.154, CI 0.136–0.171), but the monoscopic method had better inter-grader agreements in eyes without glaucoma (Supplementary Table 13).

Glaucoma likelihood

There were good agreements between ground truth and both monoscopic and stereoscopic estimates (stereo κ 0.532, CI 0.464–0.600 and mono κ 0.494, CI 0.400–0.589) (Table 2, examples are shown in Figure 4). In eyes with probable glaucoma, the accuracy of the stereo method was greater than that of the mono method (stereo κ 0.238, CI 0.101–0.375 vs. mono κ 0.118, CI 0.005–0.230) (Supplementary Table 14). There was a substantial intra-grader agreement between the

TABLE 2 The levels of agreement for glaucoma likelihood.

Viewing method	Agreement with ground truth (kappa)	Inter-grader agreement (kappa)	Agreement between two viewing viewing methods (kappa)
Stereoscopic	0.532 (0.464–0.600)	0.402* (0.377–0.427)	0.636 (0.377–0.427)
Monoscopic	0.494 (0.400–0.589)	0.359* (0.324–0.394)	

*Statistically significant.

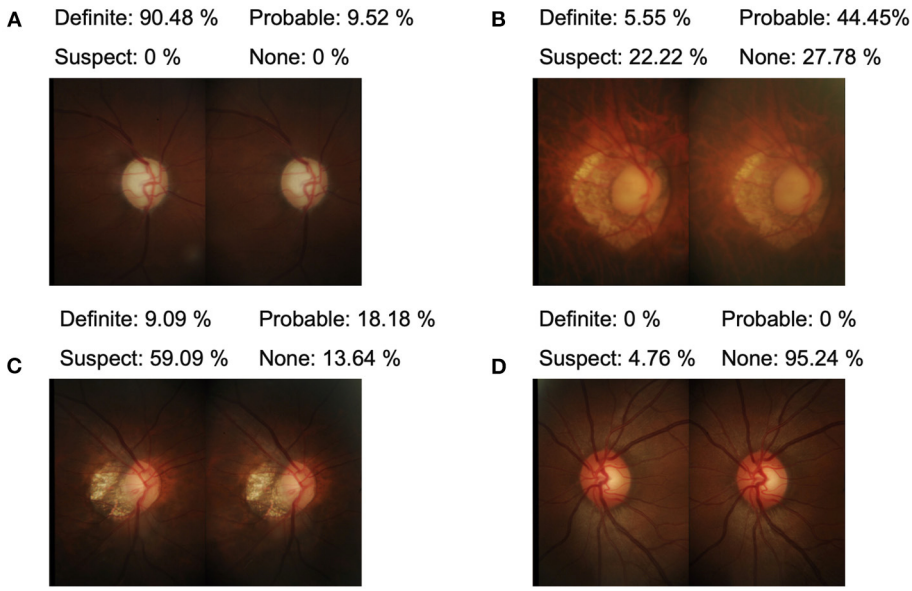

FIGURE 4
Examples of representative optic disc photographs of various glaucoma likelihood **(A)**, definite glaucoma; **(B)**, possible glaucoma; **(C)**, probable glaucoma; **(D)**, none glaucoma. The distribution of gradings is shown above corresponding images.

monoscopic and stereoscopic evaluation of glaucoma likelihood (κ 0.636, CI 0.551–0.720).

The stereoscopic method had better inter-grader agreement than the monoscopic method did (stereo κ 0.402, CI 0.377–0.427 vs. mono κ 0.359, CI 0.324–0.394), especially in eyes with definite glaucoma, but not in eyes with probable glaucoma and suspect glaucoma.

Discussion

The present study has shown that for general ophthalmologists in the real world, there were some significant differences in evaluating morphologic characteristics of the optic disc and estimating glaucoma likelihood when using stereoscopic photographs of the optic disc compared to monoscopic photographs. The results of the current study demonstrated that assessment of glaucoma likelihood with the stereoscopic method showed superior performance than the monoscopic method, especially in distinguishing eyes with probable glaucoma. The stereoscopic method had superiority in identifying glaucomatous eyes which need medical interventions. There was substantial agreement in glaucoma likelihood assessment between stereoscopic and monoscopic methods (κ 0.636). However, the stereoscopic estimates had a greater inter-grader agreement on the whole, and better accuracy in eyes with probable glaucoma. When compared with ground truth, stereoscopic photographs had a better agreement for disc size, disc color, and neuroretinal rim shape, on the whole. The stereoscopic method also presented a better inter-grade agreement for disc size, disc color, neuroretinal rim shape, and glaucoma likelihood on the whole. On the contrary, the monoscopic method showed no overall superiority for any estimated features.

We used a 4-subcategory classification of glaucoma likelihood in the present study. The grading level is incremented according to the clinical likelihood of glaucoma and various management, in which none glaucoma only needs regular

examinations, suspect glaucoma needs intensive monitoring, probable glaucoma needs treatment without setting target intraocular pressure (IOP), and definite glaucoma needs treatment with target IOP setting. Compared with the 2-subcategory classification of discriminating only glaucomatous and nonglaucomatous eyes, the more detailed classification with four subcategories helps identify the extent of risks for each individual and provides personalized management in clinical practice.

Criteria for this classification of glaucoma likelihood was based on the characteristics of the optic nerve head. Five R's Rules assist ophthalmologists to observe optic discs comprehensively in a standardized workflow, and reduce the risk of missing information (13, 14). This detailed classification enhances the ability to detect differences between stereo and mono methods as well as probably increases the difficulty of accurate grading for general ophthalmologists. In the current study, the inter-grader agreement for 4-subcategory glaucoma likelihood reached 0.4 under stereoscopic conditions and 0.35 under monoscopic conditions. In another study evaluating 4-subcategory glaucoma likelihood by 21 glaucoma specialists from multiple international centers, Kong et al. reported the κ value of inter-observer agreement reached 0.63 (17). Although expert consensus assessment demonstrated higher performance in assessment, our results may reflect optic disc assessment in clinical practice, which reaches a moderate and acceptable level. However, the inter-grader agreement might reduce when using a more refined classification system. Varma et al. reported that inter-observer agreement for 2-subcategory glaucoma diagnosis was 0.50 using the stereo method, which was assessed by six experts (10). Reus et al. reported that inter-observer agreement for 2-subcategory glaucoma diagnosis using stereo photos reached 0.72 for general ophthalmologists, and 0.45 for residents (18). Moreover, we also investigated the diagnostic accuracy, which reaches an acceptable level (mean κ 0.532) when using stereoscopic photos. Therefore, determining glaucoma likelihood with a 4-subcategory classification system after evaluating optic discs with 5R's Rules is feasible in clinical practice.

Theoretically, stereoscopic photographs provide a better understanding of the three-dimensional structure of the optic disc (7), but we noticed that some differences in estimates between stereoscopic and monoscopic methods need further explanation. For example, stereoscopic viewing provided a volumetric perspective for assessing the optic disc, which enables more precise estimates of the size, color, and shape of optic discs, and neuroretinal rim in this study. Moreover, when assessing ISNT rule consistency, stereoscopic viewing might help identify rim alterations, and especially in eyes with a high risk of glaucoma, the rim changes were more easily to be noticed in stereoscopic photos, especially for eyes with probable glaucoma. The values of CDR in stereoscopic photos were usually greater than those in monoscopic photos, and the rim widths in stereoscopic photos were usually less (9, 10). Therefore, the stereo method had a better inter-grader agreement in eyes with probable glaucoma. Furthermore, when evaluating RNFLD in eyes with definite and probable glaucoma, although inter-grader agreements were greater using the mono method, the agreements with ground truth were relatively less using the mono method, which indicated that the monoscopic method might lead to false classifications of glaucoma more easily. Similarly, although the monoscopic method showed better inter-grader agreement for disc size, disc color in eyes with probable, and for ISNT rule consistency in eyes with suspect glaucoma, they did not exceed stereo counterpart on accuracy, and even had worse agreement with ground truth. Therefore, the stereoscopic method is helpful to provide an objective evaluation. Besides, we noticed that monoscopic methods had a better inter-grader agreement for small vascular abnormalities in nonglaucoma eyes. Although this result did not influence the diagnosis of glaucoma, excessive information from stereoscopic photographs might interfere with the judgement of graders. In contrast, previous studies of comparison between monoscopic and stereoscopic methods did not evaluate the morphological features of optic discs and glaucomatous possibility comprehensively and did not evaluate the stereoscopic methods among general ophthalmologists in the real world (Supplementary Table 15).

The design of the current study applied a series of methods to standardize the evaluation process and enhance its reliability and persuasion. First of all, developing a standardized and comprehensive grading system by applying 5 R's Rules for optic discs, a detailed classification for glaucoma likelihood, and a simultaneous stereo camera can help overcome variability in the process of subjective clinical evaluation (12, 17, 19). Moreover, the number of graders and the number of evaluated photos were greater than those of previous studies. In the current study the 4-subcategory classification of glaucoma likelihood was evaluated by 21 trained general ophthalmologists, while previous studies investigated the 4-subcategory classification of glaucoma likelihood by glaucoma experts or only 2-subcategory classification (10, 17, 18, 20). Considering that level of expertise has been shown to affect stereoscopic photography grading (21), the study could reflect optic nerve head assessment in clinical practice. Furthermore, we used clinic-based photos rather than community-based photos. We excluded a large number of photos of normal optic discs. The relatively complicated conditions of optic discs and glaucoma likelihood in the present study were similar to the clinical practice in the busy clinical setting. Therefore, the methods in the current study could be reproduced in real-world clinical practice.

Considering that stereoscopic photographs provided more detailed and realistic details, these photos could be used to train residents and general ophthalmologists to achieve consistent ability and the same evaluation results, which benefits not

only image reviewing but also management of evaluation results. We also assumed that artificial intelligence models trained with stereoscopic photos might be able to provide results that are closer to the truth than those trained with monoscopic counterparts.

The strengths of this study include the relatively large number of annotated photographs and representative graders, its prospective randomized design due to the application of a cloud-based platform, and the same viewing conditions as real-world clinical settings. Therefore, considering that a great proportion of patients who visit clinics for glaucoma screening were examined by ophthalmologists with experience that might not equal that of glaucoma experts, we investigated the value of a comprehensive estimated method on stereoscopic photographs of the optic disc in real-world clinical practice. However, we still have several potential limitations. First, we did not compare ophthalmologists with less experience and expert assessment. As stereo photos could provide topographic information which enables graders to evaluate with a stereoscopic view, graders with less experience might benefit more than experts who may be able to draw reliable clinical judgments using only mono cues. On one hand, the value of experience in evaluating optic discs and estimating glaucoma likelihood was not part of our purpose. On the other hand, the conclusions of our study should not be extended to all levels of ophthalmologists. Second, due to the application of a cloud-based platform, we allowed graders to review and change their previous annotations, which introduced a risk of recall of a previously seen photograph when we evaluate the intra-grader agreement by using stereo and mono photos of the same optic discs. We used several methods to minimize this risk, for example, decreasing the number of photos used for evaluating intra-grader agreement, and setting a washout period. However, we still cannot eliminate the bias because of recall. Third, because of the limited levels of graders' training and expertise, it is inevitable that some judgments during evaluation might lack sufficient reasons and experienced estimates (20), and the repeatability of estimation with photographs of optic disc needs further investigation. Fourth, we did not calculate the single grader's intra-grader agreement of stereoscopic photos or monoscopic photos, because graders are allowed to review and revise their previous grading and annotations on the cloud-based platform, which was similar to the process of reviewing clinical information in clinical practice. Therefore, considering that once the same photo was given twice or more times the graders can evaluate photos based on previous grading and annotations by reviewing previous evaluations, we could not provide the results of this kind of intra-grader agreement in this study. Fifth, due to the intrinsic weakness of calculating kappa value, the kappa value could be amazingly low when one category in a binary variable counts almost all. For example, the accuracy of judging disc contour reaches more than 0.95, but the kappa value for agreement with ground truth was lower than 0.1.

In summary, our analysis showed that general ophthalmologists assessed optic discs with a better inter-grader agreement and diagnostic accuracy for glaucoma likelihood on the whole. The stereoscopic method had superiority in identifying glaucomatous eyes which need medical interventions. The monoscopic method showed no overall superiority for any estimated features in the present study. Stereoscopic optic disc photography is recommended for general ophthalmologists in the clinical evaluation of glaucomatous optic disc damage, and their routine use in real-world clinical settings might compensate for the lack of expertise and experience.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Peking Union Medical College Hospital Institutional Review Board. The Ethics Committee waived the requirement of written informed consent for participation. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

JY: design, definition of intellectual content, data analysis, manuscript preparation, and manuscript editing. YQ: data acquisition, manuscript preparation, and manuscript review. JZ: data acquisition, definition of intellectual content, data analysis, manuscript preparation, and manuscript editing. JC: data acquisition, data analysis, manuscript preparation, and manuscript review. ZS: data acquisition and manuscript review. YD: data acquisition, data analysis, and manuscript review. GY: design and manuscript review. DD: concepts, design, and manuscript review. YC: concepts and manuscript review. GC: concepts, design, data analysis, manuscript preparation, and manuscript editing. All authors contributed to the article and approved the submitted version.

Acknowledgments

The authors thank Wenhong Cao, Yao Chen, Yu Di, Linyang Gan, Bing Li, Gang Li, Mengda Li, Qiang Li, Xianshi Meng, Ke Song, Rui Qin, Hua Wang, Jiayu Wang, Jingyi Wang, Lichun Wang, Junyan Xiao, Shan Yang, Bilei Zhang, and Shasha

Zhang (listed in alphabetical order of their names) for helping them to set gold standards of diagnosis and annotation and to annotate images.

Conflict of interest

Authors JZ, JC, YD, and DD were employed by Visionary Intelligence Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.990611/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Examples of stereoscopic and monoscopic photographs from three glaucomatous eyes. A pair of suitable stereo glasses were strongly suggested when viewing the stereoscopic photographs.

SUPPLEMENTARY TABLE 1
The number of pairs of stereoscopic and monoscopic photographs evaluated by graders.

SUPPLEMENTARY TABLE 2
The levels of agreement for disc size. CI, confidence interval. *Statistically significant.

SUPPLEMENTARY TABLE 3
The levels of agreement for disc color. CI, confidence interval. *Statistically significant.

SUPPLEMENTARY TABLE 4
The levels of agreement for disc shape. CI, confidence interval. *Statistically significant.

SUPPLEMENTARY TABLE 5
The levels of agreement for disc contour. CI, confidence interval.

SUPPLEMENTARY TABLE 6
The levels of agreement for neuroretinal rim shape. CI, confidence interval. *Statistically significant.

SUPPLEMENTARY TABLE 7
The levels of agreement for ISNT rule consistency. CI, confidence interval; ISNT, I for inferior, S for superior, N for nasal, T for temporal. *Statistically significant.

SUPPLEMENTARY TABLE 8
The levels of agreement for CDR and comparison of different viewing methods. CDR, cup-to-disc ratio; CI, confidence interval; ICC, intraclass correlation coefficient.

SUPPLEMENTARY TABLE 9
The levels of agreement for RNFLD. CI, confidence interval; RNFLD, retinal nerve fiber layer defect. *Statistically significant.

SUPPLEMENTARY TABLE 10
The levels of agreement for beta zone. CI, confidence interval. *Statistically significant.

SUPPLEMENTARY TABLE 11
The levels of agreement for contour of beta zone. CI, confidence interval. *Statistically significant.

SUPPLEMENTARY TABLE 12
The levels of agreement for retinal and optic disc hemorrhages. CI, confidence interval.

SUPPLEMENTARY TABLE 13
The levels of agreement for small vessels. CI, confidence interval. *Statistically significant.

SUPPLEMENTARY TABLE 14
Inter-grader agreement and accuracy of each subcategory of glaucoma likelihood. CI, confidence interval. *Statistically significant.

SUPPLEMENTARY TABLE 15
Previous studies of comparison between monoscopic and stereoscopic optic disc photos.

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ORIGINAL RESEARCH
published: 09 November 2022
doi: 10.3389/fmed.2022.842864

Exploring the Association Between Resilience and Quality of Life Among Glaucoma Patients: Sleep Disturbance as a Mediating Factor

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Ophthalmology, a section of the journal Frontiers in Medicine

Received: *24 December 2021*
Accepted: *18 March 2022*
Published: *09 November 2022*

Citation:
Peng Q, Qu B, Sznajder KK, Chen Q, Fu J, He S and Yang X (2022) Exploring the Association Between Resilience and Quality of Life Among Glaucoma Patients: Sleep Disturbance as a Mediating Factor. Front. Med. 9:842864. doi: 10.3389/fmed.2022.842864

Background: Patients with glaucoma may experience many symptoms such as blindness, which seriously affect their quality of life (QoL). Resilience is playing a vital role in enhancing the QoL and well-being of patients with chronic diseases. In addition, sleep disturbance is common in patients with glaucoma, leading to a decline in their QoL. However, there is a dearth of research on whether sleep disturbance plays a mediating role between resilience and QoL among glaucoma patients.

Objective: The aim of this study is to explore the role of sleep disturbance in the relationship between resilience and QoL among glaucoma patients.

Methods: From July to December 2019, a cross-sectional survey was conducted on 215 glaucoma patients in an ophthalmic hospital in Liaoning Province. Hierarchical multiple regression (HMR) analyses and structural equation modeling (SEM) were conducted to examine the factors related to QoL and to test the hypothesis that sleep disturbance mediates the relationship between resilience and QoL among glaucoma patients.

Results: The average QoL score among glaucoma patients was 43.85 ± 14.97 as reported by the Glaucoma Quality of Life-15 (GQL-15) scale, where a higher scores indicating a poorer QoL. Resilience was found to be linked with a lower QoL score ($P < 0.01$), while sleep disturbance was associated with a higher QoL score ($P < 0.01$). When sleep disturbance was included in the model as partial mediator, the path coefficients for the association between resilience and QoL score was significantly decreased (a*b = −0.1, BCa95% CI: −0.154∼−0.045).

Conclusion: Findings of this study reflected that QoL among glaucoma patients in China was poor. Resilience was found to be an important positive factor, which could result in the improvement of QoL. Furthermore, sleep disturbance mediated

the relationship between resilience and QoL among patients with glaucoma, thereby reducing the positive impact of resilience on QoL in glaucoma patients. Efforts to improve QoL among glaucoma patients may benefit from interventions that enhance the levels of resilience and promote healthy sleep.

Keywords: sleep disturbance, resilience, quality of life, glaucoma, structural equation

INTRODUCTION

Glaucoma is a chronic lifelong disease characterized by concave atrophy of optic papilla and the loss of retinal ganglion cells, which constitutes a major concern for public health (1). According to research statistics, more than 76 million people currently suffer from this disease, with is expected to increase to 112 million by 2040 (2). Because the visual impairment caused by glaucoma is irreversible, the impact on patients is not only physiological, but also psychological. Glaucoma patients may experience psychological problems such as tension, fear, anxiety, pessimism and depression (3), which will affect the recovery of vision, and also the QoL of patients. Therefore, QoL is an important index to evaluate the treatment effect of glaucoma patients (4).

Resilience is the ability to actively adapt to adversity. It can guide individuals to alleviate negative emotions and improved QoL (5). By understanding the broaden-and-build theory of positive emotions, we can realize that positive emotions (such as happiness and interest) broaden people's thought and action, overcome the physiological effects of negative emotions, enhance resilience, and lead to the rise of emotional well-being, thereby improving individual's QoL (6, 7). Additionally, the systematic self-reflection model of resilience strengthening shows that those who have experienced hardships, or even trauma, will have greater resilience than those who did not experience (8). Therefore, when glaucoma patients experience adversities such as visual impairment, headaches and eye swelling, these uncomfortable symptoms may stimulate improved resilience, which could increase patients' treatment compliance, this improving their QoL. Additionally, most studies have suggested that people with high level of resilience had a higher QoL in general (9–12). For example, research by Craig et al. (13) has indicated the QoL of cancer patients who have higher level of resilience was significant better than those with lower level of resilience. Chen et al. (14) also reported that patients with hypertension improved their QoL by focusing actively and strategically on improving their resilience. While the association between resilience and QoL has been studied for many clinical conditions, there is a paucity of research regarding resilience and QoL among glaucoma patients in China.

Sleep disturbance is a common and severe issue among glaucoma patients. According to the previous research, more than 60% of glaucoma patients report having sleep disturbance (15). The occurrence of sleep disturbance among glaucoma patients is considered to be related to visual field damage (16) and circadian rhythm disorder (17). A growing number of studies have documented that through various mechanisms (attention transfer and cognitive change), sleep impacts the generation and regulation of emotion (18, 19). Previously published studies have showed that sleep disturbance is caused by visual field loss and is related to depression and psychological factors (20, 21). Besides, It has been confirmed that the QoL of nocturia patients who reported sleep disturbance appeared to be worse than those without, with sleep disturbance have a greater psychological impact (22). An earlier study on the QoL of pregnant women also stated that stress caused physical and mental responses that affected people's resilience. In turn, these physiological and psychological outcomes were found to be associated with sleep disturbance (23, 24). Therefore, we hypothesize that sleep disturbance will play a mediating role between resilience and the QoL of glaucoma patients.

Few studies, however, have explored the relationship between resilience and sleep disturbance among Chinese glaucoma patients, and fewer have examined the mediating effect of sleep disturbance in the association between resilience and QoL among glaucoma patients. Therefore, as noted, the aim of this study was to verify the following research hypotheses. Hypothesis 1: resilience is positive factor affecting QoL among glaucoma patients; Hypothesis 2: sleep disturbance is negatively associated with QoL; Hypothesis 3: sleep disturbance mediates the effect of resilience on QoL among glaucoma patients.

MATERIALS AND METHODS

Survey Process and Participants

From July 29 to December 30 in 2019, a cross-sectional, hospital-based investigation was carried out among glaucoma outpatients who met the criteria and be selected continuously in an ophthalmic outpatient hospital in Liaoning Province. The trained investigator conducted face-to-face interviews using a mobile phone enabled questionnaire to help the patients to fill in the questionnaire. Before the questionnaire was conducted, the informed consent of the patients was obtained.

The inclusion criteria for participating were as follows: (1) aged 18 years and above; (2) diagnosed with glaucoma; and (3) agreed to voluntary participation in the survey (25). The exclusion criteria were: (1) glaucoma patients with other systemic diseases such as cancer etc. (25); (2) current diagnosis of substance abuse or addiction; (3) lifetime diagnosis of a psychotic/affective disorder, and (4) prescribed anti-depressants, antipsychotics, or immunosuppressants (17).

Ethics Considerations

Before the investigation, all participants were fully informed the purpose and relevant contents of this study. The study was

conducted based on the Helsinki Declaration revised in 1989, and the study protocols ware also approved by the Ethics Committee of China Medical University.

Survey Instruments

Demographic Characteristics of Glaucoma Patients

Demographic characteristics of glaucoma patients were collected and included: age, gender, marital status, educational, monthly income, duration of the glaucoma disease, duration of other chronic diseases, disease types, number of operations, and family history of glaucoma/cataract. "Age" was group into "≤65 years old" or ">65 years old"; "Marital status" was dichotomized as "married" or "others"; "Educational level" was classified as "Junior high school and below" or "Senior high school and above"; "Monthly income" was divided into "<3000RMB,""3000-6000RMB," and ">6000RMB"; "Duration of glaucoma and other chronic disease" was dichotomized as "<3 years" and "≥3 years"; "The disease types" were divided into "Glaucoma" and "Glaucoma complicated with Cataract"; "The number of operations" was "0," "1," "2," "3 or more."

Quality of Life of Glaucoma Patients

The Glaucoma Quality of Life-15 (GQL-15) scale is one of the most effective tools to measure the QoL of glaucoma patients and is often used in research (26–28). There are 15 items in the GQL-15, which measure patients' peripheral vision, visual acuity, near vision, light and dark vision, glare, and outdoor activity ability. According to the degree of difficulty in completing daily life, the answers to each item are divided into five levels: no difficulty (1 point), minor difficulty (1 point), moderate difficulty (2 points), great difficulty (3 points), and extremely difficulty (4 points). In addition, there is an answer "I can't complete this daily activity because of my eyes", which is recorded as 0. The total possible score of the questionnaire was 75. The higher the score, the worse the QoL as measured by the GQL-15. The Cronbach's alpha coefficient for the scale in this study was 0.966.

Sleep Disturbance of Glaucoma Patients

The PROMIS Sleep Disturbance Short Form-8 (Promis8b) (29) was used to evaluate the sleep of study participants over the past 7 days. The scale consists of 8 items, and each item has a 5-point scale: never (1 point), seldom (2 point), sometimes (3), frequently (4 point), everyday (5 point). Items 1, 4, 5 and 6 were scored positively, while items 2, 3, 7 and 8 were reverse scored. The total score of the questionnaire was calculated and possible the score range was 8-40. After t conversion of the original score, it was divided into four levels according to the T-score: no sleep disorder ($t < 55.0$), mild sleep disturbance (55.0-59.9), moderate sleep disturbance (60.0-69.9), and severe disturbance (>70). Higher score indicated more serious sleep disturbance. The Cronbach's alpha coefficient for the scale in this study was 0.963.

Resilience of Glaucoma Patients

The Ego-resiliency scale (ER89) is used to access the levels of resilience among patients. The resilience scale has good reliability and validity and is widely used in China. There are 14 items in the Resilience Scale developed by Block and Kreman (30), which adopts 4-point scoring representing "not applicable at all" to "very applicable". Higher total scores mean higher levels of resilience. The Cronbach's alpha coefficient for the scale in this study was 0.845.

Statistical Analysis

All statistical analyses were performed by using SPSS software IBM version 23.0. *T*-tests and ANOVAs were first applied to evaluate the differences of QoL by demographic and clinical characteristics of glaucoma patients. Secondly, the correlations of sleep disturbance, resilience and QoL were examined by using the Spearman correlation. Next, hierarchical multiple regression (HMR) analysis was used to determine the predictors and mediators related to the QoL of glaucoma patients. Then, structural equation modeling (SEM) was used to asssess the mediating role of sleep disturbance between resilience and QoL in glaucoma patients, which was analyzed by AMOS 17.0. The SEM model included QoL as a dependent variable, resilience as an independent variable and sleep disturbance as a mediator variable. The results were consistent with the SEM criteria ($\chi 2/df < 5$, GFI >0.90, CFI > 0.90, RMSEA <0.08, and TLI > 0.90). The bootstrap estimate was based on 5000 random samples (a*b products) obtained from the original data, which was used to examine the mediating effect of sleep disturbance between resilience and QoL. The bias-corrected and accelerated 95% CI of each product was investigated. Statistical tests were considered significant if $P < 0.05$ (two-tailed).

RESULTS

Description of Demographic of Glaucoma Patients

Table 1 shows the demographic characteristics of glaucoma patients in this study, with an average age of 66.24 ± 12.53 years. Among the 215 patients involved in the survey, 126 (58.6%) were 65 years old and over, 117 (54.4%) were women, and the majority of glaucoma patients (94.4%) were married. In terms of educational level, 59.6% of glaucoma patients reported their highest level of educational junior high school or below. Nearly one fifth of patients (45.6%) reported a monthly income of 3000–6000 RMB, and only 18.6% of glaucoma patients have reported a monthly income of more than 6000 RMB. Most glaucoma patients had been ill for less than 3 years (63.7%). Almost half of the patients reported having at least one chronic disease (49.8%). The bivariate analysis found that glaucoma patients with high school education or above ($P < 0.05$), those with a monthly income of less than 3000 RMB ($P < 0.01$), and those without other chronic diseases ($P < 0.01$) had a higher QoL. In addition, glaucoma patients complicated with cataract exerted lower scores of QoL than glaucoma patients ($P < 0.01$). Specifically, the QoL among glaucoma patients who have family history of glaucoma or cataract was significantly lower than patients without family history ($P < 0.05$).

TABLE 1 | Demographic characteristics and clinical information of glaucoma patients and distributions of QoL.

Variables	N	%	QOL (Mean ± SD)
Age (year)			
≤65	89	41.4	42.19 ± 16.39
>65	126	58.6	45.02 ± 13.83
Gender			
Male	98	45.6	43.41 ± 15.74
Female	117	54.4	44.21 ± 14.36
Marital status			
Married	203	94.4	44.20 ± 15.07
Others	12	5.6	37.83 ± 12.13
Educational level			
Junior high school or below	128	59.6	45.67 ± 13.73
Senior high school or above	87	40.5	41.16 ± 16.35*
Monthly income			
<3000RMB	77	35.8	48.05 ± 15.30
3000–6000RMB	98	45.6	41.51 ± 13.40**
>6000RMB	40	18.6	41.47 ± 16.52**
Duration of the disease			
<3years	137	63.7	44.45 ± 14.53
≥3 years	78	36.3	42.79 ± 15.77
With other chronic diseases			
Yes	107	49.8	46.55 ± 15.84
No	108	50.2	41.17 ± 13.62**
Disease types			
glaucoma	136	63.3	46.51 ± 14.32
Glaucoma complicated with Cataract	79	36.7	39.25 ± 15.04**
Number of operations			
0	93	43.3	44.94 ± 14.03
1	84	39.5	43.86 ± 14.30
2	24	11.2	41.29 ± 17.26
3 or more	13	6.0	40.69 ± 21.31
Family history of glaucoma/cataract			
Yes	42	19.5	39.02 ± 15.33
No	173	80.5	45.02 ± 14.69*

*Significant at *P < 0.05 (two-tailed) and **P < 0.01 (two-tailed).*
Values are presented as mean ± standard deviation.

The Correlations of Sleep Disturbance, Resilience, Quality of Life

The results in **Table 2** show the correlation between sleep disturbance, resilience, and QoL. Particularly, the QoL of glaucoma patients was significantly correlated with resilience and sleep disturbance. Specifically, resilience was negatively correlated with their QoL score ($r = -0.375$, $P < 0.01$), while sleep disturbance was positively correlated with their QoL score ($r = 0.46$, $P < 0.01$). In addition, there was a negative correlation between resilience and sleep disturbance among glaucoma patients ($r = -0.268$, $P < 0.01$).

Regression Analysis of Quality of Life, Resilience and Sleep Disturbance in Glaucoma Patients

The forest plot (**Figure 1**) reflected that resilience was found to be inversely correlated with QoL scores. Conversely, sleep disturbance was positively linked with QoL scores. The final regression model explained the variance of 29.9%. The results of $\triangle R^2$ revealed that the differences in QoL explained by each variable block were 5.2, 5.1, 7.1, and 8.7%, respectively. Moreover, sleep disturbance contributed the most to the QoL among glaucoma patients. The final HMR model indicated that when sleep disturbance was added into model 4, the regression coefficient (β) between resilience and QoL increased from −0.306 to −0.226 (**Table 3**). The mediating role of sleep disturbance between resilience and QoL in glaucoma patients was confirmed by Sobel test (−0.306∼−0.226).

Mediator of Sleep Disturbance Between Resilience and Quality of Life

For the indirect effects mediated by sleep disturbance, **Figure 2** demonstrates that sleep disturbance is negatively correlated with resilience (β = −0.26) and positively correlated with QoL (β = 0.34), the results were significant ($P < 0.01$), and there was a good fitting index ($\chi 2/df < 5$, $P < 0.05$, GFI = 0.941, AGFI = 0.902, CFI = 0.969, TLI = 0.992, and RMSEA = 0.038). In addition, the direct impact of resilience on QoL was significant ($P < 0.01$), which was negative (β = −0.39). After adding sleep disturbance to the SEM model, the path coefficient for the association between resilience and QoL decreased significantly (β = −0.29, $P < 0.01$), Therefore, Sleep disturbance is regarded as a mediator in the model. From the results of the bias-corrected and accelerated bootstrap test (a*b = −0.1, BCa95% CI: −0.154∼−0.045), the significant mediating effect of sleep disturbance between resilience and QoL is confirmed. Therefore, we can calculate that resilience not only directly affects QoL, but also indirectly affects QoL through the mediating effect sleep disturbance.

TABLE 2 | The correlations of each continuous variable (*N* = 215).

Variable	Mean	SD	Range	1. QoL	2. Age	3. Resilience	4. Sleep disturbance
1. QoL	43.85	14.97	15∼75	1			
2. Age	66.24	12.53	30∼92	0.119	1		
3. Resilience	30.65	5.73	15∼56	−0.375**	−0.178**	1	
4. Sleep disturbance	22.33	7.99	9∼40	0.460**	−0.039	−0.268**	1

*Significant at *P < 0.05 (two-tailed) and **P < 0.01 (two-tailed).*
A higher QoL score meant the worse QoL among glaucoma patients.

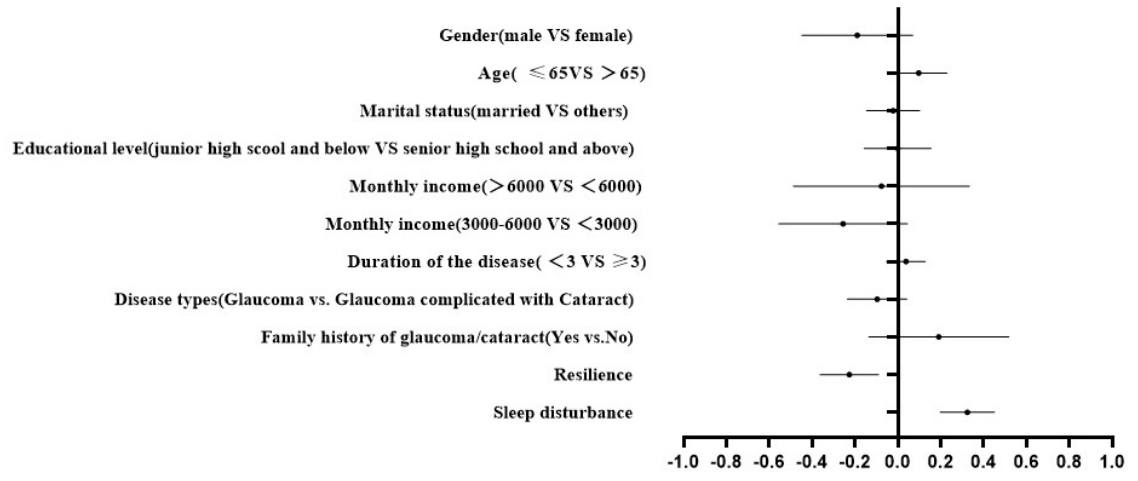

FIGURE 1 | Forest graph of the hierarchical multiple regression analysis of QoL.

TABLE 3 | The hierarchical multiple regression analysis of QoL (*N* = 215).

Variables	Model 1	Model 2	Model 3	Model 4
Block 1 Demographic characteristics				
Gender (Male vs. Female)	−0.032	−0.042	−0.072	−0.112
Age (years) (≤65 vs. >65)	−0.084*	0.145*	−0.075	0.096
Marital status (Married vs. Others)	−0.066	0.026	−0.024	0.021
Educational level (Junior high school or below vs. Senior high school or above)	−0.169*	−0.106	−0.069	−0.055
Monthly income (RMB)				
(>6000 vs. <3000)	−0.093	−0.071	0.001	−0.037
(3000–600 vs. <3000)	−0.169*	−0.177*	−0.127	−0.151*
Block 2 Clinical information				
Duration of the disease (year) (<3 vs. ≥3)		0.020	0.019	0.045
Disease types (Glaucoma vs. Glaucoma complicated with Cataract)		−0.231**	−0.199**	−0.094
Family history of glaucoma/cataract (Yes vs. No)		0.106	0.070	0.093
Block 3 Resilience			−0.306**	−0.226**
Block 4 Sleep disturbance				0.330**
R^2	0.079	0.141	0.213	0.299
Adjusted R^2	0.052	0.103	0.174	0.261
$\triangle R^2$	0.052	0.051	0.071	0.087

**Significant at *P < 0.05 (two-tailed) and **P < 0.01 (two-tailed).*

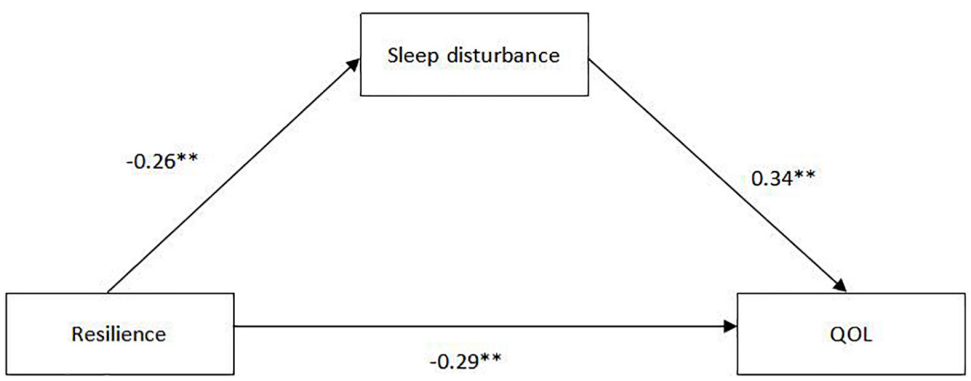

FIGURE 2 | The structural equation modeling of the relationship between resilience and QoL mediated by sleep disturbance.

DISCUSSION

There are few studies on the relationship between QoL and resilience among glaucoma patients. According to our knowledge, this study was the first attempt to examine whether sleep disturbance is a mediating factor between resilience and QoL among patients with glaucoma. Our results demonstrate that the average QoL score for patients with glaucoma was 43.85 ± 14.97, which indicates that the QoL among glaucoma patients in this study was significantly worse than the previous findings in china (28.79 ± 12.74) (31) and in Australia (30.5 ± 13.7) (32). In addition, according to our research results, the resilience of glaucoma patients (30.65 ± 5.73) was lower than that of rheumatoid arthritis patients (41.51 ± 7.07) (33). Therefore it is of upmost importance to improve the QoL for glaucoma patients.

This study revealed a significant direct correlation between resilience and QoL among patients with glaucoma, which was consistent with most previous studies. These studies showed that the QoL among patients with inflammatory bowel disease (34), recurrent coronary artery disease (35), and Parkinson's disease (36) was positively related to their resilience. Considering that the progression of glaucoma symptoms might have a negative impact on mental health, individuals with higher level of resilience may more effectively deal with the pressure brought by having a chronic disease (37). Resilience was found to be a positive psychological factor in this study, which was particularly helpful to mitigate the negative emotions of glaucoma patients and increase their psychological adaptability (38). Compared to people with lower resilience, those who had higher resilience were able to respond to problems energetically when dealing with various pressures (such as medical expenses and disease symptoms). Accordingly, when glaucoma patients faced various symptoms including visual impairment, eye distension and headache, individuals who had higher levels of resilience were more adaptable (39), which eventually improved their QoL.

Our findings illustrate that sleep disturbance has a negative impact on the QoL of glaucoma patients, and contribute the most to the QoL among the variables included this study. Therefore, meaning that glaucoma patients with severe sleep disturbance have a lower QoL. Studies have shown that sleep disturbance has become a major risk factor for the decline of QoL (40). These studies indicate that sleep disturbance reduce the QoL among patients with fibromyalgia (41), chronic kidney disease (42), and lung cancer (43). Glaucoma patients suffer from sleep disturbance due to visual impairment and pain, such as difficulty in falling asleep, waking up early, sleep interruption, difficulty sleeping, and discomfort after waking up (44). Further, sleep disturbance causes autonomic nervous disorders and aggravates glaucoma (45), thereby reducing the Qol of glaucoma patients. These findings are consistent with current the research results (46). Hence, more attention should be paid to the measurement of intraocular pressure during sleep, among patients who are undergoing glaucoma treatment in order to adopt appropriate, and correct treatment, avoid further damage of the optic nerve and visual function, which will ultimately improve the QoL of patients with glaucoma.

Our study reveals that the effect of resilience on improving the QoL of glaucoma patients was mediated by sleep disturbance. The results show that the QoL of glaucoma patients was not only directly affected by resilience, but also indirectly affected by sleep disturbance. Glaucoma patients who reported having higher resilience were less likely to have sleep disturbance, while patients without sleep disturbance had better QoL. Studies have shown that diabetic patients with lower levels of resilience and higher levels of sleep disturbance were, more likely to suffer from depression (47), thereby affecting their QoL. Guopeng Li et al. conducted a survey with pregnant women in China and found that those with high level of resilience usually had better sleep status and were less likely to suffer from sleep disturbance (48). Consequently, their QoL could also be maintained better. Similarly, Yumei Cai et al. pointed out that resilience was negatively correlated with sleep disturbance, which was associated with positive individual health status (24). However, few studies examined sleep disturbance as a mediator between resilience and QOL among glaucoma patients. This finding may be due to: positive psychological factors alleviating the changes of individual neurohormones, resulting in improved sleep. For example, the HPA axis activation of individuals with high resilience could be maintained at an optimal level and therefore these individuals may better could cope with difficulties without excessive panic, uneasiness and depression, so as to avoid psychosomatic disorders, such as sleep disturbance, and improve their QoL (49). Another highly credible explanation is that: positive psychological factors, such as high coping self-efficacy, positive emotions, cognitive flexibility, and realistic optimism, could alleviate sleep disturbance *via* the beneficial effects on physical and mental health. Therefore, individuals with higher level of resilience could better adjust their mental state, actively treat and maintain good sleep quality when facing chronic diseases such as glaucoma, ultimately improving their QoL (50).

The findings of this study have several practical significances. Based on the empirical evidence on the positive effect of resilience on the QoL of glaucoma patients and sleep disturbance mediated in the relationship between resilience and QoL among glaucoma patients, some preliminary suggestions could be drawn. Firstly, attention and efforts of glaucoma patients to improve resilience and QoL might be diverted to promoting sleep quality rather than less easily changing factors such as disease severity (51). Secondly, it is recommended that autonomous relaxation exercises can be used by glaucoma patients, including meditation, yoga and progressive muscle relaxation, which have been proven to normalize intraocular pressure, permanently reduce psychological stress and improve resilience (52, 53). it is suggested that conduct psychological counseling for particularly serious cases in order to improve coping strategies and developed higher resilience (54).

LIMITATIONS

Some limitations of this study should be explained. First, the clinical information collection of glaucoma is not comprehensive.

There is a lack of "disease severity," "drugs" and other clinical information. Second, this study lacks a control group, gathered data using a cross sectional survey, therefore the causal relationship between variables cannot be determined. In order to further confirm the results found in this study, there is a need for longitudinal research. Finally, the sample size of this study is limited and all of participants were undergoing outpatient treatments, which may limit the generalizability of the study.

CONCLUSION

This study found that people with glaucoma have a poor QoL. Improved resilience could improve the QoL of glaucoma patients, while sleep disturbance could reduce the QoL of patients. Furthermore, sleep disturbance mediated the relationship between resilience and QoL, which could reduce the QoL among glaucoma patients. Therefore, measures should be taken to improve the QoL among glaucoma patients and strengthen their resilience training, thus promoting improved QoL among glaucoma patients.

DATA AVAILABILITY STATEMENT

The original contributions presented in this study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by China Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

QP contributed to acquisition and analysis of data, drafting, and revision of the manuscript. BQ was contributed to the acquisition and interpretation of data. KS was responsible for the revision of the manuscript. QC, JF, and SH were responsible for the interpretation of the data and the study design. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by Research Project on Clinical Big Data Management and Diagnosis and Treatment of Glaucoma Patients, Shenyang Science and Technology Bureau, (Grant Number: 20-205-4-042).

ACKNOWLEDGMENTS

All authors are grateful to the participants.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers | Frontiers in Medicine

TYPE Original Research
PUBLISHED 23 November 2022
DOI 10.3389/fmed.2022.1037647

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Ophthalmology, a section of the journal Frontiers in Medicine

RECEIVED 06 September 2022
ACCEPTED 02 November 2022
PUBLISHED 23 November 2022

CITATION
Seo SB and Cho H-k (2022) Deep learning classification of early normal-tension glaucoma and glaucoma suspect eyes using Bruch's membrane opening-based disc photography.
Front. Med. 9:1037647.
doi: 10.3389/fmed.2022.1037647

Deep learning classification of early normal-tension glaucoma and glaucoma suspect eyes using Bruch's membrane opening-based disc photography

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Purpose: We aimed to investigate the performance of a deep learning model to discriminate early normal-tension glaucoma (NTG) from glaucoma suspect (GS) eyes using Bruch's membrane opening (BMO)-based optic disc photography.

Methods: 501 subjects in total were included in this cross-sectional study, including 255 GS eyes and 246 eyes of early NTG patients. BMO-based optic disc photography (BMO overview) was obtained from spectral-domain optical coherence tomography (OCT). The convolutional neural networks (CNN) model built from scratch was used to classify between early NTG and GS. For diagnostic performances of the model, the accuracy and the area under the curve (AUC) of the receiver operating characteristic curve (ROC) were evaluated in the test set.

Results: The baseline demographics were age, 48.01 ± 13.03 years in GS, 54.48 ± 11.28 years in NTG (p = 0.000); mean deviation, −0.73 ± 2.10 dB in GS, −2.80 ± 2.40 dB in NTG (p = 0.000); and intraocular pressure, 14.92 ± 2.62 mmHg in GS, 14.79 ± 2.61 mmHg in NTG (p = 0.624). Our CNN model showed the mean AUC of 0.94 (0.83–1.00) and the mean accuracy of 0.91 (0.82–0.98) with 10-fold cross validation for discriminating between early NTG and GS.

Conclusion: The performance of the CNN model using BMO-based optic disc photography was considerably good in classifying early NTG from GS. This new disc photography of BMO overview can aid in the diagnosis of early glaucoma.

KEYWORDS

Bruch's membrane opening-minimum rim width, Bruch's membrane opening-based disc photography, Bruch's membrane opening overview, deep learning, diagnosis of glaucoma, glaucoma, optical coherence tomography

Introduction

Glaucoma leads to the damage of retinal ganglion cells (RGC) and their axons, resulting in the deficit of retinal nerve fiber layer (RNFL) and the neuroretinal rim (NRR), which ultimately cause visual field (VF) loss (1). In the diagnosis of early glaucoma, early detection of structural change is more essential than detection of a functional defect (2, 3) since detectable structural change may present in advance of functional VF loss (4–6). As structural damage is minimal in early glaucoma or glaucoma suspect (GS) eyes, differentiate early glaucoma from GS is difficult based on traditional fundus photography alone. As a structural test, optical coherence tomography (OCT) is extensively used in clinical settings and is useful in the diagnosis of glaucoma in early stage. Recently, spectral-domain OCT has been used to provide a new parameter, Bruch's membrane opening-minimum rim width (BMO-MRW) along with conventional peripapillary RNFL thickness. Moreover, OCT provides BMO-based disc photography, which is called "BMO Overview" by the software. It shows the BMO-based disc margin with 12 cuts around the optic disc demonstrating each BMO and BMO-MRW at each site (**Figures 1C,F**).

BMO-MRW is the shortest distance between the inner opening of the BMO and the internal limiting membrane (**Figures 1B,E**). BMO-MRW provides a more precise assessment of the NRR than pre-existing ophthalmic parameters (7–10). Latest studies have shown that BMO-MRW demonstrated better diagnostic performance in glaucoma than preexistent NRR parameters (11–13). In our previous study, we reported that BMO-MRW might reveal normal color code classification, whereas the RNFL showed abnormal color code classification in cases of large discs and myopia (14). This previous study of ours suggested the clinical usefulness of BMO-MRW in early glaucoma or GS, particularly in cases of large disc and myopia when the diagnosis is difficult because conventional color code classification of RNFL may display false-positive results. In another our previous studies using the deep learning method, we reported that our deep learning model using the OCT parameters of BMO-MRW, peripapillary RNFL, and color classification of RNFL provided high diagnostic performance in distinguishing early normal-tension glaucoma (NTG) from GS (AUC, 0.966) (15). Interestingly, as a single parameter, BMO-MRW showed higher diagnostic performance (AUC, 0.959) than RNFL alone (AUC, 0.914) or even RNFL with its color code classification (AUC, 0.934) (15). Moreover, BMO-MRW alone showed diagnostic performance similar to that of all three OCT parameters combined. These findings suggest that the BMO-based optic disc assessment may evaluate different aspects of the optic disc compared to the conventional disc assessments in the diagnosis of glaucoma. To our knowledge, there has been no report of a study using BMO Overview in a deep learning model for the diagnosis of glaucoma.

It is more difficult to differentiate glaucoma of early stage from GS or normal subjects than the glaucoma of advanced stage (16–18). As the field of artificial intelligence (AI) is progressing rapidly these days, the deep learning model may be useful to aid clinicians in this circumstances. Many previous studies have used fundus photography in a deep learning model for the diagnosis of glaucoma (19–25). The diagnostic performance in these previous studies using fundus photography varied, with area under the receiver operating characteristic curves (AUC) of 0.82–0.986 (19–25). Nevertheless, discriminating early stage of glaucoma from GS or healthy is challenging, even using deep learning method, and there are very few studies on early glaucoma. These studies did not include only early-stage glaucoma, and thus, the AUC could vary according to the characteristics of the included subjects. Furthermore, it may be more difficult to discriminate glaucoma of early stage from GS than from a normal healthy subjects.

The prevalence of NTG is higher in Asians than in other ethnicities and NTG is the major type of primary open-angle glaucoma (mean of 76.3%) in Asians (26). Nevertheless, previous studies using deep learning methods for distinguishing glaucoma and normal control rarely included NTG, and studies investigating entirely NTG are hardly found except for our previous study (15).

In this retrospective cross-sectional study, we intended to discriminate early NTG from GS using BMO Overview with a CNN model built from scratch. We evaluated the diagnostic performance and the accuracy of our deep learning model based on convolutional neural networks (CNN or ConvNet). We aimed to investigate whether the new BMO-based disc photography could be useful in the diagnosis of early glaucoma using a deep learning model, which has not been evaluated before. Moreover, there are many previous studies using CNN model with conventional optic disc photography or fundus photography, but none using this new BMO-based optic disc photography. Furthermore, there is no consensus or diagnostic standard for interpreting this new BMO-based imaging, and thus, clinicians cannot examine its diagnostic value, but the deep learning model may aid in this task.

Materials and methods

Ethics statement

This retrospective cross-sectional, and observational study was conducted in accordance with the tenets of the Declaration of Helsinki. The present study was approved by the Institutional Review Board (IRB) of Gyeongsang National University Changwon Hospital, Gyeongsang National University School of Medicine. The acquisition of informed consent was exempted from the IRB of Gyeongsang National University Changwon Hospital due to the retrospective nature of this study.

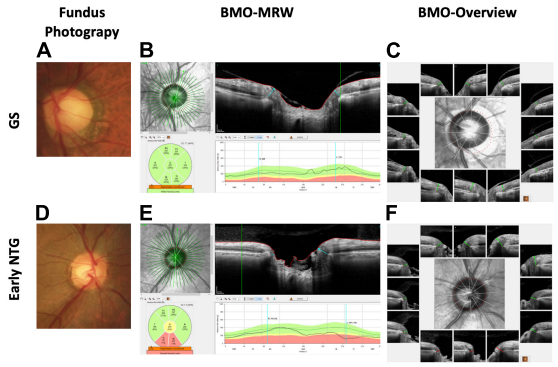


FIGURE 1
Representative cases of GS and early NTG. **(A)** The optic disc of a 70-year-old male GS patient with a spherical equivalent of −1.0 diopter from fundus photography is shown. Note that the optic disc cup seems big based on the clinical disc margin. **(B)** The BMO-MRW map shows normal color code classification in this GS case. The BMO-MRW is the minimal distance between the inner opening of the BMO and the internal limiting membrane. **(C)** BMO Overview, which is BMO-based disc photography from spectral-domain OCT. It shows the BMO-based disc margin (dotted red line) at the center with 12 cuts around the optic disc, demonstrating each BMO and BMO-MRW at each site. Note that the BMO-based disc margin includes part of the PPA, showing that the optic disc cup does not seem as big based on the BMO margin compared to the clinical disc margin. **(D)** Optic disc of a 56-year-old male NTG patient with a spherical equivalent of +1.0 diopter from fundus photography is shown. Note the thinning of the inferotemporal neuroretinal rim of the optic disc. **(E)**. BMO-MRW map shows abnormal color code classification (red) at the inferotemporal and inferonasal sector in this NTG case. **(F)** BMO Overview shows a shorter BMO-MRW (red lines) at the inferior three cuts compared to the other regions with normal lengths (green lines). Note that the optic disc cup seems markedly enlarged at the inferotemporal region based on the BMO-based disc margin (dotted red line). GS: glaucoma suspect; NTG: normal-tension glaucoma; BMO-MRW: Bruch's membrane opening-minimum rim width; OCT: optical coherence tomography; PPA: peripapillary atrophy; RNFL: retinal nerve fiber layer.

Subjects

Among a total of 726 patients, 383 patients with normal-tension glaucoma (NTG) and 343 subjects with GS were evaluated between the period of February 2016 and March 2021 in a glaucoma clinic at Gyeongsang National University Changwon Hospital, for a total of 501 eyes (501 subjects) with either early NTG (246 subjects) or GS (255 subjects) were included in the study. All subjects underwent standard ophthalmic examinations, including Spectralis spectral-domain OCT (Glaucoma Module Premium Edition, Heidelberg Engineering, Germany) and standard automated perimetry (HFA model 840; Humphrey Instruments, Inc, San Leandro, CA, USA). Only those subjects who had reliable BMO-MRW and BMO Overview test images and those who met the diagnostic criteria were included. The assessment of early NTG or GS was made by a single glaucoma specialist (H-k Cho) with consistent criteria of diagnosis.

NTG was defined when a patient had an IOP of ≤21 mmHg without treatment presenting findings of glaucomatous damage in the optic disc and corresponding defect in VF, an open angle examined by gonioscopy, and no other underlying cause for optic neuropathy other than glaucoma (27). Early NTG was defined by a mean deviation (MD) of >−6.0 dB on reliable VF tests. Pre-perimetric glaucoma patients were included in the current study to take in the very early stage of glaucoma. Pre-perimetric glaucoma was determined as cases presenting apparent localized RNFL defects on red-free fundus photography with the OCT map of the RNFL confirming the corresponding RNFL defect, but showing within normal limits on Humphrey standard automated perimetry.

GS was determined as those being followed for suspicious clinical characteristics but not definite for glaucoma, such as suspicious optic disc or RNFL changes; significant systemic, ocular, or family risk factors for glaucoma; or suspicious visual field results and intraocular pressure within the normal limits (defined as <21 mmHg on applanation tonometry). None of the GS subjects were receiving treatment for glaucoma by definition and ocular hypertensive patients under treatment were excluded from this study (28). Ocular hypertensive patients who were not receiving treatment were also excluded from this study by the definitive criteria. If both eyes met the inclusion criteria, only one eye was randomly selected.

The exclusion criteria are as follows: poor images due to eye blinking or poor fixation, history of any intraocular surgery aside from uneventful phacoemulsification, history of optic neuropathies except for glaucoma or an acute angle-closure crisis that could affect the thickness of the BMO-MRW or RNFL (e.g., optic neuritis and acute ischemic optic neuritis), and retinal disorders accompanying retinal swelling or edema and consequent BMO-MRW or RNFL swelling. The fellow eyes of unilateral glaucoma were also excluded from the GS group because of the possible effect on BMO-MRW or BMO-based optic disc assessment. Subjects were not excluded from this study by refractive error, axial length, or optic disc size.

Optical coherence tomography

Imaging of spectral-domain OCT was carried out with Spectralis OCT, Glaucoma Module Premium Edition (Heidelberg Engineering, Germany). Radial B-scans of 24 were acquired for BMO-MRW and BMO Overview. BMO overview image automatically provides BMO boundary points (the red colored dotted line around the optic disc) by the software. Only those images showing well-centered scans and accurate segmentation of the retina and scan quality scores of >20 were taken for the study. Acquirement of data and analysis of OCT scans were conducted employing the individual eye-specific axis

(FoBMO axis), which is the axis between the center of BMO area and the fovea. Applying this FoBMO axis could result in more correct analysis of Garway-Heath sectors taking into consideration of the cyclotorsion of individual eyes and thus, lead to more precise comparison to normative database than the traditional means (7). The BMO-fovea angle is the angle between the center of BMO area and the fovea.

Perimetry

Humphrey Field Analyzer (HFA model 840; Humphrey Instruments Inc, San Leandro, California, CA, United States) were used for perimetry applying a program of Swedish Interactive Threshold Algorithm standard strategy with central 30-2 mode. Reliable VF test were defined with these criteria: a fixation loss of less than 20%; a false-positive rate of <15%; and a false-negative rate of <15%.

Data preprocessing and dataset

A total of 501 eyes (501 subjects) with either early NTG (246 subjects) or GS (255 subjects) were acquired from 501 BMO Overview. The dataset consisted of BMO-based disc photographs including the disc margin (dotted line, **Figures 1C,F**). The BMO-based disc photographs were obtained by cropping the center images from BMO Overview. The cropped center regions were generated with sizes of 438 × 436 pixels using Pillow[1] in Python 3.7.6, as shown in **Figure 2A**. Among the datasets, k-fold cross validation (k = 10) was performed to compensate for the relatively small number of data set. For each fold iteration, there are 399 images are in training set, and 102 BMO-overview images are in test set. The k-fold cross validation was performed using scikit-learn (sklearn.model_selection.KFold).

Convolutional neural networks

A deep neural network (DNN) is a well-known supervised classifier containing multiple layers between the input and output layers (29). A convolutional neural network called CNN, which is a type of DNN, is known to have excellent performance in analyzing images (30). A CNN model for classifying GS and early NTG was built on the Keras Sequential API,[2] written in Python, and running on TensorFlow[3] (31). In the CNN model for image analysis, tensors of a certain shape were taken as input, and the shape of the tensors was determined by the height of the input images, width, and color channels. Our CNN model used input with dimensions of 244 × 244 × 3 and was composed of 4 convolution blocks. Each convolution block contained a maximum pool layer. The first and second hidden layers of the model had 16 and 32 filters with a kernel size of (2, 2), and a rectified linear unit (ReLU) was applied as an activation function. The third and fourth hidden layers had 64 filters with a kernel size of (2, 2), and applied a rectified linear unit (ReLU) as an activation function. The fully connected dense layer of the model had 2 units with a softmax activation function. The batch size was 10, and 100 was taken as the number of epochs in the model. For compiling the model, Nadam (32) was chosen as the optimizer and categorical cross-entropy was selected for the loss function.

Explainable artificial intelligence and Local Interpretable Model-agnostic Explanations

AI with Black-box models produce excellent accuracy and diagnostic performances, but it is hard to figure out why they made such a decision (33). Explainable AI (XAI) is artificial intelligence that can be explainable and understandable the predictions or decisions that made by the AI. The XAI algorithm aims for three things: transparency, interpretability, and explanation (34). The Local Interpretable Model-agnostic Explanations (LIME) algorithm is a well-known technique of XAI explaining the predictions of black-box machine-learning models in an interpretable way. It visualizes sections of the image that the CNN model is using to produce its final prediction. The LIME method was originally proposed by Ribeiro et al. (35). The idea of the LIME is that it is easier to interpret for a black-box model to approximate locally by a simpler glass-box. A new dataset containing permuted data and the associated predictions was created, and was used to train the new model, which was weighted by the proximity of the features in the input image to the feature of interest. As the weights were continuously updated, the fully trained new model was used to interpret and predict. Through LIME, the explanations of the predictions of black-box CNN models can be displayed directly on the image samples. The green-colored region indicates that this part of the image increased the probability for the label, and the red color region indicates a decrease in the probability for the label.

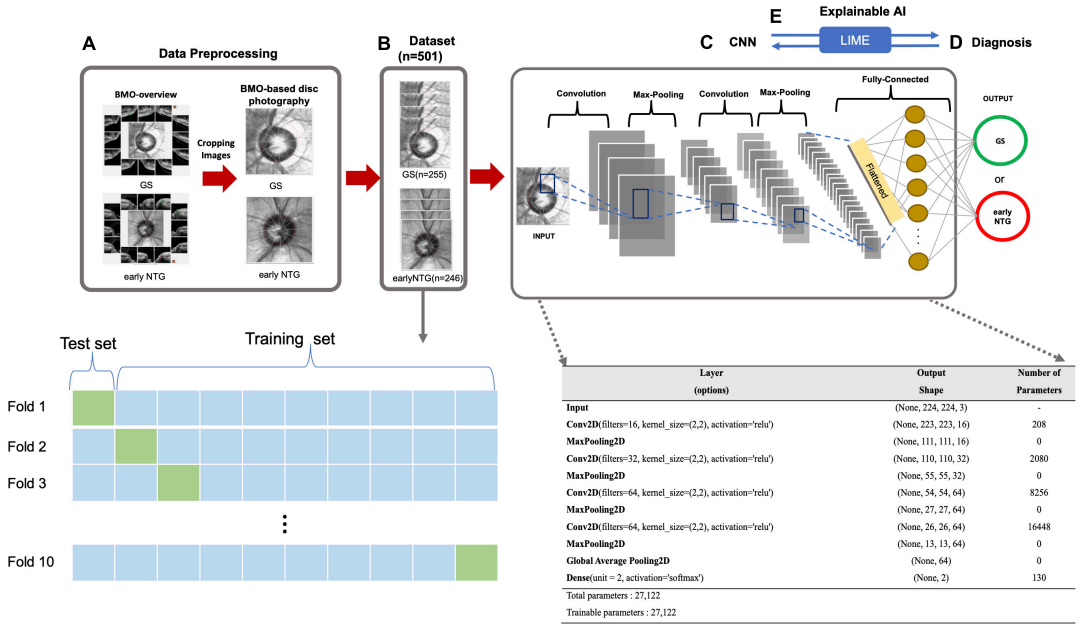
Statistical analysis

Wilcoxon-signed rank test was used to compare the baseline characteristics of the demographic data between the two early NTG and GS and groups for continuous and categorical variables. p-values of less than 0.05 were considered to be statistically significant.

1 https://pillow.readthedocs.io/en/stable/

2 https://keras.io/

3 https://www.tensorflow.org/



Layer (options)	Output Shape	Number of Parameters
Input	(None, 224, 224, 3)	-
Conv2D(filters=16, kernel_size=(2,2), activation='relu')	(None, 223, 223, 16)	208
MaxPooling2D	(None, 111, 111, 16)	0
Conv2D(filters=32, kernel_size=(2,2), activation='relu')	(None, 110, 110, 32)	2080
MaxPooling2D	(None, 55, 55, 32)	0
Conv2D(filters=64, kernel_size=(2,2), activation='relu')	(None, 54, 54, 64)	8256
MaxPooling2D	(None, 27, 27, 64)	0
Conv2D(filters=64, kernel_size=(2,2), activation='relu')	(None, 26, 26, 64)	16448
MaxPooling2D	(None, 13, 13, 64)	0
Global Average Pooling2D	(None, 64)	0
Dense(unit = 2, activation='softmax')	(None, 2)	130
Total parameters : 27,122		
Trainable parameters : 27,122		

FIGURE 2
Diagnostic workflow based on the CNN. **(A)** Data preprocessing. BMO-based disc photographs including disc margin (dotted line) were extracted from BMO Overview with a size of 438 × 436 pixels using the Pillow library in Python. **(B)** Dataset. The dataset contains a total of 2 classes with 501 eyes (501 subjects), either early NTG (246 subjects) or GS (255 subjects). 10-fold cross validation was performed to compensate for the relatively small number of dataset. For each iteration, there are 399 images in training set, and 102 BMO-overview images are in test set. **(C)** CNN. A convolutional neural network structure was built from scratch on the Keras Sequential API (https://keras.io/) for the diagnosis of early NTG. The model input was taken as a tensor with dimensions of (244, 244, 3). The first and second hidden layers of the model had 16 and 32 filters, respectively, with a kernel size of (2, 2), and a ReLU was taken as the activation function. The third and fourth hidden layers had 64 filters with a kernel size of (2, 2) and ReLUs. The model contained a fully connected dense layer with 2 units and softmax was taken as its activation function. The batch size was 10, and the number of epochs was 100 in the model. For compiling the model, Nadam (32) and categorical cross-entropy were taken as the optimizer and loss function, respectively. **(D)** Diagnosis. The AI model diagnosed the input images from the test set as either early NTG or GS. **(E)** Explainable AI. Explainable AI (XAI) is artificial intelligence that can be explainable and understandable the predictions or decisions that made by the AI. LIME was applied to understand the decisions of deep learning black-box models. By comparing with the diagnostic criteria of the clinician, the reliability in the diagnosis of the deep learning model can be given.

Results

Baseline characteristics of the datasets

A total of 501 eyes (501 subjects) out of 726 eyes (726 subjects) were included in the final analysis. The GS group included 255 eyes (255 subjects) out of 343 eyes (343 subjects) and the early NTG group included 246 eyes (246 subjects) out of 383 patients (383 subjects). The mean age of the GS subjects was 48.01 ± 13.03 years, which was significantly younger than that of the early NTG subjects at 54.48 ± 11.28 years ($p < 0.001$). The baseline intraocular pressure (IOP) was not significantly different between GS and early NTG, which was 14.92 ± 2.62 mmHg and 14.79 ± 2.61 mmHg, respectively. The mean deviation (MD) of the GS subjects, −0.73 ± 2.10 dB, was significantly higher than that of the early NTG subjects at −2.80 ± 2.40 dB ($p < 0.001$). The pattern standard deviation (PSD) was significantly lower, and the visual field index (VFI) was significantly higher in the GS subjects than in the early NTG subjects (all $p < 0.001$). The central corneal thickness (CCT) was thicker in the GS subjects than in the early NTG subjects ($p = 0.046$). However, the spherical equivalents (SE) were not significantly different between the GS and NTG subjects ($p = 0.372$). The mean SE was −1.93 ± 2.92 D in GS subjects and it was −1.80 ± 2.84 D in early NTG subjects. In GS group, mild myopia (0 to −2.0 D) consisted of 42.7% (109/255), moderate myopia (−2.0 to −6.0 D) comprised 24.3% (62/255), and high myopia (<−6.0 D) comprised 11.0% (28/255). In NTG group, mild myopia (0 to −2.0 D) consisted of 38.6% (95/246), moderate myopia (−2.0 D to −6.0 D) comprised 24.8% (61/246), and high myopia (<−6.0 D) comprised 11.0% (27/246). Approximately 35% of included subjects had more than moderate myopia (<−2.0 D) in both GS and NTG groups. The details of baseline characteristics are demonstrated in Table 1. Forty-three subjects (17.48%) with pre-perimetric glaucoma were included in the early NTG group.

Table 2 demonstrates the BMO-MRW values of the subjects with early NTG and GS. BMO-MRW values of global region were significantly thicker in the GS group than in the early NTG group (262.58 ± 41.32 and 207.42 ± 44.86 um, respectively,

TABLE 1 Baseline characteristics of glaucoma suspect and early normal-tension glaucoma subjects.

Characteristics	Values		
Diagnosis	**Glaucoma suspect**	**Early NTG**	***P-value***
Number of subjects	255 eyes (255 subjects)	246 eyes (246 subjects)	
Mean Age (year)	48.01 ± 13.03	54.48 ± 11.28	<**0.001**
Female gender (%)	138 (54.11%)	118 (47.96%)	0.147
Family history of glaucoma (%)	16 (6.27%)	27 (10.97%)	0.071
Spherical equivalent (D)	−1.93 ± 2.92	−1.80 ± 2.84	0.372
CCT (um)	546.40 ± 39.18	537.34 ± 60.06	**0.046**
Baseline IOP (mmHg)	14.92 ± 2.62	14.79 ± 2.61	0.624
VFI (%)	98.56 ± 3.94	93.32 ± 6.58	<**0.001**
MD (dB)	−0.73 ± 2.10	−2.80 ± 2.40	<**0.001**
PSD (dB)	2.13 ± 1.33	4.66 ± 2.98	<**0.001**

NTG, normal tension glaucoma; OCT, optical coherence tomography; D, diopters; CCT, central corneal thickness; IOP, intraocular pressure; VFI, visual field index; MD, mean deviation; PSD, pattern standard deviation. Results comparison with GS and early NTG are done with Wilcoxon signed-rank test. Bold font indicates significant *p*-values ($p < 0.05$).

TABLE 2 Brunch membrane opening minimum rim width of glaucoma suspect and early normal-tension glaucoma subjects.

Characteristics	Glaucoma suspect ($n = 255$)	Early NTG ($n = 246$)	*P-value*
BMO-fovea angle°	−5.61 ± 3.22	−6.04 ± 3.27	**0.033**
BMO area (mm^2)	2.45 ± 0.52	2.32 ± 0.59	**0.005**
BMO-MRW G (um)	262.58 ± 41.32	207.42 ± 44.86	<**0.001**
BMO-MRW T	191.42 ± 40.59	162.84 ± 40.18	<**0.001**
BMO-MRW TS	267.62 ± 42.73	207.98 ± 61.80	<**0.001**
BMO-MRW TI	294.68 ± 52.15	192.62 ± 67.87	<**0.001**
BMO-MRW N	275.61 ± 55.89	228.27 ± 58.47	<**0.001**
BMO-MRW NS	291.54 ± 56.98	237.10 ± 63.57	<**0.001**
BMO-MRW NI	320.79 ± 54.85	234.96 ± 64.90	<**0.001**

Values represent mean ± mean deviation. NTG, normal-tension glaucoma. BMO-MRW, bruch's membrane opening-minimum rim width. G, global. T, temporal. TS, superotemporal. NS, superonasal. N, nasal. NI, inferonasal. TI, inferotemporal. Statistical analysis between glaucoma suspect and early NTG for BMO-MRW was done by Wilcoxon signed-rank test. Bold font indicates significant *p* values ($p < 0.05$).

$p = 0.005$). The BMO-MRW values from all six Garway-Heath sectors (temporal, superotemporal, inferotemporal, nasal, superonasal, and inferonasal) were also significantly thicker in the GS group than in the early NTG group (all $p < 0.001$). The BMO area in the GS group (2.45 ± 0.52 mm^2) was significantly larger than that of the early NTG group ($p = 0.005$). Interestingly, the BMO-fovea angle was significantly different between the early NTG and GS groups ($p = 0.033$). The mean BMO-fovea angle was −5.61 ± 3.22° in the GS group and −6.04 ± 3.27° in the early NTG group. This finding indicates that the optic disc was located further away from the fovea in the early NTG group than in the GS group since the BMO-fovea angle is the angle between the BMO center and the fovea. Representative fundus photography of early NTG and GS are demonstrated in **Figures 1A,D**, respectively.

Overview of convolutional neural networks model for classifying glaucoma suspect and early normal-tension glaucoma

A CNN model for the diagnosis of early NTG with a convolutional neural network structure on the Keras Sequential API (see text footnote 2) was implemented, as shown in **Figure 2**.

BMO-based disc photographs including the disc margin (dotted line) from 501 eyes (501 subjects) with either early NTG (246 subjects) or GS (255 subjects) were collected in a glaucoma clinic at Gyeongsang National University Changwon Hospital. The BMO-based disc photographs of 246 early NTG and 255 GS were obtained by cropping the center images from BMO Overview, as shown in **Figure 2A**. The dataset contained a total of 501 eyes (**Figure 2B**). 10-fold cross validation was performed to compensate for the relatively small number of data set. For each iteration, there are 399 images are in training set, and 102 BMO-overview images are in test set. The architecture of the CNN built from scratch is demonstrated in **Figure 2C**. A convolutional neural network structure was built from scratch on the Keras Sequential API (see text footnote 2) for the diagnosis of early NTG. The model input was taken as a tensor with dimensions of (244, 244, 3). The first and second hidden layers of the model had 16 and 32 filters, respectively, with a kernel size of (2, 2), and a ReLU was taken as the activation function. The third and fourth hidden layers had 64 filters with a kernel size of (2, 2) and ReLUs. The model contained a fully connected dense layer with 2 units and softmax was taken as its activation function. The batch size was 10, and the number of epochs was 100 in the model. To compile the model for each CNN, Nadam (32) and categorical cross-entropy were chosen as the respective optimizer and loss function. The AI model diagnosed the images and output as either GS or early NTG, as shown in **Figure 2D**.

Diagnostic performances of the artificial intelligence model for discriminating glaucoma suspect and early normal-tension glaucoma

To evaluate the diagnostic performance of the AI model for discriminating early NTG and GS, accuracy, loss, and AUC of the receiver operating characteristic curve over the test set per

A

Fold	Loss	Accuracy (%)
1	0.2737	90.20
2	0.1719	94.00
3	0.2818	92.00
4	0.3044	88.00
5	0.5315	82.00
6	0.1153	98.00
7	0.7100	84.00
8	0.3103	92.00
9	0.1174	98.00
10	0.2570	92.00
Average	0.3073	91.02

B

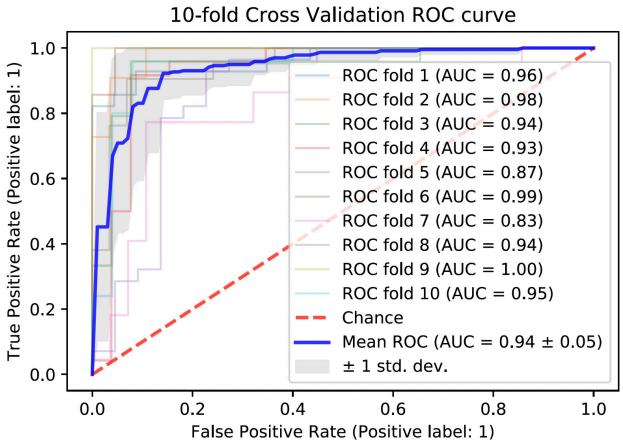


FIGURE 3
Accuracy and loss per fold and areas under the curve (AUC) for the receiver operating characteristic curves (ROC) achieved by the AI model for classifying GS and early NTG. **(A)** Accuracy and loss per each fold were evaluated. The average loss was 0.3073 (0.1153–0.7100), and average accuracy was 91.02% (82.00–98.00%). **(B)** The area under the curve (AUC) for the receiver operating characteristic curve (ROC) were calculated for the CNN model with 10-fold cross validation. The mean AUC value was 0.94 ± 0.05 (0.83–1.00). GS: glaucoma suspect; NTG: normal-tension glaucoma.

fold were calculated, as shown in Figure 3. In Figure 3A showed the losses and accuracies for the CNN model with each fold from 1 to 10. In each fold, the number of epochs was 100. The range of loss was from 0.1153 to 0.7100, and the mean loss of the model was 0.3073. The accuracy for the model ranged from 0.82 to 0.98, and the mean average accuracy was 0.9102. The area under the curve (AUC) for the receiver operating characteristic curve (ROC) were calculated for the CNN model with 10-fold cross validation. The CNN model achieved the average AUC of 0.94 ± 0.05 for classifying early NTG and GS in the test set with 10-fold cross validation, as shown in Figure 3B. The highest AUC was 1.00 and the lowest AUC was 0.83.

Inferotemporal regions were important in classifying glaucoma suspect and early normal-tension glaucoma

The black-box deep learning models are generally hard to explain why those made such predictions although they produce great performances and accuracies. The Local Interpretable Model-agnostic Explanations (LIME), a well-known technique of XAI, was used to understand the predicting its final diagnostic classification (i.e., GS or early NTG) of the CNN model in an interpretable way. The LIME algorithm reveals the area of the images that the CNN model used to extract spatial and temporal features. Inferotemporal regions of the cupping or NRR in the optic disc were considered to be predominantly influential in classifying the final diagnosis (Figure 4). Representative cases of GS and early NTG are shown in Figure 4. It shows the extraction of the top 1 and top 3 features, which are the grounds for CNN models to classify GS or early NTG. The green-colored region indicates that this part of the image increased the probability for the label, and the red-colored region indicates a decrease in the probability for the label. In the case of GS, it was mainly determined by the area around the inferotemporal region of the neuroretinal rim (see Figure 4A, green). In the case of early NTG, it was classified as early NTG by the inferotemporal region of the cupping and neuroretinal rim, as shown in Figure 4B (green).

Discussion

To our knowledge, the current study was the very initial to use BMO-based optic disc photography to discriminate early NTG from GS in a single ethnic group of Asians, where NTG is more prevalent. We found that the diagnostic performance of our CNN model built from scratch was excellent, with the mean AUC of 0.94 (0.83 – 1.00) and the mean accuracy of 0.91 (0.82 – 0.98) in discriminating early NTG from GS. Considering that it is more difficult to classify glaucoma of early stage from GS than glaucoma of advanced stage from normal controls, the results of our study are quite remarkable. Moreover, since there is no consensus or diagnostic standard for interpreting this new BMO-based optic disc photography, clinicians cannot investigate its diagnostic value in the field of glaucoma. Our CNN model has performed this task instead, which will be useful for future research and application in clinical settings.

A previous review article by Sengupta et al. (36) reported glaucoma detection results using deep learning methods with fundus images. The AUC indicating diagnostic performance

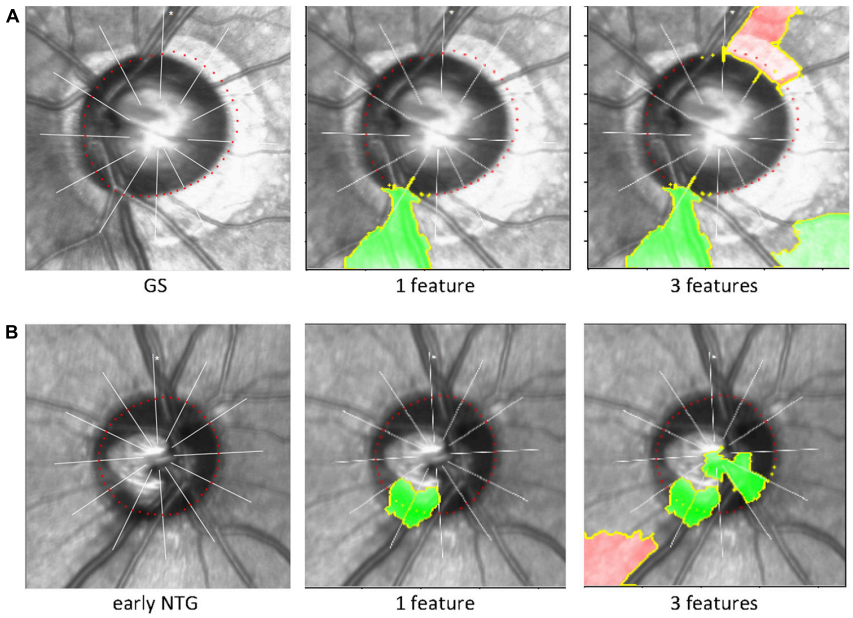


FIGURE 4
Explainable artificial intelligence and Local Interpretable Model-agnostic Explanations. The LIME algorithm, a well-known technique of explainable AI explaining the predictions of black-box machine-learning models, was used to reveal the area of the image that our CNN model used to extract spatial and temporal features and predict its final classification of the diagnosis (i.e., GS or early NTG). The inferotemporal region of the cupping or neuroretinal rim in the optic disc was considered to be predominantly influential in classifying the final diagnosis. Representative cases are shown for GS and NTG. **(A)** For GS, our CNN model identified the inferotemporal region of the neuroretinal rim (green). **(B)** For early NTG, the inferotemporal region of the cupping and neuroretinal rim were identified for classification (green). Note that the region recognized by LIME (green) includes the BMO points (the red colored dotted line around the disc) in each region for classification of either early NTG or GS. AI: artificial intelligence; LIME: local interpretable model-agnostic explanations; GS: glaucoma suspect; NTG: normal-tension glaucoma.

varied among studies from 0.82 to 0.94, and the highest one was 0.986 (36). Most of these studies used the same CNN model as that in the present study. Some studies showed much lower AUCs than in our study such as 0.82 (24), 0.831 (19), and 0.838 (23). Most of the studies showed AUCs such as 0.923 (25) and 0.926 (37), lower than that in our study, not using CNN, but using Autoencoderut and the feedforward neural network, respectively, and 0.945 (20) using the CNN. Only one study showed a higher AUC than the current study at 0.986 (21) using the CNN. However, these studies did not evaluate only the early stage of glaucoma, which is more difficult to diagnose than advanced stages of glaucoma (16–18). Furthermore, it is more difficult to discriminate glaucoma of early stage from GS than from normal subjects. Considering that our study included only early glaucoma in the discrimination from GS, the AUC results of our CNN model showed fine diagnostic performance. Furthermore, none of these studies included solely NTG for glaucoma nor classified the subtypes of glaucoma as NTG. Thus, the present study has a unique meaning that could add to the existing literature.

Since there is no consensus or diagnostic standard for interpreting this new BMO-based overview imaging yet, clinicians cannot evaluate its diagnostic ability and its value in the field of glaucoma diagnosis. Our newly developed CNN model was able to perform this task and showed that the diagnostic performance of this new BMO-based disc photography was relatively comparable or superior to conventional fundus photography used in most previous studies for the detection of glaucoma. The present study has another significant meaning in this aspect.

BMO-MRW and its BMO overview from spectral-domain OCT have become widely available to clinicians and offer merits rather than conventional optic disc analysis measurements (11–13). BMO-MRW presents a geometrically more precise evaluation of the NRR than preexistent examinations (7–10). BMO-MRW has been reported to be advantageous in correctly reflecting the amount of NRR tissue in the optic disc (38). All of the baseline BMO parameters including the BMO-fovea angle and BMO area showed significant differences between the GS and early NTG groups in our study. The BMO-fovea angle was significantly larger in the early NTG group ($-6.04 \pm 3.27°$) than in the GS group ($-5.61 \pm 3.22°$) ($P = 0.033$). It is a somewhat interesting finding because it means that the center of the optic disc defined as the BMO-based disc margin showed a greater angle from the macula in the early NTG group than

in the GS group. Acquirement of data and analysis of OCT were carried out in accordance of the individual eye-specific axis (FoBMO axis), which is the axis between the center of BMO area and the fovea. Using the FoBMO axis could result in a more precise analysis of Garway-Heath sectors regarding the cyclotorsion of individual eyes and more correct comparison with normative dataset than the traditional manner (7). The BMO-fovea angle is the angle between the center of BMO area and the fovea. There has been no previous report regarding the relationship between the BMO-fovea angle and glaucoma, especially in the early stage of glaucoma. The relative location of the optic disc from the fovea is different in each individual and it may possibly affect the development of glaucoma. Retinal nerve fibers or RGC axons could be more stretched and cause more tension in optic discs with a greater angle from the fovea than those with a lesser angle from the fovea. Thus, there could be more conformational change in the optic disc at the lamina cribrosa level in those with a greater BMO-fovea angle than in those with a lesser BMO-fovea angle. However, the spherical equivalents were similar between the GS (-1.93 ± 2.92 D) and the early NTG (-1.80 ± 2.84 D) groups ($P = 0.372$), and patients with relatively mild myopia were included in both groups. Thus, the difference in the BMO-fovea angle between the two groups was not thought to be due to the differences in myopia patients in each group. The association between the BMO-fovea angle and its effect on glaucoma needs to be confirmed in further studies.

The BMO area was significantly larger in the GS group (2.45 ± 0.52 mm^2) than in the early NTG group (2.32 ± 0.59 mm^2) ($P = 0.005$). This may be because a large optic disc with a large cup is frequently considered GS (39–42). The BMO-MRW from the global region and all 6 Garway-Heath sectors according to the FoBMO axis were significantly different between the GS and the early NTG group (all $P < 0.05$). The BMO-MRW was significantly thinner in the early NTG group than in the GS group, which indicates glaucomatous changes in the early NTG group and was also reflected in all BMO-MRW regions. The significant difference in all BMO-based parameters including the BMO-fovea angle, BMO area, and BMO-MRWs between the GS and early NTG groups may partly suggest the usefulness of BMO-based assessment in the diagnosis of early glaucoma.

A discrepancy between the clinical disc margin based on fundus photography and the BMO-based disc margin was noted in our study. It has been described in several previous studies, and initially by Chauhan et al. (7, 11, 14). The discrepancy was also noted in our previous study, "Characteristics of Patients Showing Discrepancy Between Bruch's Membrane Opening-Minimum Rim Width and Peripapillary Retinal Nerve Fiber Layer Thickness" (14). In this previous study, we found that the BMO-MRW may show normal color code classification, while the RNFL is abnormal in GS subjects, especially in patients with large discs and myopia. The discrepancy between the clinical and BMO-based disc margin, in turn, gave rise to discrepancies in the color code classification between the BMO-MRW and the RNFL. BMO-based disc margin takes peripapillary atrophy (PPA) into account. Changes in the optic disc and PPA in myopic eyes were recently described by Sung et al. (43). They found that the morphologic features of the optic nerve head were different based on the β-PPA microstructure in highly myopic eyes (43). Optic nerve head morphology varies among individuals, as does β-PPA. Some patients with β-PPA have basement membrane and some do not. BMO-based disc margin usually includes PPA without BM within the BMO area, which is the BMO-based optic disc area (**Figure 1C**, GS). This difference in the assessment of the optic disc margin actually affects the assessment of neuroretinal tissue or the neuroretinal rim, which is important in glaucoma diagnosis. The neuroretinal rim seems thinner in fundus photography based on a clinical disc margin without PPA (**Figure 1A**, GS) than the neuroretinal rim from BMO-based disc photography based on a BMO-based disc margin (**Figure 1C**, GS), especially in the inferotemporal region with a large PPA in the representative case of GS. Since the variability of optic nerve head morphology and PPA among individuals is partly considered in BMO-based disc photography or BMO-based disc assessment, we assume that the diagnostic performance may be better than conventional assessment in our studies series (14, 15), including this one. Although BMO overview is a black-and white image and does not directly provide values of NRR width (BMO-MRW), it shows BMO-based disc margin considering PPA. Therefore NRR in accordance with BMO-based disc margin can be estimated just like conventional disc photography. Moreover, BMO overview, in fact, provides both clinical and BMO-based disc margin for diagnostic information, which may be more beneficial than conventional disc photography.

Considering the relatively high prevalence of myopia in Asians (26), certain proportion of myopic subjects were included in the present study. Approximately 35% of included subjects had more than moderate myopia (<-2.0D) in both GS and NTG groups in this study. Although we did not exclude any subjects by refractive error or axial length, those high myopic patients whose images were too bad for accurate identification of BMO, and thus, cannot provide accurate BMO-based disc margin were excluded. Several recent studies reported better diagnostic performance of BMO-MRW than conventional peripapillary RNFL thickness in myopic glaucoma patients (44–46). In this regard, our study results suggest that our CNN model using BMO overview may be useful not only in general population, but also in population including considerable proportion of moderate myopia.

We used the LIME algorithm to evaluate the location our CNN model used to classify either GS or early NTG. We confirmed that our CNN model identified the proper region of the optic disc to discriminate between GS or early NTG. Our CNN model identified the inferotemporal

region of the optic disc with early NTG with thinning of the inferotemporal neuroretinal rim in the representative case (green area, **Figure 4**). This may indicate the validity and reliability of the present study since early glaucomatous changes are considered to be initiated in the inferotemporal region of the optic disc (47).

The present study had several limitations. First of all, the retrospective nature of the present study has its potential limitation. Only those who underwent BMO-MRW and BMO overview imaging and had reliable quality in both test images were included in the current study. The influence of such selection of subjects on our results is unknown. Another one is that it was a hospital-based study conducted at a referral national university hospital of the province, and thus, not a population-based design study. Those subjects included in the present study might not represent the whole population. In addition, the current study included only Korean subjects. Our study results regarding NTG, may not apply to other ethnic populations or other glaucoma types. One of the limitations is the relatively small size of sample in the current study that should be considered. Nevertheless, more than 500 subjects with early NTG and GS out of more than 720 subjects were included in the present study and this number was considered to be sufficient to train and test diagnostic performance to distinguish a single disease from single-device data. In order to compensate for the relatively small number of data set, we performed k-fold cross validation (k = 10). Through this cross-validation process, all observations (n = 501) were used for both training and test, and each observation was used for test exactly once. Therefore, this may be enough to compensate for the limitation of the small dataset, and the results were also considered to be quite good (the mean AUC = 0.94 ± 0.05).

Moreover, the diagnosis discrimination between early NTG and GS included in the dataset was made by one glaucoma specialist (H-k Cho) for more solid and consistent diagnostic standards. Different ophthalmologists may not always draw the same glaucoma diagnosis decision and not all studies were evaluated solely by glaucoma specialists. Baseline characteristics including the VF global indices and all BMO parameters, which are not available in very large datasets of more than thousands of subjects, were also inspected in the present study. Therefore, our data may provide more reliable and consistent results than other deep learning studies with larger numbers of subjects.

In conclusion, the performance of our CNN model using BMO-based optic disc photography from OCT was considerably great in classifying early NTG from GS. This new disc photography of BMO overview can aid in the diagnosis of glaucoma other than conventional disc photography. Our CNN model may be useful in clinical setting for the diagnosis of early glaucoma, which is more difficult than that of advanced glaucoma. A further multi-center study with larger patient numbers is needed to reach ultimate conclusions.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Institutional Review Board (IRB) of Gyeongsang National University Changwon Hospital, Gyeongsang National University School of Medicine. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

Both authors contributed to the design of the study conducted the study, data collection, analysis, management, interpretation, and prepared the manuscript.

Funding

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government, MSIP; Ministry of Science, ICT and Future Planning (2020R1G1A1A0100746911) to SS.

Acknowledgments

We thank Sang Soo Kang for scientific advice and discussion.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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frontiers | Frontiers in Medicine

TYPE Review
PUBLISHED 15 December 2022
DOI 10.3389/fmed.2022.1020993

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Ophthalmology, a section of the journal Frontiers in Medicine

RECEIVED 16 August 2022
ACCEPTED 28 November 2022
PUBLISHED 15 December 2022

CITATION
Martucci A, Mancino R, Cesareo M, Pinazo-Duran MD and Nucci C (2022) Combined use of coenzyme Q10 and citicoline: A new possibility for patients with glaucoma. *Front. Med.* 9:1020993. doi: 10.3389/fmed.2022.1020993

Combined use of coenzyme Q10 and citicoline: A new possibility for patients with glaucoma

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Glaucoma is the leading cause of irreversible blindness worldwide. Several risk factors have been involved in the pathogenesis of the disease. By now, the main treatable risk factor is elevated intraocular pressure. Nevertheless, some patients, whose intraocular pressure is considered in the target level, still experience a progression of the disease. Glaucoma is a form of multifactorial ocular neurodegeneration with complex etiology, pathogenesis, and pathology. New evidence strongly suggests brain involvement in all aspects of this disease. This hypothesis and the need to prevent glaucomatous progression led to a growing interest in the pharmacological research of new neuroprotective, non-IOP-lowering, agents. The aim of this paper is to report evidence of the usefulness of Coenzyme Q10 and Citicoline, eventually combined, in the prevention of glaucomatous neurodegeneration.

KEYWORDS

coenzyme Q10 (CoQ10), citicoline, glaucoma, neuroprotection, retinal ganglion cell death

Introduction

Glaucoma, the leading cause of irreversible blindness worldwide, is characterized by progressive optic nerve (ON) degeneration due to retinal ganglion cells (RGCs) death. This causes characteristic ON changes and corresponding visual field defects (1–5).

Several risk factors have been involved in the pathogenesis of the disease. Understanding the molecular and cellular changes causing glaucomatous neurodegeneration includes new and path-breaking investigations on neuroinflammation and neuroprotection. Immune response, oxidative stress and gene expression are considered possible pathogenetic mechanisms of glaucoma (6–12).

Although glaucoma is a multifactorial disease, by now, the main treatable risk factor is elevated intraocular pressure (IOP). Several clinical studies reported the importance of lowering IOP in glaucomatous patients. The Ocular Hypertensive Treatment Study reported the efficacy in preventing the onset of the disease at 5 years in 50% of healthy individuals with elevated IOP (13). The Collaborative Initial Glaucoma Treatment Study observed that both surgery and IOP-lowering medications significantly reduce perimetric progression (14). Moreover, the Collaborative Normal-Tension Glaucoma Study showed that a reduction of the IOP by 30% reduced the incidence of visual field progression in a significant percentage of patients affected by normal-tension glaucoma (15). The lowering of IOP positively influences the risk of developing glaucoma and the progression of the existing disease. However, IOP alone does not explain all the risks (16). This supports the hypothesis that other risk factors independent from IOP are involved in glaucomatous degeneration.

Several studies have shown a strong correlation between visual field damage and visual disability in patients with glaucoma, even in the early stages of the disease. Visual impairment due to glaucoma affects normal daily activities required for independent living, such as driving, walking, and reading. Decreased visual functioning due to glaucoma has many disabling consequences in patients' daily lives that, in turn, alter their quality of life (17).

New evidence strongly suggests brain involvement in all aspects of glaucoma (18). Studies using magnetic resonance imaging (MRI) have shown that the disease extends beyond the eye, altering the entire visual pathway (19, 20), indicating a connection with other neurodegenerative (17, 21–23) and mitochondrial diseases (24) as well as with disconnection syndromes (25, 26). A recent paper showed that patients with Primary Open Angle Glaucoma (POAG) exhibit a whole-brain structural reorganization that involves a variety of brain regions that take part in visual processing, motor control, and emotional/cognitive tasks. Additionally, it has been recognized a specific pattern of brain structural changes in relation to POAG clinical severity (27) that possibly justifies the glaucoma-induced functional and daily living disability (28, 29).

The need to prevent glaucomatous progression led to a growing interest in the pharmacological research of new neuroprotective, non-IOP-lowering, agents (30, 31). Citicoline and Coenzyme Q10 (CoQ10) are among the most studied and used in clinical practice for some forms of neurodegeneration. The neuromodulatory and neuroprotective properties of citicoline were extensively investigated either *in vivo* or *in vitro*. In addition, several studies supported the neuroprotective effects of CoQ10 in experimental models of ocular neurodegeneration (32).

The aim of this paper is to report evidence of the usefulness of CoQ10 and Citicoline, eventually combined, in the prevention of glaucomatous neurodegeneration.

Coenzyme Q10

Coenzyme Q10 is also known as ubiquinone as it is a coenzyme family that is ubiquitous in animals and most bacteria. Being an electron carrier from complexes I and II to complex III, CoQ10 has a fundamental role in the production of the adenosine triphosphate (ATP), but it is also an important antioxidant that protects lipids, proteins, and DNA from oxidative stress. Due to its properties, CoQ10 has been used for a long time to treat many diseases such as Leber hereditary optic neuropathy, cerebral ischemia, Parkinson's disease, and Huntington's disease (33).

Coenzyme Q10 activity has been extensively studied. *In vitro*, CoQ10 prevented the activation of the optic nerve's (ON) astrocytes induced by hydrogen peroxide. It significantly decreased two well-known processes that activate during oxidative stress: the Superoxide dismutase 2 (SOD2) and Heme oxygenase-1 (HO-1) protein expression. Hence, CoQ10 was able to prevent mitochondrial damage and the decline of ATP production (34).

Coenzyme Q10 appears an effective therapy in preventing RGCs apoptosis and loss in animal models. Intraocular administration of CoQ10 avoided RGCs death by apoptosis through the inhibition of mitochondrial depolarization by preventing the formation of the mitochondrial permeability transition pore (PTP) and reducing the glutamate increase (35). Similarly, the topical administration of CoQ10 0.1% significantly reduced staurosporine (SSP)-induced RGCs apoptosis in a rat model (36). The same effect on RGCs was reported in a model of transient ischemia where the topical administration of CoQ10 and vitamin E α–tocopherol polyethylene glycol succinate (TPGS) reduced retinal damage and prevented RGCs death possibly by inhibiting the PTP formation and cytochrome c activation. The ability of CoQ10 of reducing the accumulation of extracellular glutamate is considered one of the mechanisms underlying the protective effect on RGCs (37–39).

Moreover, in a surgically induced ocular hypertension (OHT) experimental model, Davis et al. showed a significant neuroprotective effect on RGCs using Detection of Apoptotic Retinal Cells (DARC) on a unilateral model in Adult Dark rats treated with CoQ10/TPGS micelles (40). Oral supplementation of CoQ10 was also shown to significantly increase survival of RGCs, decrease SOD-2 and HO-1 protein expression, and inactivate the astroglial and microglial cells in an animal model of OHT and in glaucomatous DBA/2J mice (41, 42). Efficacy data were not only reported in animals. In humans, the topical application of 2 drops per day of CoQ10 and vitamin E TPGS, in addition to the β-blocker monotherapy, significantly improved the visual-evoked potential (VEP) response in glaucomatous patients after 6–12 months of treatment compared to those only treated with IOP lowering medications (43).

Coenzyme Q10 bioavailability is extremely variable, and it may depend on the dosage or the delivery strategies. Achieving an optimal CoQ10 concentration is fundamental to reaching the clinical effect. Nutritional replenishment of CoQ10 requires a higher level than is available in most food. The normal level in blood is around 1 μg/ml. To increase the concentration significantly requires at least 100 mg/day which can increase the level in the blood to around 2 μg/ml or more. An increase to 2 μg/ml in the blood can be therapeutic for various conditions; this may indicate that a high blood level is needed to get CoQ10 into deficient tissues (44). Emulsified formulation and plasma lipid profiles are important factors for the absorbance of CoQ10 (38). Interestingly, a novel time-released formulation based on Miniactives® showed to be safe and to increase plasma concentration of CoQ10 during the treatment (38). Consequently, it has been reported to be a promising way to deliver the molecule (45).

Citicoline

Citicoline (cytidine-5′-diphosphocholine) is an endogenous intermediary compound in the synthesis of phospholipids' membranes, such as phosphatidylcholine. Citicoline contributes through the multifactorial mechanism of action and intervening in several metabolic pathways, including phospholipid homeostasis, mitochondrial dynamics, as well as cholinergic and dopaminergic transmission, in the complex mechanism of visual transmission (46).

Evidence of its ability to reduce glutamate-mediated excitotoxicity and oxidative stress by boosting neurotrophin levels and supporting mitochondrial activity endorsed the use of citicoline in neurodegenerative diseases (47).

Oral citicoline has been reported to increase the release of dopamine and norepinephrine and its efficacy has been proved in several neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, as well as in ischemic and traumatic brain injury. The possible neuro-enhancing effect, possibly due to the dopamine increase, justifies the improvement of visual field and electrophysiological test results obtained in glaucomatous patients (46, 47).

Oral citicoline is usually well absorbed and, after its transformation into choline and cytidine in the intestinal wall and liver, it crosses the blood-brain barrier. Hence, supplies the metabolic precursors of phospholipids and participates in the synthetic pathways of nucleic acids, proteins, phosphatidylcholine, sphingomyelin, cardiolipin, and acetylcholine, the main neurotransmitter of the cholinergic system which modulates visual processes. Beyond this, citicoline acts as a rescue recourse for cellular membrane components (46, 48).

Literature suggests that citicoline can reduce the pro-apoptotic effects and synaptic loss in neural tissues. Citicoline's ability in maintaining the proper acetylcholine metabolism and the proper levels of sphingomyelin make it a good candidate for supporting the RGCs' axonal function and consequently enhancing their survival (48). Additionally, this antiapoptotic effect seems to be also connected to the activity of the mitochondrial-dependent cell death mechanism. Indeed, citicoline prevents ischemia-induced tissue increase of free fatty acids and decreases infarct volume and brain oedema (49, 50).

Studies using optical coherence tomography showed that citicoline prevents the loss in the average retinal nerve fiber layer in glaucoma patients (47). Therefore, citicoline may have a significant impact on slowing glaucoma progression, suggesting a potential neuroprotective effect (47, 51).

Overall, these data make citicoline a candidate for the treatment of glaucomatous neurodegeneration.

Combined use of CoQ10 and citicoline: A new strategy in glaucoma treatment

Glaucoma is an extremely complex disease; therefore, it is now evident that the treatment should be targeted at different aspects of the disease, possibly by combining several molecules. However, prescribing several medications may affect the quality of life of the patients who must follow complex, and sometimes, expensive treatments. To overcome these difficulties, new combined products have been produced (**Figure 1**).

Nowadays, CoQ10 and Citicoline are among the most used molecules in glaucoma for neuroprotection. The two molecules act on different pathways leading to glaucomatous neurodegeneration. As a consequence, it is possible to speculate that CoQ10 and citicoline may have a complementary or a synergic effect as they act on different pathogenetic targets in glaucoma (**Figure 2** and **Table 1**).

Interestingly, a recent paper highlighted the complete biocompatibility of citicoline, CoQ10, and vitamin B3 tested using a viability MTT test. When the compounds were co-administered at the highest concentration tolerated by the cells (10 μM), in basal conditions, the biocompatibility was preserved, and the cell viability (hypothalamic HypoE22 cells), in all pharmacological treatments, was always > 70% compared to the untreated cells (32).

Effect on Bcl family proteins regulation

An experimental model of progressive degeneration of RGCs, induced by partial ON crush, produced a selective loss of RGCs, similar to glaucoma. Neurons in fact, although their axons were not acutely damaged, degenerated due to pro-apoptotic environmental conditions produced by the initial injury. The administration of citicoline was effective in rescuing RGCs possibly increasing retinal expression of the apoptotic regulating protein Bcl-2 as well as acting as a BDNF mimic (52).

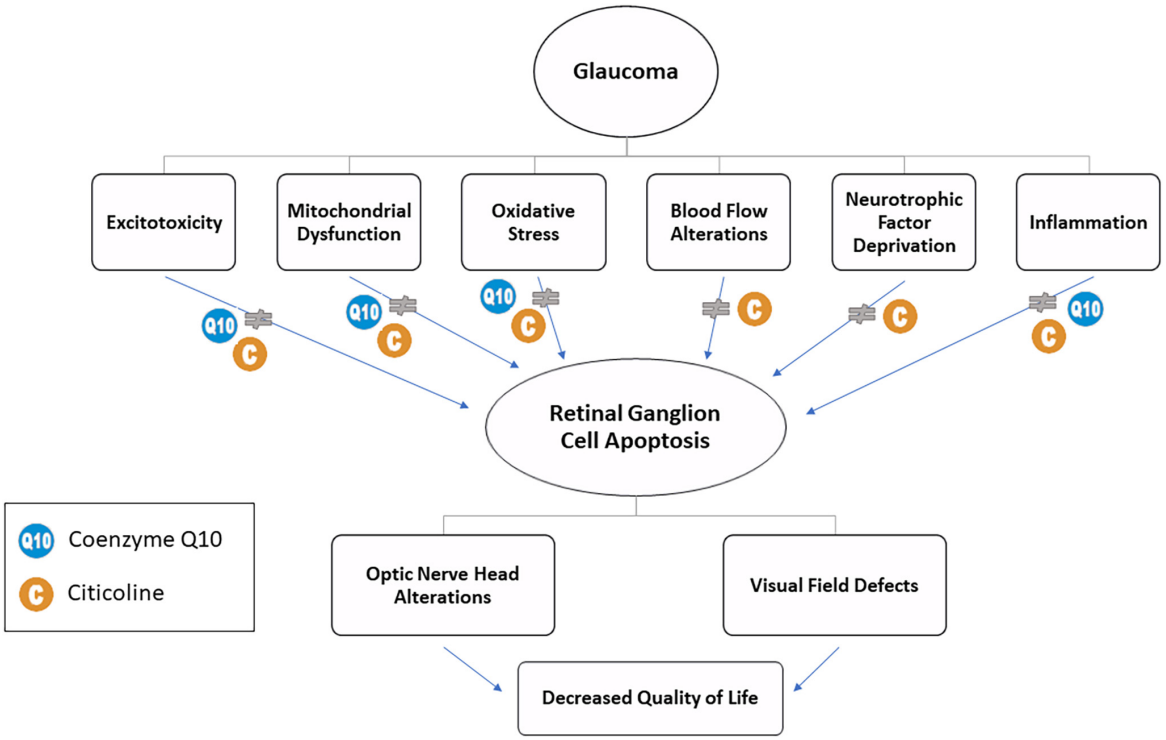

FIGURE 1
The involvement of citicoline and CoQ10 in glaucoma.

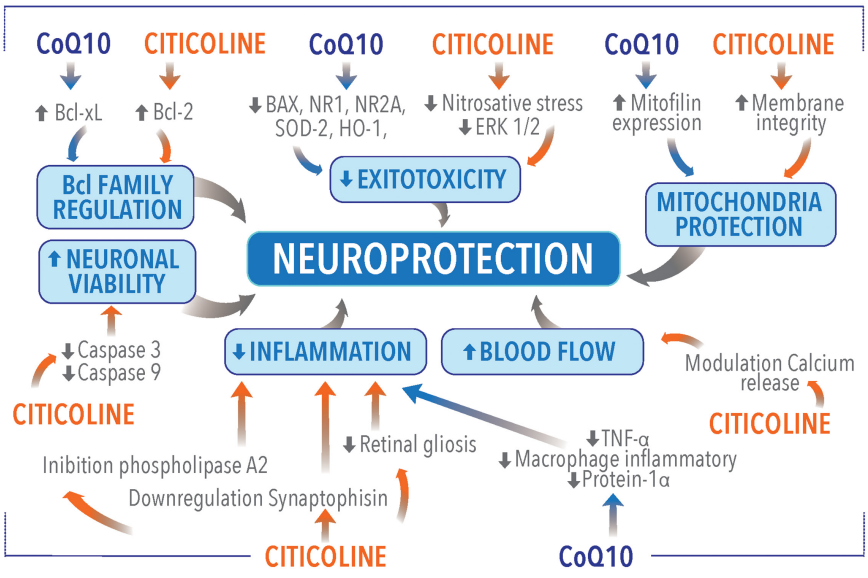

FIGURE 2
Neuroprotective molecular mechanisms of coenzyme Q10 (CoQ10) and citicoline.

In a similar experimental condition, CoQ10 promoted RGCs survival by significantly inducing Bcl-xL protein expression, a member of the BCL-2 family that exert cytoprotective and anti-apoptotic functions via several mechanisms. Bcl-xL avoids the generation of proapoptotic cytosolic Ca2+ waves, segregates a cytosolic pool of the pro-apoptotic transcription factor p53 and binds to the voltage-dependent anion channel 1, thereby inhibiting the

TABLE 1 Principal studies describing neuroprotective molecular mechanisms of coenzyme Q10 (CoQ10) and citicoline.

Mechanism	References	Outcomes
Bcl family proteins regulation	(52)	Neuroprotection provided by citicoline is due to an increased retinal expression of the apoptotic regulating protein Bcl-2 possibly mimicking brain-derived neurotrophic factor.
	(41)	CoQ10 promoted RGC survival in glaucomatous DBA/2J mice. CoQ10 significantly decreased Bax protein expression, that is a proapoptotic member of the Bcl-2 family, essential in many pathways of apoptosis.
Excitotoxicity regulation	(53)	Morphometric analysis showed a significant reduction in inner nuclear and inner plexiform layers thicknesses and ganglion cell loss after kainic acid injection, but the rate of thinning in retinal layers was reduced after citicoline treatment.
	(54)	Citicoline has a neuroprotective effect on retinal damage due to kainic acid-induced neurotoxicity.
	(42)	CoQ10 promotes RGC survival by inhibiting oxidative stress, glutamate excitotoxicity, and activation of the Bax and Bad–mediated apoptotic pathway and by preserving mtDNA content and Tfam/OXPHOS complex IV protein expression in glaucomatous DBA/2J mice.
Caspases regulation	(52)	Citicoline can reduce the expression of active forms of caspases-9 and-3 regulating apoptosis.
Effect on mitochondria	(52)	Citicoline increase the availability of nucleotides essential for the synthesis of membrane phospholipids and enhances bioenergetics and phospholipid membrane turnover in the brain.
	(34)	CoQ10 promote mitofilin protein expression, providing protection to the mitochondria, and ultimately OXPHOS capacity against oxidative stress.
	(44)	CoQ10 has bioenergetic properties. CoQ10 reduction/oxidation cycles transfer protons across the membrane forming a proton gradient essential to producing ATP.
Neurotransmitters system stimulation	(56)	Citicoline reinforces dopaminergic transmission in the retina
Membrane integrity maintenance	(46)	CDP-choline is a precursor of glycerophospholipid phosphatidylcholine which is an essential phospholipid for the maintenance of intracellular and extracellular membranes of eukaryotic organisms; these molecules are of particular relevance in surveying neuron homeostasis and functionality, by serving membranes turnover, synaptic plasticity, and neurotransmission.
	(60)	Citicoline leads to the formation of phosphatidylcholine, which is an important component of neuronal membranes and is imperative to the membrane integrity of the retinal ganglion cells.
Anti-inflammatory properties	(46)	Citicoline was found to be protective in transient cerebral ischemia through the inhibition of phospholipase A2.
	(62)	CoQ10 induced a significant reduction of TNF-α level, lessening the production of pro-inflammatory cytokines and lowering the production of macrophage inflammatory protein-1 alpha.
Cerebral blood flow improvement	(60)	Citicoline inducing calcium release from endothelial cells improves endothelial function leading to improved microvasculature and better blood flow.
	(64)	Prescription of citicoline for treatment of acute ischemic stroke is associated with hemodynamic changes in cerebral arteries.
	(63)	Citicoline is well tolerated and improves cognitive performance, cerebral blood perfusion and the brain bioelectrical activity pattern in Alzheimer's disease.
Mutual beneficial effect on molecules metabolism	(65)	Ubiquinone is necessary for the L-3-glycerophosphate oxidase of pig brain mitochondria.
	(58)	Choline oxidization is restored by the addition of ubiquinone-2 or ubiquinone-10 to the oxidase assay medium. CoQ10 was found to increase the choline oxidase activity in ubiquinone-depleted mitochondria.
	(66)	Solubilized choline dehydrogenase is capable to reduce ubiquinone.
	(68)	CoQ10 showed a neuroprotective activity, in the case of choline depletion, by inhibiting the release of glutamate in rat cerebrocortical nerve terminals.

mitochondrial permeability transition–dependent apoptotic pathway. Bcl-xL also regulates mitochondrial ATP synthesis, protein acetylation, autophagy, and mitosis (42).

Effect on excitotoxicity

Citicoline and CoQ10 have been reported to counteract excitotoxicity. The neuroprotective effect of citicoline was reported in an animal model of kainic acid (KA)-induced retinal damage (53, 54). All these studies showed that citicoline caused a significant reduction of KA-induced damage in the retinas of treated animals apparently counteracting nitrosative stress and decreasing extracellular signal-regulated kinases (ERK)1/2 activation caused by KA (55).

Similarly, CoQ10 reduces glutamate excitotoxicity and oxidative stress-mediated RGCs degeneration by preventing mitochondrial alterations in the retina in animal models. CoQ10 supports RGC survival, protected the axons in the optic nerve head (ONH), and reduced astroglial activation

by decreasing glial fibrillary acidic protein expression in the retina and ONH. CoQ10 prevented the upregulation of NR1 and NR2A, Superoxide dismutase 2 and heme oxygenase 1 protein expression, and significantly prevented apoptosis by decreasing Bax protein expression or by increasing pBad protein expression. More importantly, CoQ10 preserved mtDNA content and Mitochondrial transcription factor A (Tfam)/oxidative phosphorylation (OXPHOS) complex IV protein expression in the retina of glaucomatous DBA/2J mice (42).

Effect on caspases

Studies on animals showed that citicoline can rescue damaged RGCs through an anti-apoptotic effect and can support neurite regeneration of damaged RGCs. Similarly, to Brain-derived neurotrophic factor (BDNF) and NT-4, citicoline can reduce, in an animal model, retinal neuronal apoptosis and stimulate neurites' regeneration by lowering the expression of active forms of caspases-9 and-3 (52).

Effect on mitochondria

Animal models showed that citicoline can reverse some aging mitochondrial processes, possibly due to its ability to increase the availability of nucleotides essential for the synthesis of membrane phospholipids, phosphatidylserine and phosphatidylethanolamine, and/or to enhance brain energy metabolism (52).

Similarly, in a resonance magnetic study on volunteer humans, citicoline significantly increased the phosphocreatine, the ATP, the ratio of phosphocreatine to inorganic phosphate, and induced improvements in membrane phospholipids. This is indicative that citicoline enhances bioenergetics and phospholipid membrane turnover in the brain (52).

Mitofilin, the principal mitochondrial inner membrane protein which plays a crucial role in the preservation of mitochondrial cristae morphology, reduces in the condition of oxidative stress. In this condition CoQ10 showed partial preservation of mitochondrial morphology, increased mitochondrial numbers and mitochondrial volume density. This is possibly due to CoQ10 ability to promote mitofilin protein expression, providing protection to the mitochondria and ultimately OXPHOS capacity against oxidative stress (34).

In addition, CoQ10 plays a vital role in ATP production. In mitochondria and lysosomes, CoQ10 goes through reduction/oxidation cycles that transfer protons across the membrane forming a proton gradient essential to producing ATP (44). Thus supporting the bioenergetic role of CoQ10.

Effect on neurotransmitters system

As seen, citicoline not only exerts a neuroprotective effect but also boosts the synthesis of dopamine, acetylcholine, noradrenaline and serotonin. Studies reported that after citicoline administration retinal dopamine levels significantly increased thus possibly in part justifying the improvement of visual function in glaucomatous patients in terms of visual field and electrophysiological tests results (56).

Effect on membrane integrity

One additional target on which citicoline may act is remyelination. Disruption of the axonal membranes in glaucoma has been previously described since the early stages of the disease (19, 24, 57). Citicoline, is a precursor for phosphatidylcholine, phosphatidylethanolamine, sphingomyelin and cardiolipin, which are fundamental structural and functional components of cell membranes that ensure the correct enzymatic viability for the transport of substances across the membrane and are essential in signal transduction. Being a protagonist in maintaining membrane integrity, citicoline also plays a pivotal role in counteracting axonal degeneration in glaucoma (46, 58–60).

Effect on inflammation

Citicoline has been shown to have a positive protective effect on inflammatory diseases (61).

Citicoline has been shown to counteract the pathological downregulation of synaptophysin in the retina, restoring its anti-inflammatory properties. Moreover, citicoline is able to reduce retinal reactive gliosis, prevent apoptosis of the entire retinal components, such as photoreceptors, bipolar cells, and RGCs.

Similarly, the administration of citicoline resulted to be protective in transient cerebral ischemia through the inhibition of phospholipase A2 (PLA2), with a consequent reduction of tissue inflammation and redox imbalance (46).

Coenzyme Q10 claims some anti-inflammatory properties as well. A recent systematic review and meta-analysis reported that improving the serum level of CoQ10 induced a significant reduction of TNF-α level in the CoQ10 supplementation group compared with placebo. This may be due to the potential role of CoQ10 in lessening the production of pro-inflammatory cytokines by preventing NF-κB gene expression, reducing miR-146a and IL-1 receptor associated kinase modulation. CoQ10 may also act by lowering the production of macrophage inflammatory protein-1 alpha (62).

Effect on cerebral blood flow

Citicoline was shown to improve cerebral blood flow and velocities compared to placebo. This is possibly due to the effect on calcium release from endothelial cells that regulate nitric oxide synthesis consequently enhancing endothelial function. The proper functioning of the microvasculature and the right tissue perfusion is crucial for neuronal viability. In this context, restoring endothelial dysfunction leads to healthier microvasculature and improved blood flow, avoiding neuronal apoptosis (60, 63, 64).

Mutual beneficial effect on molecules metabolism

Data on literature, suggests that the combined use of molecules may be also beneficial for the metabolism of the molecules themselves. Barrett and Dawson (58) reported that rat liver mitochondria treated extensively with n-pentane are incapable of oxidizing choline. Choline oxidization is restored by the addition of ubiquinone-2 or ubiquinone-10 to the oxidase assay medium. The necessity for ubiquinone of the L-3-glycerophosphate oxidase of pig brain mitochondria has been also previously confirmed (65). Ubiquinone is also fundamental for Nicotinamide adenine dinucleotide (NADH) and succinate oxidase (58). Previous studies showed that solubilized choline dehydrogenase is capable to reduce ubiquinone-6 and that, in mitochondria incubated with choline until the anaerobic state was achieved, endogenous ubiquinone was reduced (66). The choline dehydrogenase is a respiratory-chain-linked enzyme that supplies electrons into the respiratory chain. It can use ubiquinone-6 as an electron acceptor once the enzyme has been solubilized and interacts with the chain in such a manner as to suggest that the possibility of reversed electron transport from choline to NAD+ strongly implicates a requirement of the choline oxidase system for ubiquinone. Remarkably, Barrett and Dawson (58) observed that most of the choline oxidase activity was reduced because of ubiquinone depletion. In this regard, CoQ10 was found to increase the choline oxidase activity in ubiquinone-depleted mitochondria, thus suggesting the importance of the presence of good levels of plasmatic choline and CoQ10 for energetic production (58).

Qu et al. (67) showed that CoQ10 decreases in the human retina with aging. In people aged over 80 retinal CoQ10 levels declined by approximately 40% compared to people under 30. This may have two main consequences: a decrease in antioxidant ability and a decrease in the rate of ATP synthesis in the retina. This would make the RGCs more vulnerable to pro-apoptotic insults.

Moreover, a study on an experimental animal model of non-alcoholic steatohepatitis (NASH) in albino rats induced by a methionine and choline-deficient (MCD) diet showed a significant increase in the brain contents of ammonia and NOx. These substances were significantly reduced by treatment with CoQ10. Concomitantly the brain-derived neurotrophic factor content, which was reduced by the diet, increased. Overall, CoQ10 showed a neuroprotective activity, in the case of choline depletion, by inhibiting the release of glutamate in rat cerebrocortical nerve terminals (68).

Conclusion

The greater efficacy of the fixed combination over the single components could therefore depend on the fact that each molecule exerts, at least in part, their activity on mitochondria (32). CoQ10 acts as an electron accepter from mitochondrial complexes I and II, thus increasing the energetic rate of cells. Additionally, citicoline maintains proper levels of cardiolipin and sphingomyelin in the cellular and axon membranes and stimulates cardiolipin production within the mitochondrial membranes. Cardiolipin is essential for the optimal activity of the enzyme complexes of the electron transport chain and for ATP production (32).

Overall, these data suggest the possible usefulness of the combined use of citicoline and CoQ10 both in terms of a putative synergistic effect and in terms of combined action on the different pathogenetic targets causing the onset and progression of glaucoma. Using combined treatment may downregulate more pro-apoptotic pathways as well as it may boost the effect on one or more pathways on which the different molecules act. In addition, it may increase patients' compliance reducing the burden of administering several medications, simplifying treatment.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

Funding

Visufarma SpA kindly provided funds for Open Access Publication Fees.

Conflict of interest

AM was medical consultant for Visufarma S.p.A.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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frontiers | Frontiers in Medicine

TYPE Original Research
PUBLISHED 16 December 2022
DOI 10.3389/fmed.2022.1009273

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Ophthalmology, a section of the journal Frontiers in Medicine

RECEIVED 01 August 2022
ACCEPTED 08 November 2022
PUBLISHED 16 December 2022

CITATION
Wang R-X, Li N and Chen X-Y (2022) Ultrasound cyclo-plasty for moderate glaucoma: Eighteen-month results from a prospective study. *Front. Med.* 9:1009273. doi: 10.3389/fmed.2022.1009273

Ultrasound cyclo-plasty for moderate glaucoma: Eighteen-month results from a prospective study

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Purpose: To evaluate the long-term clinical efficacy of ultrasound cyclo-plasty (UCP) in the treatment of moderate glaucoma and molecular effects in animal experiments.

Methods: An 18-month clinical study was conducted among 32 patients with moderate glaucoma. The primary outcome was surgical success, defined as a reduction in intraocular pressure (IOP) of greater than or equal to 20% from the baseline and an IOP value of greater than 5 mmHg at the last follow-up. The secondary outcomes were related to the quality of life, complications, and mean IOP value at each follow-up. In the animal experiment, 20 New Zealand rabbits were used to establish a high-IOP model and implement UCP. The distribution of aquaporin 4 (AQP4) in the ciliary body and the tissue changes under electron microscopy were observed after surgery.

Results: The mean patient IOP decreased from 34.9 ± 4.9 mmHg before surgery to 23.5 ± 5.2 mmHg at 18 months after UCP. No vision loss occurred in any patient. Some patients had postoperative complications, but the symptoms were mild and disappeared within 3 months after the surgery. Most patients had good postoperative quality of life. Histology showed that AQP4 remained in the ciliary muscle after UCP, and only the bilayered epithelial cells showed coagulative necrosis. Furthermore, electron microscopic observation revealed the destruction of ciliary process cells covered by ultrasound after UCP.

Conclusion: UCP is associated with mild postoperative reactions and the mild treatment of ciliary tissue and is a safe and effective method for reducing IOP in moderate glaucoma.

KEYWORDS

glaucoma, ultrasound cyclo-plasty, intraocular pressure, ciliary body, treatment

Introduction

Glaucoma is a progressive optical neuropathy caused by the accelerated degeneration of retinal ganglion cells. This degeneration severely threatens and impairs the visual pathway, eventually leading to blindness (1, 2). Therefore, it is essential to control the progression of early and moderate glaucoma and effectively protect the remaining vision. Currently, lowering the intraocular pressure (IOP) is the most important therapeutic measure to prevent and delay diminution of vision. This can be achieved clinically by reducing the inflow or increasing the outflow of the aqueous humor (1, 3, 4). Surgical destruction of the ciliary body can reduce the secretion of aqueous humor. The traditional surgical methods include cyclocryotherapy, cyclodiathermy, and diode laser ring photocoagulation (5, 6). However, due to the difficulties in precisely selecting the target ciliary body and determining the appropriate dosage, surgery often leads to damaged adjacent tissue and ocular inflammation. This increases the incidence of postoperative complications and hinders the accurate prediction of curative effects.

In recent years, the development of high-intensity focused ultrasound (HIFU) technology has advanced significantly. As a new type of non-invasive annular destruction surgery, ultrasound cyclo-plasty (UCP) can effectively reduce IOP (7). In comparison with traditional surgery, the ultrasound energy is focused using a non-optical transparent medium. The energy deposition and tissue heating at the focus are independent of cytochrome deposition and can be arbitrarily located in the intraocular tissue, thus selectively destroying the ciliary body (8, 9). The advantage of focused ultrasound is that it can focus the energy within a suitable range of tissue, and the volume of tissue destroyed is preset by the machine to avoid excessive damage (10). Thus far, many studies have shown that UCP is an effective and well-tolerated method for reducing IOP, but its therapeutic effects on moderate glaucoma are still being explored. The purpose of the present study was to examine the molecular effects and clinical efficacy of UCP for the treatment of moderate glaucoma (11).

Materials and methods

High-intensity focused ultrasound equipment

The study used an EyeOP1 device imported from France, which has been described in detail previously (12). In short, the EyeOP1 consists of a command module and a therapy device; it generates voltage through a signal generator and then raises the voltage to a certain level via an amplifier to ensure that the ultrasonic beam is emitted. The instrument's ring-shaped treatment probe contains 6 miniature piezoelectric transducers, and the contact surface is arc-shaped and rectangular. During the treatment, it acts on each sector of the ciliary body while avoiding the nasal-temporal meridian (8, 13). To stably center the probe, the suction on the bottom of the positioning ring applies a vacuum at a low level, bringing it into closer contact with the eye. The design of the probe is unique and innovative. The probe is available in 3 diameters (11 mm, 12 mm, and 13 mm), and the appropriate diameter can be selected according to the eye conditions of each individual patient.

Patients

This study was conducted in accordance with the principles of the Declaration of Helsinki and ISO 14155 standard and was approved by the local institutional review board. Written content was obtained from all enrolled patients.

This study included 32 eyes of 32 patients with moderate glaucoma who underwent UCP. The glaucoma staging was performed by glaucoma specialist based on the Hoddap - Parrish-Anderson criteria (14).

The inclusion criteria were as follows: (1) Patients diagnosed with moderate primary open angle glaucoma, (2) IOP not controlled by hypotensive medication, (3) IOP greater than or equal to 20 mmHg, (4) age more than 18 years old and less than 90 years old, (5) patients who signed the informed consent and who were able to complete all postoperative follow-up visits, (6) the type and amount of ocular hypotensive medication remained the same before and after treatment.

The exclusion criteria were as follows: (1) Eye infection in any eye in the 2 weeks before treatment, (2) any medical or treatment history or systemic disease that may affect the evaluation of the treatment efficacy, (3) pregnant or lactating women, (4) patients who underwent other procedures at the same time, and (5) patients who underwent other eye surgeries for reducing IOP within 18 months after the surgery.

A schematic diagram of the research flowchart is shown in **Figure 1**.

Preoperative examination

Each patient underwent a routine eye examination, which included uncorrected visual acuity, photography of the anterior segment, gonioscopy, perimetry, fundus photography, IOP (Goldman tonometer), ultrasound biomicroscopy (UBM), biological parameters of the eyeball (axial length, white-to-white distance), and analysis of the quality of life.

Treatment procedure

Both anesthesia and treatment were performed by the same experienced ophthalmologist. Retrobulbar anesthesia

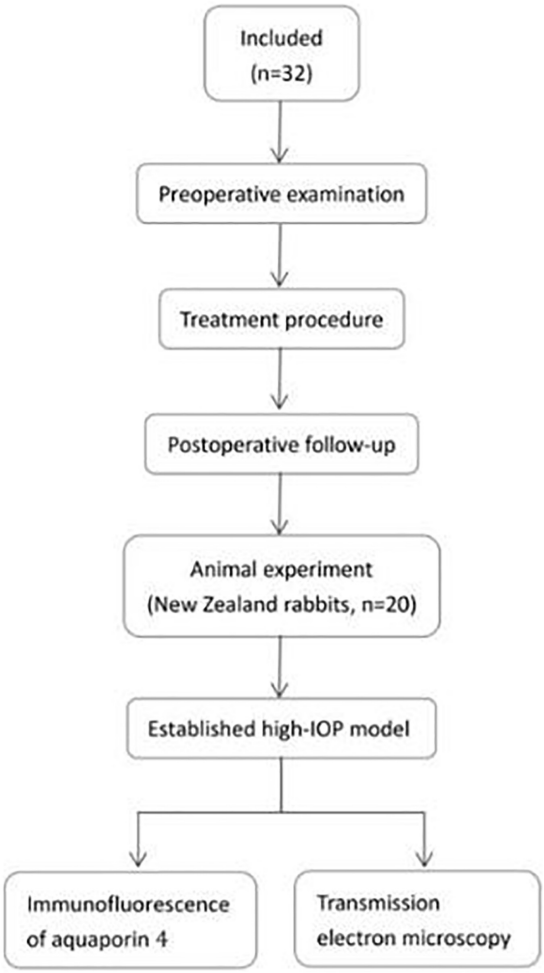

FIGURE 1
Schematic diagram of the research flowchart.

was administered in all patients. The surgeon selected the appropriate treatment procedure according to each patient's conditions to accurately determine the ultrasound dosage. Since the subjects of this study were patients with moderate glaucoma and no patient had an IOP of more than 36 mmHg, all patients were treated with 6 sectors and the exposure time was 8 s.

The specific steps were as follows. First, the patient lay supine, with the eye axis perpendicular to the horizontal line. After the instrument was started, the positioning cone was adsorbed on the ocular surface and centered. Second, the negative pressure suction was activated and filled with saline solution, stepped on the pedal to start the treatment. Finally, after the treatment, the probe and positioning cone were removed. The patients stayed in the hospital for observation for 2 h.

Tobramycin dexamethasone eye drops were added within 4 weeks after surgery, 4 times a day.

Postoperative follow-up

Follow-up visits occurred 1 day, 1 week, 1, 3, 6, 12, and 18 months after treatment. Eye examinations such as uncorrected visual acuity, photography of the anterior segment, IOP (Goldman tonometer), complication assessment, and quality of life analysis were performed at each visit.

Outcome measures

The surgical success criteria were an IOP reduction of more than or equal to 20% compared with the baseline value and an IOP of more than 5 mmHg at the last follow-up visit, without adding new glaucoma medication compared to baseline.

The Glau-QoL 36-item questionnaire was used to assess the patients' quality of life, with 7 domains: psychological wellbeing, self-image, daily life, burden of treatment, driving, anxiety, and confidence in health care. Each item had 3–9 questions, with each question's response collected on a 5-point scale. Each item was scored separately; a patient's score was the sum of the total scores of all questions, transformed into a scale from 0 to 100.

Animal experiment

All animal experiments have been approved by the ethics Committee of Xuzhou First People's Hospital. All animal testing methods were carried out in compliance with the ARRIVE guidelines.

A high-IOP model was established for 20 New Zealand rabbits. Puncture at the corneal margin, extract 0.1–0.2 ml of aqueous humor, and then inject an equal amount of compound carbomer. At 2 weeks, rabbit high IOP is basically stable. Each New Zealand rabbit was injected into the ear vein with 10% chloralhydrate (3.5 mg/kg) intravenously for general anesthesia, and one eye was treated with UCP and the other eye was used as a negative control. The rabbits were executed immediately after the treatment. Both eyeballs were removed, and the ciliary bodies were separated.

Immunofluorescence of aquaporin 4

The ciliary body tissue was fixed in 4% paraformaldehyde solution and embedded in paraffin to make 5-μm-thick sections. The tissue sections were rinsed with phosphate-buffered saline (PBS) for 30 min, treated with formaldehyde-H_2O_2 for 10 to 15 min, treated with Triton X-100 for 10 min, and blocked with normal goat serum for 4 h. After drying, the primary antibody (AQP4, 1:400) was added, and the samples were incubated overnight at 37°C. Next, the samples were rinsed with PBS, and secondary antibody (Cy3-labeled goat anti-rabbit IgG) was added. After 90 min, the samples were rinsed with PBS. Then, slides were mounted and observed under a laser confocal microscope.

TABLE 1 Patients characteristics.

Patients	32
Age, mean ± SD, year	56.8 ± 10
Sex	
Male	17
Female	15
BCVA, logMAR	0.76 ± 0.31
IOP baseline, mean ± SD	34.9 ± 4.9
Axial length	23.77 ± 0.80
White to white	11.74 ± 0.17
Lens status	
Phakic	29
Pseudophakic	3
Aphakic	0
Preoperative hypotensive medications, mean ± SD	1.7 ± 0.7

BCVA, best-corrected visual acuity; IOP, Intraocular pressure; SD, Standard deviation.

Transmission electron microscopy

The ciliary body tissue was fixed in 2.5% glutaraldehyde solution, rinsed with 0.1 M phosphate buffer, fixed with 1% osmic acid solution, and rinsed again. This was followed by gradient dehydration and epoxy resin immersion embedding. Ultra-thin sections (70 nm) were obtained using an ultra-thin cutting machine (UC7, Leica, Solms, Germany), stained with uranyl acetate and lead citrate solution, and observed under a transmission electron microscope (CM120, Philips Electronics, Mahwah, NJ, USA).

Statistical analysis

Data were analyzed using SPSS 23.0 statistical software (IBM, USA). Enumeration data, such as gender and lens status, were expressed as cases. Measurement data were expressed as means ± standard deviations (x ± s). The Wilcoxon rank-sum test was used to compare the differences between the IOP and quality of life scores during follow-up and the baseline values. The level of statistical significance was set at $p<0.05$.

Results

Patient characteristics

All 32 patients completed the surgery successfully. The patient details are described in Table 1.

Intraocular pressure

At all follow-up visits, the mean IOP value for each measurement decreased significantly from the baseline value.

TABLE 2 Intraocular pressure at baseline and during follow-up in the patients.

	Mean ± SD IOP (no patients)	Relative IOP reduction (%)	Success rate (%)	*P**-value compared with the baseline
Baseline	34.9 ± 4.9 (32)	NA	NA	NA
Day 1	25.9 ± 5.6 (32)	25.9	68.8	0.000
Day 7	22.1 ± 5.1 (31)	36.7	87.1	0.000
Month 1	22.8 ± 5.4 (29)	34.7	86.2	0.000
Month 3	23.6 ± 5.4 (28)	32.3	78.6	0.000
Month 6	23.3 ± 6.1 (29)	33.3	82.8	0.000
Month 12	24.1 ± 6.0 (26)	31.0	76.9	0.000
Month 18	23.5 ± 5.2 (21)	32.6	81.0	0.000

*Wilcoxon test. NA, not applicable; IOP, Intraocular pressure; SD, Standard deviation.

The mean preoperative IOP was 34.9 ± 4.9 mmHg; 18 months after treatment, the IOP had decreased by 32.6%, and the success rate was as high as 81%. The specific IOPs for the patients and the success rate of the surgery are shown in Table 2. In order to more clearly reflect the IOP trend, Figure 2 shows the line chart of IOP in this study.

Complications

The complications of UCP were categorized as intraoperative and postoperative. Four patients had mild pain during the operation. The postoperative complications were also mild, and most of them resolved spontaneously within 1 month. The types and numbers of complications are shown in Table 3.

Quality-of-life analysis

All patients completed the questionnaire once preoperatively and again at each postoperative follow-up visit. We analyzed the trends in each survival index, in detail, through calculations and scoring. All of the scores are shown in Table 4. In order to get a better overview of the patients' quality of life, we made a line chart, with each indicator marked in a different color (Figure 3).

Aquaporin 4 expression

AQP4 exists in the ciliary body tissue, controls the rate of the formation of aqueous humor by promoting its secretion and absorption, and plays a role in the aqueous balance and pressure regulation of ocular tissues. Therefore, observing the expression of AQP4 in the ciliary body helps to clarify the

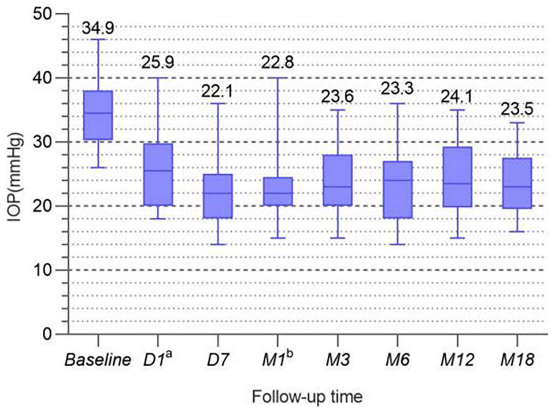

FIGURE 2
The mean IOP of all patients.

TABLE 3 Intra-operative and post-operative complications.

Description	Number of eyes (%)
Intraoperative	
Pain	4 (12.5%)
Post-operative	
Loss of visual acuity (>2 lines)	1 (3.1%)
Induced astigmatism (>1 diopter)	1 (3.1%)
Conjunctival hyperemia	3 (9.4%)
Subconjunctival hyperemia	1 (3.1%)
Corneal edema	2 (6.3%)
Superficial punctate keratitis	1 (3.1%)
Hyphema	1 (3.1%)
Aqueous flare (<7 days)	5 (15.6%)
Retinal detachment	0 (0%)
Induced cataract	0 (0%)
Phthisis	0 (0%)

surgical effect. HIFU mainly targets the epithelial cells in the ciliary body, causing coagulation necrosis due to a thermal effect in the affected area. Compared with the control group, the level of AQP4 in the ciliary process was significantly reduced after UCP treatment (**Figures 4A,B**). As seen in **Figures 4C,D,F,G** ca 50-μm local map—the UCP group also had relatively less AQP4 distal to the ciliary body. AQP4 was still present in the ciliary muscle, and only the AQP4 part of the epithelial layer was reduced (**Figures 4E,H**).

Transmission electron microscopy

The internal structure of the ciliary body epithelial cells in the UCP and control groups was observed by transmission electron microscopy. In the untreated epithelial cells, the nuclei were morphologically full with nuclear membrane wrapping, and the mitochondrial structure was basically intact (**Figure 5**). In contrast, in the UCP group, the nuclei were pyknotic, the nuclear membranes were widened, the chromatin was concentrated and aggregated, and some chromatin was collected under the nuclear membrane and border set occurred. At the same time, the mitochondria were partially destroyed and had become larger and rounder due to swelling, causing cell edema. In addition, the reduction of ribosomes was seen in the epithelial cells after UCP treatment compared with the control group.

Discussion

Although UCP is generally considered a new technology for the treatment of glaucoma, currently, it is mainly used in patients with advanced to terminal-stage disease, and the effectiveness and safety of the treatment in early and moderate glaucoma are still being explored (15). This study showed that UCP had a remarkable effect on reducing IOP in patients with moderate glaucoma, and the success rate was high. Compared with the traditional annular destruction surgery, UCP may be a step forward in the non-invasive treatment of glaucoma.

During the 18-month follow-up, we recorded the IOP values at each visit, in detail. We observed a 32.6% reduction at the last follow-up—a significant reduction from the preoperative mean (34.9 ± 4.9 mmHg) to 23.5 ± 5.2 mmHg—indicating successful IOP control. The average IOP at each follow-up visit decreased in comparison with the baseline value; these differences were statistically significant. This ideal result was due to the selective necrosis of ciliary body epithelial cells determined by the energy focusing of UCP. In this procedure, sufficient energy can be concentrated within a specific target volume, with sub-millimeter precision. The HIFU device performs annular thermal coagulation of targeted tissue through 6 cylindrical high-frequency sensors (16–18). Six ultrasound beams enter the distal end of the cornea and focus on a larger posterior plane, thereby reducing the secretion of aqueous humor and reducing the pressure of the aqueous humor on the eye wall (18, 19). Additionally, compared to the laser focal effect of diode laser ring photocoagulation, the cylindrical surface can better adapt to the geometry of the target organ while expanding the impact on the ciliary body volume, increasing the treatment area and ensuring the destruction of a sufficient number of ciliary bodies to avoid the rebound elevation of IOP (15, 20). According to our criteria for surgical success, we found that except for the first day after surgery, the average degree of IOP reduction in other follow-up results was more than 30%, and more than 70% of patients achieved success. This may be because in UCP, the destruction of the distal end of the ciliary body is not able to exert its full effect in such a short period of time (19, 21).

A small number of patients felt mild pain during the operation, which may have been related to the patients' different sensitivities to anesthetic drugs; it may also have been due

TABLE 4 Score distribution of the health-related quality of life domains for patients.

		Score						
		Mean ± SD						
	Number of patients (MD)	Psychological wellbeing	Self-image	Daily life	Burden of treatment	Driving	Anxiety	Confidence in health care
Baseline	32 (0)	59.4 ± 17.6	61.4 ± 20.8	65.2 ± 21.1	62.3 ± 17.1	40.1 ± 25.6	32.6 ± 21.1	53.1 ± 16.7
Day 1	29 (3)	59.3 ± 18.2	61.7 ± 19.8	65.9 ± 20.8	61.9 ± 16.3	40.8 ± 25.8	47.6 ± 20.1*	54.1 ± 17.9
Day 7	29 (3)	68.1 ± 16.5*	70.2 ± 19.1*	64.5 ± 20.3	62.4 ± 18.7	48.4 ± 23.4*	60.8 ± 21.9*	60.3 ± 16.1*
Month 1	26 (6)	72.3 ± 13.1*	75.2 ± 16.8*	69.0 ± 18.2*	64.6 ± 16.7	53.2 ± 26.8*	66.1 ± 24.3*	60.6 ± 16.6*
Month 3	27 (5)	73.3 ± 14.1*	70.7 ± 16.5*	71.4 ± 18.2*	63.5 ± 17.4	54.6 ± 26.1*	63.7 ± 20.9*	61.3 ± 14.8*
Month 6	25 (7)	72.8 ± 14.2*	71.4 ± 16.6*	72.4 ± 14.8*	61.2 ± 16.9	54.2 ± 20.9*	66.3 ± 19.0*	60.8 ± 14.6*
Month 12	24 (8)	73.6 ± 12.1*	70.8 ± 18.9*	73.2 ± 13.9*	62.1 ± 17.6	52.6 ± 20.6*	61.7 ± 20.6*	56.5 ± 16.2
Month 18	20 (12)	70.6 ± 11.6*	72.8 ± 17.7*	71.5 ± 15.8*	62.5 ± 14.1	51.0 ± 24.7*	61.3 ± 21.7*	53.8 ± 16.5

*$P < 0.05$. MD, Missing data, SD, Standard deviation.

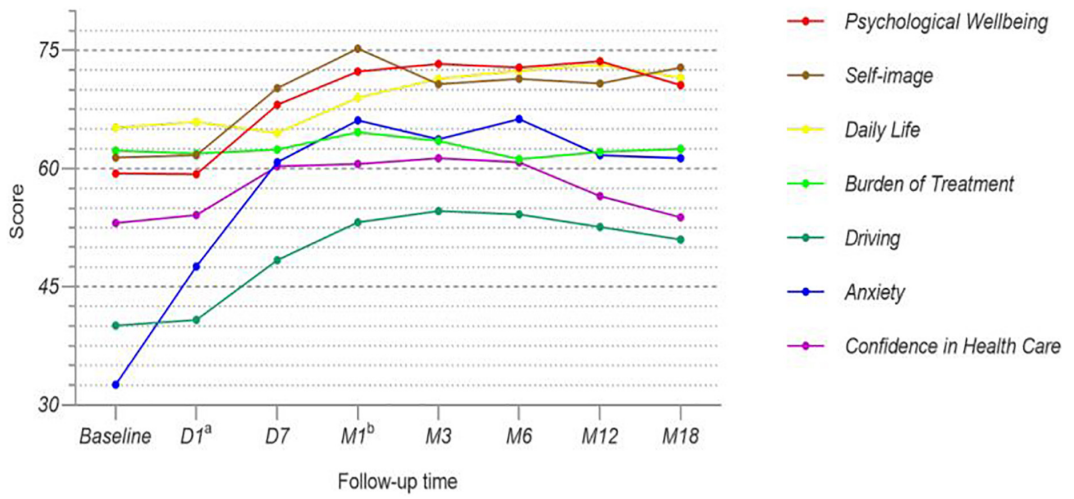

FIGURE 3
Each survival index in all patients.

to the psychological effects of patients' excessive tension. Compared with traditional glaucoma surgery, the complication rate of UCP was significantly reduced (12, 20, 22). None of the patients experienced major adverse events, such as severe hypotony or phthisis (the most severe types of complications observed after traditional annular destruction surgery). For the assurance of UCP safety, the innovative HIFU technology has the unique advantage of not only allowing for harmless propagation in living tissue but also allowing energy deposition and tissue heating independent of pigmentation. Therefore, the energy is confined to the distal end of the ciliary body, producing a controlled thermal effect on the target organ, where pigmentation may be highly variable (23, 24). In the eye, ultrasound can heat tissue at any depth or location with precise temperature control. The volume of the treated tissue can be appropriately adjusted, and the integrity of the adjacent tissue can be preserved, therefore minimizing surgical complications (25). However, some patients still experienced complications, such as conjunctival hyperemia, corneal edema, aqueous flare, and hyphema, all of which gradually disappeared within a month and did not require interventional therapy. One patient developed a mild anterior chamber inflammatory reaction with very limited signs of reaction; this also healed within a month. We performed a postoperative UBM review and found no evidence of scleral thinning or damage or adjacent tissue destruction, but some inflammatory mediators may have been synthesized and released after surgery (26, 27). Minor complications seem to be unavoidable, but their incidence was significantly reduced in this study compared to traditional surgery and did not affect the surgical outcomes.

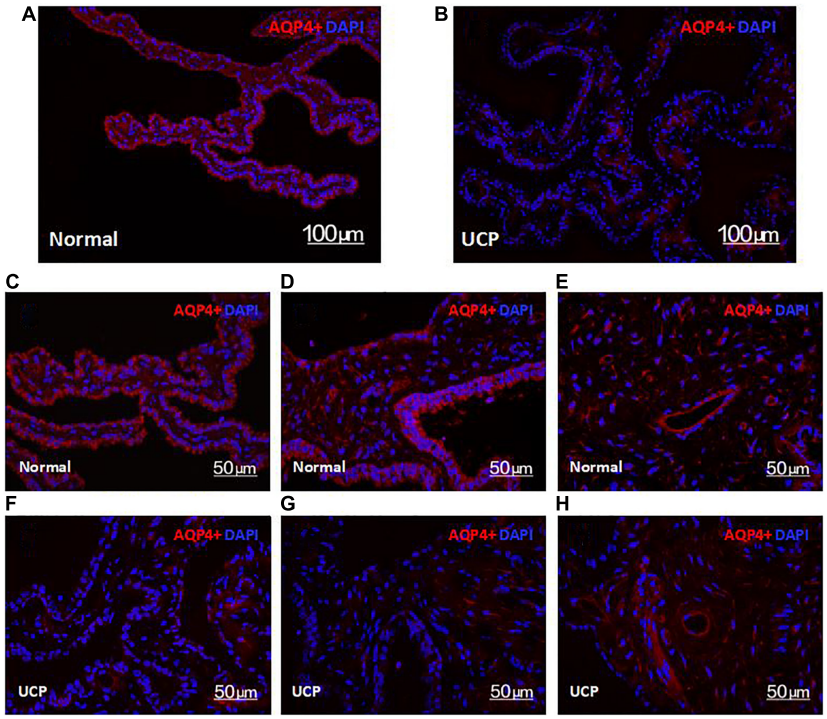

FIGURE 4
Confocal microscopy images of the expression of AQP4 in the normal ciliary body **(A)** and UCP postoperative **(B)**. Scale bars 100 μm. **(C–G)** are ciliary, processes, **(E,H)** are ciliary, muscles. Scale bars 50 μm.

Furthermore, UCP carries little potential threat to vision. The patients' visual acuities did not fluctuate significantly compared with their preoperative measurements, with the exception of one patient, who developed vision loss and astigmatism 1 year after surgery (28). This patient was found to have cataracts before the surgery, and the visual acuity may have been related to the cataract progression. This reduction in complications improves the safety profile of UCP, indicating that UCP is a more gentle and well-tolerated treatment.

In order to better understand the overall efficacy of UCP, we analyzed the patients' quality of life. We scored various survival indicators and compared the values before and after surgery (29, 30). The index score trends varied among the 7 different indicators. The largest change was in anxiety levels. A lower anxiety score indicated a higher level of anxiety. The patients' anxiety levels were very high before their surgeries, as reflected by the average score of 32.6 ± 21.1. However, their anxiety was significantly relieved on the first day after surgery and greatly reduced 1 week after surgery. At the last follow-up, the average anxiety score was stable at 61.3 ± 21.7, nearly twice that before the operations. This is a satisfactory result, indicating that UCP had a positive effect on the patients' psyches.

Large changes were also seen in the psychological wellbeing, self-image, driving, and confidence in health care domains. Although there were no statistically significant changes in these domains on the first postoperative day, the scores were significantly improved 1 week after surgery. Notably, however, in the driving and confidence in health care domains, the scores began to decrease at the last 2 follow-up visits. This may have been because some patients felt good after the treatment and were unwilling to review frequently according to the follow-up standards. There were also some patients who reduced their medication or stopped taking it altogether because their IOP remained stable, causing the scores to show a downward trend after 6 months.

For the daily life domain, the overall score increase was gradual (i.e., the rate of increase was relatively slow), and it did not reflect a change until 1 month after the operation. This may have been because it took some time for the surgical efficacy to have an impact on the patients' daily lives. However, UCP did not reduce the burden of treatment for patients. We required the type and quantity of IOP-lowering drugs to be the same for patients before and after surgery, which may have made patients feel the pressure of treatment. Overall, UCP improved the patients' quality of life to a certain extent and helped them to resist the negative emotions brought about by moderate glaucoma.

In this study, we also conducted animal experiments. Through the in-depth exploration of the tissue, we can more clearly understand the working effect of HIFU. In

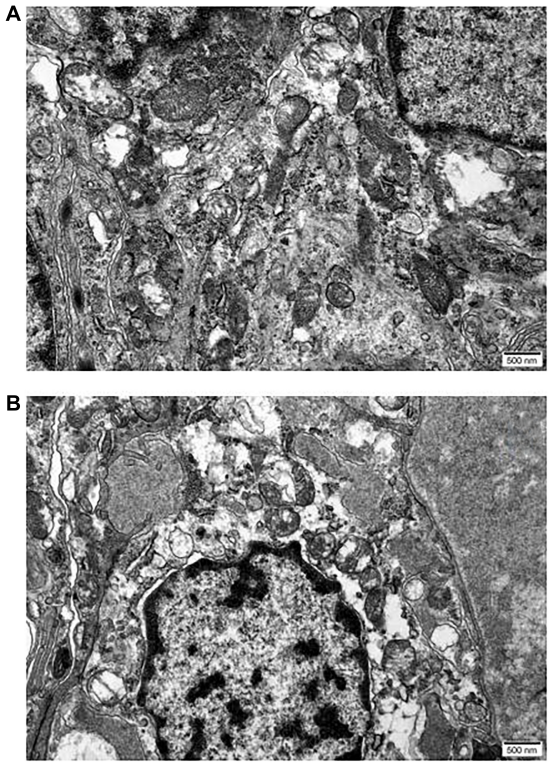

FIGURE 5
Micrographs reveal the differences between epithelia of normal ciliary body **(A)** and UCP postoperative **(B)**. Scale bars 500 nm.

immunofluorescence sections, the ciliary body regions treated with HIFU showed less AQP4 content. The degrees of pigmentation in the pigmented and non-pigmented epithelial cell layers decreased significantly compared with the control group, and some epithelial cells were removed. However, there was no significant difference in the expression levels of AQP4 in the ciliary muscle between the two groups; it was retained in both groups (**Figures 4E,H**). Only a small amount of AQP4 was lost in the epithelial cell layer in the UCP group. This is because the arc-shaped annular probe used in the operation can accurately locate the ciliary process while focusing the ultrasound on the local area, forming a small thermal coagulation range. Therefore, the maximum damage is always in the ciliary process and dose not injure the ciliary muscle or other adjacent tissues; this reduces the secretion of aqueous humor while retaining the integrity of the blood-aqueous barrier (31, 32). To clarify the extent of the tissue damage, we also analyzed the internal structural changes of the epithelial cells. The penetration of HIFU can produce mild and sustained damage to epithelial cells, resulting in cell degeneration or necrotic shedding (18). Through transmission electron microscopy, we observed that the thermal effect of ultrasonic energy conversion triggered an inflammatory response in the cell in the UCP group; the cells were edematous and the chromatin in the nuclei was condensed and shrunken. At the same time, some organelles, such as the mitochondria and ribosomes, were also damaged. The destruction of epithelial cells weakened the secretory function of the ciliary body, and the production of aqueous humor was reduced, further validating the clinical follow-up results.

Some limitations apply to the present study. Its small sample size and high long-term loss to follow-up rate constitute the limitations of this study, and further prospective randomized clinical trials are needed to confirm the long-term efficacy and safety of the surgery. At present, studies have shown that repeated UCP treatment can be performed, but the optimal time and frequency of repeated treatment remains to be elucidated. More extensive research will be carried out in this direction in the future.

Conclusion

In conclusion, UCP is an exciting innovation. With the help of computers, surgery can be implemented simply and quickly, which shortens the learning curve for surgeons and minimizes the surgical risk. This approach, which is not completely dependent on surgeons, effectively improves the safety of surgery for moderate glaucoma by weakening a functional, rather than destructive, approach regarding the ciliary body. This study should improve confidence regarding the use of UCP for the treatment of moderate glaucoma, as it indicates that the thermal coagulation effect of HIFU on the ciliary body is an effective method to reduce IOP and control the progression of the disease.

Data availability statement

The original contributions presented in this study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

This animal study was reviewed and approved by the Medical Ethic Committee, the First Affiliated Hospital of Anhui Medical University.

Author contributions

R-XW: conceptualization, methodology, formal analysis, writing—original draft, data curation, and visualization. NL: investigation, validation, and data curation. X-YC: validation, writing—review, supervision, project administration, and

funding acquisition. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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frontiers | Frontiers in Medicine

TYPE Original Research
PUBLISHED 26 May 2023
DOI 10.3389/fmed.2023.1157773

OPEN ACCESS

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RECEIVED 03 February 2023
ACCEPTED 04 April 2023
PUBLISHED 26 May 2023

CITATION
Pinazo-Durán MD, Zanón-Moreno V, García–Villanueva C, Martucci A, Peris-Martínez C, Vila-Arteaga J, García-Medina JJ, Andrés–Blasco I, Gallego–Martínez A, Nucci C and García–Feijoo J (2023) Biochemical–molecular–genetic biomarkers in the tear film, aqueous humor, and blood of primary open-angle glaucoma patients. *Front. Med.* 10:1157773. doi: 10.3389/fmed.2023.1157773

Biochemical–molecular–genetic biomarkers in the tear film, aqueous humor, and blood of primary open-angle glaucoma patients

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Introduction: Glaucoma is a chronic neurodegenerative disease, which is the leading cause of irreversible blindness worldwide. As a response to high intraocular pressure, the clinical and molecular glaucoma biomarkers indicate the biological state of the visual system. Classical and uncovering novel biomarkers of glaucoma development and progression, follow-up, and monitoring the response to treatment are key objectives to improve vision outcomes. While the glaucoma imaging field has successfully validated biomarkers of disease progression, there is still a considerable need for developing new biomarkers of early glaucoma, that is, at the preclinical and initial glaucoma stages. Outstanding clinical trials and animal-model study designs, innovative technology, and analytical approaches in bioinformatics are essential tools to successfully uncover novel glaucoma biomarkers with a high potential for translation into clinical practice.

Methods: To better understand the clinical and biochemical-molecular-genetic glaucoma pathogenesis, we conducted an analytical, observational, and case-comparative/control study in 358 primary open-angle glaucoma (POAG) patients and 226 comparative-control individuals (CG) to collect tears, aqueous humor, and blood samples to be processed for identifying POAG biomarkers by exploring several biological pathways, such as inflammation, neurotransmitter/neurotrophin alteration, oxidative stress, gene expression, miRNAs fingerprint and its biological targets, and vascular endothelial dysfunction, Statistics were done by using the IBM SPSS 25.0 program. Differences were considered statistically significant when $p \leq 0.05$.

Results: Mean age of the POAG patients was 70.03 ± 9.23 years, and 70.62 ± 7.89 years in the CG. Malondialdehyde (MDA), nitric oxide (NO), interleuquin (IL)-6, endothelin-1 (ET-1), and 5 hydroxyindolacetic acid (5-HIAA), displayed significantly higher levels in the POAG patients vs. the CG ($p < 0.001$). Total antioxidant capacity (TAC), brain derived neurotrophic factor (BDNF), 5-hydroxy tryptamine (5-HT), solute carrier family 23-nucleobase transporters-member 2 (*SLC23A2*) gene, and the glutathione peroxidase 4 (*GPX4*) gene, showed significantly lower levelsin the POAG patients than in the CG ($p < 0.001$). The miRNAs that differentially expressed in tear samples of the POAG patients respect to the CG were the hsa miR-26b-5p (involved in cell proliferation and apoptosis), hsa miR-152-3p (regulator of cell proliferation, and extracellular matrix expression), hsa miR-30e-5p (regulator of autophagy and apoptosis), and hsa miR-151a-3p (regulator of myoblast proliferation).

Discussion: We are incredibly enthusiastic gathering as much information as possible on POAG biomarkers to learn how the above information can be used to better steer the diagnosis and therapy of glaucoma to prevent blindness in the predictable future. In fact, we may suggest that the design and development of blended biomarkers is a more appropriate solution in ophthalmological practice for early diagnosis and to predict therapeutic response in the POAG patients.

KEYWORDS

primary open-angle glaucoma, glaucoma neurodegeneration, biomarkers, molecules, genes, miRNAs

1. Introduction

Glaucoma is a kind of progressive eye disease that is precisely characterized by optic nerve degeneration (OND), which manifests itself in the ocular fundus as optic disc cupping due to retinal ganglion cell (RGC) loss, and the corresponding visual field (VF) defects, being these the structural and functional reference landmarks of the disease (1–3). Glaucoma is a neurodegenerative disease that affects millions, and is the first global cause of irreversible blindness. In Europe, primary open-angle glaucoma (POAG) increased odds correlated to aging. Moreover, POAG prevalence is expected to grow in Europe because strength rises in the older population (4, 5). POAG is the most prevalent glaucoma form, characterized by intraocular pressure (IOP) elevation (the most relevant risk factor for the disease), with a typical anterior chamber angle appearance, and no other ocular identifiable comorbidity that may be causing ocular hypertension (OHT) (6, 7). Aging African–Caribbean and Hispanic races, myopia, thinner cornea, familial glaucoma history, and other conditions are important risk factors for the development and progression of POAG, which have to be kept in mind when gathering information for a complete clinical history (6, 8–10). Moreover, in a recent Spanish–Portuguese population study, overweight/obesity, migraine, asthma, and smoking have been shown to be significant risk factors for conversion from OHT to POAG (11). Imaging techniques for glaucoma, such as anterior and posterior optical coherence tomography (OCT) and OCT angiography (OCTA), OCT elastography, the oximetry, and hyperspectral image, fluorescence lifetime imaging ophthalmoscopy, and detection of apoptotic RGCs (12, 13), have been arising in the past years. Also, the VF fully automated innovating techniques (1–3, 5), as the combination of perimetry with the application for the colorimetric analysis of optic nerve head (ONH) images (which topographically assesses the cup and the presence of hemoglobin), the Laguna-ONhE, improved using five deep learning models (14), have been recently described.

The IOP reduction diminished the risk of progression in the glaucoma patients involved in The Ocular Hypertension Treatment Study (OHTS) (15, 16) and is currently the only affordable glaucoma therapy. First-line treatment is constituted by the hypotensive eye drops of prostaglandin analogs and β-blockers (1, 10, 17). However, the glaucoma progression rate and the risk of visual impairment are arduous to foresee individually because a vital part of the glaucoma patients appropriately receiving hypotensive medical–laser–surgical treatment still undergo visual impairment and blindness, while others remain stable even with having higher IOP (18–20). Because of this, up-today, glaucoma has no cure.

Experimental models of glaucoma have been extensively used (21–26). Pre-clinical glaucoma models have been mainly carried out in rodents, which have successfully reproduced the human glaucoma milestones: specific RCGs and axons damage and death, anterior and posterior eye segment changes, and elevated IOP (22, 23, 25, 26). Therefore, in animal glaucoma models, it has been demonstrated that raised chronic IOP always precedes glaucomatous OND. Many animal models of OHT have mimicked POAG by developing moderated and sustained IOP increases that induced apparent retinal and optic nerve damage (22, 23, 25, 26).

Elevated IOP induces cellular stress with the result of mitochondrial failure, autophagy, and apoptosis (27–30). Aging is

a pivotal risk factor for oxidative stress (OS) and dysregulation of inflammation (INF) (13, 25, 27–31). Moreover, morphologic and functional changes in the trabecular meshwork are mediated by OS and INF (13, 32). It has also been suggested that systemic endothelial and autonomic dysfunction are present in POAG (33) as well as reduced blood flow and vascular dysfunction (VD) have been recognized as glaucoma features (34). In addition, the role of neurotransmitters such as glutamate, glycine, dopamine, serotonin, etc., and neurotrophins, such as the brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), and neurotrophin-4/5 (NT4/5) in glaucoma, has also been extensively investigated (35, 36). The POAG gene candidates have also been described. Myocilin (MYOC), optineurin (OPTN), WD repeat domain 36, (WDR36), cytochrome P450, family 1, subfamily B, polypeptide 1 (CYP1B1), glutathione S-transferase mu 1 (GSTM1), and Neurotrophin (NTF4) have been widely linked to POAG (37, 38). The small single-stranded microRNAs (miRNAs) can regulate the expression of mRNAs/proteins within a cell and have been extensively implicated in numerous diseases, including the most prevalent ocular disorders (39). Some specific miRNAs regulate IOP (40), and others have been proposed for the diagnosis of glaucoma (41). Despite the aforementioned facts, the molecular and cellular hallmarks underlying OND in glaucoma still remain unsolved.

There is a relevant need for finding/validating imaging, biochemical, molecular, and genetic glaucoma biomarkers that help improve the clinical diagnosis for early detection, as well as for monitoring glaucoma therapy and disease progression.

In this study, we described some validated classical biomarkers for POAG diagnosis and treatment and potential new biomarkers with higher sensitivity based on clinical and experimental glaucoma research.

2. Materials and methods

2.1. Focused question

To better understand the clinical and molecular–genetic glaucoma pathogenesis, we conducted a collaborative multicenter, analytical, observational, case–comparative/control study of 625 participants, classified according to the inclusion/exclusion criteria, that were initially recruited from the glaucoma sections at the ophthalmological departments of the following hospitals: General of Valencia; "Dr. Peset" of Valencia; University of Rome "Tor Vergata" Rome, Italy; University and Polytechnic "La Fe" of Valencia; "Morales Meseguer" of Murcia; and Clinic "San Carlos" of Madrid. The study volunteers agreed to participate in the study and signed the informed consent form. In this study, we adhered to the principles of the Declaration of Helsinki is universally known in science and the Ethics Committee standards of the study centers (2020–2022). All clinical research requirements to maintain the data privacy of our study participants were explicitly met.

2.2. Eligibility criteria

Ophthalmologists from the glaucoma sections performed a systematized ocular examination of the potential participants of both sexes, aged 40–80 years, who had an appointment for the eye clinic. A total of 625 individuals were selected by a non-random consecutive sampling procedure. Most suitable study participants were chosen after ensuring their health status and ocular condition in agreement with the inclusion and exclusion criteria, as shown in Table 1. The suitable volunteers were classified into two groups: (1) patients with POAG diagnosis (POAGG; $n = 380$) and (2) individuals without glaucoma, as a comparative–control group (CG; $n = 245$). The whole sample of POAG patients was under glaucoma treatment (hypotensive eye drops, laser, or glaucoma surgery), depending on the glaucoma stage and personal characteristics. The final sample size of our study participants was (358 POAG patients and 226 CG individuals). The reduction from the initial number of the recruited sample was due to volunteer dropout, clinical findings/unique or unusual complications that recommended excluding the participant, and/or sampling transport or laboratory processing.

TABLE 1 Inclusion and exclusion criteria for the study participants.

Inclusion	Exclusion
Individuals aged 40–80 years	Individuals under 40 years of age
Accurate diagnosis of POAG for the corresponding group	Other glaucoma type
Non-glaucomatous healthy individuals for the comparative–control group of participants (CG)	Patients experiencing other ophthalmological diseases and/or comorbidities. Patients receiving local or systemic treatment that may interfere with the study. Eye laser surgery in the previous 12 months.
Precise and complete data on the medical history. Psycho-physical status that permits for participation in the study	History, including previous diagnoses that do not fit with the study purpose Unfeasibility of having a thorough and complete clinical history and being unable to participate.

POAG, primary open-angle glaucoma; CG, comparative controls.

2.3. Proceedings of the clinical approaches

Each participant was interviewed to determine the social and demographic factors, systemic comorbidities, and glaucoma medications. Data corresponding to this part were recorded as DEMO in a Microsoft Excel spreadsheet that was explicitly reviewed by the study coordinators.

Ophthalmological examination was performed by combining the IOP measurements (using Goldmann applanation tonometry Haag–Streit AT 900; Haag–Streit Köniz, Switzerland), morphological measurements [indirect gonioscopy through a slit lamp (IMAGEnet, Topcon, Barcelona, Spain) with the Goldmann 3-mirror lens, to demonstrate an open anterior chamber angle; ocular fundus exploration through a slit-lamp with a 78 D lens; and optical coherence tomography (OCT) examination (Cirrus spectral–domain OCT, Carl Zeiss Meditec, Inc., Madrid, Spain)], as well as the functional VF performance, using the 24-2 Swedish interactive threshold algorithm, Humphrey field analyzer (Carl Zeiss Meditec, Inc., Madrid, Spain). In this context, best-corrected visual acuity (BCVA) obtained by the logarithm

of the minimum angle of resolution (LogMAR) for each eye and the IOP determination (three consecutive times) were registered during the ophthalmological visit. It has to say that only the ophthalmologists involved in the present study were responsible for the measurements. Normal IOP is considered as the IOP two standard deviations (SDs) above the normal, i.e., 21 mm Hg, and the IOP above this level was defined as OHT. The IOP values were registered in millimeters of mercury as the mean ± SD for three determinations for each participant's eye. To determine the central corneal thickness (CCT), the hand-held ultrasonic pachymetry (Reichert® iPac®; Reichert/Ametek, Munich, Germany) was used, and three independent measurements were performed in a random sequence of 3 min from each other. Normal CCT was estimated at 533 μm and registered as the mean ± SD of three determinations for each participant's eye. For the CG, the IOP had to be lower than 21 mmHg, with normal visual fields, optic disc, and retinal nerve fiber layer (RNFL) in absence of another ocular or systemic disease. Moreover, glaucomatous OND was considered when including specific ONH alterations, such as neuroretinal rim thinning, splinter hemorrhages, peripapillary nerve fiber loss, asymmetry of cupping between patient eyes, and parapapillary atrophy, among others. We recorded the data into a Microsoft Excel spreadsheet as "OPHTHAL," which was reviewed by two independent ophthalmologists.

Three types of biosamples were collected from the study participants for the programmed experiments, tears, aqueous humor, and blood. (1) Reflex tears were collected (30 μl) from the two study groups by a gentle rubbing of the inferior meniscus and external canthus of each eye without instilling anesthetics, as previously described (42–44), by using a Microhematocrit capillary tube, appropriately labeled, which was immediately transferred into micro-Eppendorf tubes and stored at −80°C until processing. (2) Aqueous humor was collected (100 μl) at the time of glaucoma surgery, under an operating microscope by means of a Rycroft cannula, through the incision for performing classic trabeculectomy, valvular procedures, or microincisional glaucoma surgery (MIGS), with special care to avoid contamination. Samples were immediately frozen at −85°C until processing, as described elsewhere (45, 46). Also, at the time of surgery, the aqueous humor was collected from individuals programmed for non-complicated cataract surgery with the same described protocol (27–29, 40–42) that were considered the comparative–control participants. (3) Peripheral blood samples were collected, from the two study groups, under fasting conditions at 8:00 a.m. from the antecubital vein into 4.5 ml ethylene-diamine-tetra-acetic acid (EDTA) or sodium citrate vacutainer tubes (Becton Dickinson, Auckland, New Zealand), as an anticoagulant. The EDTA tube (purple cap) was used to analyze gene expression. The sodium citrate tube (purple cap) was centrifuged at 3,000 rpm/10 min to obtain the plasma fraction, which was aliquoted and stored at −80°C until processing to diverse analyses, as previously shown (47, 48). All experiments were performed in duplicate at the laboratories of the Ophthalmic Research Unit "Santiago Grisolía" and the Department of Surgery of the University of Valencia (Valencia, Spain).

The biochemical, molecular, and genetic actors that were assayed in this study were malondialdehyde (MDA), total antioxidant capacity (TAC), nitric oxide (ON), interleukin-6 (IL-6), endothelin 1 (ET1), brain-derived growth factor (BDNF), serotonin or 5-hydroxy tryptamine (5-HT), and its metabolite the 5 hydroxyindolacetic acid (5-HIAA), specific genes related to antioxidant status (SLC23A2 gene and GPX4 gene), and specific miRNAs.

The proceedings are described below.

- *Determination of lipid peroxidation by-products.* This was measured by the MDA/TBARs Assay Kit (Reference: KB-03-016, BioQuochem, Asturias, Spain), based on the measurement of thiobarbituric acid reactive substances (TBARS) formed after the reaction of MDA with thiobarbituric Acid (TBA). The assay was done under high-temperature conditions (90°C for 60 min), and the product of the reaction was measured in a colorimetric way using a 532 nm absorbance value for the plasma samples. The concentration was calculated by extrapolating all data in the standard curve as reported (27–31, 45–47).
- *Determination of TAC.* This was measured by the antioxidant assay kit (Reference: 709001, Cayman Chemical Company, Ann Arbor, MI, USA) based on the antioxidant capacity to inhibit the 2, 2′-azino-di-[3-ethylbenzthiazoline sulphonate] oxidation to 2, 2′-azino-di-[3-ethylbenzthiazoline sulphonate] radical solution by the metmyoglobin, as published (27–31, 46–49). The reaction's product was measured colorimetrically using a 750-nm absorbance value for the plasma samples. The concentration was calculated by extrapolating all data in the standard curve.
- *Determination of NO.* The total nitric acid was determined using a commercial preparation by R&D Systems. This essay was based on the enzymatic conversion of nitrate to nitrite by means of nitrate reductase enzyme. After the reaction, the colorimetric determination of the nitrite is carried out by Griess' reaction, which is based on a two-stage diazotization reaction: (1) acidification of NO_2 to produce a nitrosating agent and (2) reaction of this agent with sulfanylic acid to produce a diazonium ion which will be joined to N-(1-naphthyl) ethylenediamine to form a chromophore which absorbs light at 540–570 nm, and which is measurable in the aqueous humor samples (50).
- *Determination of 5-HT and 5-HIAA.* Samples were diluted 1:3 v/v in 0.2N perchloric acid, filtered through a 0.2 μm Nylon microfilter (Costar, Cambridge, MA) by centrifugation (10,000 rpm for 5 min at 4°C), and analyzed by high-performance liquid chromatography (HPLC) with electrochemical detection (ECD), according to a modified method of Audhya, Adams, and Johansen (51). Analyses were performed using a Gilson Medical Electronics HPLC system (Middleton, WI) with an LC-234 auto-injector equipped with an LC3-07 delivery pump and with an LC-142 electrochemical detector under reversed phase conditions with a Supelcosil LC 7.5 cm × 4.6 cm, 3-mm column (Supelco; Sigma-Aldrich, Bellefonte, PA). The software used was a 712 HPLC system controller data version 1.30 management (Gilson Medical Electronics). Compounds were eluted isocratically over an 18-min runtime at a 1 ml/min flow rate. The mobile phase consisted of 70 mM potassium dihydrogen phosphate buffer (pH adjusted to 3.0 with phosphoric acid), 1 mM 1-hepatosulfonic acid, 107.5 mM sodium EDTA, and 10%

methanol. Sample injection was 20 ml, and the electrochemical detector was recorded with a glassy carbon working electrode set at +0.75 V. Identification was performed by comparison with standard retention times determined by injections of standard mixture run at given intervals between sample analyses. Quantification was made using the calibration curve standards with 5-HT ($r = 0.0004$) and 5-HIAA ($r = 0.0003$). Samples were injected in duplicate, and the amount of each compound was expressed as mean ± SE in ng/ml of the corresponding blood or aqueous humor sample.

- *Determination of IL-6.* Tears, aqueous humor, and plasma samples were used to determine the cytokine expression in POAG patients vs. the CG. This was performed using the Human IL-6 ELISA Kit (Ref: EH2IL6, Invitrogen, Vienna, Austria), which was based on an *in vitro* enzyme-linked immunosorbent assay (ELISA) for the quantitative measurement of human IL-6.An IL-6 molecule is binding for two antibodies: (1) monoclonal antibody specific for IL-6 and (2) acetylcholinesterase:Fab' conjugate. The enzymatic activity of acetylcholinesterase is measured at 450 nm. The concentration of the IL-6 is proportional to the amount of bound conjugate as widely reported (42–44, 52, 53).
- *Determination of ET1.* We used a commercial kit for human endothelin-1 (BI-20052, Biomedica Gruppe, Vienna, Austria). Using this kit, by the action of the Endothelin Converting Enzyme-1 (ECE-1), the pro-ET molecule (or big ET) is split into two fractions: the active ET (21 amino acids) and an inactive C-terminal fragment. Using a double-antibody sandwich technique, the active ET binds the two antibodies. A conjugate is formed, producing a yellow color as an indicator, which absorbs at 450 nm. The intensity of this color is proportional to the amount of bound conjugate, which in turn is proportional to the ET concentration in the aqueous humor samples as described (54).
- *Determination of BDNF.* We used the Human BDNF Immunoassay Kit (DBD00, R&D Systems Inc., MN, USA). The kit is based on a double-antibody sandwich technique. The BDNF present in samples is bound to the two antibodies, forming a conjugate. This conjugate reacts with a substrate solution, and the color is developed proportionally to the BDNF concentration. The intensity of the color is measured by spectrophotometry at 540 nm for the aqueous humor samples, as described (55).
- *Gene expression assays.* Whole blood samples were obtained from each participant and collected into EDTA tubes. Total RNA was isolated from blood samples by the Trizol method. Then, 300 ng of total RNA (integrity number: RIN > 7) were converted into cDNA by reverse transcription using the High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems, Foster City, CA, USA). The relative SLC23A2 and GPX4 gene expression was analyzed by real-time PCR using a 7900HT Sequence Detection System (SDS; Applied Biosystems®, Madrid, Spain). TaqMan gene expression assays were used for both target (SLC23A2, GPX4) and internal control (18S rRNA) genes (Applied Biosystems®, Spain). Samples were assayed in duplicate. The expression values were calculated by the double delta Ct formula as previously reported (56, 57). The results were expressed as fold changes in gene expression for each group and subgroup at baseline.
- *miRNA expression assays.* On the day of processing, samples were defrosted and prepared for RNA extraction using the miRNeasy Mini Kit (QIAGEN Inc., Hilden, Germany). Briefly, purification is based on spin column chromatography using a proprietary resin as the separation matrix. Small RNAs are separated from other cellular components, such as proteins without using phenol or chloroform. The quality and quantity of total RNA obtained from tears were assessed using a Bioanalyzer 2100 (Agilent® Technologies, Inc., Santa Clara, CA, USA) and the RNA 6000 Nano Kit (Agilent® Technologies, Inc.). RNA libraries were prepared using NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (#E7300 y #7580; New England BioLabs®, Inc., Ipswich, MA, USA), according to the manufacturer's protocol (https://international.neb.com/protocols/2018/03/27/protocol-for-use-with-nebnext-small-rna-library-prep-set-for-illumina-e7300-e7580-e7560-e7330). According to the guidelines for low RNA concentration samples, the adapters and RT primers were diluted 1:2 with nuclease-free water, and 15 cycles were used for the amplification by PCR. The indexed libraries were purified using the QIAquick® PCR Purification Kit (#28104, QIAGEN®, Hilden, Germany). Library quality control was assessed using a 4200 TapeStation (Agilent® Technologies, Inc.) and High Sensitivity D1000 Kit (Agilent® Technologies, Inc.). The miRNA fraction of each library (120–200 bp) was collected using the Pippin Prep System (Sage Science, Inc., Beverly, MA, USA) following the manufacturer's guidelines and using 3% agarose dye free gel cassettes with internal standards (Marker P) (Sage Science #CDP3010). miRNAs were quantified using a 4200 TapeStation (Agilent® Technologies, Inc.) and High Sensitivity D1000 Kit (Agilent® Technologies, Inc.) prior to normalization and pooling. Sequencing was performed on a NextSeq 500 System (Illumina, Inc., San Diego, CA, USA) with a Mid-Output flow cell for 150-cycle reads, obtaining about 3.5 million reads per sample. FASTQ file quality was assessed using the FASTQC tool (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Adapters and low liability reads were removed. At this point, non-coding RNAs previously described in the ENSEMBL database were selected and characterized. Statistical analyses were performed using Limma and edgeR packages deposited in Bioconductor (www.bioconductor.org). A predictive analysis based on receiver operating characteristic (ROC) curves was performed to select those miRNAs showing an area under the curve (AUC) >0.75. Subsequently, an analysis of the main components (PCA) was performed. The proceeding for searching the target genes of the selected miRNAs was reported elsewhere (58–60).

2.5. Statistical processing

All data were statistically processed using the IBM SPSS 25.0 program. The normal distribution of the quantitative variables was

verified using the Shapiro-Wilk test (subgroups) and Kolmogorov-Smirnov test (main groups). Qualitative variables were described by absolute and relative frequencies. Quantitative variables were defined using the mean and standard deviation (normal distribution) or median and interquartile range (non-normal distribution). Differences between quantitative variables were analyzed using the Student's *t*-test for independent samples and ANOVA (normal variable) or the Mann–Whitney and Kruskal–Wallis U test (non-normal variable). The association between qualitative variables was determined using the Chi-square test or Fisher's exact test. The correlation between two quantitative variables was analyzed using the Pearson correlation coefficient (normal distribution) or the Spearman correlation coefficient (non-normal distribution). The differences were considered statistically significant when $p \leq 0.05$.

3. Results

Absolute number of study participants was 584, which was subdivided into POAG patients ($n = 358$) and CG individuals ($n = 226$). The mean age was 70.03 ± 9.23 years in the POAGG and 70.62 ± 7.89 years in the CG. Gender distribution was 70% women in the POAG and 52% in the CG.

All POAG patients showed IOP elevation, increased optic disc excavation, ONH damage, and/or altered visual fields, and these participants were all under glaucoma treatment. The ophthalmological parameters of the study participants are shown in Table 2. Moreover, individuals conformed to the control–comparative group of non-glaucoma eyes and did not show any of the above glaucoma hallmarks.

TABLE 2 Ophthalmological characteristics of the study participants.

Clinical variables	POAGG	CG	*p*-value
IOP (mm Hg)	19 ± 2	15 ± 2	<0.0001
CCT (mm)	534 ± 31	579 ± 38	<0.0001
Average C/D ratio	0.6 ± 0.02	0.04 ± 0.04	<0.0001
Vertical C/D ratio	0.6 ± 0.1	0.1 ± 0.01	<0.0001
Average RNFL thickness (μm)	73 ± 12	91 ± 13	<0.0001
Rim area (μm^2)	5 ± 0.9	1 ± 0.6	<0.0001
RGCs density	66 ± 10	92 ± 9	<0.0001

POAGG, primary open-angle glaucoma group; CG: comparative–control group; IOP, intraocular pressure; CCT, central corneal thickness; C/D, cup/disk; RNFL, retinal nerve fiber layer; RGCs, retinal ganglion cells. Comparison between groups was statistically significant.

3.1. Oxidative stress

When the antioxidant defense mechanisms fail, all biomolecules (lipids, proteins, nucleic acids, etc.) are susceptible to be attacked by reactive oxygen species (ROS), and probably lipids are the most prone to undergo oxidation, in a process named oxidative stress (OS). Main lipid peroxidation by-products, the total antioxidant status, and the nitrosative stress markers were determined in the study participants' aqueous humor; the results are highlighted in Figure 1.

The MDA levels were significantly higher in the aqueous humor from the POAGG than in the CG ($p < 0.001$). The TAC was considerably lower in the aqueous humor from the POAGG compared to the CG (<0.001). As a marker of nitrosative stress, the ON levels were significantly higher in the aqueous humor from the glaucoma patients than in the comparatives ($p < 0.001$).

3.2. Neuroinflammation

Cytokines are small proteins relevant to cell signaling. Pivotal pro-inflammatory cytokines, including IL-1, IL-6, and TNF-α, exert their effects through type I cytokine receptors (CCR1).

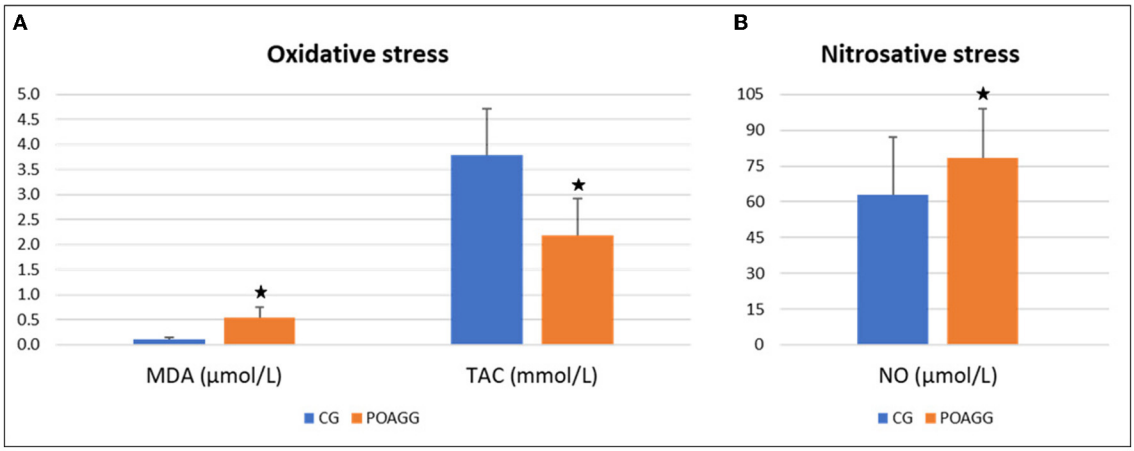

FIGURE 1
Oxidative stress markers in aqueous humor. POAGG, primary open-angle group; CG, comparative group; MDA, malondialdehyde; TAC, total antioxidant capacity; NO, nitric oxide. The star shows significant differences ($p < 0.05$).

These cytokines are essential for coordinating cell-mediated immune response.

In this study, the cytokine levels were assayed by the IL-6 expression in the tear film, aqueous humor, and plasma samples of our study participants, and the results are shown in Figure 2. Data showed that IL-6 was significantly higher in the tear samples of the glaucoma patients vs. the comparatives ($p < 0.0001$). The IL-6 levels were significantly higher in the aqueous humor of the POAGG vs. the CG ($p < 0.001$). Finally, the plasma IL-6 was significantly higher in the POAGG than in the CG ($p < 0.001$).

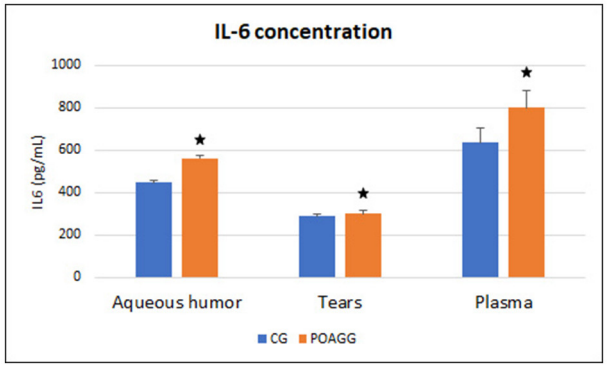

FIGURE 2
Interleukin-6 concentration in aqueous humor, tear, and plasma samples. POAGG, primary open-angle group; CG, comparative group. The star shows significant differences ($p < 0.05$).

3.3. Vascular endothelial dysfunction

The vasoconstriction is modulated by the endothelial cells, acting as counterparts of the opposed molecule, the NO, that, in turn, modulates vasodilation. The ET1 is a vasoconstrictor that contributes to the vascular tone and regulates cell proliferation (activating two receptors, ETA, and ETB). ET1 expression levels were significantly higher in the aqueous humor from the POAGG in comparison with the GG individuals operated on cataracts ($p = 0.001$), as compiled in Table 3.

TABLE 3 Biochemical parameters on the vascular endothelium, neurotrophins, and neurotransmitters in the aqueous humor of the participants.

Parameter	POAGG	CG	*p*-value
ET-1 (ng/ml)	2.75 ± 0.30	1.35 ± 0.16	0.0001
BDNF (pg/ml)	95.75 ± 14.85	111.30 ± 11.43	0.001
5-HT (ng/ml)	2.838 ± 220.61	3.076 ± 98.76	0.820
5-HIAA (ng/ml)	22.87 ± 6.51	19.10 ± 4.66	0.016

POAGG, primary open-angle glaucoma group; CG, comparative–control group; ET-1, endothelin-1; BDNF, brain-derived neurotrophic factor; 5-HT, 5-hydroxy tryptamine/serotonin; 5-HIAA, 5-hydroxyindoleacetic acid. All biochemical parameters were statistically significant, except the 5-HIAA that lacked significance.

3.4. Neurotrophins

Neurotrophins or neurotrophic growth factors are essential molecules involved in the development, maintenance, and function of the nervous system. The BDNF is a protein encoded by the BDNF gene with the central role of regulating plastic changes in the adult brain, including regulation of trafficking, phosphorylation, synapsis strength, etc.

The assayed BDNF concentration in the aqueous humor (Table 3) demonstrated significantly lower values of the assayed neurotrophin in the POAGG than in the CG ($p = 0.001$).

3.5. Neurotransmitters

Neurotransmitters are chemical messenger molecules released by neurons from the synaptic vesicles into the synapse and from here to the subsequent neurons. Serotonin, an indolamine, is an inhibitory neurotransmitter that regulate mood, anxiety, appetite, pain, sleep patterns, and sexuality. Serotonin is a precursor of melatonin. Melatonin has an inhibitor effect on the levels of nitric oxide.

Regarding the plasma serotonin levels in the study participants, when comparing the POAGG with the CG, 5-HT concentration was noticeably lower in the glaucoma patients ($p < 0.001$). Both the study groups had an inverse correlation between age and plasma 5-HT levels in (Pearson correlation coefficient: −0.191; $p = 0.028$). In addition, when analyzing the 5-HT plasma values with the perimetric evaluation of the right and left eyes (RE/LE) of the POAGG, plasma serotonin levels were significantly lower in the glaucomatous eyes (RE: 125.72 ± 65.46 ng/ml; LE: 128.84 ± 73.91 ng/ml; ($p < 0.001$)].

The aqueous humor concentrations of the assayed neurotransmitter are included in Table 3. 5-HT was noticeably lower in the glaucoma patients than in the comparatives ($p = 0.820$). In addition, 5-HIAA concentration, as a serotonin breakdown product, displayed significantly higher values in the aqueous humor of the POAGG vs. the CG (0.016). The 5-HT turnover (5-HIAA/5-HT) was higher in the GG than in the glaucoma patients (POAGG: 14.050 ng/ml vs. CG: 12.684 ng/ml; $p = 0.598$). In addition, the correlation between 5-HT and 5-HIAA was assessed using Pearson's correlation coefficient, and the levels of 5-HT and 5-HIAA were associated with POAGG: Pearson = −0.756 ($p = 0.021$; as compared to the CG of patients operated of cataracts: Pearson = −0.613 ($p = 0.028$).

3.6. Gene expression

The role of OS in POAG has been widely investigated. Moreover, many studies have shown the effect of antioxidant supplementation in glaucoma. Goyal et al. (55) demonstrated that vitamin C levels in the aqueous humor of POAG patients were significantly lower than those obtained from comparative individuals operated on cataracts. Similarly, our group found

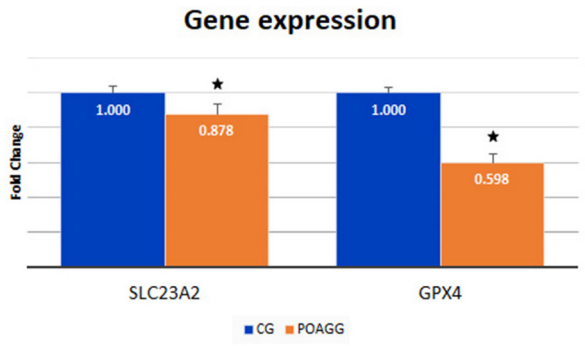

FIGURE 3
Expression of SLC23A2 and GPX4 genes in blood samples of patients with primary open-angle glaucoma and healthy subjects. POAGG, primary open-angle group; CG, control group. The star shows significant differences ($p < 0.05$).

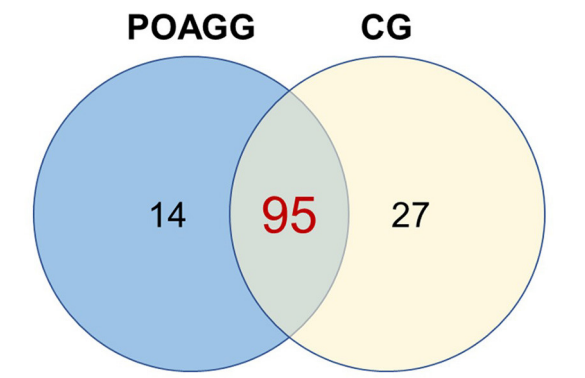

FIGURE 4
Number of miRNAs identified in tear samples from primary open-angle glaucoma and ocular hypertension patients. POAGG, primary open-angle group; CG, comparative group.

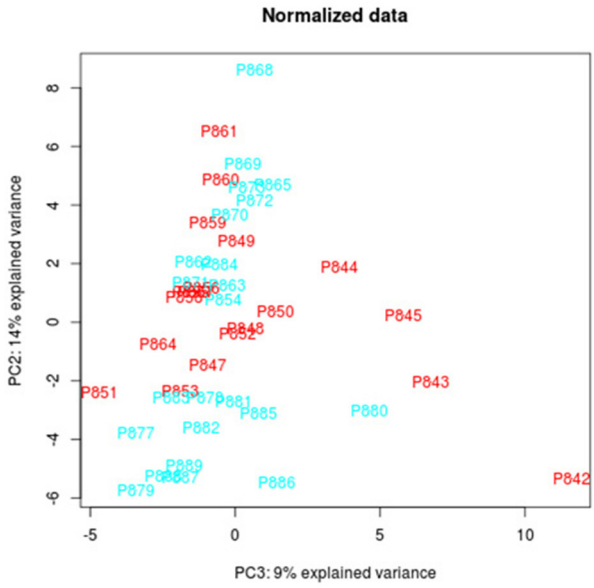

FIGURE 5
Principal component analysis (PCA) from all the miRNAs identified in the tear samples from the 2 study groups.

substantially lower levels of vitamin C, GPX, and other antioxidants in the aqueous humor of POAG patients (56). In this study, we assayed the expression levels of two genes involved in OS, the solute carrier family 23—nucleobase transporters—member 2 (SLC23A2) gene, which is the main effector of L-ascorbic acid transmembrane transporter activity, and the glutathione peroxidase 4 (GPX4) gene, also named GSHPx-4, that is implicated in catalyzing the reduction of hydrogen peroxide (H_2O_2) by glutathione with the primary purpose of protecting the cells against oxidative attack. We determined the SLC23A2 gene expression in the aqueous humor of the POAG patients, which was significantly lower than that in the CG ($p < 0.001$) (Figure 3). Also, the GPX4 gene expression levels in the aqueous humor of the POAGG were significantly lower than in the CG ($p < 0.001$) (Figure 3).

3.7. Micro-RNAs fingerprint

The total RNA extraction in the tear film of the study participants, followed by the construction of libraries, and the analyses by next-generation sequencing (NGS) were carried out, and 122 miRNAs were identified in tears, of which 95 were expressed in both POAG patients and comparative subjects (Figure 4). Bioinformatic analyses permitted to create the PCA of all miRNAs that were identified in tear samples of the participants (Figure 5). Moreover, the more specific PCA (Figure 5) permitted to identify the miRNAs that differentially expressed between groups, and those with the higher area under the curve (Table 4) were hsa-miR-26b-5p (involved in cell proliferation and apoptosis), hsa-miR-152-3p (regulator of cell proliferation, and extracellular matrix expression), hsa-miR-30e-5p (regulates autophagy and apoptosis), and hsa-miR-151a-3p (regulates myoblast proliferation). Figure 6 shows the expression of these miRNAs in tears of POAG patients vs. comparative subjects. In Table 4, miRNAs with the higher statistical power for the differential expression between groups (fold-change between groups) are shown. In Table 5, surrogated biological actions of the four miRNAs identified in the tears of the study participants are reflected.

We designed a new illustration (Figure 7) with the 4 miRNAs with higher area under the curve and their glaucoma-related target genes, identified in the following databases: https://mirdb.org/ and https://www.targetscan.org/.

In summary, we prepared a new figure to reflect the expression trend of the biomarkers obtained from the tears, plasma, blood, and aqueous humor samples of the study participants (Figure 8).

4. Discussion

In this study, we explored some classical and emergent biomarkers of POAG in 584 men and women volunteers pertaining to a Spanish ophthalmological population aged 40–80 years, with

TABLE 4 miRNAs with the higher statistical power for the differential expression between groups (fold change between groups).

miRNA ID	Fold change§ (POAGG vs. CG)	*p*-value*	AUC
hsa-miR-26b-5p	1.809	0.012	0.81693
hsa-miR-30e-5p	1.867	0.005	0.76201
hsa-miR-151a-3p	0.749	0.009	0.75972
hsa-miR-152-3p	1.685	0.004	0.75743

miRNA, microRNA; ID, identification; POAGG, primary open-angle glaucoma group; CG, comparative group; AUC, area under the curve. §CG was formed by ocular hypertension patients and was set as the reference group. *Significant when $p = 0.05$.

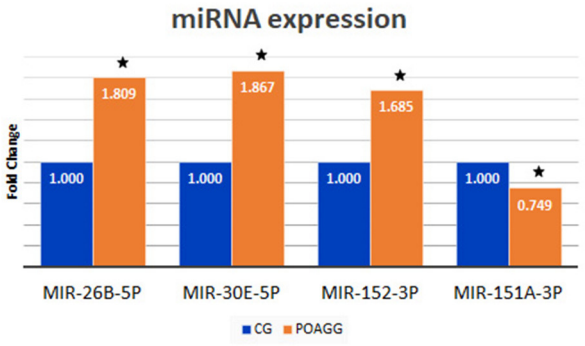

FIGURE 6
Tear expression of the 4 miRNAs with higher area under the curve from the 2 study groups. POAGG, primary open-angle group; CG, comparative group. The star shows significant differences ($p < 0.05$).

TABLE 5 Biological processes significantly associating primary open-angle glaucoma and miRNAs.

BP ID	BP name	N	*p*-value*
GO:0009101	Glycoprotein biosynthetic process	21	0.001
GO:1901137	Carbohydrate derivative biosynthetic process	27	0.002
GO:0030154	Cell differentiation	193	0.006
GO:0007166	Cell surface receptor signaling pathway	138	0.009
GO:0009628	Response to abiotic stimulus	50	0.011
GO:0016477	Cell migration	61	0.018
GO:0048870	Cell motility	63	0.024
GO:1902533	Positive regulation of intracellular signal transduction	41	0.029
GO:1901135	Carbohydrate derivative metabolic process	79	0.03

miRNA, microRNA; BP, biological process; ID, identification; GO, gene ontology. *Significant when $p = 0.05$.

the primary goal of disclosing the importance of identifying glaucoma biomarkers with the highest sensitivity and specificity to pick out people at risk of glaucoma development and progression, to better fight against glaucoma OND and blindness.

To update the clinical and biochemical-molecular-genetic glaucoma pathogenesis, we collected tears, aqueous humor, and blood samples (whenever possible, one or more than this type of sample per patient) from the POAGG and GC study participants to be processed for identifying POAG biomarkers. For this purpose, we explored several biological pathways: apoptosis, inflammation, neurotransmitter/neurotrophin alteration, oxidative stress, specific gene expression, micro-RNAs fingerprint and its biological targets, and vascular endothelial dysfunction. Thus, we obtained the following biochemical, molecular, and genetic biomarkers that showed a differential expression profile between the groups of participants: MDA, TAC, ON, IL-6, ET1, BDNF, 5-HT, 5-HIAA, the SLC23A2 and GPX4 genes, and the miRNAs hsa-miR-27a-3p, hsa-miR-152-3p, hsa-miR-151a-3p, and hsa-miR-1307-3p.

Scientific evidence supports the role of both oxidative and nitrosative stress in POAG pathogenesis (27–32, 61–63). The following are among the most relevant hypothesis. (1) The trabecular meshwork morphology and function are significantly compromised by ROS. (2) Resistance to aqueous humor outflow in the anterior eye chamber is higher when ROS are present. (3) Main OS parameters are noticeably altered in tears and in aqueous humor of POAG patients. (4) Some specific antioxidant enzymes and vitamins are significantly reduced in the aqueous humor of POAG patients, (5) Oxidative DNA damage significantly correlates with elevated IOP in POAG patients. (6) Specific candidate genes are involved susceptibility to ROS-induced damage in POAG. (7) The anterior and posterior eye segments suffer OS attack in the glaucoma course. In this study, it has been found among the participants that a disbalance between the pro-oxidants and antioxidants in the aqueous humor of the POAG patients with respect to the CG (see Figure 1). In addition, the mean age of the glaucoma participants was 70.03 ± 9.23 years, which is a relevant risk factor for POAG, as well as for OS (27–29, 64). The OS and its downstream effectors have been involved in ocular diseases, such as dry eyes, corneal dystrophies, uveitis, cataracts, diabetic retinopathy, age macular degeneration, retinitis pigmentosa, toxic neuropathies, and others. Despite this, the precise signaling pathways regulated by ROS are, however, not widely known.

Pathogenic glaucoma milestones are the progressive damage and apoptotic death of the RGCs and degeneration of the ONH axons (1, 3, 5, 65). Epidemiological and animal model studies fully demonstrated that neuroinflammation plays a vital role in POAG (21.24). The OS can induce neuroinflammation in the glaucoma course, through a variety of biological pathways, among them: the secretion of pro-inflammatory cytokines from the retinal glia (13, 65). The OS can activate the inflammasome (66–68). Pro-inflammatory cytokines increase phagocytosis in glaucoma eyes (69). In this study, we demonstrated that the IL-6 showed significantly differential expression values in tears, aqueous humor, and plasma from the POAGG concerning the CG.

Endothelial cells intervene in regulating blood flow. Changes in the endothelial cells and the blood vessels are fully involved in the pathogenic mechanisms of a wide variety of diseases: diabetes, stroke, cardiopathies, venous thrombosis, chronic kidney failure, tumor growth, and metastasis (70). Endothelial dysfunction early occurs in the vascular complications associated with the above diseases and has been linked to the decreased bioavailability

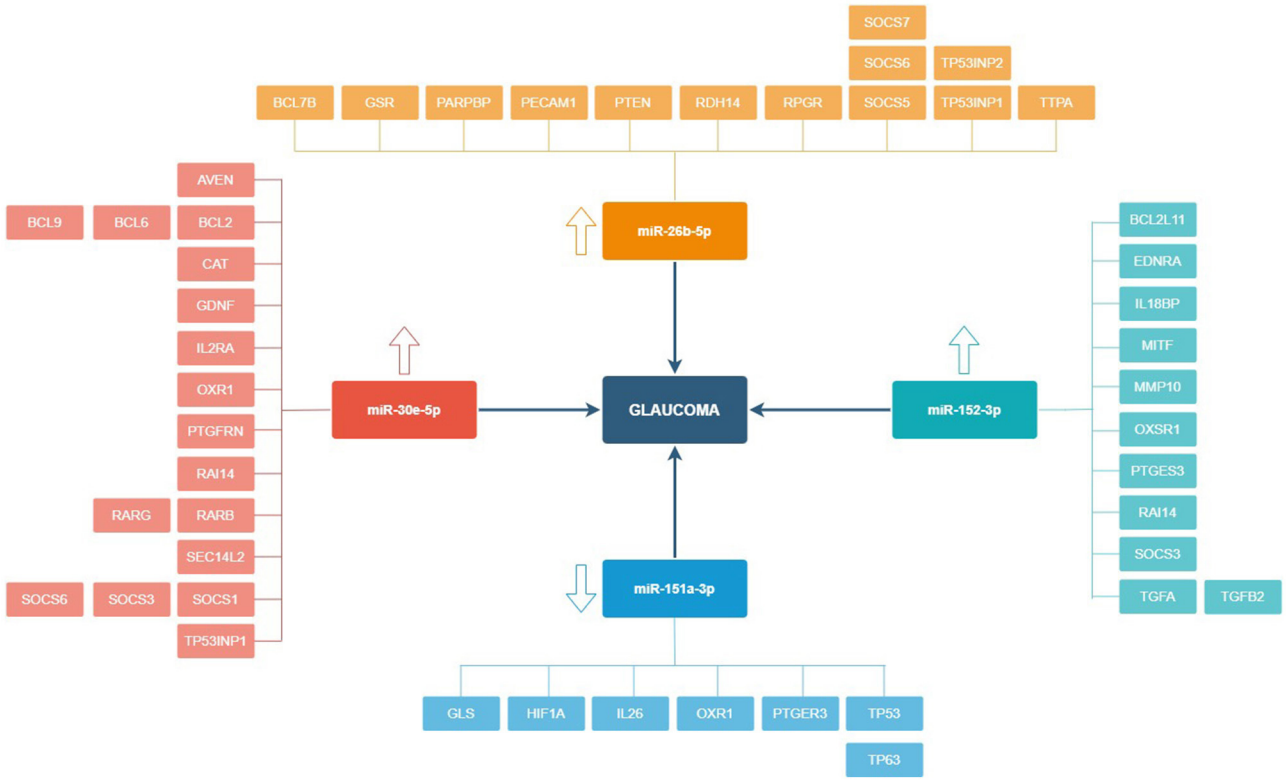

FIGURE 7
miRNAs and main target genes in relation to primary open-angle glaucoma. The 4 miRNAs included in this figure are those with significantly different expression between groups and with greater area under the curve. Target genes have been obtained from the MicroRNA Target Prediction Database (https://mirdb.org) and TargetScanHuman (https://www.targetscan.org/vert_80).

of specific vasodilators, such as NO. Nevertheless, it has been well established that the relevant role of the enhanced endogenous activity of ET-1, a vasoconstrictor, pro-inflammatory, and mitogenic endothelial peptide in specific human diseases related to endothelial dysfunction (71). Despite the importance of the vascular endothelium and the role of ET-1 and NO, scarce but exciting research has been done in relation to POAG pathogenesis (72, 73).

In this study, we have shown that ET1 and NO levels were significantly higher in the aqueous humor from the POAGG than that in GG, as reflected in Table 3 and Figure 1. It has been described that increased ET-1 availability is rather a consequence of reduced NO levels (74). We venture that an imbalance between ET1/NO can be involved in developing endothelial dysfunction and altered modulation of ocular blood flow. and aqueous humor homeostasis.

A wide variety of molecular signals promote RGC and optic fibers death in glaucoma OND, among them it has to be considered: OS, INF, mitochondrial failure, excitotoxicity, neurotrophin deprivation, axonal transport dysfunction, apoptosis, neuroglia alterations, synaptic loss, etc. (31, 35) Trying to understand the involvement of neurotrophins in POAG could help improve knowledge for better eye and vision care in glaucoma. In this study, we have found that BDNF levels in the aqueous humor (refer to Table 3) were significantly lower in the POAGG than in the CG.

Neurotransmitters are essential for visual function. Although specific neurotransmitters, such as the cholinergic drugs, have been used in hypotensive glaucoma treatment (75), less is known about the role of other neurotransmitters in enhancing neuron survival and vision care in glaucoma. It has been recently reported that Coenzyme Q10 (an electron carrier from complexes I and II to complex III, with an essential role in adenosine triphosphate synthesis, and an important antioxidant) and Citicoline (a nootropic agent con numerous beneficial effects in the CNS), eventually combined, in the prevention of glaucoma OND (76). Herein, we have shown that the expression levels of serotonin in plasma samples and aqueous humor were noticeably lower in the POAGG vs. the CG. Furthermore, when analyzing the inhibitory neurotransmitter plasma levels with the perimetric evaluation of both eyes of the study participants, plasma serotonin concentration was significantly lower in the glaucoma eyes than in the CG. We propose that serotonin deserves further research for potential use as a diagnostic glaucoma biomarker.

Gene expression of some genes related to OS has been studied in this study. Previously, our group and others found significantly lower levels of vitamin C, GPX, and other antioxidants in the aqueous humor of POAG patients (56, 57) demonstrating that vitamin C levels in the aqueous humor of POAG patients were significantly lower than those obtained from comparative individuals operated of cataracts. Majsterek et al. (77) reported significantly lower levels of GPS in the aqueous humor of POAG

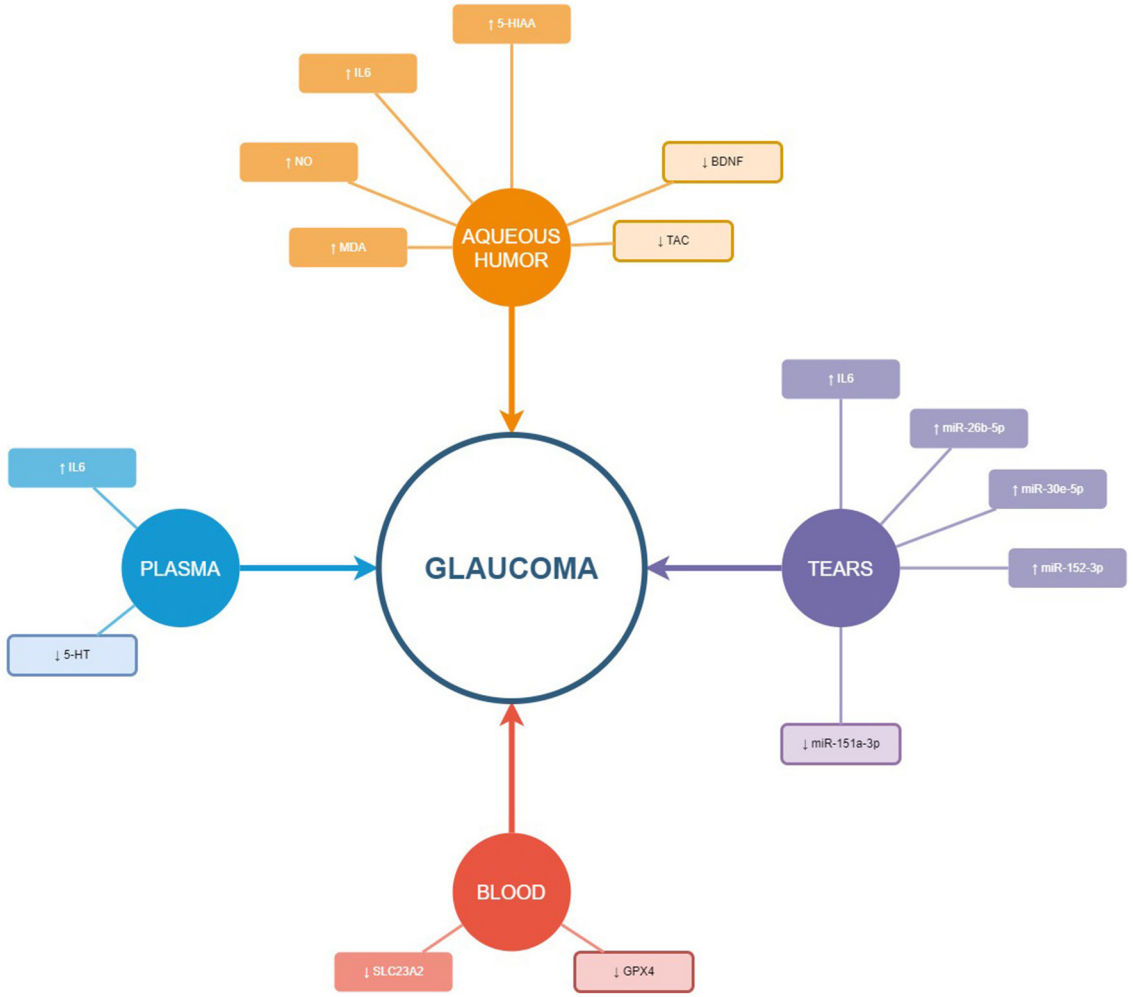

FIGURE 8
Schematic illustration of the biomarkers studied in the different samples (aqueous humor, tears, plasma, and blood). The up-arrow shows over expression and the down-arrow under expression of the biomarker in glaucoma compared to control or comparative group. The miRNA included in this figure are those 4 with significantly different expression between groups and higher area under the curve.

patients compared with the CG. These results support the critical role of genes related to the OS physiopathology and antioxidant defenses in POAG.

miRNAs are small non-coding RNA molecules that regulate gene expression, and have been involved in health and disease, including glaucoma (78). In this study, we have successfully profiled the tear miRNAs fingerprint from POAG patients with respect to the CG, and 4 specific miRNAs were the strongest candidates to be diagnostic glaucoma biomarkers. Those miRNAs are: hsa-miR-26b-5p (involved in cell proliferation and apoptosis), hsa-miR-152-3p (regulator of cell proliferation, and extracellular matrix expression), hsa-miR-30e-5p (that regulates autophagy and apoptosis), and hsa-miR-151a-3p (regulator of myoblast proliferation). In Figure 6, the differential expression of these miRNAs in tears of POAG patients vs. comparative subjects can be fully appreciated. Also, new biotherapies for POAG can arise from the aforementioned identified miRNAs.

Regarding the biological samples (79) used in this study, we emphasize that tears are painless and relatively easy to collect, store, manipulate, and process, and are instrumental samples to identify biochemical, molecular, and genetic biomarkers, that differentially express themselves in the POAG patients and the comparative-control individuals. In contrast, aqueous humor requires to be collected in surgical conditions. Still, more volume of sample (100 μl) than in the case of the reflex tears (30 μl) can be collected from each eye at the very beginning of surgery, which benefits the number of molecules to be determined. This also happens with the blood samples, which need to be done by the nursing staff who can collect 10–20 ml from each person.

Study limitations are as follows. (1) Volunteers may have underreported their personal and familiar data for the clinical history (either consciously or because of recall bias). (2) Glaucoma medications were recorded but not included in the final statistics. (3) This study produced much information that was statistically processed by generating a large volume of data. Thus, we concentrated on the primary study purposes, and some of the information and data acquired were finally excluded from the final statistical processing. Correspondingly, some actions were carried out in order to reduce the study limitations and to achieve the best results. (1) It was better to revise and confirm the patient data

collection. (2) Any discrepancy in the recruitment of participants, data screening, and results obtained was discussed by our team. We know that the study results could have reflected any of the described limitations. Nevertheless, to confirm coherence in the collected information, both the data scrubbing and normalization were independently performed by two researchers. With the aforementioned actions, we warrant the strength of our data power.

5. Conclusion

Studies to date have been adding classical biomarkers for POAG. Based on our present study, several potential solid biomarker candidates have emerged in relation to the most relevant POAG pathogenic mechanisms: OS, INF, vascular endothelium, neurotrophins and neurotransmitters, specific genes, and miRNAs. Therefore, the following biochemical, molecular, and genetic biomarkers have been considered for diagnosis, management, and assessment of therapeutic effects of POAG: MDA, TAC, NO, IL-6, ET-1, 5-HT, and 5-HIAA, SLC25A2 gene, GPX4 gene and the miRNAs hsa-miR-26b-5p, hsa-miR-152-3p, hsa-miR-30e-5p, and hsa-miR-151a-3p. Viewing the complexity of the pathogenic mechanisms of POAG, we suggest that the design and development of blended biomarkers is a more appropriate solution in ophthalmological practice for early diagnosis and to predict therapeutic response in glaucoma patients.

Data availability statement

The data for this article is available in Dryad Digital Repository: Zanon-Moreno, Vicente; Pinazo-Duran, Dolores (2023), miRNAs in primary open-angle glaucoma and ocular hypertension, Dryad, Dataset, https://doi.org/10.5061/dryad.bcc2fqzj1, link: https://datadryad.org/stash/share/Uyjq8RwQTXQ-agNmK1uR1Kl9v6uh9bZ7lR7DjxCq7sU.

Ethics statement

The studies involving human participants were reviewed and approved by CEIC University Hospital Doctor Peset, Valencia. The patients/participants provided their written informed consent to participate in this study.

Author contributions

CN and JG-F: study coordinators. All authors contributed to the article and approved the submitted version.

Funding

The Spanish Network of Inflammatory Diseases: REI-RICORS (RD21/0002/0032) of the Institute of Health Carlos III (ISCIII), Spanish Government, Madrid (Spain) has founded part of this study through the financial support given to the research activities of the members pertaining to this Network. In addition, The Foundation for the Promotion of Health and Biomedical Research of Valencia Region, FISABIO, Valencia (Spain), provided the fundus for open access publication fees through the Polisabio Innovative Research Projects Grant BIOMEGA-2.0/2023 (group leader: IA-B). Part of this study was also supported by the research fundus of the Department of Surgery, Faculty of Medicine and Odontology of the University of Valencia (MP-D).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Glossary

5HIAA, 5-Hydroxy-indoleacetic acid; 5HT, 5-Hydroxy tryptamine/serotonin; AUC, area under the curve; AVEN, apoptosis and caspase activation inhibitor; BCL2, B-Cell CLL/Lymphoma 2; BCL2L11, Bcl-2-Like Protein 11; BCL6, B Cell CLL/Lymphoma 6; BCL7B, B-Cell CLL/Lymphoma 7 Protein Family Member B; BCL9, B-Cell CLL/Lymphoma 9; BCVA, best corrected visual acuity; BDNF, brain-derived neurotrophic factors; CAT, catalase; CCT, central corneal thickness; cDNA, complementary DNA; CG, comparative-control group; CLOCK, circadian locomotor output cycles protein kaput; CYP1B1, cytochrome P450, family 1, subfamily B, polypeptide 1; ECD, electrochemical detection; ECE-1, endothelin converting enzyme 1; EDNRA, endothelin receptor type A; EDTA, ethylene-diamine-tetra-acetic acid; ELISA, enzyme-linked immunosorbent assay; ET1, endothelin 1; GDNF, glial cell-derived neurotrophic factor; GLS, glutaminase; GPX4, glutathione peroxidase 4; GSR, glutathione S-reductase; GSTM1, glutathione S-transferase mu 1; HIF1A, hypoxia inducible factor 1 subunit alpha; HPLC, high-performance liquid chromatography; IL1, interleukin 1; IL17A, interleukin 17A; IL18BP, interleukin 18 binding protein; IL26, interleukin 26; IL2RA, interleukin 2 receptor subunit alpha; IL6, interleukin 6; IL6ST, interleukin 6 cytokine family signal transducer; INF, inflammation; IOP, intraocular pressure; LE, left eye; LogMAR, logarithm of the minimum angle of resolution; MDA, malondialdehyde; MIGS, microincisional glaucoma surgery; miRNA, microribonucleic acid; MITF, microphthalmia-associated transcription factor; MMP10, matrix metallopeptidase 10; MYOC, myocilin; NT3, neurotrophin 3; NT4/5, neurotrophin 4/5; OCT, optical coherence tomography; OCTA, optical coherence tomography angiography; OHT, ocular hypertension; ON, nitric oxide; OND, optic nerve degeneration; OPTN, optineurin; OS, oxidative stress; OXR1, oxidation resistance 1; OXSR1, oxidative stress-responsive kinase 1; PARPBP, PARP1 binding protein; PECAM1, platelet and endothelial cell adhesion molecule 1; POAG, primary open-angle glaucoma; POAGG, primary open-angle glaucoma group; PTEN, phosphatase and tensin homolog; PTGER3, prostaglandin E receptor 3; PTGFRN, prostaglandin F2 receptor inhibitor; PTGS2, prostaglandin-endoperoxide synthase 2; RAI14, retinoic acid-induced 14; RARB, retinoic acid receptor beta; RARG, retinoic acid receptor gamma; RARRES1, retinoic acid receptor responder 1; RDH14, retinol dehydrogenase 14; RE, right eye; RGC, retinal ganglion cells; RIN, RNA integrity number; RNFL, retinal nerve fiber layer; ROC, receiver operating characteristic; RPGR, retinitis pigmentosa GTPase regulator; SD, standard deviation; SEC14L2, SEC14-like lipid binding 2 (tocopherol-associated protein 1); SLC23A2, solute carrier family 23 member 2; SOCS1, suppressor of cytokine signaling 1; SOCS3, suppressor of cytokine signaling 3; SOCS5, suppressor of cytokine signaling 5; SOCS6, suppressor of cytokine signaling 6; SOCS7, suppressor of cytokine signaling 7; TAC, total antioxidant capacity; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; TGFA, transforming growth factor alpha; TGFB2, transforming growth factor beta 2; TNF-α, tumor necrosis factor alpha; TP53, tumor protein P53; TP53INP1, tumor protein P53; inducible nuclear protein 1; TP53INP2, tumor protein P53 inducible nuclear protein 2; TP63, tumor protein P63; TTPA, alpha tocopherol transfer protein; VD, vascular dysfunction; WDR36, WD repeat Domain 36.

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