• Clinical Research •

Corneal nerve changes by anti-glaucoma medications examined by *in vivo* confocal microscopy

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Abstract

● **AIM:** To evaluate the effects of antiglaucoma eye drops on corneal nerves by *in vivo* confocal microscopy (IVCM).

● **METHODS:** This study comprised 79 patients diagnosed with glaucoma and 16 healthy control individuals. Among the glaucoma patients, 54 were treated with medication, while 25 remained untreated. Central corneal images were evaluated by IVCM, and then ACCMetrics was used to calculate the following parameters: corneal nerve fiber density (CNFD), branch density (CNBD), fiber length (CNFL), total branch density (CTBD), fiber area (CNFA), fiber width (CNFW), and fractal dimension (CNFrD). The correlation between IVCM parameters and drugs was evaluated using non-parametric measurements of Spearman's rank correlation coefficient.

● **RESULTS:** The CNFD was reduced in glaucoma groups compared to healthy subjects (P<0.01). Patients using anti-glaucoma medications exhibited poorer confocal parameters compared to untreated patients. As the number of medications and usage count increased, CNFD, CNBD, CNFL, CTBD, CNFA, and CNFrD experienced a decline, while CNFW increased (all P<0.01). For the brinzolamide-therapy group, there was a significant decrease in CNFD and CNFL compared to the other monotherapy groups (P<0.001). In the absence of medication, CNFD in males was lower than that in females (P<0.05). Among patients under medication therapy, CNFD remained consistent between males and females.

● **CONCLUSION:** Antiglaucoma eye drops affect the microstructure of corneal nerves. IVCM and ACCMetrics are useful tools that could be used to evaluate the corneal nerve changes.

● **KEYWORDS:** glaucoma therapy; corneal nerve fibers; *in vivo* confocal microscopy; ACCMetrics

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INTRODUCTION

Glaucoma represents the primary cause of irreversible visual impairment worldwide[1]. The estimated prevalence of 3.54% was observed among individuals within the age of 40 to 80y[2]. Glaucoma is a chronic eye disease and patients require lifelong management. Topical medication is the mainstay of treatment for effective intraocular pressure (IOP) regulation[3-4]. Moreover, an extensive body of clinical and experimental evidence supports the view that prolonged use of topical medications is strongly correlated with the onset and progression of ocular surface toxicity[5-8]. The cornea is one of the structures that are profoundly impacted[9]. Numerous research studies have examined the corneal nerve characteristics of glaucoma patients undergoing medical therapy. These studies observed a decrease in the density, quantity, and length of nerve fibers in individuals with glaucoma following medication administration[10-14]. Anti-glaucoma medications containing preservatives may adversely impact the overall health of the ocular surface, contributing to conditions like neurotrophic keratopathy, which specifically affects the corneal nerves[11]. The specific relationship between corneal nerve changes and the type of drug administered, as

well as the number of drug drops used, remains unexplored to date. Furthermore, no study has assessed mono-therapy groups based on either the drug class or the type of active compound.

Confocal microscopy serves as a non-invasive imaging technique extensively employed in the medical domain. It demonstrates exceptional reproducibility, remarkable resolution, and high sensitivity[15]. This methodology facilitates the acquisition of high-resolution imaging from all layers of human corneal tissue, enabling comprehensive qualitative and quantitative analysis of *in vivo* corneal nerve fibers. It is extensively employed for the evaluation of corneal nerve innervation in a variety of pathological states, including viral keratitis[16], diabetes mellitus, and neurodegenerative disorders[17-20]. Despite the significant clinical relevance of confocal microscopy in the management of corneal nerve pathologies, the adoption of automated software tools for corneal nerve analysis remains limited. To date, analysis of corneal nerves visualized through *in vivo* confocal microscopy (IVCM) has predominantly been reliant on manual or semi-automated modalities[21]. These methodologies entail considerable time investment, necessitate laborious efforts, and exhibit limitations in terms of objectivity[22-23]. The TFOS DEWS II "Pain and Sensation" Subcommittee Report emphasized the need for the integration of automated quantitative measures in IVCM analysis, as it would significantly enhance research methodology and the interpretation of finding[24]. As of now, there is a lack of studies implementing automated quantitative IVCM measurements to elucidate the correlation between corneal nervous changes and antiglaucoma treatment in the existing medical literature. Consequently, this investigation employed ACCMetrics, a state-of-the-art and fully automated software, to characterize corneal nerves across diverse treatment regimens for anti-glaucoma interventions. This signifies a crucial reference point in the implementation of a comprehensive treatment strategy for glaucoma.

SUBJECTS AND METHODS

Ethical Approval This study was performed following the principles of the Declaration of Helsinki and with the approval of the Ethics Committee of Qingdao Eye Hospital [No. (2022)08]. All of the subjects signed informed consent.

Patients In this study, 79 patients diagnosed with glaucoma and 16 healthy control individuals were included. Of the glaucoma patients, 54 were treated with medication, while 25 remained untreated. The eye drops used for lowering IOP included tafluprost 0.0015%, brimonidine tartrate 0.2%, brinzolamide 1%, and timolol 0.05%. The patients were initially divided into five groups based on the number of medication classes used by each participant: untreated group (37 eyes), one-drug treatment group (22 eyes), two-drug treatment group (37 eyes), three-drug treatment group (23 eyes), or four-drug treatment group (20 eyes). The patients were initially divided into five groups based on the daily dosage of eye drops used by each participant: untreated group (37 eyes), one-drop treatment group (6 eyes), two-drop treatment group (11 eyes), three-drop treatment group (15 eyes), or four-drop or more treatment group (70 eyes). The single-drug treatment groups consist of the brinzolamide group (5 eyes), the brimonidine group (6 eyes), the tafluprost group (6 eyes), and the timolol group (5 eyes).

Inclusion Criteria 1) Primary open angle glaucoma (POAG): the patient with POAG exhibits an open angle during gonioscopy, and demonstrates optic disc changes characterized by a cup-to-disc ratio of ≥0.7 in either eye or an asymmetry of ≥0.3 between the two eyes, and presents reliable visual field test results indicating a cluster of ≥3 points on the pattern deviation plot with a sensitivity below 5%; 2) receiving anti-glaucoma drugs for at least 6mo; 3) healthy control individuals needed to demonstrate a fully normal eye examination, including normal results on clinical tests for the ocular surface.

Exclusion Criteria 1) prior to or currently using ophthalmic medications other than anti-glaucoma eye drops (such as artificial tears); 2) a history of contact lens use; 3) a history of previous ocular surgery; 4) recent occurrence of ocular inflammation; 5) previously diagnosed with xerophthalmia; 6) presence of an allergic constitution; 7) diagnosed with diabetes; 8) presence of autoimmune diseases; 9) complicated by severe cardiovascular and cerebrovascular diseases.

Clinical Examinations The subjects underwent a standardized ophthalmic examination at Qingdao Eye Hospital, which included visual acuity, IOP, and ocular biometrics. Visual acuity was evaluated using the internationally recognized logarithmic visual acuity chart. A slit lamp biomicroscope was utilized to evaluate the anterior segment. IOP measurement was performed using a non-contact tonometer Canon TX-20P. Visual field data were collected using the automated perimetry (Humphrey Field Analyzer II; Carl Zeiss Meditec, Dublin, CA, USA). Ophthalmic B-ultrasound was employed for examining the vitreous, while fundus photography and optical coherence tomography were used for assessing the retina and macular region respectively. The visual fields were assessed using a Goldman perimeter. Ocular biometry measurements including central cornea thickness (CCT), lens thickness (LT), axial length (AL), and anterior chamber depth (ACD) were obtained using the IOLMaster 700 device (Carl Zeiss Meditec AG, Jena, Germany).

***In Vivo* Confocal Microscopy** All subjects underwent laser scanning IVCM with the Heidelberg Retina Tomograph III Rostock Corneal Module (Heidelberg Engineering GmBH, Heidelberg, Germany)[25-26]. Before IVCM evaluation, a drop

of 0.4% benoxinate hydrochloride (Chauvin Pharmaceuticals, Surrey, UK) was used to anesthetize all eyes, and a drop of Viscotears (Carbomer 980, 0.2%; Novartis, North Ryde, NSW, Australia) was applied as a coupling medium between the contact cap of the objective lens and the cornea. Throughout the examination, the subjects were instructed to focus their gaze on a target position that allowed for examination of the central cornea. A rate of 3 frames per second was used for recording digital images in sequence mode. The images of the nerve plexus were captured using a frame size of 400×400 μm. Five images were randomly selected for quantitative analysis.

ACCMetrics The images were automatically analyzed and quantified using ACCMetrics software[27-29]. The following seven parameters were measured and reported by the software: 1) corneal nerve fiber density (CNFD), the total number of nerves (/mm^2); 2) corneal nerve branch density (CNBD), the number of branches emanating from major nerve trunks (/mm^2); 3) corneal nerve fiber length (CNFL), the total length of all nerve fibers and branches (mm/mm^2); 4) corneal nerve total branch density (CTBD), the total number of branches (/mm^2); 5) corneal nerve fiber area (CNFA), the total nerve fiber area (mm^2/mm^2); 6) corneal nerve fiber width (CNFW), the average nerve fiber width (mm/mm^2); 7) corneal nerve fractal dimension (CNFrD), a measure of the structural complexity of corneal nerves.

Statistical Analysis Data were analyzed using SPSS version 26 (IBM Corp, Armonk, NY, USA). The distribution of variables was assessed using the Shapiro-Wilk *W* test. The evaluation of gender differences between groups was conducted using χ^2 test. The assessment of age differences between groups was performed using an analysis of variance (ANOVA). The Kruskal-Wallis test and Mann-Whitney test were used to assess differences that did not follow a normal distribution. The correlation between IVCM parameters and drugs was evaluated using non-parametric measurements of Spearman's rank correlation coefficient. If the probability values were less than 0.05, statistical significance was assumed. Non-normally distributed data are described using the median and interquartile range, while normally distributed data are described using the mean and standard deviation.

RESULTS

There were no statistically significant differences between the glaucoma patients and controls in terms of gender and age (P>0.05; Table 1).

The CNFD was reduced in glaucoma groups compared to healthy subjects (P<0.01; Table 2). Patients undergoing anti-glaucoma medication treatment displayed poorer confocal parameters relative to their untreated counterparts. The representative morphologies of the corneal nerve fibers in each group were shown in Figures 1 and 2. The changes in corneal nerve parameters were not correlated with IOP and visual field progression (Table 3).

Table 1 Demographic features of subjects enrolled in the study

Parameters	Healthy controls	Primary open angle glaucoma	
		Untreated group	Medication group
Patients	16	25	54
Eyes	16	37	102
Age (mean±SD), y	66.36±2.49	66.08±7.72	65.71±7.96
Gender (M/F)	6/10	10/15	22/32

M: Male; F: Female.

With an increase in both the number of medications classes and drops, there was a reduction in CNFD, CNBD, CNFL, CTBD, CNFA, and CNFrD, whereas CNFW exhibited an upward trend.

There was no significant difference in the values of seven corneal nerve parameters between the untreated group and the one-drug treatment group. There was no significant difference for CNBD, CNFL, CTBD, CNFA, CNFW, and CNFrD between the three-drug treatment group and the four-drug treatment group. For CNFW, there was no statistically significant difference observed between the three-drug treatment group and the two-drug treatment group. However, a significant difference was observed between the three-drug treatment group and the untreated group (Table 4).

There was no statistically significant difference for the seven corneal nerve parameters between the untreated group, the one-drop treatment group, and the two-drop treatment group. In terms of CNBD, CTBD, and CNFA, there was no significant difference observed between the three-drop treatment group and the two-drop treatment group. However, a significant difference was found between the three-drop treatment group and the untreated group. For CNFA and CNFW, the comparison between the three-drop treatment group and the four-drop treatment group demonstrated a lack of significant difference. Significant differences in CNFW were observed only between the untreated group and the four-drop treatment group, as well as between the two-drop treatment group and the four-drop treatment group (Table 5).

According to the correlation analysis, CNFD, CNBD, CNFL, CTBD, CNFA, and CNFrD negatively correlated with medication types and usage count, while CNFW showed strong positive correlations with medication types and usage count (Table 6).

When utilizing a solitary medication, patients in the brinzolamide group demonstrated a significant decrease in CNFD when compared to groups of brimonidine alone, tafuprost alone, and timolol alone (P<0.001). In comparison to the timolol group, patients in the brinzolamide group exhibited a statistically significant decrease in CNFL (Table 7).

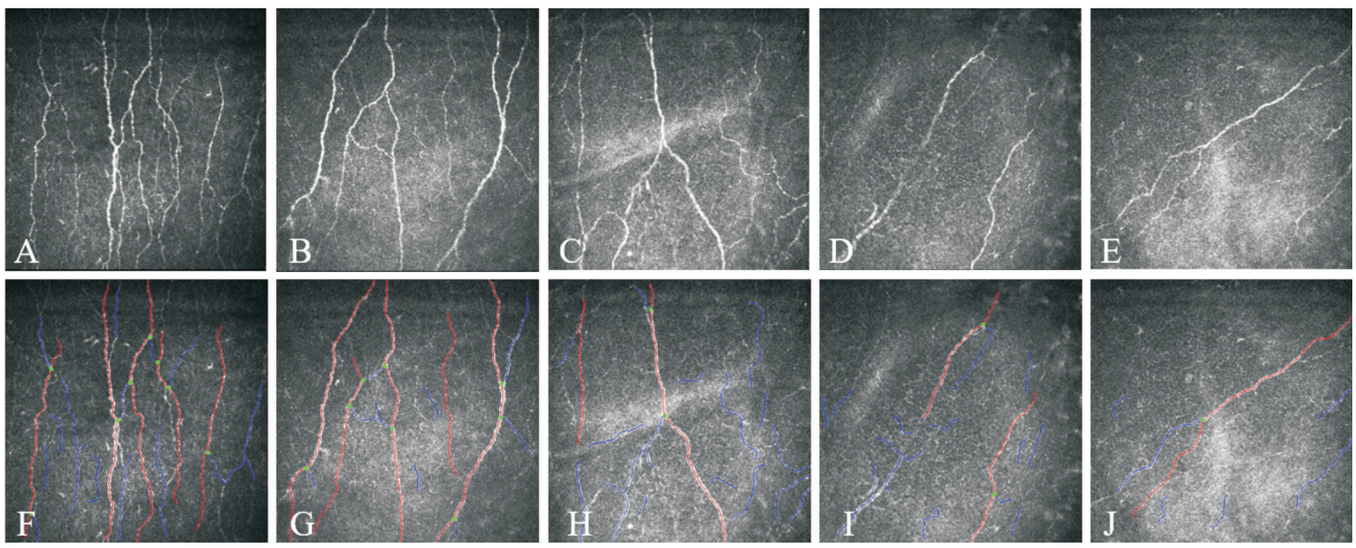

Figure 1 Confocal images of corneal nerves with different amounts of drugs A-E: Typical images of the corneal nerve obtained through *in vivo* confocal microscopy; F-J: Automated image analysis using ACCMetrics software: main nerve fibers were indicated in red, nerve branches in blue and branch points in green; A, F: Untreated group; B, G: One-drug treatment group; C, H: Two-drug treatment group; D, I: Three-drug treatment group; E, J: Four-drug treatment group.

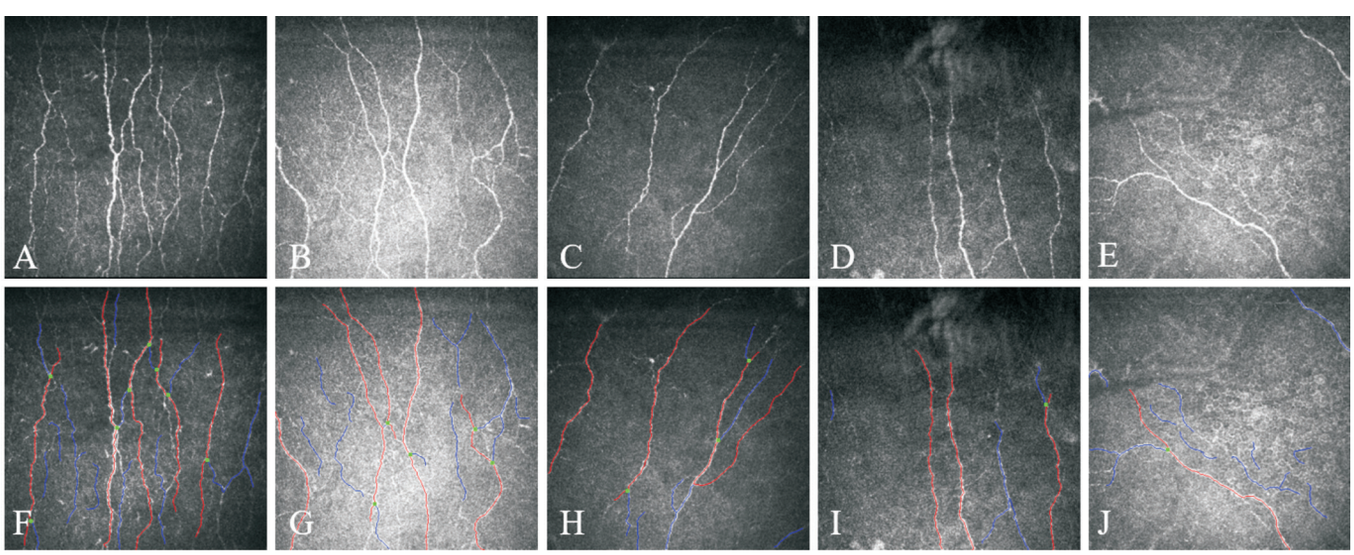

Figure 2 Confocal images of corneal nerves with different amounts of drops A-E: Typical images of the corneal nerve obtained through *in vivo* confocal microscopy; F-J: Automated image analysis using ACCMetrics software: main nerve fibers are indicated in red, nerve branches in blue and branch points in green; A, F: Untreated group; B, G: One-drop treatment group; C, H: Two-drop treatment group; D, I: Three-drop treatment group; E, J: Four-drop or more treatment group.

Table 2 *In vivo* confocal microscopy of corneal nerves in glaucoma groups and controls

Parameters	Healthy controls	Primary open angle glaucoma	
		Untreated group	Medication group
CNFD (/mm^2)	31.25 (31.25, 37.50)	31.25 (25.00, 31.25)[a]	18.75 (12.50, 18.75)[a,b]
CNBD (/mm^2)	50.00 (25.00, 62.50)	43.75 (25.00, 62.50)	18.75 (6.25, 31.25)[a,b]
CNFL (mm/mm^2)	15.11 (13.23, 16.58)	15.36 (13.38, 17.30)	10.26 (7.48, 12.98)[a,b]
CTBD (/mm^2)	75.00 (32.81, 93.74)	62.50 (37.50, 87.49)	18.75 (31.25, 50.00)[a,b]
CNFA (mm^2/mm^2)	0.007 (0.005, 0.009)	0.008 (0.006, 0.010)	0.006 (0.004, 0.008)[a,b]
CNFW (mm/mm^2)	0.022 (0.021, 0.023)	0.022 (0.021, 0.023)	0.022 (0.021, 0.024)[a,b]
CNFrD	1.47 (1.46, 1.50)	1.49 (1.47, 1.50)	1.44 (1.41, 1.48)[a,b]

CNFD: Corneal nerve fiber density; CNBD: Corneal nerve branch density; CNFL: Corneal nerve fiber length; CTBD: Corneal nerve total branch density; CNFA: Corneal nerve fiber area; CNFW: Corneal nerve fiber width; CNFrD: Corneal nerve fractal dimension. [a]*P*<0.01 *vs* healthy controls, [b]*P*<0.01 *vs* untreated group using Kruskal-Wallis test.

Table 3 Correlation between IOP, visual field, and *in vivo* confocal microscopy corneal nerve parameters

Parameters	IOP (mm Hg)		MD (dB)	
	r	*P*	*r*	*P*
CNFD	0.001	0.977	0.071	0.066
CNBD	0.018	0.642	0.020	0.605
CNFL	0.013	0.745	0.035	0.356
CTBD	0.027	0.488	0.003	0.948
CNFA	0.014	0.719	0.021	0.579
CNFW	0.014	0.719	-0.058	0.131
CNFrD	-0.008	0.826	0.013	0.731

IOP: Intraocular pressure; MD: Mean defect; Db: decibel; CNFD: Corneal nerve fiber density; CNBD: Corneal nerve branch density; CNFL: Corneal nerve fiber length; CTBD: Corneal nerve total branch density; CNFA: Corneal nerve fiber area; CNFW: Corneal nerve fiber width; CNFrD: Corneal nerve fractal dimension.

Table 4 *In vivo* confocal microscopy automated morphometric analysis between medication types and corneal nerves

Parameters	Untreated	1 drug	2 drugs	3 drugs	4 drugs or more	*P*
CNFD (/mm^2)	31.25 (25.00, 31.25)	25.00 (25.00, 31.25)	18.75 (18.75, 18.75)^{a,b}	12.50 (12.50, 12.50)^{a,b,c}	6.25 (6.25, 6.25)^{a,b,c,d}	<0.001
CNBD (/mm^2)	43.75 (20.31, 62.50)	37.50 (20.31, 56.25)	18.75 (12.50, 31.25)^{a,b}	12.50 (6.25, 18.75)^{a,b,c}	6.25 (6.25, 12.50)^{a,b,c}	<0.001
CNFL (mm/mm^2)	15.36 (13.38, 17.30)	14.31 (11.64, 16.52)	10.85 (9.01, 12.94)^{a,b}	8.67 (6.63, 10.33)^{a,b,c}	6.23 (4.73, 7.81)^{a,b,c}	<0.001
CTBD (/mm^2)	62.50 (37.50, 87.49)	50.00 (31.25, 81.24)	34.37 (25.00, 50.00)^{a,b}	25.00 (12.50, 37.50)^{a,b,c}	18.75 (12.50, 29.69)^{a,b,c}	<0.001
CNFA (mm^2/mm^2)	0.008 (0.006, 0.010)	0.007 (0.005, 0.009)	0.006 (0.004, 0.008)^{a,b}	0.005 (0.004, 0.007)^{a,b,c}	0.004 (0.003, 0.006)^{a,b,c}	<0.001
CNFW (mm/mm^2)	0.022 (0.021, 0.023)	0.021 (0.020, 0.023)	0.022 (0.021, 0.024)	0.022 (0.021, 0.024)^{a,b}	0.023 (0.022, 0.024)^{a,b,c}	<0.001
CNFrD	1.49 (1.47, 1.50)	1.48 (1.45, 1.50)	1.45 (1.42, 1.48)^{a,b}	1.42 (1.39, 1.46)^{a,b,c}	1.40 (1.36, 1.44)^{a,b,c}	<0.001

CNFD: Corneal nerve fiber density; CNBD: Corneal nerve branch density; CNFL: Corneal nerve fiber length; CTBD: Corneal nerve total branch density; CNFA: Corneal nerve fiber area; CNFW: Corneal nerve fiber width; CNFrD: Corneal nerve fractal dimension. ^{a}*P*<0.01 *vs* untreated, ^{b}*P*<0.01 *vs* 1 drug, ^{c}*P*<0.01 *vs* 2 drugs, ^{d}*P*<0.01 *vs* 3 drugs using Kruskal-Wallis test.

Table 5 *In vivo* confocal microscopy automated morphometric analysis between the usage count and corneal nerves

Parameters	Untreated	1 drop	2 drops	3 drops	4 drops or more	*P*
CNFD (/mm^2)	31.25 (25.00, 31.25)	25.00 (25.00, 31.25)	25.00 (25.00, 31.25)	18.75 (18.75, 25.00)^{a,b,c}	12.50 (6.25, 18.75)^{a,b,c,d}	<0.001
CNBD (/mm^2)	43.75 (20.31, 62.50)	46.87 (26.56, 62.50)	31.25 (18.75, 50.00)	25.00 (12.50, 37.50)^{a,b}	12.50 (6.25, 23.44)^{a,b,c,d}	<0.001
CNFL (mm/mm^2)	15.36 (13.38, 17.30)	14.39 (11.87, 17.30)	15.06 (12.88, 16.63)	11.45 (10.01, 13.39)^{a,b,c}	8.92 (6.769, 11.18)^{a,b,c,d}	<0.001
CTBD (/mm^2)	62.50 (37.50, 87.49)	71.87 (37.50, 100.00)	50.00 (31.25, 81.24)	43.75 (25.00, 56.25)^{a}	25.00 (18.75, 43.75)^{a,b,c,d}	<0.001
CNFA (mm^2/mm^2)	0.008 (0.006, 0.010)	0.007 (0.005, 0.009)	0.007 (0.006, 0.009)	0.006 (0.004, 0.007)^{a}	0.005 (0.004, 0.007)^{a,b,c}	<0.001
CNFW (mm/mm^2)	0.022 (0.021, 0.023)	0.022 (0.021, 0.023)	0.021 (0.020, 0.023)	0.022 (0.021, 0.023)	0.023 (0.021, 0.024)^{a,c}	<0.001
CNFrD	1.49 (1.47, 1.50)	1.48 (1.45, 1.49)	1.48 (1.47, 1.50)	1.46 (1.43,1.48)^{a,c}	1.43 (1.39, 1.46)^{a,b,c,d}	<0.001

CNFD: Corneal nerve fiber density; CNBD: Corneal nerve branch density; CNFL: Corneal nerve fiber length; CTBD: Corneal nerve total branch density; CNFA: Corneal nerve fiber area; CNFW: Corneal nerve fiber width; CNFrD: Corneal nerve fractal dimension. ^{a}*P*<0.01 *vs* untreated, ^{b}*P*<0.01 *vs* 1 drop, ^{c}*P*<0.01 *vs* 2 drops, ^{d}*P*<0.01 *vs* 3 drops using Kruskal-Wallis test.

In the context where medication treatment was not provided, it was observed that male individuals displayed relatively lower levels of CNFD in comparison to their female counterparts (*P*<0.05). Among patients receiving medication for glaucoma, the parameters of the corneal nerve remained consistent between males and females (Table 8).

DISCUSSION

During chronic treatment for glaucoma patients, anti-glaucoma medications have the potential to induce impairment in corneal nerves, thereby potentially interrupting the efficacy of treatment. The accurate and dependable quantification of corneal nerve structural condition serves as the foundation for assessing the impact of drugs on corneal nerves.

In this study, the comparative evaluation of corneal nerve changes in patients with glaucoma was conducted using IVCM. To our knowledge, this is the first study to use IVCM combined with ACCMetrics analysis in anti-glaucoma drugs.

In our study, we found a reduced CNFD in glaucoma patients compared to healthy individuals, indicating a possible early indicator of corneal nerve alterations. The results indicated no

Table 6 Correlations between anti-glaucoma medications and corneal nerve parameters

Parameters	The number of medication types		The number of daily usage count	
	r	*P*	*r*	*P*
CNFD	-0.92	<0.01	-0.73	<0.01
CNBD	-0.63	<0.01	-0.48	<0.01
CNFL	-0.76	<0.01	-0.56	<0.01
CTBD	-0.49	<0.01	-0.36	<0.01
CNFA	-0.40	<0.01	-0.25	<0.01
CNFW	0.23	<0.01	0.21	<0.01
CNFrD	-0.61	<0.01	-0.41	<0.01

CNFD: Corneal nerve fiber density; CNBD: Corneal nerve branch density; CNFL: Corneal nerve fiber length; CTBD: Corneal nerve total branch density; CNFA: Corneal nerve fiber area; CNFW: Corneal nerve fiber width; CNFrD: Corneal nerve fractal dimension.

Table 7 In vivo confocal microscopy automated morphometric analysis in a single medication

Parameters	Brinzolamide group	Brimonidine group	Tafluprost group	Timolol group	*P*
CNFD (/mm^2)	25.00 (18.75, 25.00)	25.00 (25.00, 25.00)[a]	25.00 (25.00, 31.25)[a]	28.12 (25.00, 31.25)[a]	<0.001
CNBD (/mm^2)	25.00 (25.00, 43.75)	31.25 (15.62, 53.12)	46.87 (26.56, 62.50)	31.25 (23.44, 45.31)	<0.001
CNFL (mm/mm^2)	11.86 (10.47, 14.33)	14.56 (12.86, 16.43)	14.39 (11.87, 17.30)	15.93 (12.95, 16.98)[a]	<0.001
CTBD (/mm^2)	43.75 (31.25, 62.50)	50.00 (25.00, 84.37)	71.87 (37.50, 100.00)	50.00 (35.94, 81.24)	<0.001
CNFA (mm^2/mm^2)	0.006 (0.005, 0.008)	0.007 (0.005, 0.009)	0.007 (0.005, 0.009)	0.008 (0.006, 0.009)	<0.001
CNFW (mm/mm^2)	0.022 (0.021, 0.023)	0.022 (0.020, 0.024)	0.022 (0.021, 0.023)	0.021 (0.020, 0.022)	<0.001
CNFrD	1.46 (1.45, 1.48)	1.48 (1.46, 1.51)	1.48 (1.45, 1.49)	1.48 (1.47, 1.50)	<0.001

CNFD: Corneal nerve fiber density; CNBD: Corneal nerve branch density; CNFL: Corneal nerve fiber length; CTBD: Corneal nerve total branch density; CNFA: Corneal nerve fiber area; CNFW: Corneal nerve fiber width; CNFrD: Corneal nerve fractal dimension. [a]*P*<0.01 *vs* brinzolamide group, [b]*P*<0.01 *vs* brimonidine group, [c]*P*<0.01 *vs* tafluprost group using Kruskal-Wallis test.

Table 8 *In vivo* confocal microscopy automated morphometric analysis between genders

Parameters	Untreated group				Medication group			
	Females	Males	*Z*	*P*	Females	Males	*Z*	*P*
CNFD (/mm^2)	31.25 (25.00, 31.25)	25.00 (25.00, 31.25)	-2.18	0.029	18.75 (12.50,18.75)	18.75 (12.50, 18.75)	-0.399	0.69
CNBD (/mm^2)	43.75 (25.00, 62.50)	43.7472 (25.00, 62.50)	-0.091	0.928	18.75 (6.25,31.25)	18.75 (9.37,31.25)	0.927	0.354
CNFL (mm/mm^2)	15.41 (13.32, 17.55)	15.10 (13.52, 17.27)	-0.452	0.651	10.26 (7.56,12.66)	10.27 (7.34, 13.41)	0.333	0.379
CTBD (/mm^2)	62.50 (32.81, 87.50)	68.75 (31.25, 87.49)	0.642	0.521	31.25 (18.75,50.00)	31.25 (18.75, 56.25)	0.826	0.409
CNFA (mm^2/mm^2)	0.007 (0.005, 0.009)	0.0079 (0.0054, 0.0098)	0.466	0.641	0.006 (0.004, 0.008)	0.005 (0.004, 0.008)	-1.146	0.252
CNFW (mm/mm^2)	0.022 (0.021, 0.023)	0.0219 (0.0206, 0.0232)	0.583	0.59	0.022 (0.021, 0.024)	0.022 (0.021, 0.024)	-0.794	0.427
CNFrD	1.49 (1.47, 1.50)	1.49 (1.47, 1.50)	0.984	0.325	1.45 (1.41, 1.47)	1.44 (1.40, 1.48)	-0.072	0.943

CNFD: Corneal nerve fiber density; CNBD: Corneal nerve branch density; CNFL: Corneal nerve fiber length; CTBD: Corneal nerve total branch density; CNFA: Corneal nerve fiber area; CNFW: Corneal nerve fiber width; CNFrD: Corneal nerve fractal dimension.

correlation between IOP and corneal nerve parameters, as well as no correlation between visual field progression and corneal nerve parameters. Subsequently, the glaucoma patients were stratified into five groups based on the number of medication classes utilized by each participant, namely: untreated, one-drug treatment, two-drug treatment, three-drug treatment, or four-drug treatment. There was no significant difference in the values of seven corneal nerve parameters between the untreated group and the one-drug treatment group. This showed that the use of only one drug has little effect on the corneal nerves of the patients. Regarding CNFD, there were significant differences among all groups except for the untreated group and the single-drug treatment group. This finding suggests that CNFD served as a highly sensitive indicator. Pairwise comparison showed that there was no significant difference for CNBD, CNFL, CTBD, CNFA, CNFW, and CNFrD between the three-drug treatment group and the four-drug treatment group. It can be observed that the effect on the corneal nerve remained unchanged after the number of drugs reached a certain value. For CNFW, there was no statistically significant difference observed between the three-drug treatment group and the two-drug treatment group. However, a significant

difference was observed between the three-drug treatment group and the untreated group. This finding demonstrates that a significant impact on CNFW can only be observed through the use of three or more drugs. Next, the subsequent subgroup analysis based on the daily dosage of eye drops revealed no statistically significant difference in the seven corneal nerve parameters between the untreated group, the one-drop treatment group, and the two-drop treatment group. The findings pointed to the conclusion that only one or two drops of medicine have little effect on the corneal nerves of the patients. Pairwise comparison among the groups demonstrated that there were no statistically significant differences in CNFD and CNFL between the untreated group, the one-drop treatment group, and the two-drop treatment group. However, significant differences were observed among the remaining groups. In terms of CNBD, CTBD, and CNFA, there was no significant difference observed between the three-drop treatment group and the two-drop treatment group. However, a significant difference was found between the group treated with three drops and the untreated group, suggesting that three or more drops of drugs had a notable effect on CNBD, CTBD, and CNFA. For CNFA and CNFW, after reaching a certain threshold of drop count, the comparison between the three-drop treatment group and the four-drop treatment group demonstrated a lack of significant difference, emphasizing that no further changes occurred in the effect on the corneal nerve. Significant differences in CNFW were observed only between the untreated group and the four-drop treatment group, as well as between the two-drop treatment group and the four-drop treatment group. As indicated by our findings in the study, it could be inferred that a sufficient number of drug drops must be administered for any change to occur in CNFW. According to the correlation analysis conducted in this study, CNFD, CNBD, CNFL, CTBD, CNFA, and CNFrD demonstrated significant negative correlations with both the number of drugs and also with the number of drug drops. This means that anti-glaucoma drugs lead to the degeneration of corneal nerves. On the contrary, CNFW exhibited a strong positive correlation with both the number of drugs and also with the number of drug drops. This implied that the administration of anti-glaucoma drugs results in the induction of hypertrophy in corneal nerve fiber. In light of our obtained data, it could be concluded that the quantity of administered drugs and the frequency of ocular administration played essential roles in the relationship between anti-glaucoma drugs and corneal nerves. As the number of drugs and drops increased, the severity of the corneal nerve injury escalated. These findings aligned with prior research on this subject[10,30]. Martone *et al*'s[11] study revealed a decrease in basal nerve fiber count and an increase in tortuosity during anti-glaucoma medical treatment. The findings of an Italian study also demonstrated that treated individuals with POAG exhibited a reduced number of nerves and increased tortuosity compared to untreated POAG individuals[31]. Labbé *et al*'s[13] study indicated a significant reduction in basal nerve fiber density and branch number among patients receiving multiple treatments, while no discernible difference was observed between glaucoma and dry eye disease (DED) patients. However, the precise correlation between alterations in corneal nerve morphology and both the specific drug type and dosage regimen remains unexplored to date. Moreover, there is a noticeable scarcity of studies evaluating monotherapy groups categorized by either drug class or active compound type.

To further investigate the variations in corneal nerve parameters among different medications used for treating glaucoma, the corneal nerve parameters were compared among different drugs in a single drug setting. Patients in the brinzolamide group demonstrated a significant decrease in CNFD when compared to groups of brimonidine alone, tafuprost alone, and timolol alone. In comparison to the timolol group, patients in the brinzolamide group exhibited a statistically significant decrease in CNFL.

An additional intriguing discovery was that, in the absence of medication, men displayed lower CNFD levels compared to women. Cao *et al*'s[32] study yielded similar results to this finding. The potential relationship between hormones and this result may be worth exploring. The administration of β-estradiol in an animal experiment investigating corneal nerve regeneration in mice was found to enhance the density of subbasal nerve fibers[33]. On the other hand, chronic smoking can reduce the number of fibers in the corneal nerve[34]. It is worth noting that there is typically a higher prevalence of smoking among men than women in China, thereby potentially contributing to this observed outcome. After being treated with glaucoma drugs, the parameters of the corneal nerve were found to be similar in both men and women. The findings suggest that anti-glaucoma medications exert an impact on corneal nerve function in both genders, with a more pronounced reduction in corneal nerve fiber density observed among women. The existence of this trend notwithstanding, further studies are required to ascertain whether CNFD truly functions as a sex-sensitive indicator.

In this study, we conducted the first-ever exploration of the effects of anti-glaucoma drugs on both CNFW and CNFrD. Following prior research, elevated levels of CNFW have been observed in DED. The persistent inflammation that occurs in DED has the potential to induce swelling of nerve fibers or trigger the release of neurotrophic factors[35]. In the present study, it was observed that patients who underwent drug treatment exhibited elevated levels of CNFW. It was suggested

that the administration of anti-glaucoma drugs may potentially trigger chronic inflammation and result in abnormalities of corneal nerve fibers. In this study, we observed a significant decrease in CNFrD among glaucoma patients who underwent multi-drug therapy for the first time, which was an important finding. CNFrD was employed as a measure to assess the complexity of corneal nerves[36]. The decrease in CNFrD indicated the presence of neurodegeneration.

Additionally, we acknowledge certain limitations associated with our study. The temporal sequence in which alterations in nerve fiber parameters initially occur cannot be explained, nor whether these parameters exhibit variability in response to the duration of treatment. As a consequence of the strict limitations of the inclusion and exclusion criteria, the sample size is small. Due to the predominantly elderly participants and the absence of a comprehensive comparison across different age groups, this study was unable to establish a causal relationship between corneal nerve alterations and aging.

To summarize, the number of medication classes and the daily dosage of eye drops were factors that may contribute to alterations in corneal nerves in patients with glaucoma receiving pharmacological treatment. In women, anti-glaucoma drugs exhibit a significantly greater reduction in CNFD compared to men. Therefore, in patients who need use drug therapy to control the progression of glaucoma, emphasis should be placed on treatment strategies specifically aimed at protecting corneal nerves. On the flip side, it is also suggested that the breakthroughs in drug formulation research and the utilization of sustained-release devices for drug administration were particular importance.

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