A Positive Association Between Intrinsically Photosensitive Retinal Ganglion Cells and Retinal Nerve Fiber Layer Thinning in Glaucoma

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Purpose. To assess the integrity of intrinsically photosensitive retinal ganglion cells (ipRGCs) using the pupillary light reflex in glaucoma patients.

Methods. A cross-sectional study was conducted, including 76 eyes from 38 patients with primary open-angle glaucoma and 36 eyes from 18 control subjects. The patients were tested in the dark with light stimuli using the Ganzfeld system, and the pupil diameter was measured with the assistance of an eye tracker consisting of two infrared cameras fit to an eyeglass frame. To preferentially stimulate ipRGCs, we used a 1-second 470-nm flash with a luminance of 250 cd/m^2. To stimulate different retinal photoreceptors (cones and rods), we used a 1-second 630-nm flash with a luminance of 250 cd/m^2. Standard automated perimetry (SAP), matrix frequency-doubling technology (FDT), and high-definition optical coherence tomography (Cirrus HD-OCT) were also performed. The correlation between the ipRGC-mediated sustained response following the pupillary light reflex and the structural and functional changes in glaucoma patients was analyzed using generalized estimating equation.

Results. An association was observed between the average retinal nerve fiber layer (RNFL) thickness, as measured by Cirrus HD-OCT, and the sustained pupillary response to the blue flash ($P = 0.024$). The severity of glaucoma, based on the mean deviation of SAP (Hodapp-Anderson-Parrish system), was also associated with the sustained response to the blue flash ($P = 0.006$).

Conclusions. This study showed a correlation between the mean RNFL thickness and the pupillary light response. A decrease in the number of ipRGCs is potentially related to the reduced RNFL thickness.

Keywords: intrinsically photosensitive retinal ganglion cells, pupil light reflex, retinal ganglion cells, non–image-forming visual functions, glaucoma

Glaucoma is the most common type of optic neuropathy and represents a leading cause of irreversible blindness worldwide.[1,2] The disease is considered a degenerative and progressive optic neuropathy that leads to structural and functional changes in the optic nerve and retinal ganglion cells (RGCs).[3] The majority of RGCs are involved in cortical image processing; however, a small proportion of RGCs, called intrinsically photosensitive RGCs (ipRGCs), are not related to the thalamo-cortical pathway of image processing and project their axons into the lateral geniculate nucleus, pretectal olivary nucleus, and suprachiasmatic nucleus.[4,5] Loss of the ganglion cell population potentially results in loss of function and/or a decreased number of ipRGCs.[4,6] However, descriptions of the relative preservation of the number of ipRGCs are available.[7,8]

In 2002, ipRGCs were described as a new type of photoreceptor[5,9–13] that expresses the photopigment melanopsin (also known as opsin 4, or OPN4). These cells account for approximately 1% to 3% of the total RGC population in the human retina.[14] Previous studies have indicated that ipRGCs are responsible for non–image-forming tasks, such as the pupillary light reflex,[15,16] entrained circadian rhythms,[13,17–19] memory modulation, and behavioral mood regulation.[20]

The ipRGC population is most sensitive to short-wavelength, blue light (480 nm)[21] and contributes directly to the postillumination pupil response of sustained constriction (>6 seconds) after the offset of high luminance (250 cd/m^2).[17] Investigating this specific class of RGCs, Kankipati and colleagues[22] reported that a reduction in the number of ipRGCs was related to the postillumination pupillary response in patients with glaucoma. By measuring ipRGC function during postillumination pupil responses, Feigl et al.[23] revealed that patients with advanced glaucoma demonstrated reduced ipRGC function compared with patients with early glaucoma and normal subjects, serving as indirect evidence of the role of this ganglion cell subtype in the mechanism of progressive disease. Although previous studies have correlated the post-

illumination pupil response (based on measuring specific ipRGC functions) with visual field test mean deviation (MD) values in glaucoma patients,[22] no studies have investigated the association between glaucomatous structural damage, as measured based on retinal nerve fiber layer (RNFL) thickness, and the ipRGC-mediated pupillary response.

In the present study, we sought to investigate the integrity of ipRGCs by measuring the pupillary light reflex of patients with glaucoma and to correlate this measurement with glaucomatous functional and structural damage.

Methods

Study Participants

This cross-sectional study adhered to the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board of the Federal University of São Paulo (CEP 262.470). In addition, written informed consent was obtained from all participants.

We prospectively enrolled 112 eyes from 56 patients (76 eyes with primary open-angle glaucoma and 36 eyes from control subjects) attending the outpatient clinics in the Ophthalmology Department of the Federal University of São Paulo. All patients underwent a complete ophthalmological examination, including a medical history review, best-corrected visual acuity testing, slit-lamp biomicroscopy, intraocular pressure measurement, gonioscopy, dilated funduscopic examination, and refraction. The exclusion criteria included less than 40 or more than 80 years of age; best-corrected visual acuity of less than 0.2 logMAR; lens opacity greater than 0.5 (cortical opacity, nuclear opalescence, posterior subcapsular opacity, or changes in nuclear color) according to the Lens Opacity Classification System III (LOCS III)[24]; corneal, retinal, or orbital disease; and previous ophthalmic surgeries. Subjects were also excluded if they presented with a spherical refractive error greater than ±5D or a cylindrical error greater than 3D or if they were unable to cooperate during the psychophysical tests. Only patients with an open angle on gonioscopy were included. An open angle was defined as having a relatively normal appearance, with 180° or more of appositional angle closure.[25,26]

Patients were classified as having glaucoma if they had at least three repeatable, consecutive, abnormal visual field test results, which were defined as a pattern standard deviation (PSD) outside the 95% normal confidence limits or a glaucoma hemifield test (GHT) result outside the normal limits matching the appearance of the optic disc. Patients were also considered glaucomatous if they demonstrated signs of glaucomatous optic neuropathy based on optic disc stereophotographs. Evidence of glaucomatous damage in the optic disc stereophotographs consisted of localized or diffuse neuroretinal rim loss or RNFL defects.

Patients were also classified according to the severity of the disease based on the Hodapp-Anderson-Parrish system.[27] This system categorizes the disease into five different stages based on a combination of standard automated perimetry (SAP) MD and one of the following: a pattern deviation probability plot (indicating deviation from a normalized visual field pattern); a dB plot (stages 2 to 4); or, for stage 1, either a corrected PSD/PSD (CPSD/PSD) or GHT results. In this system, a mild defect corresponds to SAP MD $\geq$ –6 dB, a moderate defect corresponds to SAP MD between –6 and –12 dB, an advanced defect corresponds to SAP MD between –12 and –20 dB, a severe defect corresponds to SAP MD $>$ –20 dB, and end-stage disease is considered if the patient is unable to perform the Humphrey visual field test. In our study, we considered all patients with SAP MD $>$ –12 dB as having advanced glaucoma.[27]

Additionally, for the purposes of the analysis, subjects were classified into two groups based on whether or not using α-2 adrenergic agonist agents.

Pupillary Light Reflex Assessment

Stimuli. The protocol used for measuring the pupillary response to a stimulus was based on a method previously developed by Park et al.[28] To optimize and preferentially stimulate the ipRGCs, we used 470-nm (blue) flashes with a luminance of 250 cd/m^2 and a 1-second duration. To stimulate different retinal photoreceptors (cones and rods) without direct stimulation of the ipRGCs, we used 1-second 640-nm (red) flashes with a similar luminance. The interval for each stimulus was 60 seconds. The peak amplitude was calculated as the maximum pupil constriction and was expressed relative to the baseline value (peak amplitude = baseline diameter – smallest pupil diameter). The sustained response was expressed as the pupil diameter at 6 seconds after the flash offset relative to the baseline.[28]

Procedure. Monocular tests were performed on both eyes in a randomly selected order. The patient was first adapted to the dark for 10 minutes. Next, alternating red and blue flashes, with a luminance of 250 cd/m^2, were presented to the patient. A red flash was presented first with 1-second duration, followed by a blue flash 60 seconds after the offset of the red flash. The intervals between stimuli allowed the pupil size to return to baseline and prevented fatigue before the presentation of the next stimulus. Stimuli were generated via corresponding light-emitting diodes (LEDs) using the Ganzfeld system (RETIport; Roland Consult, Brandenburg, Germany), and responses were recorded using an eye-tracking camera system with an infrared LED (Arrington Research, Scottsdale, AZ, USA).[28–30] Figure 1 illustrates the peak and sustained responses expected for the blue and red flashes in control patients.

Standard Automated Perimetry (SAP)

All patients underwent a monocular SAP examination using the Humphrey Field Analyzer II perimeter (Carl Zeiss Meditec, Inc., Dublin, CA, USA). The SAP was performed using the 24-2 program, including the Swedish interactive threshold algorithm (SITA) standard protocol and a standard Goldmann size III stimulus (diameter 0.43°). Three tests were performed on different days (during a period of up to 2 weeks). Visual fields with more than 33% fixation losses or false-negative errors or more than 15% false-positive errors were excluded; the only exception was the inclusion of visual fields with more than 33% false-negative errors when the field test indicated advanced disease. Visual fields were further reviewed for the following artifacts: eyelid and rim artifacts, fatigue effects, inappropriate fixation, inattention, and evidence that the visual field results were caused by a disease other than glaucoma.

Frequency-Doubling Technology (FDT)

FDT was implemented using the Humphrey Field Analyzer II (Carl Zeiss Meditec, Inc.) and the 24-2 SITA strategy. We performed three tests on different days (during a period of up to 2 weeks). A reliable visual field test was defined as less than 33% fixation losses, less than 33% false-negatives and less than 15% false-positives. The FDT results were reviewed for the following artifacts: lid and rim artifacts, fatigue effects, inappropriate fixation, and inattention.

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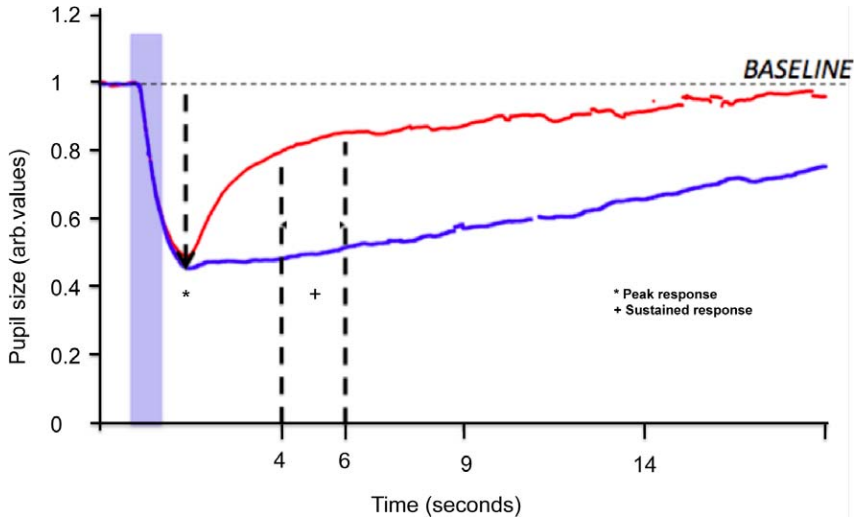

FIGURE 1. Illustration showing the peak and sustained responses expected for the blue and red flashes in healthy patients.

Stereophotographs

Stereoscopic photographs were obtained nonsimultaneously (FF450 plus IRU Retina Camera, Visupac Software version 4.4, Carl Zeiss Meditec AG, Jena, Germany) and analyzed by three ophthalmologists specializing in glaucoma using an estereovisor. Both optical discs from each patient were analyzed, and glaucomatous damage was classified in accordance with the Disc Damage Likelihood Scale (DDLS).[31]

Optical Coherence Tomography (OCT)

The peripapillary RNFL thickness was analyzed using a Cirrus HD-OCT (software version 5.2, model 4000, Carl Zeiss Meditec, Inc.), which uses a superluminescent diode scan with a center wavelength of 840 nm and an acquisition rate of 27,000 A-scans per second at an axial resolution of 5 μm. RNFL measurements were obtained using a circular sweep of a fixed diameter of 3.45 mm around the optic disc. All information was reviewed and confirmed for the absence of movement artifacts, for good centering on the optic disc, and for a signal strength > 7. Scans were also evaluated to assess the adequacy of the algorithm in RNFL detection. Scans without overt algorithm failure in the detection of the retinal borders were exclusively included in the study.

Statistical Analysis

Descriptive statistics, means, and standard deviations were calculated for normally distributed variables. We used skewness/kurtosis testing and histograms to assess normality. For variables with a nonparametric distribution, the Wilcoxon rank-sum test was used.

Generalized estimating equation (GEE) models adjusted for within-patient, intereye correlations were used to compare the glaucoma and control groups.[32] Because both eyes of each glaucoma patient and control subject were included in this analysis and given that the eyes of the same subject are expected to display a certain degree of intercorrelation with respect to the results, the GEE allowed us to make adjustments for these within-patient, intereye correlations.[32] The GEE models were also used to compare the pupillary light reflex, RNFL thickness, and SAP MD and FDT MD results between the groups.[32]

To obtain the correlation coefficients for the relationship between the pupillary light reflex and the RNFL thickness, linear regression was performed. Whenever both eyes were eligible, the right eye was arbitrarily chosen for this specific analysis.

All statistical analyses were performed with commercially available software SPSS (version 17, IBM, Armonk, NY, USA). The α level (type I error) was set at 0.05.

RESULTS

This study included 112 eyes from 56 participants; 76 eyes were from 38 subjects with glaucoma, and 36 eyes were from 18 control subjects. The mean (±standard deviation) ages of the glaucoma and control groups were 60.5 ± 11.2 and 56.2 ± 7.5 years, respectively ($P = 0.052$). Table 1 presents the demographic and clinical variables for the two groups. A significant difference in the mean RNFL thickness was noted between the two groups ($P < 0.001$). Figure 2 presents the mean RNFL thickness (μm) distribution for the two groups.

Significant differences in the SAP and FDT MD values were also noted between the two groups ($P < 0.001$ and $P < 0.001$, respectively). Among the glaucoma patients, 24 eyes (31.6%) exhibited advanced glaucoma, 12 eyes (15.8%) exhibited moderate glaucoma, and 40 eyes (52.6%) exhibited mild glaucoma according to the Hodapp-Anderson-Parrish system.[27] Figure 3 presents the distribution of the SAP MD values for the groups.

The mean sustained responses to the blue flash at 250 cd/m^2 were 0.417 ± 0.120 and 0.462 ± 0.073 ($P = 0.064$) for the glaucoma and control groups, respectively. The mean peak responses to the blue flash at 250 cd/m^2 were 0.505 ± 0.080 and 0.546 ± 0.055 for the glaucoma and control groups, respectively ($P = 0.520$). Additionally, the mean sustained response to the red flash at 250 cd/m^2 was 0.125 ± 0.080 for the glaucoma group, and the value was 0.170 ± 0.084 for the control group ($P = 0.264$). Finally, the mean peak responses to the red flash at 250 cd/m^2 were 0.485 ± 0.072 and 0.513 ± 0.057 for the glaucoma and control groups, respectively ($P = 0.692$).

When considering the entire cohort, an association was noted between the mean RNFL thickness and the sustained response to the blue flash with a luminance of 250 cd/m^2 ($P = 0.024$; $R^2 = 0.403$; Fig. 4). A poorer sustained response to the blue flash was associated with a thinner RNFL, on average; however, this correlation was not observed for the red flash with a luminance of 250 cd/m^2 and the RNFL thickness ($P =$

Table 1. Demographic and Clinical Variables for the Eyes in the Control and Glaucoma Groups

	Control Subjects ($N = 18$)	Glaucoma Subjects ($N = 38$)	*P* Value
Age, y*	56.2 ± 7.5	60.5 ± 11.2	0.052†
Ancestry, %			0.214§
European	13 (23.2%)	23 (41.1%)	
African	5 (8.9%)	15 (26.8%)	
Sex, %			0.789§
Female	14 (25%)	30 (53.6%)	
Male	5 (8.9%)	7 (12.5%)	
Average IOP, mm Hg*	14.11 ± 2.00	16.69 ± 2.91	<0.001‡
Visual acuity, logMAR*	0.03 ± 0.06	0.11 ± 0.09	0.660†
MD, FDT, dB*	−0.78 (−5.46 to 3.76)	−7.03 (−23.28 to 2.02)	<0.001‡
MD, SAP, dB*	−1.48 (−7.82 to 1.52)	−9.44 (−32.28 to 2.49)	<0.001‡
Average PSD, dB*	3.05 (0.97 to 15.28)	3.27 (1.1 to 9.2)	<0.001‡
Average RNFL thickness, μm*	99.42 ± 8.93	76.79 ± 16.21	<0.001†
Cup:disc ratio*	0.35 ± 0.08	0.80 ± 0.15	<0.001‡
Pachymetry, μm*	544.72 ± 29.75	527.87 ± 36.79	0.019†

IOP, intraocular pressure.
* Mean (SD).
† *t*-test.
‡ Wilcoxon's rank-sum test.
§ Pearson's χ^2 test.

0.821; $R^2 = 0.089$). Moreover, no significant correlation between the RNFL thickness and the peak response to blue or red flashes with a 250 cd/m^2 luminance was observed ($P = 0.267$; $R^2 = 0.355$ and $P = 0.340$; $R^2 = 0.181$, respectively). Figure 5 presents the results of the analyses of the correlation between the RNFL thickness and the peak response to blue flashes with a luminance of 250 cd/m^2.

No correlation was noted between the SAP MD and the sustained response to blue or red flashes with a luminance of 250 cd/m^2 ($P = 0.978$ and $P = 0.230$, respectively) when considering the entire cohort. In addition, no significant correlation was observed between the SAP MD and the peak response to a blue or red flash with a luminance of 250 cd/m^2 ($P = 0.832$ and $P = 0.245$, respectively) when considering the entire cohort.

The FDT MD did not correlate with the sustained response to blue or red flashes with a luminance of 250 cd/m^2 ($P = 0.429$ and $P = 0.079$, respectively). Moreover, no significant correlation between the FDT MD and the peak response to blue or red flashes with a luminance of 250 cd/m^2 ($P = 0.102$ and $P = 0.160$, respectively) was observed.

When the severity of glaucoma was considered based on the Hodapp-Anderson-Parrish system,[27] an association was observed between the severity of glaucoma and the sustained response to blue flashes at 250 cd/m^2 ($P = 0.006$).

Age did not demonstrate a significant effect on the sustained response to the blue flash with a luminance of 250

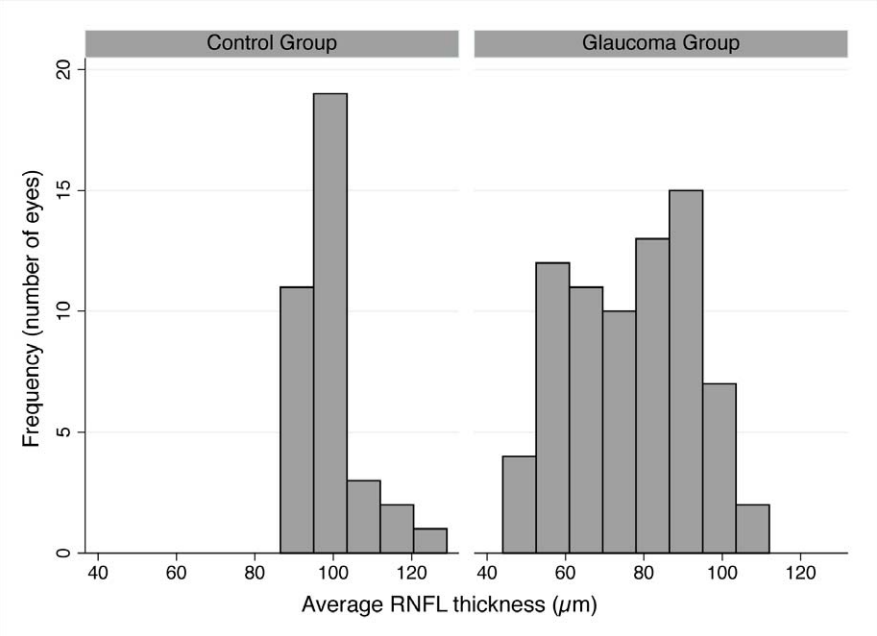

Figure 2. Histogram depicting the frequency distribution of the average RNFL thickness in the control and glaucoma groups.

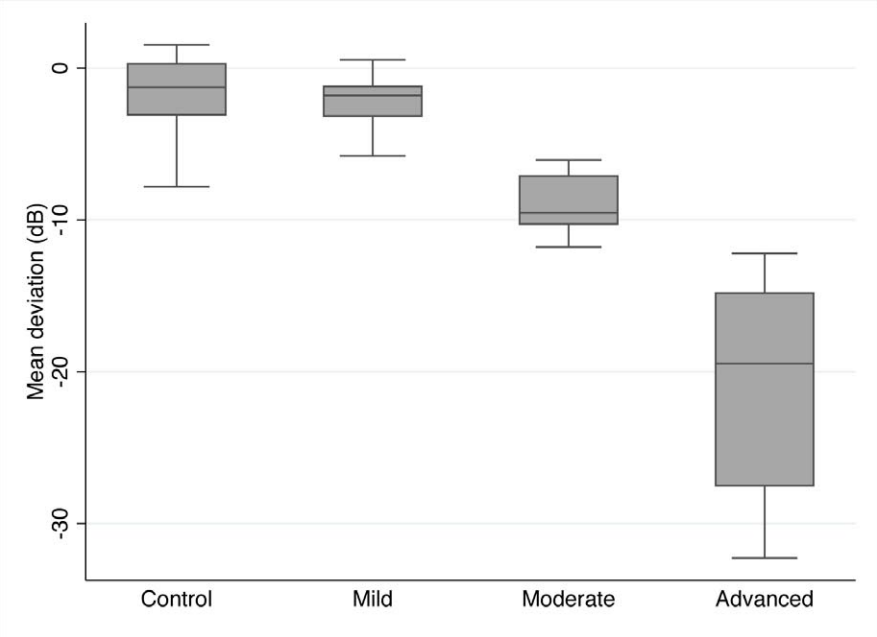

FIGURE 3. Box plot depicting the MD (dB) distribution for SAP in the control and glaucoma groups according to the Hodapp-Anderson-Parrish system.[27]

cd/m^2 in either the control or the glaucoma group ($P = 0.730$ and $P = 0.270$, respectively). Figure 6 shows examples of the peak and sustained pupil responses to blue or red flashes with a luminance of 250 cd/m^2 for one glaucoma patient and one control subject matched by age and sex.

In total, eight of 38 glaucoma patients were receiving α-2 adrenergic agonist agents. For the glaucoma group not using α-2 adrenergic agonist agents ($n = 30$ patients), a significant correlation between the RNFL thickness and the sustained response to the blue flash with a luminance of 250 cd/m^2 ($P = 0.007$; $R^2 = 0.318$) was found. However, an association between the RNFL thickness and the peak response to blue and red flashes with a luminance of 250 cd/m^2 or the sustained response to red flashes with a luminance of 250 cd/m^2 was not observed ($P = 0.134$, $P = 0.343$ and $P = 0.340$, respectively). In contrast, for the glaucoma group using α-2 adrenergic agonist agents ($n = 8$ patients), there was a significant correlation between the RNFL thickness and the sustained response to the blue flash with a luminance of 250 cd/m^2 ($P = 0.010$; $R^2 = 0.203$). However, no correlation between the RNFL thickness and the peak response to the blue flash with a luminance of 250 cd/m^2 or the peak and sustained responses to the red flash

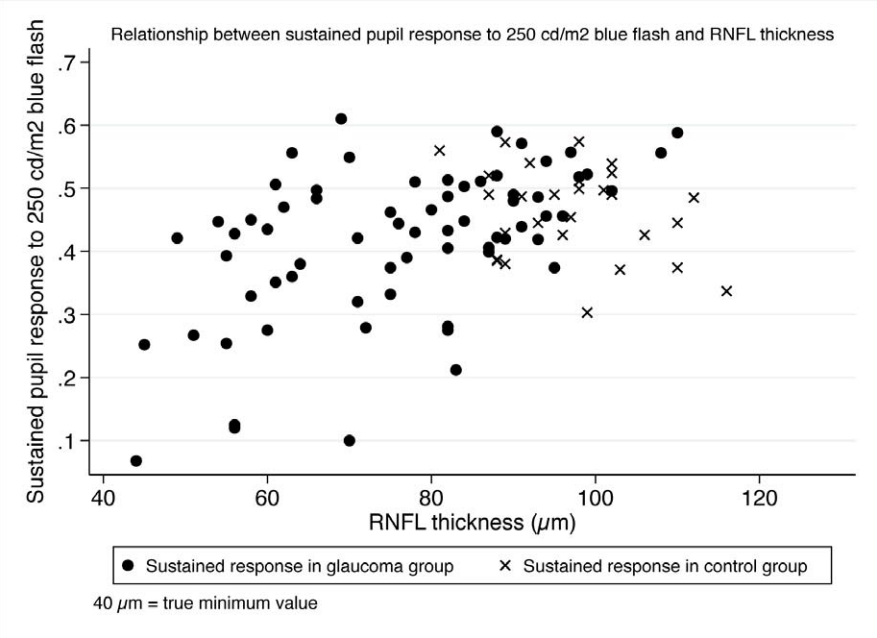

FIGURE 4. Scatter plot depicting the association between the sustained pupil response to the blue flash at 250 cd/m^2 and the RNFL thickness in the control and glaucoma groups.

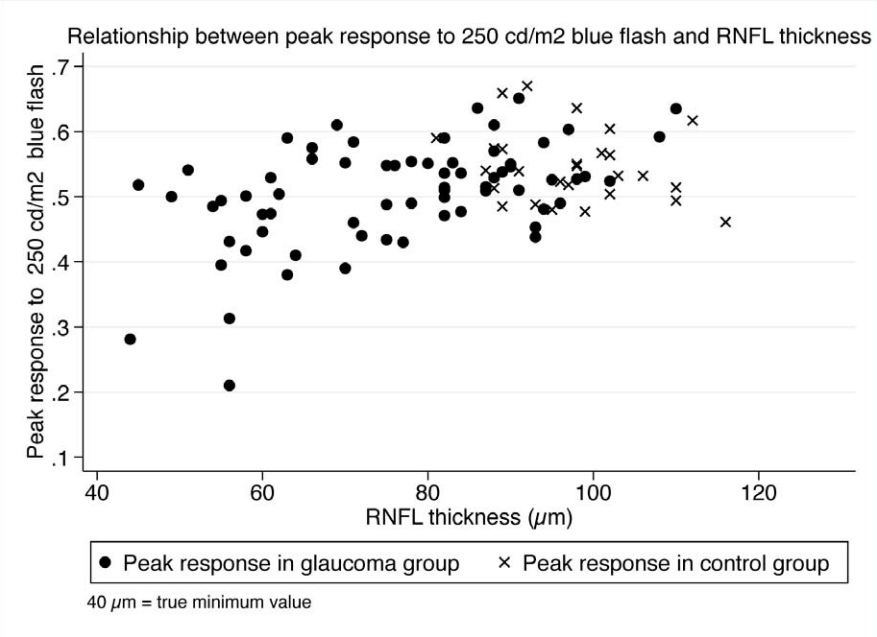

FIGURE 5. Scatter plot demonstrating the association between the peak response to the blue flash at 250 cd/m^2 and the RNFL thickness in the control and glaucoma groups.

with a luminance of 250 cd/m^2 were observed ($P = 0.060$, $P = 0.800$ and $P = 0.202$, respectively). Furthermore, when the glaucoma group using α-2 adrenergic agonist agents was included in the GEE analysis, no significant effect was observed for this group ($P = 0.052$).

DISCUSSION

This study demonstrated a significant correlation between the RNFL thickness and the sustained response to blue flashes with a luminance of 250 cd/m^2 during the pupillary light reflex. Furthermore, a significant correlation was noted between the severity of glaucoma and the sustained pupillary response to the blue flash with a luminance of 250 cd/m^2. To our knowledge, this is the first study to demonstrate an association between the sustained pupillary light response and the RNFL thickness in glaucoma patients.

Previous studies have described a reduction in the postillumination pupil reflex in glaucoma patients.[22,23] For instance, Feigl et al.[23] demonstrated that moderate and severe glaucoma patients exhibit a dysfunctional ipRGC-mediated postillumination pupil response. Based on their study, ipRGC function measured according to the pupillary response may become a clinical method for measuring glaucoma progression. In addition, Kankipati et al.[22] found that MD values were correlated with the postillumination response. However, in the current study, we did not observe a correlation between the SAP MD and the sustained response to a blue flash. One potential reason for this disagreement is that the average MD of the patients in research by Kankipati et al.[22] was –12.44, whereas the average MD in our sample was –9.44 (ranging

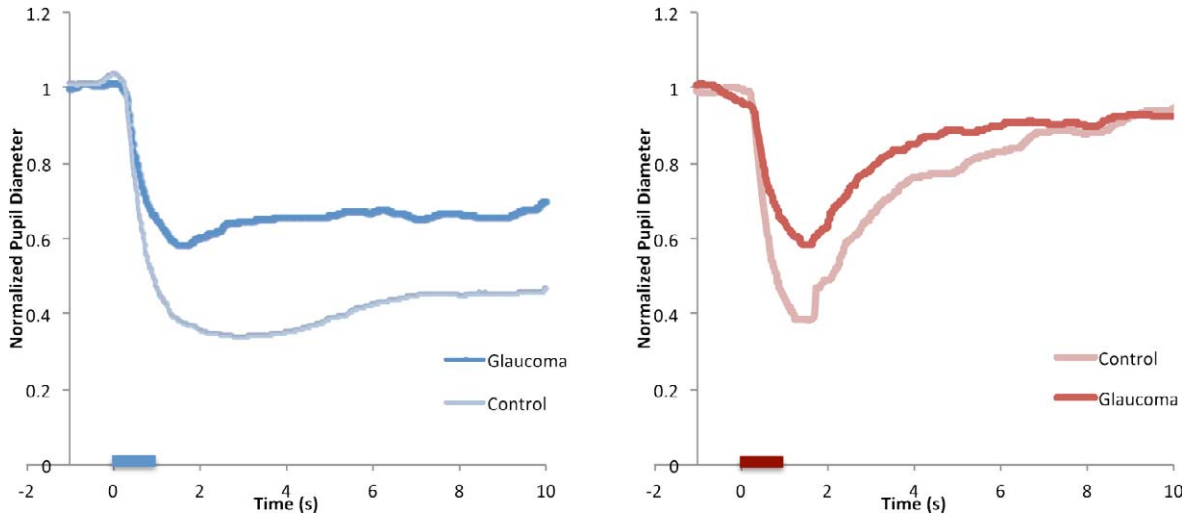

FIGURE 6. Examples of peak and sustained pupil responses to 250 cd/m^2 blue (*left*) and red (*right*) flashes in the right eye of a 50-year-old patient with advanced glaucoma and one control subject matched by age and sex.

from −32.28 to 2.49). This difference implies that our sample contained a higher proportion of mild glaucoma cases, which might not have yet displayed impaired ipRGC function.

Different studies have addressed the correlation between the structural and the functional changes in glaucoma.[33–36] Before functional loss can be detected, a large number of RGCs must be lost. Studies on the cadavers of subjects with glaucoma estimated that at least 25% to 35% of RGCs would need to be damaged for statistically significant abnormalities to appear on automated perimetry.[37] Hence, although the structural changes of glaucoma are usually accompanied by functional losses, patients may exhibit structural changes in the optic nerve (e.g., altered RNFL thickness) before functional changes (e.g., changes in SAP MD).[32,34] Furthermore, the ipRGCs have demonstrated unique resistance to injury in previous studies,[38–40] suggesting that only in advanced glaucoma we can find dysfunctions in this subtype of RGCs. We believe that is the reason why this present study did not find a correlation between SAP and FDT MD values and the pupil reflex (the sustained response to blue flashes). It is likely that patients with an initial defect did not present a difference in their sustained response to blue flashes compared with the control group because they did not have damaged ipRGCs at this stage of the disease.

Nevertheless, when considering the severity of the disease (group effect), a significant correlation was noted between SAP MD and the sustained response. In other words, exclusively considering cases of advanced glaucoma (SAP MD worse than −12 according to the Hodapp-Anderson-Parrish system[27]) will result in a worse sustained response. This result is in agreement with previous studies suggesting that the sustained response was reduced in patients with advanced glaucoma compared with healthy age-matched subjects.[22,23]

Another important topic that should be reported is that different studies have correlated the pupillary reflex with structural and functional damage in glaucoma.[41–45] The pupillary light reflex is initiated by RGCs and therefore is directly correlated with RGC function. With a better evaluation of the pupil response parameters using computerized automated pupillography, it is possible to correlate the pupillary light reflex with a different test whose results can be altered by RGC damage, such as SAP or OCT. For these reasons, Chang et al.[46] studied the correlation between the pupillary light reflex and both MD and the RNFL thickness. They included 148 glaucoma patients and 71 controls matched by age, and using a pupillometer, they analyzed different stimulus patterns. These authors showed a very strong correlation between the pupil response and both functional assessment based on SAP MD values ($P < 0.001$; $R^2 = 0.83$) and structural damage based on the RNFL thickness ($P < 0.001$; $R^2 = 0.67$).[46] These findings support the idea that pupillary responses to light are representative of ganglion cell function. However, this pupillary test has not been used to specifically isolate the ipRGCs; the detection of pupil response abnormalities in this test may be a useful functional test for detecting glaucoma damage but not for analyzing ipRGCs population, which is responsible for non–image-forming tasks.

According to physiological and anatomical classification, approximately 12 different types of RGCs exist. The ipRGCs are a subgroup of RGCs, and although glaucomatous disease is characterized by the death of all types of RGCs, each type of RGC demonstrates unique resistance to injury or damage.[38–40] The underlying mechanism of ipRGC resistance to injury is uncertain. Certain authors have reported that the ipRGC subtype exhibits a large soma size and long, sparsely branching dendrites throughout the retina, suggesting that these cells are potentially more resistant to injury.[10] Additionally, if RGC death were to occur in the central retina more than in the peripheral retina, then the increased ipRGC survival reported might be explained by differences in the retinal distribution of nonmelanopsin (highest density in the central retina) and ipRGCs (highest density in the peripheral retina).[10] Moreover, preliminary studies have confirmed that ipRGCs intensively stain with antibodies against both mitochondrial and cytochrome c oxidase, and these characteristics are associated with an abundant mitochondrial population within these cells, supporting their resistance property.[47] The role of pituitary adenylate cyclase-activating polypeptide (PACAP), a neuropeptide specifically expressed by ipRGCs, has also been discussed and examined by different studies on experimental models of monosodium glutamate toxicity and ocular ischemia as a neuroprotective factor.[8] In the present study, the 250 cd/m^2 blue flash stimulus specifically isolated the activity of ipRGCs, as demonstrated in previous studies.[7,28,48] However, the real mechanism of damage in these cells was not investigated; thus, further studies should be conducted to better understand the resistance properties of these cells.

It is also important to consider that patient age could influence the pupillary response. However, previous studies have shown that the postillumination response did not significantly decrease with age[49] and that only the pupil diameter was reduced in an elderly population.[49] In addition, we did not detect a significant difference in age between the two groups in our study. Finally, age might affect the pupil response and pupil diameter, but we did not observe any significant differences between the two groups. Furthermore, our study determined measures based on the patient baseline (e.g., peak amplitude = baseline diameter − smallest pupil diameter); in this way, the pupil measures could have been less affected by the possible influence of the age of each patient. Indeed, previous studies have also suggested that the tendency for the amplitude of the postillumination pupil reflex to decrease with age is not significant.[22]

Moreover, it is important to emphasize that certain patients (8 of 38 subjects in the glaucoma group) were receiving α-2 adrenergic agonist agents. This class of medication (e.g., brimonidine tartrate) is frequently used to reduce the intraocular pressure in patients with glaucoma by reducing aqueous humor production.[50] Additionally, different studies have reported the action of brimonidine on the pupil diameter.[51,52] Brown et al.[51] reported that the drug could inhibit the release of norepinephrine from the sympathetic terminals by acting on presynaptic α-2 adrenergic receptors. As a result, the contraction of dilator muscle mediated by norepinephrine binding to α-1 receptors is decreased, which in turn inhibits mydriasis.[52] This effect is more evident under scotopic conditions, as norepinephrine is the main mediator of nocturnal pupil dilation when unopposed by the acetylcholine-mediated sphincter muscles. In photopic situations, α-2 adrenergic agonist agents show no substantial effect, as they do not affect the sphincter muscles.[52] Hence, the most important effect of α-2 adrenergic agonist agents is mydriasis inhibition (antimydriasis effect), which we did not evaluate in the present study. Furthermore, all measures (peak and sustained responses) were collected based on the patient baseline (relative measures) to decrease the chances of drug influences. The difference between two measures (the baseline pupil diameter and the smallest pupil diameter) was used to determine the peak response of each patient. Additionally, the sustained response was expressed as the pupil diameter at 6 seconds after the flash offset relative to the baseline, as suggested by previous studies.[28] α-2 adrenergic agonist agents would not influence any of the measures once the relative peak and sustained responses were calculated. Moreover, certain studies have reported the occurrence of tachyphylaxis with continuous use of α-2 adrenergic agonist

agents in glaucoma patients.[53] Therefore, the chronic use of α agonists may also decrease the pupillary response to this drug. However, even considering these points, we also performed the analysis for the glaucoma patients separately, and the same correlation was found. In particular, the RNFL thickness was associated with the sustained response to blue flashes with a luminance of 250 cd/m^2 in both the group using and the group not using α-2 adrenergic agonist agents.

The main clinical finding in our study was the significant association between the pupillary response and the mean RNFL thickness. Thus, clinical examination of the pupillary response could be used as an additional tool for monitoring disease progression and assessing patient prognosis. In addition, abnormal pupillary responses in patients with advanced glaucoma are potentially associated with other symptoms, such as changes in circadian rhythm; additional studies should be performed to elucidate the effect of pupillary responses on the quality and pattern of sleep in these patients.

Certain specific drawbacks of our study should be mentioned. First, our study was limited by its small sample size. This investigation should be repeated in larger populations, and different disease categories should be used in clinical practice. Second, this study was cross-sectional, which did not allow correlation of the results of pupillometry with the development of disease. Third, although we reduced the duration of stimulation to 1 second,[28] we still found patients with certain difficulties in tolerating the flashes, and especially flashes with high luminance, with these patients experiencing difficulty in keeping their eyes open. Fourth, the sustained response was expressed as the pupil diameter 6 seconds after the flash offset, a method used in previous research,[28] but it is possible that using the average measurement between 6 and 8 seconds could better reduce variability, making the measurement less prone to artifacts and noise. Finally, we found a relatively weak association between the RNFL thickness and the sustained pupillary response. This suggest that this approach may not be an effective approach to detect glaucoma or progression of the disease compared to existing approaches. Further, a complete evaluation would also need to include a more thorough assessment of test-retest variability.

In conclusion, this study observed a correlation between a reduced RNFL thickness and a reduced sustained pupillary response to a blue flash with 250 cd/m^2 luminance. The reduced amplitude of the sustained pupil response suggests a decrease in the number and/or activity of the ipRGCs. Additionally, in the present study, the severity of glaucoma that reflects a SAP MD worse than −12 dB was associated with worse pupillary response. Therefore, these associations can potentially be used in the future to correlate this response with the progression of glaucoma disease.

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