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Journal

Investigative Ophthalmology & Visual Science, 55(1)

ISSN

0146-0404

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Publication Date

2014-01-29

DOI

10.1167/iovs.13-12921

Peer reviewed

Estimation of Retinal Ganglion Cell Loss in Glaucomatous Eyes With a Relative Afferent Pupillary Defect

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Submitted: July 26, 2013
Accepted: November 10, 2013

Citation: Tatham AJ, Meira-Freitas D, Weinreb RN, Marvasti AH, Zangwill LM, Medeiros FA. Estimation of retinal ganglion cell loss in glaucomatous eyes with a relative afferent pupillary defect. *Invest Ophthalmol Vis Sci*. 2014;55:513–522. DOI:10.1167/iovs.13-12921

Purpose. To estimate retinal ganglion cell (RGC) losses associated with a relative afferent pupillary defect (RAPD) in glaucoma.

Methods. A cross-sectional study was conducted including both eyes of 103 participants from the Diagnostic Innovations in Glaucoma Study. A total of 77 subjects had glaucoma in at least one eye and 26 were healthy. Pupil responses were assessed using an automated pupillometer that records the magnitude of RAPD as an "RAPD score." Standard automated perimetry (SAP) and optical coherence tomography (OCT) also were performed. Retinal ganglion cell counts were estimated using empirical formulas that combine estimates from SAP and OCT. The estimated percentage RGC loss was calculated using the combined structure function index (CSFI).

Results. There was good correlation between RAPD magnitude and intereye differences in estimated RGCs ($R^2 = 0.492$, $P < 0.001$), mean deviation ($R^2 = 0.546$, $P < 0.001$), retinal nerve fiber layer thickness ($R^2 = 0.362$, $P < 0.001$), and CSFI ($R^2 = 0.484$, $P < 0.001$). Therefore, a high RAPD score is likely to indicate large asymmetric RGC losses. The relationship between intereye difference in RGC counts and RAPD score was described best by the formula; RGC difference = 21,896 + 353,272 * RAPD score. No healthy subjects had an absolute RAPD score > 0.3, which was associated with asymmetry of 105,982 cells (or 12%).

Conclusions. Good correlation between the magnitude of RAPD and intereye differences in mean deviation and estimated RGC counts suggests pupillometry may be useful for quantifying asymmetric damage in glaucoma. (ClinicalTrials.gov number, NCT00221897.)

Keywords: glaucoma, pupils, pupillograph

Glaucoma is an optic neuropathy characterized by progressive loss of retinal ganglion cells (RGCs) and reduction in visual field sensitivity. Although glaucoma usually is bilateral, asymmetry is common and patients often have intereye differences in IOP,[1] retinal nerve fiber layer (RNFL) thickness,[2] and standard automated perimetry (SAP) sensitivity.[1,3,4] A relative afferent pupillary defect (RAPD) is an important marker of asymmetric impairment of the afferent visual system and, in glaucomatous subjects, is likely to indicate significant asymmetric optic nerve damage.[5,6]

An RAPD can be detected clinically using the swinging flashlight test, during which each eye is illuminated alternately and the velocity and amplitude of pupillary responses compared.[7–9] An RAPD is present when there is asymmetry of the light reflex and this can be quantified by placing neutral density filters in front of the normal eye and repeating the swinging flashlight test until the pupil responses become symmetric.[10] Previous studies have reported that the swinging flashlight test can detect an RAPD in one- to two-thirds of patients with glaucomatous optic neuropathy.[11,12] However, this test is subject to interobserver variation and interpretation may be difficult in eyes with anisocoria, or dark irides; also, small RAPDs may go undetected.[5,13,14] An objective assessment of the pupillary response is possible using automated pupillometry.[5] Computerized assessment of the pupil is advantageous, as it is objective, allows averaging of multiple responses, and provides a method of quantifying parameters that are not visible to the naked eye.[5,15] Automated pupillometers are able to detect RAPDs with greater sensitivity than the swinging flashlight test.[13,16] For example, Lankaranian et al.[13] found an RAPD in 39 of 70 patients (56%) with glaucoma using a pupillometer, compared to only 20 of 70 patients (29%) using the swinging flashlight test. Other studies have shown that the severity of an RAPD is correlated with the difference in visual field loss between eyes[17–19] and with the anatomic extent of retinal abnormalities, such as retinal detachment and macular degeneration.[20–22] These studies suggest that assessment of the pupillary response might be a useful measure of glaucomatous damage.

Although RAPDs are common in glaucomatous patients, little is known about the quantity of RGC loss and the asymmetry that must be present before an RAPD becomes detectable. An animal study found unilateral loss of 25% to 50% of RGCs was required for an RAPD of 0.6 log units, measured using neutral density filters.[8] However, this was an experimental study where RGC loss was induced using macula diode laser rather than as a consequence of glaucomatous optic neuropathy. Direct counting of RGCs in vivo is not yet possible; however, empirical formulas may be used to estimate the number of RGCs and these estimates have shown good

correlation to histologic counts in experimental glaucoma models.[23] We recently proposed a method for estimating RGC loss from a combination of SAP and RNFL assessment with optical coherence tomography (OCT).[24–26] The combined RGC estimates performed significantly better than isolated structural and functional parameters for staging the disease and monitoring glaucomatous progression.[24–26]

The aim of this study was to estimate the number of RGCs in glaucomatous patients and determine the intereye RGC difference associated with an RAPD measured using automated pupillometry. As small RAPDs may be present in healthy subjects,[14] pupil responses in glaucomatous patients were compared to a control group of healthy subjects.

Methods

Study Participants

This was a cross-sectional study involving participants from a prospective longitudinal study designed to evaluate optic nerve structure and visual function in glaucoma; the Diagnostic Innovations in Glaucoma Study (DIGS) at the University of California, San Diego (UCSD). Methodologic details have been described previously.[27] The UCSD Human Subjects Committee approved all protocols, and methods adhered to the Declaration of Helsinki.

At each visit subjects underwent comprehensive ophthalmologic examination, including visual acuity, slit-lamp biomicroscopy, IOP measurement, gonioscopy, dilated funduscopic examination, simultaneous stereoscopic optic disc photography (Kowa WX3D; Kowa Optimed, Inc., Torrance, CA), and SAP using the Swedish interactive threshold algorithm (SITA Standard 24-2). Only subjects with open angles on gonioscopy were included. Subjects were excluded if they presented with a best-corrected visual acuity less than 20/40, spherical refraction outside ±5.0 diopters (D), and/or cylinder correction outside 3.0 D, or any other ocular or systemic disease that could affect the optic nerve or the visual field. Details of the methodology used to grade optic disc photographs at the UCSD Optic Disc Reading Center have been provided elsewhere.[27–29]

The study included both eyes of 103 participants. Participants were defined depending on the diagnosis in their worse eye as healthy or glaucomatous. Eyes were classified as glaucomatous if they had repeatable (≥2 consecutive) abnormal SAP test results on the 24-2 program of the Humphrey visual field analyzer (Carl Zeiss Meditec, Inc., Dublin, CA). An abnormal SAP result was defined by a pattern standard deviation outside the 95% confidence limits (CI) or a glaucoma hemifield test result outside the reference range. Eyes with suspect glaucoma were defined as those with suspicious neuroretinal rim thinning or RNFL defects on masked stereophotographic assessment, without repeatable abnormal SAP results. Eyes with suspect glaucoma also included those with IOP > 21 mm Hg but with healthy-appearing optic discs and without repeatable abnormal SAP results. Healthy subjects were recruited from the general population through advertisements, and from the staff and employees of UCSD. Healthy eyes had IOP ≤ 21 mm Hg with no history of increased IOP and normal SAP.

Each subject was required to have Cirrus spectral domain OCT (SD-OCT, software version 6.0; Carl Zeiss Meditec, Inc.), SAP, and automated pupillometry, and for each subject all tests were performed within a 4-month interval. As pupil responses may be influenced by medications affecting the autonomic nervous system, patients using systemic or topical cholinergic or anticholinergic medications, including pilocarpine, were not included in the study.

Imaging and Standard Automated Perimetry

The Cirrus SD-OCT was used to acquire RNFL measurements in the study. Cirrus SD-OCT uses a superluminescent diode scan with a center wavelength of 840 nm and an acquisition rate of 27,000 A-scans per second. The optic disc cube 200 × 200 protocol was used to acquire RNFL thickness measurements. This protocol is based on a 3-dimensional scan of a 6 × 6 mm area centered on the optic disc in which information from a 1024 (depth) × 200 × 200 point parallelepiped is collected. The parapapillary RNFL thickness measurements were calculated from a 3.46-mm diameter circular scan (10.87-mm length) automatically placed around the optic disc. The reported average RNFL thickness corresponded to the 360° measure automatically calculated by the OCT software. The Cirrus SD-OCT images were included if the signal strength was greater than 7, if movement artifacts were absent, and there was good centering on the optic disc.

Patients underwent SAP testing using the SITA Standard 24-2 strategy. All visual fields were evaluated by the UCSD Visual Field Assessment Center (VisFACT).[30] 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 fields with false-negative errors of more than 33% when the field showed advanced disease. Visual fields exhibiting a learning effect (i.e., initial tests showing consistent improvement on visual field indices) also were excluded. Visual fields were reviewed further for the following artifacts: eyelid and rim artifacts, fatigue effects, inappropriate fixation, evidence that the visual field results were caused by a disease other than glaucoma, and inattention.

Pupillometry

Pupil responses were tested using the RAPDx (Konan Medical USA, Inc., Irvine, CA), a new binocular infrared computerized pupillometer. The device measures bilateral pupil responses to a novel sequence of monocularly presented visual stimuli. The pupillometer stimulus is generated using a single LCD screen with a central physical barrier creating two optical channels. The screen displays a target (green cross) for patient fixation and during testing each portion of the screen can be enabled selectively to achieve separate stimulation of each eye. The screen is viewed at infinity through a pair of 50-mm objective lenses providing an approximate 25° field of view in each eye. Eyes also are illuminated by a pair of infrared emitting diodes, with peak emission at 880 nm, mounted at a 35° angle.

Under infrared conditions information regarding the "dark" pupil diameter is captured as camera pixels and this measurement converted to millimeters using a scaling factor. The stimulus then is presented as a series of trials, either to the full field of each eye or limited to predetermined regions. The size, color, intensity, and length of time of each stimulus are controlled automatically via proprietary software. The pupillometer has an inbuilt pupil tracking and blink detection system using 60 Hz digital cameras, each with a resolution of 240 × 240 pixels/frame, for approximately 25 pixels/mm. If a blink obscures the pupil during recording the test is repeated automatically. The device also includes novel synchronizing circuitry to tie presented stimuli timing to the recording to optimize spatial (pupil dimension data), temporal (latency data), and other pupil response metrics.

For the purposes of this study, only the full field stimulus testing strategy was used. The full field stimulus extends to

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approximately 18° from fixation. Each trial consisted of a period of stimulation followed by a period of darkness during which the cameras record continuously. The total time of each trial was 2.0 seconds plus a 100-ms posttrial rest period during which no images were acquired. The full field white stimulus was presented for 200 ms of the 2.1-second duty cycle and there was a total of 18 trials (nine for each eye) using this stimulus for a total test time of 37.8 seconds. The right eye was stimulated first, followed by the left, then the right, with continued stimulation alternating between eyes. The white stimulus had a luminance of 384 cd/m^2, and there was a nominal background luminance of 0.01 cd/m^2. Testing was conducted under dark room conditions with an illuminance of <0.5 lux. The color chromaticity coordinates of the LCD white stimulus were $x = 0.313$, $y = 0.329$, and the background consisted of a very low white setting.

The pupillometer includes proprietary analysis software, which was used to parse the generated pupil diameter waveforms into specific metrics. The repetitions from each eye were averaged (median) before analysis to minimize noise inherent in the pupil responses. Parameters measured by the pupillometer include prestimulus pupil diameter (in millimeters), minimum pupil diameter following the stimulus (in millimeters), response latency (time in milliseconds between stimulus onset and time when pupil velocity has reached 50% of the peak velocity of constriction), time to peak constriction (in milliseconds), and response amplitude. The response amplitude is the maximal contraction of the pupil as a percentage of the prestimulation size, that is, the prestimulus pupil diameter minus the minimum pupil diameter, divided by the prestimulus pupil diameter. An RAPD is defined as a difference in average pupillary constriction when each eye is stimulated monocularly.[9,13] An index of the direction and magnitude of pupil response asymmetry, known as the RAPD score, is generated automatically by the RAPDx device. The RAPD score is calculated as the difference in the amplitude of pupil constriction between stimulation of the two eyes using the following formula[31]:

$$RAPD\,score = 10^{*}\log_{10}(od/os)$$

Where *od* is the mean response amplitude in both eyes, in response to right eye stimulation, and *os* is the mean response amplitude in both eyes in response to left eye stimulation. An RAPD score of 0 would be expected for a healthy subject. A positive value indicates a relative abnormality of the left afferent system and a negative value indicates a relative abnormality of the right afferent system.[31] The RAPD score is useful as it confers information regarding the direction as well as the magnitude of an RAPD. However, to investigate the effect of potential confounders, such as average disease severity, the absolute RAPD score also was calculated as an overall measure of asymmetry of the afferent visual pathways, regardless of which eye was affected. The arithmetic difference in response amplitude on stimulation of the better eye and on stimulation of the worse eye also was calculated.

Estimation of Retinal Ganglion Cell Number

The estimates of RGC counts were obtained according to the model developed by Medeiros et al.[24,25,32] based on empirical formulas derived by Harwerth et al.[23] for estimating RGC counts from SAP and OCT. The model uses information from structural and functional tests to derive a final estimate of the RGC count in a particular eye. The model has been described in previous publications.[24,25,32] The following formulas were used to estimate the number of RGC somas in an area of the retina corresponding to a specific SAP test field location at eccentricity *ec* with sensitivity *s* in dB:

$$m = (0.054^{*}[ec^{*}1.32]) + 0.9$$

$$b = (-1.5^{*}[ec^{*}1.32]) - 14.8$$

$$gc = ([\{s - 1\} - b]/m) + 4.7$$

$$\mathrm{SAP}rgc = \Sigma 10^{(gc^{*}0.1)}$$

In the above formulas, *m* and *b* represent the slope and intercept, respectively, of the linear function relating ganglion cell quantity (*gc*) in decibels to the visual field sensitivity (*s*) in decibels at a given eccentricity. To account for the total number of RGCs in an area of the retina, the cell density derived from each perimetry measurement was considered to be uniform over an area of retina corresponding to an area of 6° × 6° of visual space that separates test locations in SAP. The estimate of RGC count (SAP*rgc*) was obtained by adding the estimates from all SAP locations. The structural part of the model consisted of estimating the number of RGC axons from OCT RNFL thickness measurements. The model took into account the effect of aging on axonal density, and the effect of disease severity on the relationship between the neuronal and nonneuronal components of the RNFL thickness estimates obtained by OCT. To derive the total number of RGC axons from the global RNFL thickness measurement obtained by OCT (OCT*rgc*), we applied the following formulas:

$$d = (-0.07^{*}age) + 1.4$$

$$c = (-0.26^{*}MD) + 0.12$$

$$a = average\,RNFL\,thickness^{*}10870^{*}d$$

$$\mathrm{OCT}rgc = 10^{([\log\{a\}^{*}10 - c]^{*}0.1)}$$

In the above formulas, *d* corresponds to the axonal density (axons/μm^2), *c* is a correction factor for the severity of disease to take into account remodeling of the RNFL axonal and nonaxonal composition, and MD is the SAP mean deviation. These calculations provide an estimate of the number of RGCs from two sources, one functional and one structural. A combined calculation of RGC counts was performed according to the following formula:

$$Estimated\,RGC\,count = (1 + MD/30)^{*}\mathrm{OCT}rgc$$

$$+(-MD/30)^{*}\mathrm{SAP}rgc$$

The rationale for using a weighting system for deriving the final RGC count is described by Medeiros et al.,[24,25,32] but in essence it relies on the fact that the accuracies of clinical perimetry and imaging tests are inversely related to disease severity. Age-corrected estimates of RGC number in healthy eyes have been determined previously using the above formulas.[24] Using these models it was possible to calculate the combined structure function index (CSFI) as an estimate of the percentage of RGC loss.

$$CSFI = [(expected\,number\,of\,RGCs$$

$$-estimated\,number\,of\,RGCs)$$

$$/(expected\,number\,of\,RGCs)]^{*}100$$

Table 1. Demographic and Clinical Characteristics of Healthy Subjects Compared to Those With Glaucoma in at Least One Eye

	Healthy Subjects, $N = 26$	Subjects With Glaucoma in at Least 1 Eye, $N = 77$	*P* Value
Age, y*	50.0 ± 15.2	68.9 ± 12.3	<0.001
Sex	14 (54%) female	41 (53%) female	0.958
Response amplitude, %†			
Worse eye stimulated	29.4 (30.4) 27.8–32.6	26.9 (27.7) 23.5–30.8	<0.001
Better eye stimulated	29.5 (31.7) 27.6–32.9	27.5 (27.2) 24.2–30.3	0.002
Intereye difference	0.6 (0.6) 0.4–0.9	1.6 (1.0) 0.5–1.9	<0.001
Response latency, ms†			
Intereye difference	4.9 (4.2) 2.2–5.3	6.3 (4.6) 1.4–9.2	0.652
Peak constriction, ms†			
Intereye difference	7.6 (5.5) 3.1–7.9	11.1 (10.2) 3.6–16.6	0.007
Absolute RAPD score†	0.10 (0.09) 0.05–0.14	0.26 (0.16) 0.08–0.30	0.002
SAP MD, dB*			
Worse eye	−0.4 ± 1.1	−5.0 ± 5.7	<0.001
Better eye	0.3 ± 0.9	−2.1 ± 4.1	0.004
Intereye difference	0.6 ± 0.5	3.0 ± 3.1	<0.001
RNFL thickness, μm*			
Worse eye	94.5 ± 10.3	74.6 ± 15.4	<0.001
Better eye	94.5 ± 10.5	80.9 ± 12.8	<0.001
Intereye difference	4.0 ± 3.6	10.0 ± 7.9	<0.001
Estimated RGC count, 1000s cells*			
Worse eye	1032 ± 191	599 ± 211	<0.001
Better eye	1071 ± 211	724 ± 205	<0.001
Intereye difference	61 ± 49	148 ± 123	<0.001
CSFI, %*			
Worse eye	3 ± 11	33 ± 22	<0.001
Better eye	0 ± 11	18 ± 21	<0.001
Intereye difference	5 ± 4	17 ± 14	<0.001

Response amplitude, maximal contraction of the pupils as a percentage of the prestimulation pupil size; Response latency, time between stimulus onset and pupil velocity reaching 50% of peak velocity of constriction; Peak constriction, time between beginning of stimulus and peak constriction; RAPD score, $10*\text{Log}_{10}$ (mean response amplitude of both eyes [in response to right eye stimulation]/mean response amplitude of both eyes [in response to left eye stimulation]).

* Mean ± SD, *t*-test.

† Mean, (median), interquartile range, Wilcoxon-rank sum test.

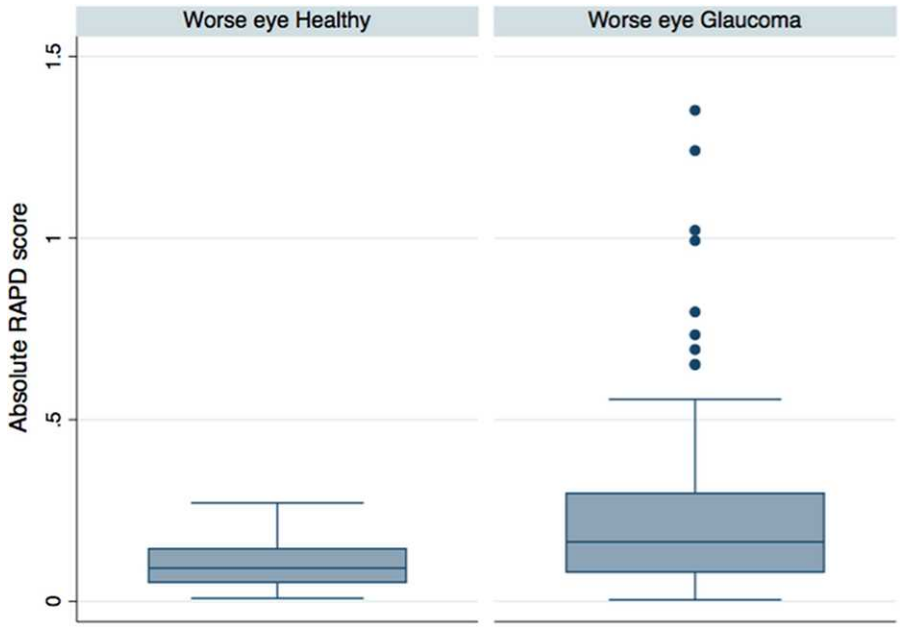

Figure 1. Box plots showing the median, interquartile range, and outside values of the absolute RAPD scores in healthy subjects and those with glaucoma in at least one eye.

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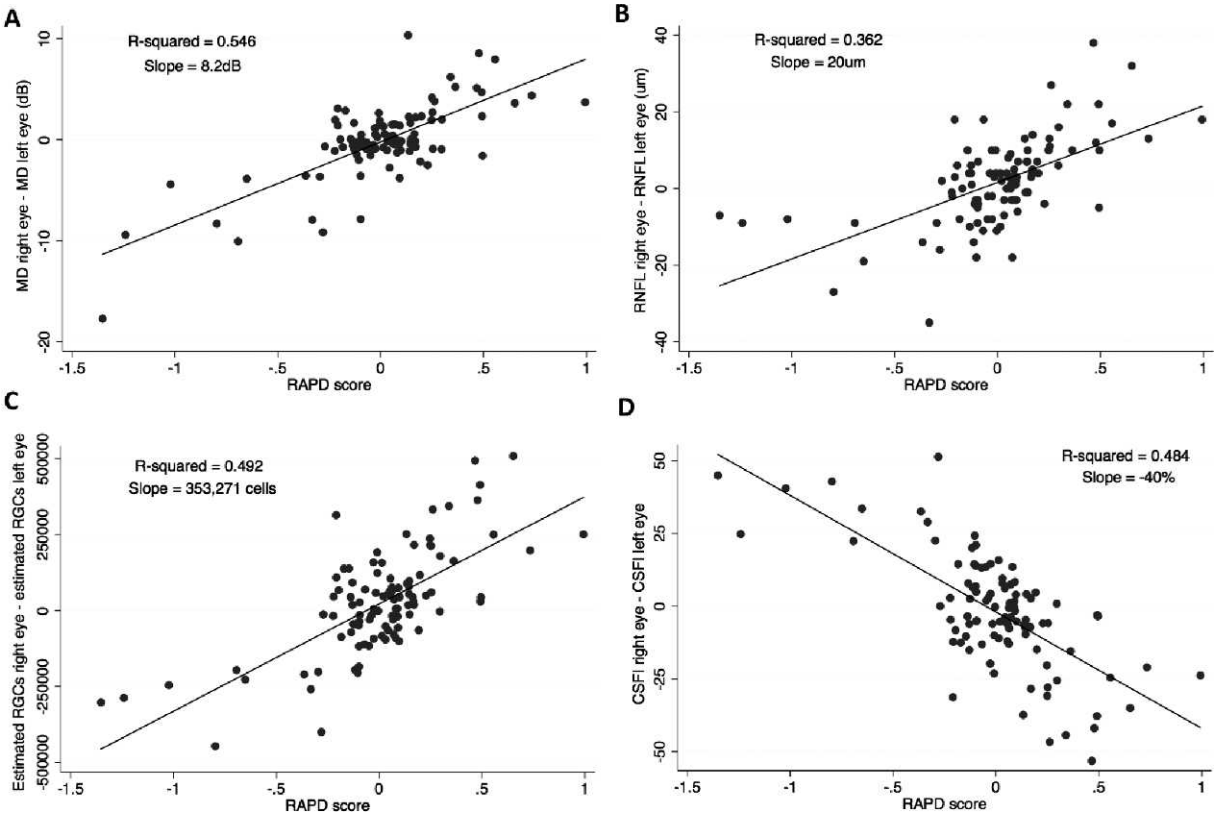

FIGURE 2. Scatter plot showing RAPD score association with intereye differences in SAP MD (**A**), RNFL thickness (**B**), estimated number of RGCs (**C**), and CSFI (**D**). All intereye differences were calculated by subtracting left eye values from right eye values. The ordinary least squares regression lines are shown.

Statistical Analysis

Normality assumption was assessed by inspection of histograms and using Shapiro-Wilk tests. Summary statistics included the mean ± SD for normally distributed variables and the mean, median (interquartile range) for nonnormal variables. Student's *t*-tests were used for group comparison for normally distributed variables and the Wilcoxon rank-sum test for continuous nonnormal variables.

The relationship between RAPD score and intereye differences (right eye minus left eye) in SAP MD, RNFL thickness, estimated number of RGCs, and CSFI was examined using scatter plots and linear regression. Determination of fit was assessed using R^2 statistics. The relationship between absolute RAPD score and absolute intereye differences in MD, RNFL thickness, estimated number of RGCs, and CSFI also was examined using linear regression analysis. The average MD, RNFL thickness, RGC estimate, and CSFI were included in the respective analyses to evaluate the effect of disease severity on asymmetry needed for an RAPD. The effect of age was examined as a covariate. All statistical analyses were performed with commercially available software (STATA, version 12; StataCorp LP, College Station, TX). The α level (type I error) was set at 0.05.

RESULTS

The study included both eyes of 103 subjects. There were 77 subjects with glaucoma in at least one eye and 26 healthy subjects. Of those with glaucoma, 52 had glaucoma in both eyes, 23 had glaucoma in one eye and suspected glaucoma in the other, and two had glaucoma in one eye and a putative healthy fellow eye. The demographic and clinical characteristics of participants are summarized in Table 1.

The absolute RAPD score was significantly higher in those with glaucoma than in healthy participants ($P = 0.002$). The mean (median, interquartile range) absolute RAPD score was 0.10 (0.09, 0.05–0.14) in healthy subjects compared to 0.26 (0.16, 0.08–0.30) in those with glaucoma. Figure 1 shows the distribution of absolute RAPD scores in healthy and glaucomatous subjects. Only two healthy subjects had an absolute RAPD score ≥ 0.2 and none had an RAPD score ≥ 0.3. Of 77 subjects with glaucoma, 32 (42%) had an absolute RAPD score ≥ 0.2 and 19 of 77 subjects (25%) had an absolute RAPD score ≥ 0.3 (Fig. 1). The mean (median, interquartile range) absolute RAPD score in subjects with glaucoma in both eyes was 0.25 (0.14, 0.07–0.30), compared to 0.30 (0.18, 0.08–0.49) in subjects with glaucoma in one eye and suspect glaucoma in the other. The two subjects with glaucoma in one eye and a healthy fellow eye had absolute RAPD scores of 0.14 and 0.25.

Subjects with glaucoma also had significantly lower response amplitudes ($P < 0.001$) and a greater intereye difference in response amplitude than healthy subjects ($P <$

TABLE 2. Results of Univariate Regression Analysis of RAPD Score Compared to Intereye Difference (Δ, Right Minus Left Eye) in SAP MD, RNFL Thickness, RGC Estimate, and CSFI

Intereye Difference, Δ	Constant	Coefficient	R^2	*P* Value
MD-Δ, dB	−0.21	8.23	0.546	<0.001
RNFL thickness-Δ, μm	1.61	19.99	0.362	<0.001
RGC estimate-Δ, cells	21,896	353,272	0.492	<0.001
CSFI-Δ, %	−1.97	−40.0	0.484	<0.001

Table 3. Results of Multivariate Regression Analysis of Absolute RAPD Score and Absolute Intereye Difference (Δ) in SAP MD, Including the Covariate of Average MD-Δ ($R^2 = 0.618$, $P < 0.001$)

	Coefficient	95% CI	*P* Value
Absolute RAPD score	6.47	5.41–7.53	<0.001
Average MD-Δ*	−0.25	−0.31 to −0.19	<0.001
Constant	0.27	−0.07–0.61	0.003

* Average MD-Δ was not significant in the regression analysis for absolute RAPD score and RGC estimate ($P = 0.244$), RNFL thickness ($P = 0.299$), or combined structure and function index ($P = 0.445$).

0.001, Table 1). Patients with glaucoma had, on average, a 26.9% reduction in pupil diameter on stimulation of the worse eye, compared to a 29.4% to 29.5% reduction in pupil diameter on stimulation of either eye in healthy subjects (Table 1). There was no significant difference in the intereye latency between healthy subjects and those with glaucoma ($P = 0.652$). However, those with glaucoma had a greater intereye difference in time to peak constriction than healthy subjects ($P = 0.007$). There was no significant relationship between age and absolute RAPD score in healthy subjects ($R^2 = 0.020$, $P = 0.315$) or those with glaucoma ($R^2 = 0.002$, $P = 0.563$).

Subjects with glaucoma had greater asymmetry (intereye differences) in MD, RNFL thickness, estimated RGC counts, and CSFI than healthy subjects ($P < 0.001$ for all comparisons) (Table 1). Patients with glaucoma had a mean estimated RGC count of 598,645 ± 211,062 cells in the worse eye and 723,532 ± 205,344 cells in the better eye compared to 1,031,521 ± 190,906 cells in the worse eye of healthy participants (Table 1). The median (interquartile range) intereye difference in estimated RGC counts was 100,795 (49,479–215,555) cells in patients with glaucoma in at least one eye, compared to 50,456 (19,155–74,846) cells in healthy participants. The median (interquartile range) intereye difference in CSFI was 13% (6%–25%) in patients with glaucoma compared to 5% (3%–7%) in healthy subjects.

There was good correlation between the RAPD score and intereye differences in estimated RGC counts ($R^2 = 0.492$, $P < 0.001$), CSFI ($R^2 = 0.484$, $P < 0.001$), MD ($R^2 = 0.546$, $P < 0.001$), and RNFL thickness ($R^2 = 0.362$, $P < 0.001$, Fig. 2, Table 2). The relationship between estimated intereye RGC difference and RAPD score was best described by the formula:

$$\textit{Intereye RGC difference} = 21,896 + 353,272 * \textit{RAPD score}$$

Similar linear regression models were estimated to model the relationship between RAPD score and intereye differences in MD, RNFL thickness, and CSFI (Tables 2, 3). Expected intereye differences in RNFL thickness, RGC estimates and CSFI, for a range of RAPD scores are shown in Table 4. The expected differences shown in Table 4 are the average values of the differences associated with a positive and negative RAPD score, that is, the average of the estimated intereye difference for RAPD scores of 0.3 and −0.3.

Multivariate regression analysis indicated that the average MD was a significant influence on the relationship between absolute intereye MD difference and absolute RAPD score ($P < 0.001$, Table 3). The relationship between absolute intereye difference in MD and RAPD score was described best by the formula:

$$\textit{Absolute intereye MD difference} = 0.27 + 6.47 * \textit{absolute RAPD score} - 0.25 * \textit{average MD}$$

Age also was examined by inclusion in the multivariable model, but was found to offer no additional predictive value ($P = 0.084$). The effect of disease severity on the relationship between absolute RAPD score and intereye differences in estimated numbers of RGCs, RNFL thickness, and CSFI was examined using a similar method. Indices of disease severity, including average MD ($P = 0.244$) and average RGC estimate ($P = 0.926$), had no significant influence on the relationship between absolute RAPD score and intereye RGC differences. Disease severity also had no influence on the relationship between absolute RAPD score and intereye RNFL difference or intereye CSFI differences. Expected intereye differences in MD for a range of RAPD scores are shown in Table 4. Examples of patients included in the study are shown in Figures 3 and 4.

Discussion

The results of our study demonstrated that the magnitude of RAPD, quantified using a commercially available pupillometer, shows good correlation with measures of disease asymmetry in glaucoma (Fig. 2). Subjects with more asymmetric pupillary light responses (those with high or low RAPD scores, or high absolute RAPD scores) were found to have greater intereye differences in structural and functional measures. Furthermore, using empirically derived formulas, it was possible to estimate the number of RGCs in each eye, and, therefore, estimate the quantity of RGC asymmetry likely to be associated with RAPDs of various sizes.

The results indicated that greater asymmetry of pupil response is associated with greater intereye difference in estimated numbers of RGCs. For example, the results of the regression analysis in Table 2 suggested that each 0.1 increase in RAPD score asymmetry is likely to correspond to an increase in RGC asymmetry of approximately 35,000 cells. Using this model, one can estimate that an RAPD score of 0.5 (or −0.5) is likely to represent an intereye RGC difference of approximately 176,636 cells, or an intereye difference in CSFI of 20%, whereas an absolute RAPD score of 1.1 (or −1.1) is likely to

Table 4. Average Expected Intereye Differences (Δ) in SAP MD, RNFL Thickness, Estimated RGC Number, CSFI for Given Values of RAPD Score

	MD-Δ, dB				
Absolute RAPD Score	If Average MD = −5 dB	If Average MD = −15 dB	RNFL Thickness-Δ, μm	Estimated RGC Number-Δ, Cells	CSFI-Δ, %
0.3	3.5	6.0	6.0	105,982	12
0.5	4.8	7.3	10.0	176,636	20
0.7	6.0	8.5	14.0	247,290	28
0.9	7.3	9.8	18.0	317,945	36
1.1	8.6	11.1	22.0	388,599	44

The RNFL thickness, estimated RGC number and CSFI estimates are the average of estimates derived from positive (relative abnormality of the left afferent pathway) and negative (relative abnormality of the right afferent pathway) RAPD scores.

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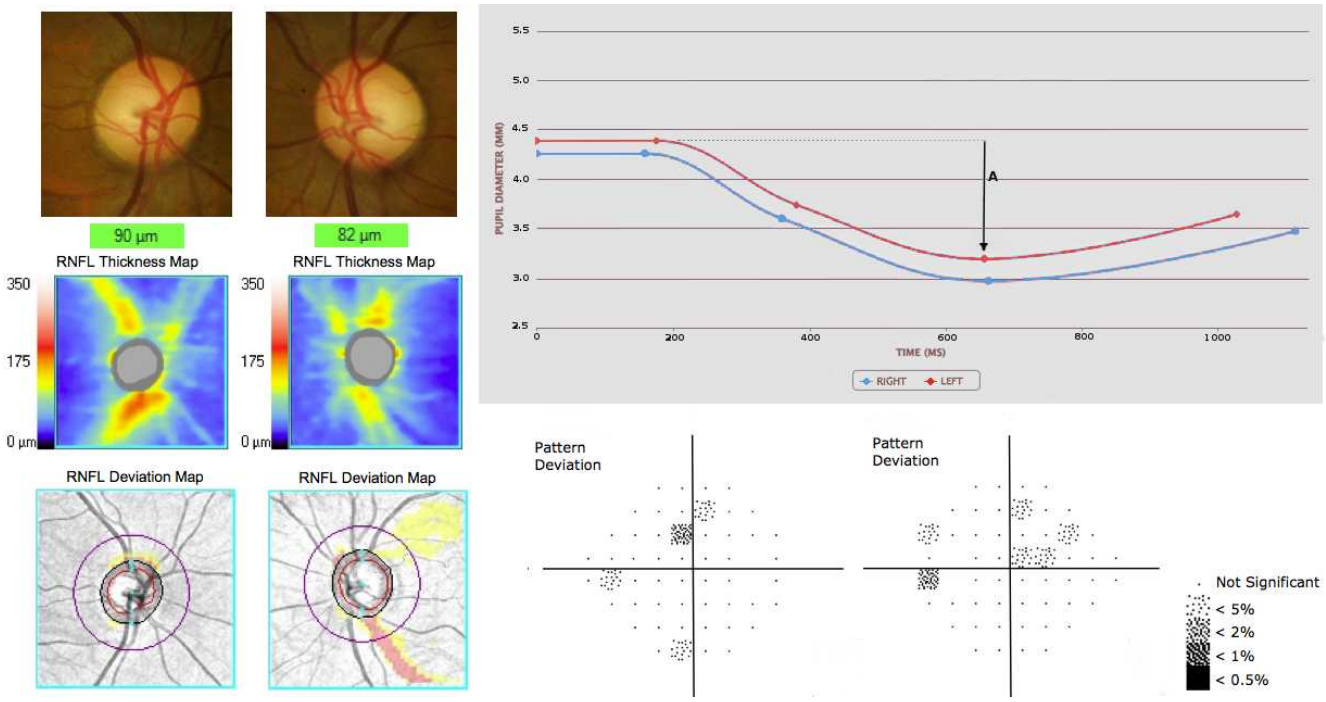

Figure 3. Example of a 56-year-old patient with early glaucoma in the left eye. Pupillography shows the average pupil responses on right and left eye stimulation, including response amplitude (denoted with letter *A*). The *blue trace* shows the average of right and left pupil diameters on right eye stimulation, and the *red trace* the average pupil diameters on left eye stimulation. The MD in the right eye and left eyes was 0.73 and –0.39 dB, respectively. The estimated RGC count was 1,005,524 in the right eye and 864,756 in the left eye. The average response amplitude on right eye stimulation was 0.30 compared to –0.27 on left eye stimulation, giving an RAPD score of $10^{*}\text{Log}_{10}(0.30/0.27) = 0.46$. This indicates a relative abnormality of the left afferent pathway.

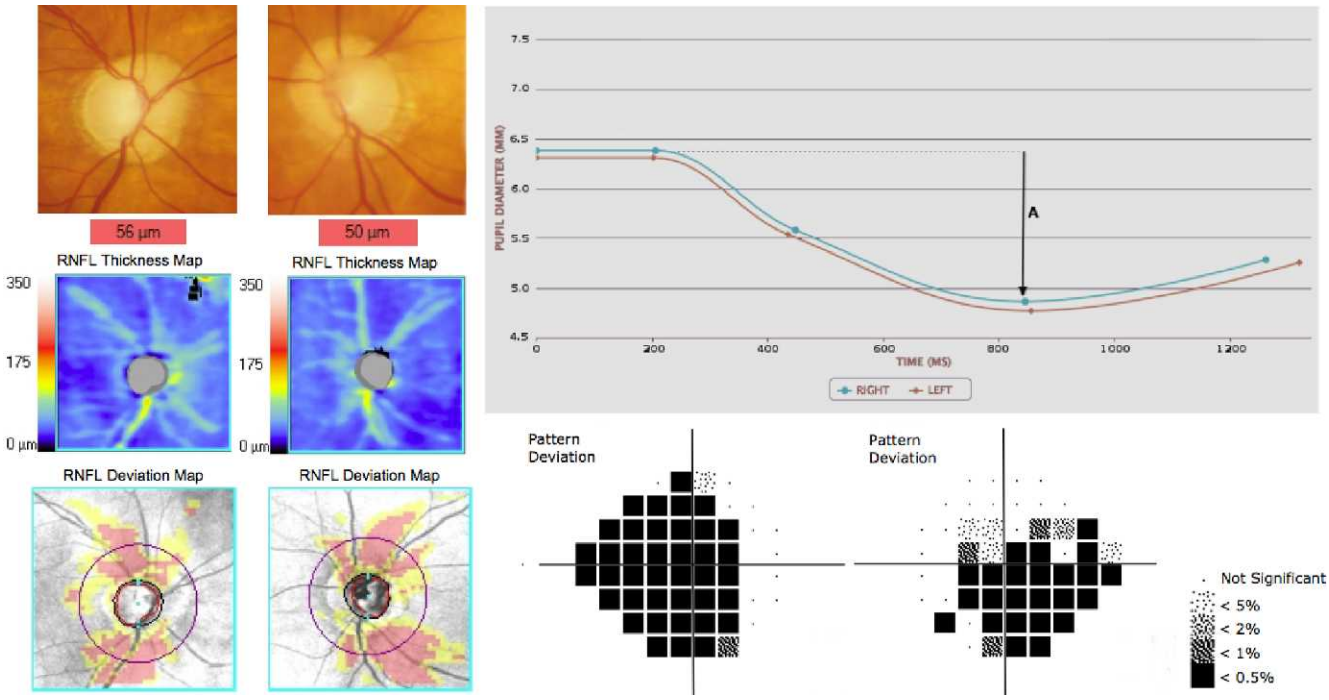

Figure 4. Example of a 70-year-old patient with advanced glaucoma in both eyes. Pupillography shows the average pupil responses on right and left eye stimulation, including response amplitude (denoted with letter *A*). The mean deviation in the right eye and left eyes was –23.56 and –15.79 dB, respectively. The estimated RGC count was 111,000 in the right eye and 237,996 in the left eye. The relative afferent pupillary defect score was –0.10, indicating a small relative abnormality of the right afferent pathway.

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represent an intereye RGC difference of 388,599 cells, or an intereye difference in CSFI of 44% (Table 4).

These results showed good agreement with a previous histologic study, in which Kerrison et al.[8] examined histologic RGC counts in primates with RAPDs. Unilateral RGC loss of at least 25% was required for a 0.6 log unit RAPD measured using neutral density filters. Although we quantified pupil response asymmetry using a pupillometer rather than neutral density filters, the model indicates that an RAPD score of 0.6 (or −0.6) corresponds to a predicted intereye CSFI difference of approximately 24%. This is remarkably similar to the results of the histologic study as the CSFI represents the estimated percentage loss of RGCs. The equivalent predicted intereye difference in RGC estimate for an RAPD score of 0.6 (or −0.6) was approximately 211,963 cells.

The estimates of RGC loss associated with asymmetric pupil responses (Table 4) also are similar to the results of a previous clinical study in nonglaucomatous human subjects.[33] Lagrèze and Kardon[33] examined the pupil responses of 36 subjects with a variety of neurologic diagnoses. The number of RGCs in each eye was estimated by overlying templates of known RGC density on Goldmann and Humphrey visual fields. A 1.0 log unit RAPD was associated with asymmetric RGC losses of 30% to 40%, compared to an estimated loss of 40% of RGCs for a similar magnitude RAPD in our study. The good agreement between the results of Lagrèze and Kardon[33] and our study is particularly interesting given the range of diagnoses included in the former. An important difference, however, is that Lagrèze and Kardon[33] used visual field sensitivity alone to estimate RGC counts. In glaucomatous subjects, estimating RGC numbers from only the functional domain may lead to underestimation of neural losses, as some eyes may lose large numbers of RGCs before a SAP defect develops.[24,26,32] Therefore, in our study RGC estimates were derived from SAP and SD-OCT.

Previous studies have investigated the intereye RNFL thickness and MD differences associated with pupil response asymmetry.[6,34,35] Using the swinging flashlight test, two studies reported an intereye difference in RNFL thickness of 17% to 27%, or an intereye difference in SAP MD of 9.5 to 12 dB, was needed for an RAPD of at least 0.3 to 0.6 log units.[6,34] These estimated losses were greater than those predicted by our analysis. For example, we found an RAPD score of 0.3 was associated with an intereye difference in MD of only 6.0 dB if the average MD in both eyes was −15 dB and only 3.5 dB if the average MD was −5 dB. The difference between studies likely is due to automated pupillometry detecting smaller intereye differences in disease than the swinging flashlight test.[13,31] Indeed, using a similar pupillometer, Chang et al.[35] found an RAPD score of 0.3 was associated with an average intereye difference in SAP MD of only 2.6 dB and an intereye difference in RNFL thickness of only 3.2 μm.

An important finding of our study was that the intereye difference in MD associated with a particular RAPD score was influenced by disease severity. For example, Table 4 indicated that when the average MD is −5 dB, an intereye difference in MD of approximately 4.8 dB is likely to result in an absolute RAPD score of 0.5. In contrast, in those with worse average MD, a greater intereye difference in MD is needed for the same magnitude RAPD score. If the average MD is −15 dB, an intereye difference in MD of 7.3 dB is likely to be required for an absolute RAPD score of 0.5. The intereye difference in MD needed for an RAPD is likely to depend on the severity of disease, as the SAP sensitivity thresholds are obtained and reported using a logarithmic scale (dB). The result is that in early disease large RGC losses can occur before there is a significant change in MD.[24–26,32,36] Therefore, in early disease a small difference in MD between eyes may represent a relatively large difference in RGC counts between eyes. In contrast, in later disease small further RGC losses can result in large decreases in SAP sensitivity. It follows that in subjects with advanced disease, small differences in MD between eyes may represent relatively small intereye differences in RGC counts. In addition, as RGC counts decrease with increasing severity, the absolute difference in RGC counts between eyes also is likely to decrease.[25,32] This concept is supported by the finding that the relationship between RAPD score and intereye differences in the linear units of estimated RGC counts and RNFL thickness was not significantly affected by disease severity.

The RAPD score, like MD, is a logarithmic scale. The logarithmic scale is useful in automated pupillography, as it allows better comparison of results to clinical grading of the RAPD using the swinging flashlight test and neutral density filters, which also is reported using a logarithmic scale. The logarithmic scale is also useful for measuring small degrees of asymmetry; however, in eyes with large degrees of asymmetry the scale is compressed, which may lead to loss of information. For this reason, we also assessed the relationship between intereye differences in RGC estimates and pupil response, measured as the difference in response amplitude between eyes. These results were similar to those obtained using the RAPD score.

Although the results of our study suggested that the RAPD score might be a useful objective tool for quantifying asymmetric damage in glaucoma, as glaucoma usually is a bilateral disease, used alone the RAPD score is unlikely to be useful for the detection of glaucoma. Asymmetric disease was infrequent in our sample, with only 13 of 77 subjects with glaucoma (17%) having an intereye MD difference $\geq$ 5 dB. Therefore, it is not surprising that only 19 of 77 subjects with glaucoma (25%) had an absolute RAPD score $\geq$ 0.3 and only 32 of 77 (42%) had a score $\geq$ 0.2. We found, after adjusting for differences in age between healthy subjects and those with glaucoma, the absolute RAPD score had a sensitivity of only 50% for a specificity of 80% for distinguishing those with glaucoma from healthy subjects (area under the curve [AUC] = 0.68, 95% CI 0.58–0.78). It is possible that a combination of other pupil response measurements using different stimuli might perform better; however, investigating the diagnostic ability of the device was not the primary objective of the current study.[35,37]

A challenge of using pupillometry to detect disease asymmetry is that pupillometry is able to detect small degrees of pupil asymmetry that may be physiological. Therefore, healthy subjects often have small RAPDs by pupillometry that are not seen on clinical examination.[14] For example, Wilhelm et al.[14] found an RAPD between 0.08 and 0.22 log units was present on pupillometry in 43 (42%) of 102 healthy individuals. In contrast, larger RAPDs (greater than 0.3 log units) were present in fewer than 2% of healthy subjects.[14] We examined the distribution of RAPD scores in our healthy control group and found an absolute RAPD score of <0.3 to be common in healthy subjects, whereas no healthy subjects had an absolute RAPD score $\geq$ 0.3 (Fig. 1). These findings suggested that an absolute RAPD score of >0.3 might be a useful indicator of asymmetry; however, further testing in larger numbers of healthy subjects is needed to corroborate this. This would be an intuitive value as an RAPD of 0.3 log units is the threshold of RAPD detectability using the swinging flashlight test.[14] The intereye difference in RGC estimates associated with an RAPD score of 0.3 (or −0.3) was 105,982 cells, or a CSFI intereye difference of 12%. Although most subjects with glaucoma had RAPD scores < 0.3, glaucomatous eyes with asymmetry in MD, RNFL thickness, and estimated RGC counts tended to have higher RAPD scores (Fig. 2). An example of a subject with early

glaucoma, but a relatively high RAPD score of 0.46 is shown in Figure 3. This patient had only early visual field loss, but the RAPD score indicated a relative abnormality of the left afferent pathway. This is consistent with the SD-OCT finding of RNFL loss in the left eye and the estimated intereye RGC difference of 140,768 cells. Although this patient had relatively early glaucoma, the RAPD score was elevated due to disease asymmetry.

The design of this study has limitations. First, the magnitude of an RAPD also depends on factors, such as the brightness and location of the stimulus.[38] Even in healthy eyes, the strength of the afferent signal varies depending on the location of the stimulus in the visual field. This largely is a reflection of the variation in RGC densities in the retina, but also may be due to differences in decussation of fibers at the optic chiasm and midbrain. To minimize the effect of stimulation of different retinal regions, only the large full field flash stimulus was used in this study. Second, the RGC estimates used in the study were derived from empirical formulas and the true number of RGCs in these eyes is not known. The formulas, however, have been validated in multiple previous studies and have been found to provide estimates close to histological RGC counts.[24,32] A further limitation is that healthy subjects in our study were younger than those with glaucoma. The number of RGCs is known to decrease with age and, therefore, it is possible that the absolute RAPD score also might alter with age. We analyzed the effect of age on RAPD score in the healthy and glaucomatous subjects, but there was no significant relationship. Given that aging is a bilateral process, this finding is expected, and the difference in age between subjects and controls was not likely to have affected the relationship between RAPD score and RGC estimates. The swinging flashlight test was not performed in this study and, therefore, the relationship between RGC asymmetry and RAPD assessment using neutral density filters is not known. Previous studies, however, have shown good correlation between clinical measurements of RAPD and automated pupillometry,[33] and between the RAPD score and measures of structure and function. These results suggested that the RAPDx provides an accurate measure of the pupillary light reflex. Another important issue is that specific RGC subtypes (e.g., the intrinsically photosensitive melanopsin containing RGCs or ipRGCs[33,39]) may be involved in the pupillary light response. Although individual RGC subtypes were not targeted specifically in this study, previous investigations have shown ipRGCs have a uniform distribution and, therefore, loss is likely to be proportional to total RGC loss.[33,39,40] It also is important to note that this was a study of glaucomatous subjects and the results may not be applicable to RAPDs due to other disease processes. Lastly, some subjects with asymmetric glaucoma, whom one might expect to have a high RAPD score, in fact had a low score. Figure 4 shows a subject with bilateral advanced glaucoma with an intereye MD difference of almost 8 dB and estimated intereye RGC difference of 126,996 cells. Despite advanced glaucoma and evidence of disease asymmetry, the RAPD score was only 0.1. The model predicts that an RAPD score of 0.1 would be associated with an intereye RGC difference of only 35,327 cells. The difference in the predicted RGC estimates is likely to be due to the fact that the model only explains approximately 50% of RAPD score variability. Although we attempted to control for confounding factors that could influence pupillary responses in this study, it is likely that other uncontrolled factors associated with subject variability may have a role explaining the imperfect relationship between measures of neural damage and RAPD.

In conclusion, our study has demonstrated good correlation between the magnitude of an RAPD and the intereye difference in estimated numbers of RGCs. Therefore, a large RAPD is likely to indicate a large degree of RGC asymmetry. Although the RAPD is a useful measure of asymmetry, subjects with severe symmetrical disease are likely to have normal RAPD scores, even in the presence of large bilateral RGC losses. An RAPD score of 0.3, which was not present in any healthy subjects, is likely to be present in eyes with an intereye difference in RGCs of at least approximately 106,000 cells, or an intereye difference in CSFI of 12%.

Acknowledgments

Supported in part by National Institutes of Health/National Eye Institute Grants EY021818 (FAM), EY11008 (LMZ), EY14267 (LMZ), EY019869 (LMZ), and Core Grant P30EY022589 (LMZ); Brazilian National Research Council-CNPq Grant 200178/2012-1 (DMF); and grants for participants' glaucoma medications from Alcon, Allergan, Pfizer, Merck, and Santen.

Disclosure: **A.J. Tatham**, None; **D. Meira-Freitas**, None; **R.N. Weinreb**, Carl-Zeiss Meditec (F, C), Heidelberg Engineering (F), Optovue (F), Kowa (F), Nidek (F), Topcon (F, C); **A.H. Marvasti**, None; **L.M. Zangwill**, Carl-Zeiss Meditec (F), Heidelberg Engineering (F); **F.A. Medeiros**, Carl-Zeiss Meditec (F), Heidelberg Engineering (F), P

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