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Relationship Between Flicker Modulation Sensitivity and Retinal Ganglion Cell Related Layer Thicknesses

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Purpose: Early detection of structural changes in retinal ganglion cells (RGCs) and corresponding changes in visual function is important in early degenerative diseases of the retina, but the sensitivity of both measurements is limited by the inherent variability in healthy subjects. This study investigates the relationships between RGC-related layer thicknesses and foveal and parafoveal flicker modulation sensitivity (FMS) across photopic and mesopic light levels in healthy subjects.

Methods: Photopic and mesopic FMS was measured in 56 young adults, at the point of fixation and at an eccentricity of 5 degrees, in each of the four quadrants. Spectral-domain optical coherence tomography (SD-OCT) was used to measure retinal thicknesses. Relationships between foveal and parafoveal FMS and the retinal thickness in the corresponding region were examined after adjusting for confounding variables.

Results: Total macular and inner retinal layer (IRL) thicknesses in the parafoveal ring were significant predictors of photopic ($P = 0.034$) and mesopic ($P = 0.034$) parafoveal FMS, respectively. The superior peripapillary retinal nerve fiber layer (pRNFL) thickness was a contributing factor to the inferior parafoveal FMS (photopic: $P = 0.006$ and mesopic: $P = 0.021$) and the inferior pRNFL thickness was also a contributing factor to the superior parafoveal FMS (photopic: $P < 0.001$ and mesopic: $P = 0.015$).

Conclusions: The pRNFL thicknesses predict parafoveal FMS for both mesopic and photopic conditions in healthy eyes.

Translational Relevance: The measurement of rapid flicker sensitivity in the parafoveal retina together with the pRNFL thickness profiles measured before the onset of disease, may provide a more sensitive biomarker for detecting loss of sensitivity caused by the earliest neurodegenerative changes in the eyes.

Introduction

The ability of the visual system to detect temporal and spatial modulations in luminance contrast is strongly dependent on the normal functioning of ganglion cells (RGCs) and their axons in the retina.[1–4] In glaucoma and other neurodegenerative diseases characterized by loss of RGCs and axon fibers,[5] both contributing to the thinning of the retinal nerve fiber layer (RNFL),[6] visual tests using temporally modulated (flickering) stimuli have been shown to be sensitive for early detection of neurodegenerative damage.[7–13] In agreement with the often reduced sensitivity and greater visual discomfort at lower light levels described in patients with glaucoma[14] and healthy older subjects,[15] rapid flicker sensitivity has also been shown to be reduced across the visual field under both photopic and mesopic lighting.[16] The flicker tests used in this study have been shown to have sufficient sensitivity to reveal the effects of normal aging of rod and cone-specific pathways, both in central vision and in the parafoveal retina.[17,18]

The relationship between loss of visual sensitivity to certain stimulus attributes and structural changes in RGCs and their axons within the retina has been shown to be the hallmark in the diagnosis of glaucoma.[19,20] Confirmation of structural damage in the retina adds

significantly to any evidence of functional loss based on visual field tests, and vice versa.[21] Although previous studies in glaucoma have suggested that, in some cases, structural changes may be detectable earlier than functional changes, other studies have also argued that such outcomes are largely the result of high variability in functional tests and poor signal-to-noise ratio in standard automated perimetry (SAP), when compared to imaging tests, such as optical coherence tomography (OCT).[22,23] Signal changes in visual field tests that may be clinically important are often small compared with the variability between successive tests ("noise"). The ability of a test to discriminate healthy from diseased eyes is determined by the signal-to-noise ratio that can be achieved.[24,25] The latter indicates the sensitivity of the test to detect gradients of damage within a visual field and is affected by the variability of the measurements and the dynamic range of the technique.[24] The extent to which the within- and intersubject variabilities contribute to the lack of test efficiency remains poorly understood. Test-specific, within-subject variability limits the smallest changes in sensitivity that can be considered statistically significant and is of great importance in monitoring subject-specific changes in progressive diseases or the outcome of treatment. Intersubject variability, on the other hand, limits the smallest changes in sensitivity needed to classify the subject's performance as being outside the age-matched, normal range. The latter is usually much larger and includes the within-subject variability.[26] The relationship between structural and functional changes in glaucoma remains particularly controversial largely because of large intersubject variability in both structural parameters and functional performance. Results from four key studies which compared axonal loss in post mortem optic nerve head tissue in patients suspected with glaucoma with loss of visual field sensitivity in perimetric tests concluded that the former precedes significant changes in visual field sensitivity detected by SAP.[27–30] An extensive re-assessment of the same results by Hood[21] revealed limitations in these studies and argued convincingly that significant loss in visual field sensitivity could be demonstrated before loss of RGCs and their axons could be detected reliably. Because glaucoma is a slowly developing disease, Hood also points out that many RGCs and their axons may exhibit poor performance, even when not missing and therefore not showing up in either imaging or post mortem RGC counts.[21] Another recent study provides further convincing evidence that true functional changes precede and also appear to predict thinning of the RNFL in glaucoma.[31] The study recommends the development of improved tests of visual performance with reduced variability in repeated measurements to allow for reliable detection of smaller functional changes. A reduction in inter-subject variability in normal vision is important, if functional changes attributable to poorer performing RGCs and their retinal axons are to be detected prior to cell and / or axonal death. In addition to reduced signal size, poorer performing neurons generate more noise with an inevitable reduction in signal / noise ratio and hence higher thresholds. This is only one of several parameters that contribute to the measured within-subject variability.[24] Differences in neuronal density in healthy eyes is also likely to contribute to increased intersubject variability. It can therefore be argued that all these changes can cause an overall decrease in signal to noise (S/N) ratio and hence higher thresholds over localized regions in the visual field.[24,25] Improved tests may include the use of different measures of visual performance, such as contrast sensitivity, red/green and yellow/blue chromatic sensitivity,[32] motion detection,[33] and rapid flicker sensitivity.[17]

Despite previous perimetric studies, little is known about the structure-function relationships in early stages that precede glaucoma and in healthy subjects. Significant relationships have been found between peripapillary retinal nerve fiber layer (pRNFL) thickness and peripheral grating resolution acuity as well as differential light sensitivity in healthy subjects above 50 years of age.[34] The relationships between spatial contrast sensitivity and retinal thicknesses measured by spectral-domain OCT (SD-OCT) have been investigated in glaucoma[35–37] and also in healthy subjects.[38,39] Some studies have examined the relationships between rapid flicker modulation sensitivity (FMS) measured across the visual field and RNFL thicknesses in patients with glaucoma.[13,40–42] In contrast, fewer if any studies investigated how RGC-related layer thickness parameters (e.g. inner retinal layer, ganglion cell complex [GCC], and pRNFL) relate to FMS in normal healthy eyes.

When large stimuli are used, rapid FMS is likely to be mediated largely by magnocellular RGCs, which tend to exhibit high sensitivity to low spatial frequencies and higher temporal frequencies.[1–3,43] Although there is no guarantee that a set of stimulus parameters can be found to isolate fully either magnocellular or parvocellular pathways, it is generally agreed when large stimuli are presented in the periphery of the visual field, the threshold detection of rapid luminance flicker is mediated largely by magnocellular pathways. It is also generally agreed that both pathways can contribute to other suprathreshold visual functions.[44] Detection of rapid flicker in human vision at threshold must involve the pooling of signals from the most sensi-

tive ganglion cells. It is reasonable to assume that the smallest modulation thresholds that can be measured are therefore determined by both the number and the normal functioning of these RGCs and their retinal axons. The latter are metabolically extremely active and particularly vulnerable to energy insufficiency.[5,45] Although the density of ganglion cells varies in healthy eyes, their functional integrity remains normal. This may not, however, be the case in the earliest stages of glaucoma when subtle changes, such as the degeneration of RGC dendrites and mitochondrial insufficiency, prior to gross axonal and soma loss have been demonstrated.[46] Such early changes discovered in examinations of post mortem eyes recovered from patients with glaucoma were also shown to have been accompanied by reduced visual sensitivity.[46] It is reasonable to assume that rapid flicker sensitivity, either in central vision or in the near periphery may be affected by these early neural changes and also by intersubject variability in the relative numbers of RGCs in healthy normal eyes. It is therefore important to establish whether differences in RGC-related parameters that can be measured using clinical retinal imaging techniques in healthy eyes correlate directly with measures of maximum sensitivity in functional tests.

In order to minimize the effects of normal aging, particularly in relation to the optics of the eye, it is of advantage to measure visual performance in young healthy eyes and to investigate how such measures correlate with RGC-related layer thickness parameters, which are largely determined by variations in ganglion cell density. The hypothesis tested in this study is that the thickness of the RNFL also correlates with FMS in healthy subjects when test variability is minimized. To test our hypothesis, we examined the relationships between flicker sensitivity measured under mesopic and photopic stimulus conditions and parameters, such as the thicknesses of macular inner retinal layer (IRL), macular ganglion cell complex (mGCC) and pRNFL in healthy normal eyes. The FMS was measured in central vision and also in the near periphery. The findings from this study may help us to establish whether significant differences in RGC-related layer thickness parameters impact rapid flicker sensitivity in healthy eyes.

Such findings may increase our understanding of how degraded neuronal performance, as reflected in RGC-related thickness parameters, affects visual function in normal eyes. If a functional relationship between RGC-related thickness parameters and FMS can be established in healthy normal eyes, the intersubject variability in FMS tests can be significantly reduced with immediate effect on the signal to noise ratio that can be achieved.

Materials and Methods

Subjects

This observational and cross-sectional study included 56 healthy young adults, age range 20 to 31 years. Participants were students of the Faculty of Optics and Optometry, Complutense University of Madrid (Spain), and had no current or previous eye disease. The Ethics Committee of the Hospital Clínico San Carlos (Madrid, Spain) approved the study protocol. The design followed the tenets of the Declaration of Helsinki, and informed consent was obtained from all subjects.

All participants received a full ocular examination to detect loss of visual function or the presence of clinically recognized disease. Ophthalmic assessments included best-corrected visual acuity (BCVA), subjective refraction, slit-lamp biomicroscopy of the anterior segment and fundus examination performed in the University Optometry Clinic. Normal retina health was evaluated by color fundus photography and posterior SD-OCT assessment. The eye with the highest BCVA was selected for measurements. If both eyes had the same BCVA, the right eye was selected.

Inclusion criteria required study participants to have a BCVA of 0.00 logMAR or better, a refractive error no greater than 3.50 diopters (D) of sphere or 1.50 D of cylinder, normal trichromatic color vision as assessed using the Colour Assessment and Diagnosis (CAD) test (City Occupational Ltd., London, UK) and normal findings in the ocular examination. Exclusion criteria included systemic disease, such as diabetes, previous ocular surgery, lens opacities LOCS III classification grade 1 or greater, medications, glaucoma, amblyopia, retinal abnormality, or any other ophthalmological pathology.

Spectral Domain Optical Coherence Tomography

The retinal thickness at the macula and the pRNFL were measured in the selected eye in all subjects using the 3.3 iVue OCT system (Optovue Inc., Freemont, CA, USA). Data were obtained using the Retina Map Scan protocol for macular thicknesses and the Glaucoma Scan protocol for GCC and RNFL thickness. Scans were taken through undilated pupils under dark room lighting and only high-quality images with a Scan Quality Index >65 were included.

Macular thickness measurements were restricted to the 5 × 5 mm grid used in the Early Treatment Diabetic Retinopathy Study (ETDRS). In this study,

we analyzed only the central and parafoveal sections of the ETDRS grid: the central fovea with a diameter of 1 mm and the parafoveal ring with an outer diameter of 3 mm and an inner diameter of 1 mm. Mean macular thicknesses were recorded for each of the three retinal segmentations measured automatically in the central fovea and the parafoveal ring: (1) the total retinal layer, from the internal limiting membrane to the retinal pigment epithelium; (2) the IRL from the internal limiting membrane to the outer limit Of the inner plexiform layer (IPL) including the macular RNFL, ganglion cell layer, and IPL; and (3) the outer retinal layer (ORL) from the outer limit of the IPL to the retinal pigment epithelium. The total, IRL, and ORL thicknesses were obtained by averaging the corresponding thickness measurements made in each of the four quadrants of the parafoveal ring. In the central fovea, only the total thickness was recorded.

The GCC scanning protocol was used to measure macular GCC thickness. This region covers a zone of 7 × 7 mm centered 1 mm temporal to fovea. The mGCC thickness included the IPL, the ganglion cell layer and the nerve fiber layer. The superior and the inferior mGCC thicknesses taken above and below the horizontal meridian and the mGCC overall thickness were also measured. The pRNFL thickness was measured automatically along a circle of 3.45 mm diameter centered at the optic nerve head. The data provided separate estimates of the mean pRNFL thickness when restricted to the superior and the inferior hemispheres and its average over the whole circle.

Flicker Modulation Sensitivity

FMS was measured using the Flicker-*Plus* test supplied with the Advanced Vision and Optometric Tests (AVOT), City Occupational Ltd., London UK. The test measures rapid flicker sensitivity for a number of user-defined stimulus conditions. The test also provides standard protocols designed for clinical studies. This investigation used the standard protocols for mesopic (rod-enhanced) and photopic (cone-enhanced) stimulus conditions.[17,18]

Briefly, the two protocols measure flicker modulation thresholds at five discrete locations in the visual field with stimuli that differ in spectral composition, size, retinal illuminance, and temporal modulation frequency is order to favor either rods or cones. The viewing distance is 1 m for both protocols. Monocular thresholds were measured foveally (0 degrees) and at four parafoveal locations selected diagonally away from fixation at an eccentricity of 5 degrees in each of the four quadrants. Flicker modulation thresholds were measured at each location using randomly interleaved 2-down/1-up adaptive staircases and a statistically efficient, five-alternative forced-choice procedure with a chance probability of 1 in 25. The subject was provided with a bespoke numeric keypad with five keys arranged to map the five screen locations of the stimulus. Following each stimulus presentation, the subject's task was to indicate the location of the stimulus by pressing the appropriate button. The visual stimuli were uniform discs and had the same mean luminance as the uniform background (i.e. 0.5 cd/m^2 for the mesopic and 24 cd/m^2 for the photopic protocols). Each stimulus generated a burst of flicker presented randomly at one of five possible locations in the visual field. The photopic protocol used stimulus sizes of 0.5 degrees in central vision and 1 degree in the periphery at a temporal modulation frequency of 15 Hz. The hard-edged temporal presentation lasted for 344 ms. The mesopic protocol used a disc of 0.75 degrees for central vision and 1.5 degrees in the periphery at a temporal modulation frequency of 5 Hz presented for 600 ms. The spectral composition of the light used in the two stimulus conditions was selected to produce a scotopic to photopic (S/P) luminance ratio of 0.8 for the cone-enhanced and 8 for the rod-enhanced conditions. The choice of stimulus sizes, retinal illuminances, temporal modulation frequencies, stimulus presentation times, and differences in spectral content, as reflected by the S/P ratios, ensure that rods are favored in the mesopic protocol and middle- and long-wavelength cones respond best in the photopic protocol. Another important advantage of the new flicker test is invariance of flicker modulation amplitude with variation in prereceptoral filters in the eyes. Because the relative spectral composition of the test stimulus and the adjacent background remains unchanged in each stimulus condition, only luminance signals are involved and any prereceptoral filters in the eye do not affect the flicker modulation amplitudes.

The latter can be described as, $\delta L/L_{mean}$, where L_{mean}, equals the luminance of the uniform background and represents the mean luminance of the stimulus during one cycle. The δL represents the peak luminance difference between the stimulus and the background during the cycle. The reciprocal of the temporal modulation amplitude was used as a measure of FMS. For comparison with other studies, the FMS values were plotted on a logarithmic scale. The FMS values measured at each of the four parafoveal locations (at an eccentricity of 5 degrees) were averaged for each subject to produce a measure of overall parafoveal FMS for each of the two stimulus conditions. Further, average FMS values for the two superior and the two inferior parafoveal locations were calculated to obtain the superior parafoveal and

inferior parafoveal FMS for each participant in each protocol. Global FMS was the average of the values measured at parafoveal and foveal visual field locations.

The learning mode of the Flicker-*Plus* test preceded the actual test and used suprathreshold stimuli. The 100% correct response scores were required in order to proceed with the full test. This ensured that every subject who passed the learning mode understood how to respond to each stimulus during the full test. The order of the photopic and mesopic flicker testing was randomized. A spectrally calibrated, neutral density filter was used to lower the luminance of the display by 1.0 log unit so as to ensure that the calibration of the display remained valid for the very low light levels used in the mesopic protocol. Each test was preceded by 2 minutes of light adaptation to the corresponding background luminance for the selected protocol. Before each test, pupil sizes were measured at the photopic or the mesopic light level used using a Colvard infrared pupillometer (Oasis Medical, Glendora, CA, USA). Pupil size was needed to assess changes in retinal illumination which can affect FMS.[47,48] Screen luminance (in cd/m^2) and pupil area (in mm^2) provided the information needed to calculate retinal illuminance in trolands.

Analysis of FMS Results Versus RGC-Related Parameters

To analyze the correlations between the measured structure-function variables, we related the location of the flickering stimuli in the visual field to the corresponding regions on the retina. In the macular region, RGCs are displaced radially away from the foveola so that the photoreceptors receiving a stimulus in the foveal region and the RGCs mediating the signal response are not co-localised.[49,50] Different numerical models have been developed to account for this displacement when analyzing the spatial correspondence between retinal thickness or RGC density estimates and visual sensitivity at specific locations in the visual field measured using SAP.[49,51] In the present study, the parafoveal flicker points which use larger stimulus sizes were located diagonally on a circle of 10 degrees diameter (approximately 3 mm), which corresponds approximately to the parafoveal ETDRS ring. As the thicknesses of the four quadrants of the parafoveal ring were averaged and related to overall parafoveal FMS (the mean of 4 parafoveal FMS tests), the effect of RGC displacement in the macular region on the structure/function relationship is minimized. The central foveal flicker point (with photopic and mesopic stimulus diameters of 0.5 degrees and 0.75 degrees, respectively) was referred to in relation to the central foveal ETDRS field (diameter 3.3 degrees). The two parafoveal flicker locations in the superior visual field and the two located in the inferior field match the inferior and superior hemispheres selected for mGCC and pRNFL measurements. In addition, global correspondences were also considered. The steps involved in this analysis are described below.

Statistical Analysis

According to prior sample size for power calculations to detect statistical significance for an anticipated correlation coefficient of 0.40, an alpha risk of 5.0%, and a power of 85%, a minimum sample size of 54 subjects is required. Two more participants (56 subjects) were recruited for the study.

The normality of the data was checked by using the Kolmogorov-Smirnov test. The demographics and baseline characteristics of the study participants were evaluated using traditional descriptive methods. Continuous variables are shown as mean and SD. Paired Student *t*-test was used to compare normally distributed variables, such as FMS and Holm-Bonferroni post hoc correction was applied for multiple comparisons.

The linear associations between global and sectoral FMS (dependent variables) with the corresponding overall and regional retinal thicknesses (independent variables) were examined using the Pearson correlation coefficients and the Holm-Bonferroni corrected *P* values were calculated for testing the same hypothesis in the three sections of each retinal thickness variable (total, ORL, IRL, and / or overall, superior, and inferior). A log-log coordinates scale was used to enhance the linearity of the structure-function relationship.[52,53] Foveal central FMS was correlated to foveal central thickness. Overall parafoveal FMS was correlated to the average macular thicknesses (total, ORL, and IRL) of the parafoveal ring. The average parafoveal FMS values in the inferior field were correlated to averages of superior mGCC and superior pRNFL thicknesses, and the average parafoveal FMS values in the superior field were correlated to averages of inferior mGCC and inferior pRNFL. Further, the average global FMS was correlated to overall mGCC and pRNFL thicknesses.

Forward stepwise multiple linear regression was performed adjusting for covariates, such as age, gender, BCVA, refractive error, retinal illuminance, and the retinal thickness corresponding to each FMS dependent variable. All outcomes were reported with the corresponding beta coefficients and *P* values corrected using the Holm-Bonferroni adjustment. All statistical

tests were performed with Statgraphics Centurion XVI; Statpoint Technologies, Inc., The Plains, VA, USA). The statistical significance level was set at $P < 0.05$.

Results

Table 1 provides the demographic and ocular characteristics of the study participants. As Table 2 shows, mean central fovea FMS and parafoveal FMS (overall, superior, and inferior) were 0.2 and approximately 0.1 log units higher under photopic than mesopic light conditions, respectively (all $P < 0.001$).

Pearson correlation coefficients for the association between foveal or parafoveal FMS and the thickness in each corresponding retinal area are shown in Table 3. There were no significant correlations between either photopic or mesopic FMS for the central stimulus and the central foveal thickness. Overall parafoveal FMS showed significant correlation with the thickness in the parafoveal ring, specifically photopic FMS with total ($P = 0.034$) and ORL thickness ($P = 0.048$), and mesopic FMS with ORL ($P = 0.048$) and IRL thickness ($P = 0.050$). Parafoveal FMS in the inferior visual field showed significant correlation with the pRNFL thickness of the superior hemisphere (photopic: $P = 0.006$ and mesopic: $P = 0.042$). Parafoveal FMS in the superior visual field showed significant correlation with the pRNFL thickness of the inferior hemisphere (photopic: $P < 0.001$ and mesopic: $P = 0.015$). These correlations were

Table 1. Demographic and Ocular Characteristics of the Study Participants

Characteristic	Overall
Eyes, *n*	56
Age, y	24.8 ± 2.4 (20, 31)
Sex, male/female	18 / 38
BCVA, logMAR	−0.14 ± 0.08 (−0.30, 0.00)
Spherical equivalent, D	1.41 ± 0.08 (1.22, 1,56)
Pupil size, mm	
Photopic	3.29 ± 0.54 (2.40, 4.60)
Mesopic	6.24 ± 0.78 (4.00, 7.60)
Color threshold, CAD units	
Red/green	1.29 ± 0.23 (0.84, 1.89)
Yellow/blue	1.11 ± 0.27 (0.63, 1.96)
Retinal thickness, µm	
Central fovea	257.2 ± 15.0 (234.0, 295.0)
Parafoveal ring	308.5 ± 11.1 (282.0, 331.3)
Overall mGCC	96.0 ± 5.3 (86.0, 106.0)
Overall pRNFL	99.8 ± 7.7 (84.0, 124.0)

BCVA, best corrected visual acuity measured using logMAR letter charts; CAD units, standard normal Colour Assessment and Diagnosis units for the CAD test; pRNFL, peripapillary retinal nerve fiber layer; mGCC, macular ganglion cells complex.
Mean ± SD (min, max).

Table 2. Foveal and Parafoveal Flicker Modulation Sensitivity (FMS) Measured in the 5 Degrees Central Visual Field at Photopic and Mesopic Light Conditions

	Flicker Modulation Sensitivity (Log Units)		
Stimulus Locations	Photopic	Mesopic	*P* Value
Central fovea	1.46 ± 0.13 (1.13, 1.74)	1.26 ± 0.12 (1.00, 1.49)	<0.001
Parafovea			
Overall	1.40 ± 0.08 (1.21, 1.56)	1.31 ± 0.09 (1.10, 1.52)	<0.001
Superior	1.41 ± 0.07 (1.25, 1.56)	1.32 ± 0.09 (1.10, 1.54)	<0.001
Inferior	1.38 ± 0.09 (1.16, 1.56)	1.31 ± 0.09 (1.11, 1.53)	<0.001
Global	1.41 ± 0.08 (1.22, 1.56)	1.30 ± 0.09 (1.13, 1.50)	<0.001

Mean ± SD (min, max).

Table 3. Pearson Correlations of Foveal or Parafoveal FMS Measured Under Photopic and Mesopic Light Conditions Versus the Thickness in Each Corresponding Retinal Area

	Flicker Modulation Sensitivity			
	Photopic		Mesopic	
Retinal Thickness	r	*P* Value	r	*P* Value
Central fovea	0.20	0.134	0.23	0.089
Parafoveal ring				
Total	**0.32**	**0.034**	0.30	0.053
ORL	**0.32**	**0.048**	**0.27**	**0.048**
IRL	0.15	0.256	**0.32**	**0.050**
mGCC				
Overall	0.13	0.700	0.11	0.438
Superior	0.21	0.360	0.16	0.690
Inferior	0.09	0.526	0.14	0.582
pRNFL				
Overall	0.01	0.922	0.07	0.597
Superior	**0.39**	**0.006**	**0.31**	**0.042**
Inferior	**0.48**	**<0.001**	**0.37**	**0.015**

IRL, inner retinal layer; ORL, outer retinal layer; mGCC, macular ganglion cells complex; pRNFL, peripapillary retinal nerve fiber layer; Superior, superior hemisphere; Inferior, inferior hemisphere.

Significant r coefficients and *P* values are shown in bold.

positive, meaning that increased retinal thickness correlated with higher FMS. There were no significant correlations between either the superior or the inferior FMS and the corresponding thickness of the inferior or superior hemisphere of the mGCC at both luminance conditions. Further, neither the photopic nor the mesopic global FMS showed significant correlations with estimates of overall mGCC or pRNFL thicknesses.

Table 4 shows the significant independent predictors of FMS identified after forward stepwise multiple linear regression analyses were conducted to control for every factor. Retinal illuminance and total thickness in the parafoveal ring were significant predictors of photopic parafoveal FMS ($P = 0.008$). IRL thickness in the parafoveal ring emerged as the only independent contributor to mesopic parafoveal FMS ($P = 0.034$), which increased approximately 1.0 log unit for each 1.0-log micron increase in IRL thickness (Fig. A). The superior pRNFL thickness was a contributing factor to the inferior parafoveal FMS (photopic: $P = 0.006$ and mesopic: $P = 0.021$) and the inferior pRNFL thickness was a contributing factor to the superior parafoveal FMS (photopic: $P < 0.001$ and mesopic: $P = 0.015$). The estimated effects of a 1.0 log micron increase in superior or inferior pRNFL thickness resulted in 0.7 to 0.5 log units increase in FMS for both protocols (Figs. B, 1C).

Discussion

This study was designed to examine relationships between RGC-related layer thickness parameters and FMS to gain insight into the neuronal changes that impact flicker sensitivity in healthy eyes. The main finding was that both the macular IRL and the pRNFL thicknesses correlate well with the parafoveal FMS measured at 5 degrees eccentricity in the superior and inferior visual fields. These findings suggest that in normal, healthy young subjects, sensitivity to rapid flicker is limited by RGC density.

Previous studies have found significant FMS changes across the visual field at photopic[9,10,12] and mesopic light conditions in glaucoma when compared with controls.[16] Fluctuations in retinal illuminance can have a large effect on FMS in healthy subjects.[48] Differences in pupil size (see Table 1) may have contributed significantly to increased intersubject variability in retinal illuminances under both photopic and mesopic conditions employed in this study. Although the differences between the mean FMS results measured with the rod- and cone-enhanced stimulus conditions were statistically significant ($P < 0.001$), the measured differences were small and can be altered by simply changing the spatiotemporal and spectral properties of either protocol. The small differences in FMS values between the two protocols may, however, increase significantly in older subjects and particularly in those with early stage diseases of the retina who are likely to exhibit a greater decrease in sensitivity in the mesopic range.[16]

To our knowledge, the relationship between RGC-related layer thickness parameters and temporal flicker sensitivity has not been examined previously in healthy subjects. In this study, a stepwise multiple regression analysis was conducted to control for potential confounding factors. No significant association was detected between the central fovea thickness and the central fovea FMS measured at photopic and mesopic light levels. This finding can be explained by taking into account how the structure-function relationship varies with retinal location.[54] In order to produce a similar functional change, a greater reduction in RGC density is expected in central vision when compared with the more peripheral retina.

When examining results measured at parafoveal locations, retinal illuminance and macular total thickness in the parafoveal ring (at 5 degrees eccentricity)

Table 4. Forward Stepwise Multiple Regression Models Showing the Independent Predictor Variables Related to Parafoveal Flicker Modulation Sensitivity Measured at Photopic and Mesopic Luminance Levels

Dependent Variable	Predictor Variable	β Coefficient	*P* Value
Overall parafoveal FMS			
Photopic	Retinal illuminance	0.1656	0.008
	Total parafoveal	1.2343	
Mesopic	IRL parafovea	1.0369	0.034
Inferior parafoveal FMS			
Photopic	Superior pRNFL	0.7009	0.006
Mesopic	Superior pRNFL	0.5694	0.021
Superior parafoveal FMS			
Photopic	Inferior pRNFL	0.6003	<0.001
Mesopic	Inferior pRNFL	0.5238	0.015

β coefficient represents the change in the dependent variable for one unit of change in the predictor variable; IRL, inner retinal layer; pRNFL, peripapillary retinal nerve fiber layer.

emerged as independent predictors of photopic FMS ($P = 0.008$). The macular IRL thickness was found to be a unique, independent predictor accounting for variability in parafoveal FMS in the mesopic protocol ($P = 0.034$). Structure-function relationships between macular IRL thickness and spatial contrast sensitivity measured at photopic and mesopic light level were also found in healthy subjects, both young and old.[38] In contrast to these findings, mGCC[38] and pRNFL[38,39] thickness parameters were not found to be related to spatial contrast sensitivity in healthy subjects.[38,39] In the present study, we also did not find significant correlation between mGCC and FMS. Results from other studies also show that the pRNFL performed better in discriminating between healthy and early glaucomatous eyes than the mGCL and any other retinal macular layers, when assessed separately.[55] The study also identified the mGCL as least useful in discriminating between healthy and glaucomatous eyes.

Relationships between RNFL thickness and the mean defect measured in flicker perimetry[13,41] and between RNFL thickness and flicker sensitivity and mean defects[40] have been described in glaucoma and healthy controls.[42] In the present study, we found a relationship between the sectoral structural pRNFL thickness and the parafoveal FMS at corresponding locations. Both protocols show that the thinning of the pRNFL results in loss of FMS. The superior pRNFL thickness was a contributing factor to the inferior parafoveal FMS (photopic: $P = 0.006$ and mesopic: $P = 0.021$) and the inferior pRNFL thickness is a contributing factor to the superior parafoveal FMS (photopic: $P < 0.001$ and mesopic: $P = 0.015$). Studies carried out at photopic light levels found lower sensitivity to 16 Hz flicker in the superior temporal when compared to the inferior temporal visual field. The effect was greater in older when compared to younger normal individuals.[56] In another study, the superior nasal field was reported to have better flicker sensitivity when compared to the inferior temporal field in healthy eyes.[57] In this study, we have not found significant differences between the superior and inferior parafoveal FMS in either protocol. Flicker sensitivities measured in the superior parafoveal visual field locations showed the strongest correlations with the pRNFL thickness measured in the inferior hemisphere. Although our study involved only normal healthy subjects, it is of interest to note that in other studies RGC axons in the inferior hemisphere have been found to be most susceptible to glaucomatous damage.[58,59]

Figure C also provides useful information in relation to the thinning of pRNFL needed to indicate loss of sensitivity. Because the inferior pRNFL thickness in this sample of normal, healthy young subjects ranges from 141 to 78 µm, a large loss of fibers would be needed to diagnose the onset of disease in most subjects, except for those with values close to the lower normal limit. An examination of Figure C suggests that during the earliest stages of disease when RGC thickness changes are small, measurements of FMS and the corresponding pRNFL thickness in the inferior retina can be used to detect glaucomatous damage. A knowledge of the subject's pRNFL thickness before or at the onset of glaucomatous changes, can in principle be used to provide a better estimate of the lowest FMS limit that can be considered normal for the subject's pRNFL thickness. The linear trends shown in Figure C can be corrected for so that the additional intersubject variability that arises from normal variations in pRNFL thickness is removed. This approach

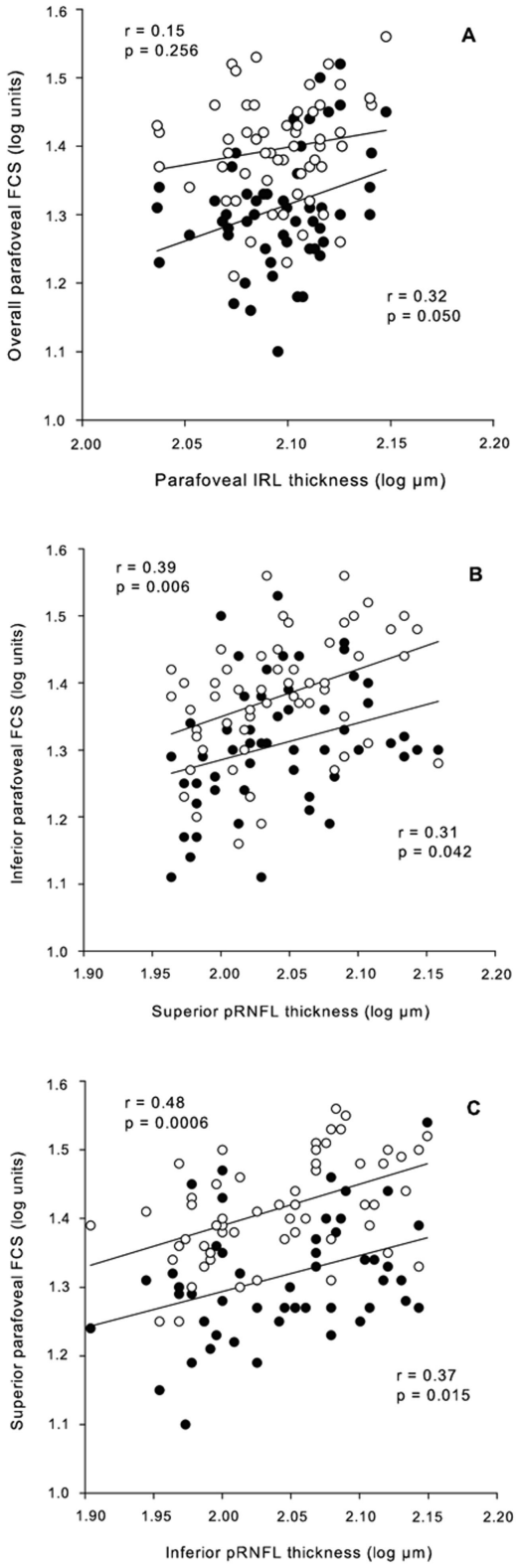

Figure. Relationships between the parafoveal flicker modulation sensitivity and the retinal thickness in the macula and peripapillary areas. (**A**) Overall parafoveal FMS in relation to inner retinal layer thickness in the parafoveal ring, (**B**) FMS in the inferior parafoveal visual field location in relation to pRNFL thickness in the superior hemisphere, and (**C**) FMS in the superior parafoveal visual field location in relation to pRNFL thickness in the inferior hemisphere. In the graphs, *open circles* represent photopic FMS and *solid circles* mesopic FMS; r, Pearson's correlation coefficient.

can make the measurement of FMS more effective in predicting the presence of disease. The measured FMS can be compared directly to the lower, normal FMS limit expected for the corresponding pRNFL thickness (see Fig. C). This combined approach, which requires the measurement of both pRNFL thickness and FMS, may provide a sensitive predictor of glaucomatous changes in the retina. In order to implement this approach, one needs to have a measurement of pRNFL thickness ahead of disease onset to be of value in establishing normal upper limits of expected sensitivity when using the Flicker-*Plus* test. This is simply because a heightened degree of autoregulation may occur in RGCs prior to cell death. RGCs may well behave differently under stress and this could disrupt the structure/function relationship that is otherwise found in normal healthy subjects. The remaining intersubject variability in FMS at a known pRNFL thickness (see Fig. C) is at least in part attributable to within-subject variability. It therefore becomes even more important to reduce both within and intersubject variability in visual psychophysical tests so that even smaller changes in visual sensitivity become statistically significant. There are at least two improvements one can make to the present the Flicker-*Plus* test to achieve this aim.

An important limitation of this test is the expected intersubject variation in retinal illuminance due to individual differences in pupil size and the corresponding variation in FMS. Development of enhanced FMS tests, which maintain constant retinal illuminance, would undoubtedly reduce the observed intersubject variability and make the test more specific, as has already been suggested.[31] FMS measurements are affected by both within- and intersubject variability. The coefficient of variability in repeated FMS tests in the same subject is expected to be significantly smaller than the intersubject variability. The latter is around 17% based on the photopic FMS data (shown in Fig. C after correction for the linear trend attributed to pRNFL thickness). The intersubject variability at any pRNFL thickness includes the within-subject variability. The variability in repeated measurements of pRNFL thickness in the same subject is again expected to be small, but the intersubject variability is approximately 14% (based on the pRNFL data shown for the inferior retina in Fig. C). The detection of small, significant changes in FMS or pRNFL is directly affected by the corresponding intersubject variabilities. Within-subject variability in FMS can

be reduced by improving the staircase procedure and by averaging repeated measurements. The intersubject variability in FMS can also be reduced by controlling for pupil size changes and variation in retinal illuminance. This analysis shows that it may be possible to reduce significantly both the within- and the intersubject variability in the data shown in the Figure to make the combined pRNFL and FMS a more sensitive biomarker for detection of the earliest glaucomatous changes.

In conclusion, this study demonstrates that the pRNFL thicknesses correlate well with parafoveal photopic and mesopic FMS in healthy eyes. The measurement of FMS using the current Flicker-*Plus* test demonstrates high sensitivity for detection of RGC related changes, both in the macula and the RNFL, but the intersubject variability remains high. This realization justifies further efforts to enhance the performance of visual psychophysical tests by reducing both within and intersubject variability. Future investigations using improved functional tests are needed to demonstrate the potential advantages using pRNFL parameters to make rapid flicker tests more sensitive for the early detection of neurodegenerative diseases of the retina.

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