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Evaluation of photoreceptor function in inherited retinal diseases using rod- and cone-enhanced flicker stimuli

Running title: Rod/ cone function in inherited retinal diseases

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Disclosure:

John L Barbur is an inventor of AVOT tests (some employed in this study); an employee of City, University of London; and a director of City Occupational Ltd. (a City University spin out company setup to manufacture and supply AVOT tests).
Other authors: None

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Abstract

Purpose: Clinical assessment of rod and cone photoreceptor sensitivity often involves the use of extended dark adaptation times to minimise cone involvement or the use of bright adapting backgrounds to saturate rods. In this study we examine a new rod / cone sensitivity test which requires minimal dark adaptation. The aim was to establish whether rod/cone sensitivity losses can be measured reliably in patients with retinal diseases that selectively affect rods or cones when compared to age-matched subjects with normal vision.

Methods: Flicker modulation thresholds (FMTs) were measured psychophysically using cone- and rod-enhanced stimuli located centrally and in four quadrants at 5^{o} retinal eccentricity in 20 patients (age range: 10 – 41 years) with cone-dominated (Stargardt's disease or Macular dystrophy; n = 13) and rod-dominated (Retinitis Pigmentosa; n = 7) disease. These data were compared against age-matched normals tested with identical stimuli (Hathibelagal et al., 2020).

Results: Across all retinal locations, cone FMTs in cone-dominated diseases (Median ± IQR: 32.32 ± 28.15% for central location) were greater than a majority (83%; 49/59) of corresponding rod FMTs (18.7 ± 3.29%; p = 0.05) and cone FMTs of controls (4.24 ± 2.00%). Similarly, rod FMTs in rod-dominant disease (14.99 ± 22.58%) were greater than a majority (88%; 29/39) of the corresponding cone FMTs (9.09 ± 10.33%) (p = 0.13) and rod FMT of controls (6.80 ± 2.60 %).

Conclusions: Cone-specific deficits were larger than rod-specific deficits in cone-dominated diseases and vice versa in rod-dominated disease. These results suggest that the new method of assessing photoreceptor sensitivity has potential application in detecting specific rod/cone losses without the need for dark adaptation.

Keywords: rod, cone, temporal contrast sensitivity, Stargardt's dystrophy, retinitis pigmentosa

1. Introduction

Hereditary retinal diseases can be either classified as rod - dominated (e.g., Retinitis Pigmentosa and Rod-cone dystrophy) or cone - dominated diseases (e.g., Cone-rod dystrophy and Stargardt's disease) based on the predominant type of photoreceptor sensitivity loss.[1] These diseases cause a loss of visual function with consequences for the quality of life.[2] Treatment options for hereditary retinal diseases have been limited but recent advances have resulted in a number of new therapies that are currently in clinical trials to determine their efficacy.[3-7] The need to detect changes in sensitivity that fall outside normal age limits to estimate disease severity and to monitor either the natural progression of the disease or the effectiveness of treatment have therefore become more important.[3-7] In general, any test of the visual function should be rapid, easy to execute, sensitive and reliable to identify small alternations in functionality, and potentially act as clinical markers/endpoints[8, 9] to monitor disease progression[10-14] and treatment outcomes.[6] In the context of inherent retinal diseases, full field and multifocal electroretinography (ERG) is currently the most commonly used objective test to measure rod and cone photoreceptor sensitivity.[15-19] For instance, multifocal ERGs can be useful in the diagnosis of local cone deficits in Stargardt's disease[20] when compared to the more diffuse dysfunction encountered in generalized cone dystrophy. However, ERG techniques do not provide information on functional vision[15, 21] and typically requires long dark adaptation times[15-19] and the use of a bright flickering target[22] which can be uncomfortable for some patients. Other tests measure either cone or rod function, but not both. For example, contrast sensitivity[23, 24], colour vision[25, 26] and visual acuity are typical measures of cone functions in central vision. Perimetry can identify changes in retinal sensitivity in rod–specific diseases[27], but the isolation of rod and cone-specific responses are poor. While such an isolation of photoreceptor function is possible with dark-adapted chromatic perimetry[28-31], adaptometry[32-35] and silent substitution techniques,[36-39] these procedures are tedious and typically require 15 - 30 minutes of dark adaptation.[21] They have therefore remained laboratory procedures for most part and are yet to be reliably translated into a clinical setting for testing patients with visual impairment.[9]

A novel psychophysical approach – the Flicker-*plus* test executed on the Advanced Vision Optometric Tests (AVOT) setup – involving the measurement of monocular flicker modulation thresholds (FMTs), was recently described by our group for testing of rod and cone-mediated

vision with minimal adaptation time.[40] FMTs describe the smallest modulation thresholds at the corresponding temporal frequency employed in the test needed to detect rapid flicker on 71% of presentations. The stimulus causes no change in time-averaged retinal illuminance and the modulation depth is quantified using Michelson contrast. The stimuli for evaluating the functionality of the two types of photoreceptors in this test is based on exploiting the well-known differences in rod and cone sensitivities to different spatiotemporal properties such as temporal frequency, retinal illuminance, size, duration and spectral composition.[40] Normative data of rod/cone FMTs across a wide age range were also described in that study.[40] Central and parafoveal (5^o) rod and cone-enhanced FMT remained invariant up to 45 years of age, however beyond that age, both rod and cone FMT increase at a faster rate with increasing age and more specifically rod FMTs increased at a faster rate than cone FMT.[40] Interestingly, there was no difference in cone and rod FMTs across the four parafoveal locations (superonasal, superotemporal, inferonasal and inferotemporal).[40] Values higher than the upper limits of this normative database may signal deficits in flicker processing of subjects and could potentially be used to identify patients with cone and rod photoreceptor disease. The present study evaluates the capability of the Flicker-*plus* test in identifying selective deficits of cone and rod-photoreceptor functions in patients with the aforementioned cone-dominant and rod-dominant diseases. This study tests the following two complementary hypotheses related to cone and rod FMTs in these patients: 1) Cone FMTs in patients with cone-dominated diseases will be significantly higher than the corresponding rod FMTs and higher than the upper limit of cone FMT's of age-matched controls; rod FMTs of these patients may not be significantly different from that of age-matched controls. 2) Rod FMTs in patients with rod-dominated diseases will be higher than the corresponding cone FMTs and higher than the upper limit of rod FMT's of age-matched controls; cone FMTs of these patients may not be significantly different from that of age-matched controls.

2. Methods

Twenty patients with rod- or cone photoreceptor-dominated disease participated in this study. These subjects were recruited from the outpatient department of the Vitreo-retinal services of the L V Prasad Eye Institute (LVPEI), Hyderabad, India. The protocol and ethics for the study were approved by the Institutional Review Board at the LVPEI, Hyderabad, India. All the procedures in the study were conducted in accordance with the tenets of the Declaration of Helsinki. Written

informed consent was obtained from all the participants before they took part in the study. The written consent was provided by the parents or the local guardian for participants aged <18 years. Participants who are diagnosed as having Retinitis Pigmentosa (rod–dominated; n = 7; 5 males and 2 females; Mean ± 1SD age: 32.4 ± 13.5yrs) and or Stargardt's disease/macular dystrophy (cone–dominated; n =13; 8 males and 5 females; Mean ± 1SD age: 23.3 ± 12.2yrs) were included in the study. The diagnosis was confirmed by retina specialists, if at least one of the three following criteria were met: 1) Presence of retinal flecks or Bulls' Eye maculopathy for cone-dominated disease and presence of arteriolar attenuation and bony spicules appearance for rod – dominated disease during the clinical presentation; 2) Fundus autofluorescence (FA) revealing a peripheral ring of hyperfluorescence spots around the central macular region of hypofluorescence confirming the presence of Stargardt's disease[41]; 3) Full-Field electroretinography responses showing impaired rod or cone-specific responses. None of the patients had any systemic syndrome associated with the ocular pathology. Only participants aged ≥10 years were recruited as a pilot study in our lab found that older participants were more reliable and consistent in their test responses when compared to their younger counterparts.

The Advanced Vision Optometric Tests (AVOT) is commercially available equipment developed at the City, University of London[40, 42] that supports a number of psychophysical assessments of visual functions. The AVOT software runs on a laptop computer with Windows operating system. The user interface is displayed on the laptop monitor while the visual stimuli are displayed on a second monitor that is fully calibrated for luminance and chromaticity. In the experimental set up available at LVPEI, the stimulus monitor is a 24" calibrated visual display (EIZO, Model ColorEdge CS2420; EIZO Corporation, Japan) that is separated from the laptop display by a black curtain, such that the patient can only see the stimulus monitor without any stray light from the latter. The calibration of the display was performed using a photometer (Mavo-Monitor USB, Gossen, Germany) and custom-built program (LUMCAL; City Occupational, Ltd., London, UK). The stimulus is controlled by the experimenter using the Flicker-*plus* module, which runs on the laptop. The room light was turned off while the test was carried out.

For assessing cone thresholds, the central (0^o) test stimulus was 30' in angular subtense and the four parafoveal test stimuli at 5^o eccentricity were 60' each, all at 1m viewing distance. The

photopic luminance of the display was 24 cd/m^2. The CIE chromaticity co-ordinates, scotopic/photopic (S/P) ratio, temporal frequency and presentation duration were (0.58, 0.32), 0.9, 14.9Hz and 334ms, respectively. For assessment of rod thresholds, the central stimulus subtended 45' while the four parafoveal stimuli subtended 90', all at 1m viewing distance. The CIE chromaticity co-ordinates, S/P ratio, temporal frequency and presentation duration for rod-enhanced stimuli were (0.18, 0.077), 9.0, 5Hz and 600ms, respectively. As part of a related study carried out during the development of the test, different stimulus sizes have been investigated. The improvement in rod threshold for stimulus sizes greater than 45' in central vision was minimal. We wanted to ensure that the stimulation of the retina was restricted to small regions and to avoid the averaging of responses, in patients with localized changes in sensitivity. Even with the 45' size, the area stimulated may be significantly larger as a result of micro fluctuations in fixation during the stimulus. Although the stimulus presentation time during the Flicker-plus test is only 600 ms for the rod condition, the fluctuation in eye movement while attempting fixation can be as large as 30 - 45 min of arc[43]. It is therefore reasonable to expect that a region of ~ 90 min arc may be stimulated with a stimulus diameter of 45 min of arc. The photopic luminance of the uniform background was 0.5 cd/m^2 and this was achieved by the subjects' wearing spectrally calibrated neutral density filters. The temporal profile of the stimulus was sinusoidal with equal time-averaged luminance and it was the same for both rod and cone stimuli. The calibration of the display was also adjusted automatically by the program to take into account the spectral transmittance characteristics of the filters. For both stimuli, the background and the target always had the same spectral composition to eliminate potential inaccuracies in contrast computations caused by spectrally selective, pre-receptoral filters in the eye.[44] The order of the rod and cone tests was randomized. Adaptation times of ~15s for cones and 90s for rods were employed in all tests. Preliminary tests revealed that the use of natural pupil size and extended dark adaptation times of up to 15minutes does not cause significant changes in the measured thresholds. The rod/cone flicker test is not intended to provide full isolation of each class of photoreceptors but to produce large differences in sensitivity between the two major photoreceptor classes (rod and cone). Therefore, cone (S and M cone) intrusions may still be present.

Only participants who had visual acuity of at least 20/200 (logMAR 1.0) or better with spectacle correction were recruited for this study to ensure adequate fixation stability on a well-defined

fixation target located in the centre of the display and flanked by diagonal peripheral guides, all pointing towards the centre of the display. In addition, a square target imaged at the centre of the screen preceded each stimulus presentation. The combination of guides and the briefly presented fixation target made it easier for the subject to keep his / her point of regard on the centre of the screen during each stimulus (Figure 1). Each presentation was followed by an auditory beep. The tests were carried out monocularly and only eyes which met the inclusion criteria were tested. Based on the previous pilot study in healthy controls, the repeatability of FMT measurements was estimated to be ~2%.

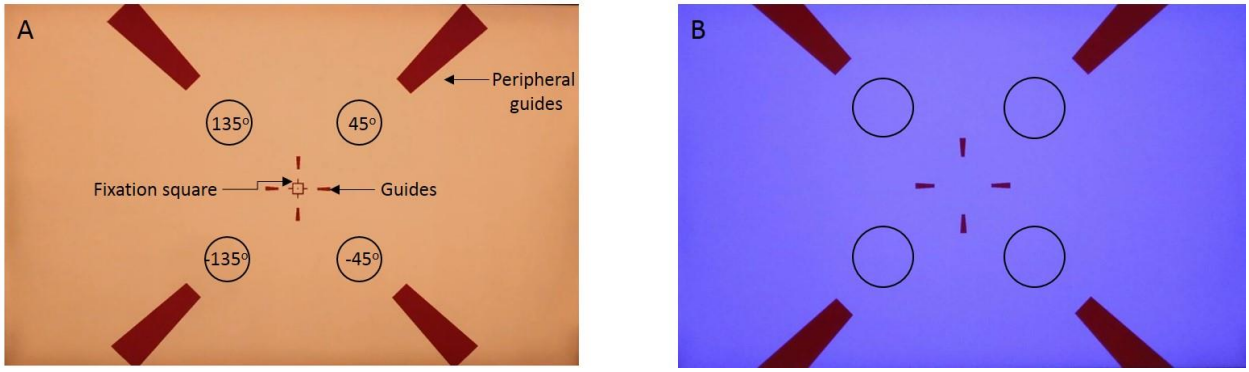

Figure 1. Schematic of the cone (left panel) and rod-enhanced (right) test conditions used for the Flicker-plus test. *The numbers in panel A indicate the position in degrees, where the stimulus would appear in one of the parafoveal locations. ((±45° and ±135°). The central stimulus (0°) is not shown in the figure. However, it would appear on the place where fixation square is shown (panel A). There are also central and peripheral guides to aid fixation. Note that the actual size of the stimulus is not shown in the figure, it is only for representation and also the original stimulus does not have any outline.*

The stimulus was presented either centrally or in one of the four quadrants (45° - Upper Right; 135° - Upper Left; -135° – Lower Left and -45° - Lower Right). The participant's task was to indicate the location of the stimulus by pressing raised buttons on a numeric keypad, which mirrored the five test locations. Participants were instructed to press the sixth button, if they were unable to locate the target, in which case, the program randomly assigned the response to one of the five locations. In instances (25%, 5/20 participants), where the participant was unable to use the keypad, the examiner pressed the appropriate key, based on the participant's verbal response. FMTs are measured at each of the five locations in the visual field using five randomly interleaved 2-down 1-up adaptive staircases wherein the step size varied commensurate with the subject's response to arrive at the threshold quickly. The staircases terminated at 9 reversals each and the threshold was taken as the average of the last 6 reversals of each staircase.

Data analysis was carried out using SPSS software (IBM SPSS, version 25; IBM Corp., Armonk, NY, USA). The figures were created using ggplot2 package built in R 3.6.3 (http://www.r-project.org/) under R studio 1.2.5001 (RStudio, Boston, MA, USA) and SPSS. The data was not normally distributed as tested by Shapiro Wilk test ($p < 0.05$). Therefore, non-parametric tests were used for comparison between flicker modulation thresholds for rod and cone-dominated diseases. The rod and cone FMT in patients with inherited retinal diseases will be compared against the age-matched database. [40]

3. Results

Twenty-two subjects that passed the inclusion criteria were recruited for the study. Amongst them, two participants were unable to complete the learning mode and were not included in the main study. Therefore, a total of 20 subjects finally participated in the study - the testability rate of the Flicker-*plus* test for the current study was therefore ~91% (20/22). The mean (±1SD) age of the participants was 25±12 years. There were ten subjects in whom both eyes were tested, only one eye from each subject was randomly included for analysis. Randomization was achieved by applying the formula "RANDBETWEEN (0, 1)" formula in Microsoft Excel (2013). In instances when rows corresponding to participants were assigned "0" (zero), the right eye was selected and in case of "1" (one) the left eye was chosen. Mean of two eyes in the same subject can be obtained when intraclass correlation between the two eyes is close to 1[45]. However, in the current dataset, only two of the 10 participants had an intraclass correlation close to 1 (≥ 0.90). Therefore, the mean response was not utilized to keep it consistent across all subjects. Twenty eyes of 20 subjects (7 females, 13 males) were included for the final analysis. A sub-analysis involved estimation of the Coefficient of Repeatability (CoR) to compare the differences in the independent measures of rod and cone FMTs between the two eyes of the same subject. The mean (± SD) CoR across the subjects was 7.89 % (±4.59 %). Table 1 shows the clinical characteristics of patients who met the inclusion criteria and were recruited for the study. In general, the time taken for completion of the test ranged between 10-15 minutes for each condition.

Table 1: *Clinical characteristics of the patients recruited in the study*

	Age, (years)	Sex	BCVA (logMAR)		Fundus findings	ERG/FA findings
			OD	OS	Both eyes	Both eyes
1	**41**	F	0.70 (6/30)	0.50 (6/19)	Pigmentary changes, arteriolar attenuation	Rod: Absent; cone: almost extinguished; left eye less affected
2	**35**	F	0.10 (6/7.5)	0.10 (6/7.5)	Bony spicule pigmentation, attenuated arteries and veins	Rod: Absent; Cone: slightly present
3	**39**	M	0.0 (6/6)	0.0 (6/6)	Arteriolar attenuation pigmentary changes	-
4	**10**	M	0.40 (6/15)	0.40 (6/15)	RPE changes all over with attenuated arteries	Both responses are extinguished
5	**39**	M	0.10 (6/7.5)	0.10 (6/7.5)	Bony spicules, Arteriolar attenuation, disc pallor+, Stable	-
6	**17**	M	0.10 (6//7.5)	0.20 (6/9.5)	Arteriolar attenuation pigmentary retinal degeneration	-
7	**40**	M	0.10 (6/7.5)	0.10 (6/7.5)	Bony spicules, Arteriolar attenuation, disc pallor+, Stable	-
8	**37**	F	0.90 (6/48)	0.90 (6/48)	Macular scar	-
9	**36**	M	0.20 (6/9.5)	0.80 (6/38)		Auto hypofluorescence with surrounding hyper autoflourscence
10	**13**	F	1.00 (6/60)	1.00 (6/60)	RPE changes and Flecks	-
11	**21**	M	0.90 (6/48)	0.90 (6/48)	RPE Atrophic patch	-
12	**48**	M	0.60 (6/24)	0.20 (6/9.5)	Arteriolar attenuation, dystrophic patch	Auto hypofluorescence with surrounding hyper autoflourscence
13	**11**	M	1.20 (6/95)	1.00 (6/60)	Mild attenuation; RPE hypo and hyper pigmentary changes	Auto hypofluorescence with surrounding hyper autoflourscence
14	**30**	F	0.90 (6/48)	0.90 (6/48)	Multiple whitish yellow spots +	Auto hypofluorescence with surrounding hyper autoflourscence
15	**30**	M	0.80 (6/38)	0.60 (6/24)	RPE changes and Flecks	-
16	**12**	F	0.80 (6/38)	0.80 (6/38)	Fundus normal	-
17	**11**	F	0.70 (6/30)	0.90 (6/48)	Macular degeneration patch	-
18	**10**	M	0.70 (6/30)	0.80 (6/38)	Macular degeneration patch	Auto hypofluorescence with surrounding hyper autoflourscence
19	**27**	M	0.90 (6/48)	0.90 (6/48)	Bull's eye maculopathy	Auto hypofluorescence with surrounding hyper autoflourscence
20	**12**	M	0.60 (6/24)	0.60 (6/24)	Fundus normal	Auto hypofluorescence with surrounding hyper autoflourscence

Table 2 shows the median [interquartile range (IQR)] cone and rod FMTs obtained in cone- and rod-dominated disease along with the age-matched values of controls from Hathibelagal et al (2020).[40] Ninety-nine percent (185/187) of the central and parafoveal cone and rod photoreceptor FMTs in the patients with cone- and rod-dominated diseases were higher than the corresponding

median values of age-matched controls, irrespective of the disease type (Table 2). Mann Whitney U-test revealed borderline significant differences between central cone FMTs [Median: 32.32% (IQR: 28.15%)] and the corresponding rod-FMTs [18.7 %, (3.3%); p = 0.05) in the cone-dominated disease (Figure 2). None of the comparisons in the parafoveal test locations were significantly different from each other (p > 0.05), although there was a qualitative trend for the cone FMTs to be larger than the corresponding rod FMTs (Figure 2A). None of the rod FMTs were significantly different when compared to the corresponding cone FMTs in rod-dominated diseases (p > 0.05). However, the qualitative trend of higher rod FMTs in comparison to cone FMTs in rod-dominated disease can be noticed in the Figure 2B.

Table 2: *Comparison of median (IQR) central and parafoveal cone vs rod FMT in the cone and rod-dominated diseases against the normative database[40]. IN, IT, ST and SN correspond to inferonasal, inferotemporal, superotemporal and superonasal parafoveal locations.*

Stimuli	Cone-dominated disease					Rod-dominated disease				
	Central FMT (%)	Parafoveal FMT (%)				Central FMT (%)	Parafoveal FMT (%)			
		IN	IT	ST	SN		IN	IT	ST	SN
Cone FMT	32.3 (28.2)	13.6 (28.4)	15.4 (30.2)	29.3 (28.0)	26.8 (31.3)	9.1 (10.3)	12.8 (20.2)	13.7 (7.9)	9.6 (8.0)	12.0 (6.0)
Rod FMT	18.7 (3.3)	10.3 (5.7)	10.9 (3.6)	13.0 (4.8)	14.9 (13.1)	15.0 (22.6)	16.7 (22.6)	13.6 (26.3)	14.5 (19.5)	19.1 (9.3)
Normative database Cone FMT[40]	4.2 (2.0)	4.4 (1.5)				4.2 (2.0)	4.4 (1.5)			
Normative database Rod FMT [40]	6.8 (2.6)	5.5 (1.3)				6.8 (2.6)	5.5 (1.3)			

Even while the median values did not reveal statistically significant differences in the cone and rod-FMT's in the two disease types, the ratio of cone to rod FMTs revealed photoreceptor-specific disease patterns (Figure 3). Cone/rod FMT ratio of >1.0 indicates that the deficits in cone photoreceptors were relatively more than those in rods and a ratio of < 1.0 indicates the reverse. Eighty-three percent (49/59) of the test locations showed cone/rod FMT ratios > 1.0 in cone-dominant diseases (Figure 3A) and 74.6% (44/59) of the test locations had cone/rod FMT ratio between 1.0 and 4.0. Only one individual exhibited cone/rod FMT ratio greater 4.0 in at least 4 of the testing locations. Eighty-eight percent (29/33) of the test conditions showed these ratios to be < 1.0 in the rod-dominant disease (Figure 3B), while 48.4% (16/33) and 39.4% (13/33) of the test locations had cone/rod FMT ratio between 0.5 - 1.0 and ≤ 0.5 respectively. These ratios were in

the expected direction of photoreceptor functionality loss depending on the dominance of the disease type.

A correlation analysis was carried out to ascertain if there was any relationship between central rod/cone FMTs and visual acuity. The central cone and rod FMTs were poorly and statistically insignificantly correlated with the high-contrast logMAR acuity of patients in cone-dominated (cone: r = 0.37; p = 0.21; rod: r = -0.21; p = 0.50) and rod-dominant (cone: r = 0.40; p = 0.36; rod: r = 0.19; p = 0.69) disease.

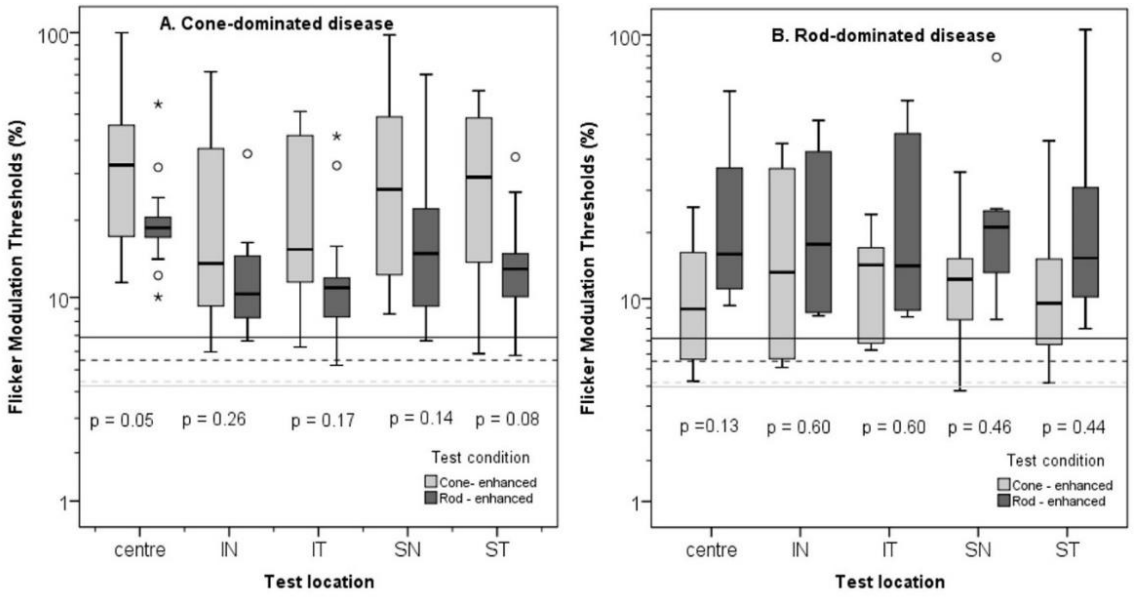

Figure 2. *Box and whisker plots of cone and rod FMTs obtained from the central and parafoveal (IN, IT, SN, ST) positions for cone- (panel A) and rod-dominated disease (panel B).The thick horizontal line in each box plot indicate the median, the upper and lower end of the box indicates the interquartile range, the open circles represent the outliers and asterisk shows the extreme values. The solid and dashed horizontal lines refer to the central (gray for cone and black for rod) and parafoveal average age-matched flicker threshold values, respectively, from Hathibelagal et al (2020)[40]. P-values indicate the comparison between two tests conditions at each of the stimulus location in both the diseases.*

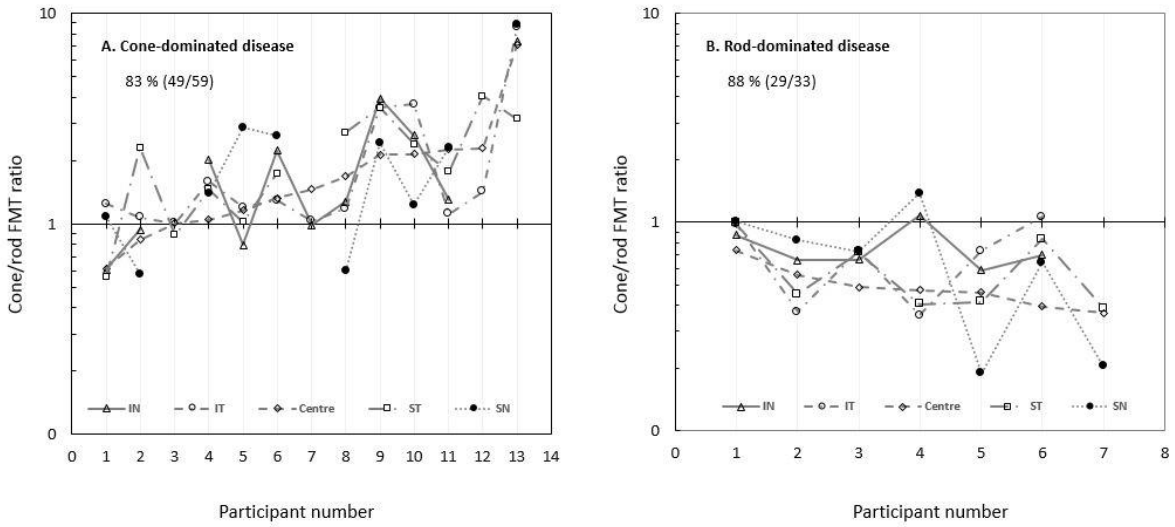

Figure 3. *Ratio of cone to rod FMT in cone-dominated (panel A) and rod-dominated (panel B) diseases plotted for each subject that participated in this study. The solid horizontal lines in each of the panels at 1.0 indicate that rod and cone FMTs were equal. Ratios >1 indicates cone FMT were greater than rod thresholds, indicating cone dysfunction and ratio <1 indicates rod FMT were higher than cones, indicating rod dysfunction. The different symbols indicate the five test locations namely centre (C), inferonasal (IN), inferotemporal (IT), superonasal (SN), and superotemporal (ST) quadrants. The number in panel A indicates the percentage of test locations with cone/rod FMT ratio > 1 and number in panel B indicate the percentage of test locations with cone/rod FMT ratio < 1.*

4. Discussion

This study evaluated a new Flicker-*plus* test designed to measure cone- and rod-mediated flicker sensitivity in patients with either cone-or rod-dominant diseases of the retina. The results reveal two principal findings. First, irrespective of the disease type (cone- or rod-dominated disease), both the rod and cone thresholds were higher than the corresponding, age- and ethnicity-matched normative values reported earlier[40] (Table 2). Second, cone FMTs were greater than rod FMTs in cone-dominated disease and the effect reversed in rod-dominated disease (Figures 2 and 3). The results from this study also confirm earlier findings which show generalized flicker deficits in patients with inherited retinal degenerations.[46, 47] More specifically, flicker deficits have been reported in patients with Stargardt's disease at all temporal frequencies (up to 50Hz) except for an intermediate range of frequencies (~5-15Hz).[46, 48] Loss of sensitivity at high temporal frequencies have been reported in patients with retinitis pigmentosa.[46, 47] The larger cone FMTs relative to rods (Figure 2) measured in this study and cone/rod FMT ratios above unity in cone-dominated disease

(Figure 3A) are consistent with previously reported studies, which also show greater cone losses relative to rods in patients with cone dystrophy.[17, 19, 23] Larger rod FMTs relative to cones (Figure 2) and below unity cone/rod FMT ratios in rod-dominated disease diseases (Figure 3) are also in line with reports from previous studies.[18, 49] Smaller response amplitudes in rod-specific electroretinogram signals and accelerated loss in rod function when compared to cone responses have been reported in patients with the rod-dominated disease such as Retinitis Pigmentosa.[15, 50] In general, flicker sensitivity losses in retinal degenerations have been attributed to loss of quantum catching ability in the photoreceptors due to low photopigment density or the change in temporal properties of rods and cones in response to flickering stimuli.[46]

The observation of both the rod and cone FMTs being poorer than age-matched controls, irrespective of disease type, indicates the absence of normal function in all photoreceptors, even when clinically the disease is labelled as either rod- or cone-photoreceptor specific. Rod deficits have been shown to be present in cone-dominated diseases such as progressive cone dystrophy[19] and Stargardt's disease.[14] Histopathological studies in some patients with cone dystrophy have shown abnormalities in rod morphology such as the rod outer segment enlargement[51], which may adversely affect rod photoreceptor function, even in a cone-dominated disease. Changes that may occur in proteins acting at the rod photoreceptor segments[52] may lead to rod dysfunction in the cone dystrophies. Analogously, longer dark adaptation times for rods in patients with Stargardt's disease points towards rod dysfunction, potentially attributed to the accumulation of lipofuscin in the retinal pigment epithelium (RPE) layer that may interfere with the visual pigment regeneration process.[53] The presence of cone deficits in rod-dominant disease such as Retinitis Pigmentosa may arise from cone cell death in this disease, perhaps due to increased oxidative stress or release of rod-derived toxins or microglial activity.[54]

The lack of significant correlation between high contrast visual acuity and cone/rod FMT ratio is not surprising as it has been well established previously that high contrast visual acuity fails to reflect early-stage photoreceptor loss in patients with inherited retinal diseases[55] and, more particularly, rod FMTs. This is consistent with a previous study that showed no significant relationship between FMTs and visual acuity in normal subjects[56]. However, the same group also showed that there is a significant relationship between FMT and visual acuity in patients with

macular pathology such as age-related macular degeneration.[57] The differences between the results of the present study and previous findings could be attributed to differences in the disease cohort (inherited retinal diseases in the present study versus age related macular degeneration in the study by Brussee et al (2018)[57]), younger age group (Average age: 26.8 ± 13.4 years (present study) versus 77 years[57]) and the flicker frequency (5 & 15 Hz (present study) versus 8 Hz[57]).

Inherited retinal diseases typically have bilateral presentation[58] and it is therefore expected that the FMTs will be elevated in both eyes of the patient, relative to age-matched controls. One may also be tempted to interpret the difference in FMT between the two eyes of the same subject as a measure of test "repeatability". Such an interpretation is based on the assumption that the disease severity between the two eyes are similar. Large inter-eye variability in FMTs were observed in this study, may well be indicative of varying levels of disease severity in the two eyes and location-specific differences in disease pattern between the eyes. Therefore, caution must be exercised before such an interpretation is made.

Overall, this study demonstrates that with the appropriate choice of light level and spectral and spatiotemporal parameters, it is possible to measure rod- and cone-specific thresholds without the need for either high retinal illuminance levels or full dark adaptation. The cone/rod FMT ratio metric would be useful in differentially identifying cone versus rod dominant disease types in a clinical scenario, when there is uncertainty in the diagnosis. The Flicker-*Plus* test is easy to carry out and has the additional advantage of measuring rod and cone-specific sensitivity at five discrete locations in central vision using small stimuli. This test is not intended to replace the existing diagnostic technology such as electroretinography, but to add further value that would aid improved diagnosis, management, follow-up and the overall understanding of disease pathophysiology. As is often the case in a challenging diagnosis, multiple investigations need to be carried out by specialized personnel to confirm the presence/absence of a disease, which include objective techniques such as ERG combined with psychophysical tests. Genetic testing for genotyping and/or para-neoplastic panels for anti-retinal autoantibodies can also provide further diagnostic value. The shared inter-professional collaboration can therefore to better diagnosis and management of the disease.

The new test has some obvious limitations. First, the protocol employed measured peripheral FMTs in each of the four quadrants, but only a single retinal eccentricity of 5°. This choice of eccentricity was to ensure that the protocol remained consistent with age-matched normative data. Changes in the FMTs at further eccentricities remain unknown, but can be explored, if required. The staircase procedures employed require a minimum of five stimulus locations, but both the eccentricity and the number of peripheral locations tested can be altered. Although more eccentricities can be tested in the same run, the time required to complete the test using randomly interleaved staircases is directly proportional to the number of stimulus locations involved. Changes in the FMTs at further eccentricities remain unknown, but can be explored, if required. The test is likely to be of value for use in the clinic, but further research is needed to investigate the optimum number of retinal locations and eccentricities to be investigated in relation to the time needed to complete the test. Second, the presence of eccentric fixation in the patients that participated in this study was not tested using techniques such as scanning laser ophthalmoscope[59]. However, none of the participants were noted to have any obvious eccentric fixation in their clinical records. This was also supported by the lack of any abnormal head posture while fixating on the center of the screen. Therefore, any impact of eccentric fixation on the FMTs reported here are likely to be negligible. However, those subjects who may have experienced small eccentric fixation, we expect that the strong fixation stimulus and guides minimized potential drifts in fixation during the stimulus. The third limitation is the lack of real-time, eye fixation monitoring during the test. While such monitoring would be desirable, it is unlikely to significantly affect the peripheral thresholds reported here, particularly when the peripheral locations are selected randomly during the test. The use of extended guides and appropriate fixation stimulus at the centre of the screen combined with constant reinforcement to maintain fixation during the testing process minimized the tendency of subjects to saccade to the peripheral stimuli. In addition, goal-directed saccades towards the peripheral stimuli are best elicited with high contrast targets and are less likely to occur when the stimuli are close to threshold. One of the caveats of the rod/cone flicker test is that it produces large differences in sensitivity between the two photoreceptor classes (rod and cone) but does not provide full isolation of rods and cones which could add to the test variability. This study is preliminary and employs a small sample size. The test would need to be evaluated on a larger cohort to gain greater understanding of its suitability as a functional biomarker in clinical trials. Additionally, genetic testing of the participants to identify the

genotypes would strengthen the validation of the rod/cone test. Despite these limitations, the results demonstrate that in principle, rod-enhanced and cone-enhanced stimuli can be used to separate rod- and cone-mediated responses and to reveal the corresponding lack of sensitivity in diseases of the retina which affect preferentially either rods or cones.

5. Conclusions

The Flicker-*plus* test can be used to quantify rod and cone-specific preferential loss of sensitivity at several locations in the visual field, in patients with suspected loss of photoreceptor function, without the need for dark adaptation. Notwithstanding the disease type (cone or rod-dominated), both cone and rod thresholds are higher than the age-matched FMT. However, the higher magnitude of photoreceptor-specific losses corresponds to the photoreceptor that is predominantly affected in any particular disease. Further studies are needed to optimize the test parameters for clinical use and also to investigate the usefulness of the new test in detecting changes in photoreceptor sensitivities in other retinal diseases.

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