Current Eye Research, 32:555–564, 2007

ISSN: 0271-3683 print / 1460-2202 online
DOI: 10.1080/02713680701418405

informa
healthcare

Measurement of Macular Pigment Optical Density Using Two Different Heterochromatic Flicker Photometers

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ABSTRACT *Purpose*: To compare macular pigment optical density using two different heterochromatic flicker photometers. *Methods*: We measured macular pigment optical density in 121 healthy subjects using heterochromatic flicker photometry. *Results*: The mean (±SD) macular pigment optical density measured using the Maculometer was 0.394 (±0.170), and that using the Densitometer was 0.395 (±0.189). The difference in measurements on each instrument was influenced by age and macular pigment levels. *Conclusions*: On average, there is no difference in measurements provided by these two instruments. The Maculometer tends to underestimate macular pigment in older subjects and/or those with higher macular pigment compared with the Densitometer.

KEYWORDS age-related maculopathy; critical flicker frequency; heterochromatic flicker photometry; macular pigment; retinal eccentricity

INTRODUCTION

Age-related macular degeneration (AMD), the late stage of age-related maculopathy (ARM), results in loss of central and color vision and is the most common cause of blindness in the elderly population in the Western world.[1] Although the pathogenesis of AMD remains unclear, there is a growing consensus that cumulative short-wavelength visible light damage and/or oxidative stress play a role.[2]

Macular pigment (MP) is composed of the xanthophyll carotenoids: lutein (L), zeaxanthin (Z), and meso-zeaxanthin (MZ).[3,4] L and Z are of dietary origin, whereas MZ is formed in the retina after conversion from L.[3] There is evidence to support the view that MP plays an important role in preventing the development of ARM by filtering out high-energy (short-wavelength) visible light before it reaches the photoreceptors or the retinal pigment epithelium (RPE)[5] and by acting as a free radical scavenger.[6] Therefore, accurate measurement of MP *in vivo* is important in the assessment of the role this pigment may play in the protection against ARM.

Heterochromatic flicker photometry (HFP) was the first technique, and remains one of the most widely used techniques, for measuring MP optical density *in vivo*. HFP is a subjective psychophysical method, which requires the subject

Received 7 March 2007
Accepted 23 April 2007

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to make iso-luminance matches between green and blue flickering lights. The log ratio of the amount of blue light absorbed centrally, where MP peaks, to that absorbed at a peripheral retinal locus (the "reference point," where MP optical density is assumed to be zero), gives a measure of the individual's MP optical density. This method has been validated against the absorption spectrum of MP *in vitro*.[7] Measurement of MP optical density by HFP also correlates well with results obtained by reflectometry and autofluorescence, both of which are objective techniques for measuring MP optical density *in vivo*.[8] However, in this study by Delori et al., despite good correlation between the methods, they found that reflectometry systematically underestimated MP optical density and that autofluorescence systematically overestimated MP optical density when compared with measurement by HFP.[8]

In this study, we compared MP optical density values obtained with two HFP instruments: the Eyemet Maculometer, and the Macular Metrics Densitometer. The Maculometer has a reference point at 5.5 degrees retinal eccentricity, whereas the reference point is at 7 degrees retinal eccentricity in the Macular Metrics Densitometer. Of note, it has been reported that MP is detectable at up to 8 degrees retinal eccentricity[5,9,10] and, in theory, therefore, the Maculometer (and other instruments using a similar reference point) may underestimate MP optical density, due to its more centrally located reference point. This study was designed to compare MP optical density measurements obtained using two HFP instruments with different reference locations and to explore the significance of measuring MP optical density using such different reference points.

MATERIAL AND METHODS

Subjects

One hundred twenty-one healthy subjects were recruited for this study. Subjects were recruited after a locally advertised poster campaign and by word of mouth. This study was approved by the Research Ethics Committee of Waterford Institute of Technology, and subjects were required to sign an informed consent document prior to participation. All experimental procedures adhered to the tenets of the Declaration of Helsinki.

Inclusion criteria for participation in this study were age between 20 and 70 years; no clinical evidence of ocular pathology; visual acuity 6/12 or better. The following information was recorded for each subject: demographic details; general health status and medication use; family history of ARM; personal smoking history. Examination included visual acuity (Snellen and LogMAR); body mass index [BMI (calculated as kg/m^2)]; MP optical density measurement by HFP (using the Eyemet Maculometer, and the Macular Metrics Densitometer, see below); iris and fundus photography (without pupil dilation), using a nonmydriatic NIDEK Handy NM100 (Fremont, California, USA) type D camera.

Measurement of Macular Pigment by HFP

We used two different HFP instruments to measure the MP optical density of each subject. For the purpose of this study, we assume that flicker perception is dominated by the edges of the disk-shaped stimuli used in each instrument,[11] although other research has suggested that this may not be the case.[12] The instruments used in this study were the Eyemet Maculometer, developed by Professor John Mellerio of the University of Westminster, London, UK (for a more detailed description of this instrument and its method of use, please refer to Mellerio et al.[13]); and the Macular Metrics Densitometer, developed by Professor Billy Wooten of Brown University, Providence, Rhode Island, USA (for a more detailed description of this instrument and its method of use, please refer to Wooten et al.[14]).

Instrumentation

The Eyemet Maculometer

The Maculometer is a small, portable HFP instrument, capable of measuring MP optical density at a single retinal locus (0.5 degree retinal eccentricity). The Maculometer uses light emitting diodes (LEDs) as light sources for the foveal and parafoveal targets. LEDs are used as light sources in this instrument because they are easily powered by simple power supplies, are small and inexpensive, and emit near monochromatic light. The LEDs used in the Maculometer alternate in square-wave counterphase between one that emits green light, at a wavelength of 530 nm, and one that emits blue light, at a wavelength of 470 nm.

The Maculometer uses a foveal target of 1 degree diameter (i.e., 0.5 degree retinal eccentricity) with the reference location at 5.5 degrees retinal eccentricity (parafoveal target of 10 degrees inner diameter and 1

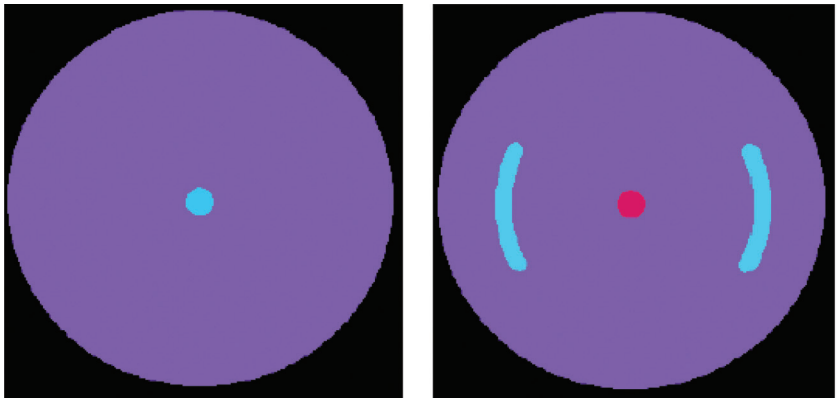

FIGURE 1 **Maculometer; showing the foveal test field on the left and the parafoveal test field on the right (for illustration only, not drawn exactly to scale).**

degree width). These targets are presented on a background adapting field provided by eight blue LEDs with a maximum wavelength of 428 nm. The background adapting field is necessary to saturate the S-cones, so that they play no part in the determination of the end point of blue/green minimum flicker matching. The Maculometer provides for central fixation by presenting the foveal target as a circular disk in the center of the viewing field and the parafoveal target as two arcs around a central fixation point (diameter 1 degree) (see Fig. 1). The test is carried out at a viewing distance of 330 mm, under conditions of normal office light (ambient illuminance: 252 lux, as measured with an Iso-Tech ILM 350 Lux Meter, RS Components, Corby, United Kingdom). The flicker frequency for each target is fixed. The Maculometer uses a sufficiently high flicker frequency to exclude rod and/or S-cone involvement in minimum flicker matching. The parafoveal target uses a flicker frequency of 13 Hz, which is above the critical fusion frequency (CFF) of the rods. The foveal target uses a flicker frequency of 18 Hz, which is also above the critical fusion frequency of the S-cones, preventing involvement of any such cones that may be unadapted by the blue background field. The luminance of the LEDs in the Maculometer is varied in an independent manner (i.e., the green LED stays at a constant luminance, while the luminance of the blue LED is varied). The luminance of the blue LED is varied by the subject until the end point of matching luminance, or minimum flicker, is observed. The log ratio of the foveal to parafoveal luminance values at minimum flicker yields a reading of MP optical density.

The Macular Metrics Densitometer

The Densitometer is a physically larger instrument than the Maculometer, and it is not portable. The Densitometer is slightly modified from a device described by Wooten, Hammond, Land, and Snodderly.[14] The Densitometer also uses LEDs as light sources, but the luminance of both the green (550 nm) and the blue (460 nm) LEDs are varied in a yoked manner. This means that, as the luminance of the green LED increases, the luminance of the blue LED decreases, and vice versa. This is an important feature of this instrument as it avoids

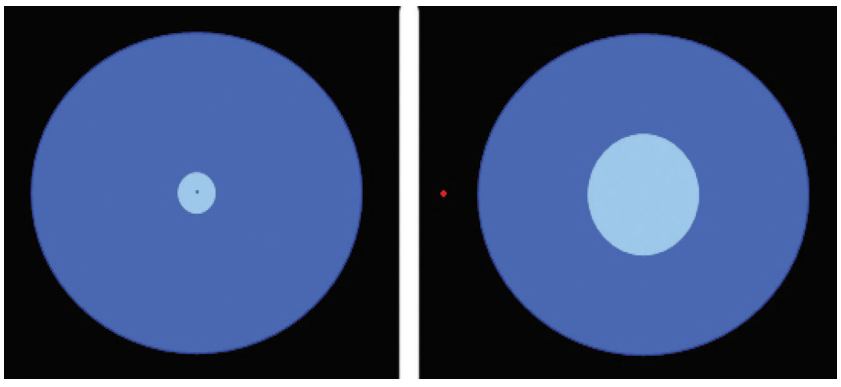

FIGURE 2 **Densitometer; showing the foveal test field on the left and the parafoveal test field on the right (for illustration only, not drawn exactly to scale).**

any change in the overall luminance of the target, which may be confusing for some subjects during the test.

Similar to the Maculometer, the illumination of the blue and green LEDs is alternated in square-wave counterphase. The emitted blue light is maximally absorbed by MP, whereas the emitted green light is not absorbed by this pigment. The Densitometer uses a reference location at 7 degrees and, by presenting the test field at various eccentricities, can be used to map a subject's MP spatial profile. The target used to measure MP optical density at 0.5 degree retinal eccentricity is a centrally located circular stimulus of 1 degree diameter, with a central fixation spot, at which the subject was encouraged to fixate. The 7 degrees reference target, on the other hand, uses an eccentrically located red LED, 5 minutes in diameter, as the fixation spot. This is presented to the left-hand side of a blue/green flickering circular disk, which has a diameter of 2 degrees and is centered at an eccentricity of 7 degrees from the red fixation LED. Both the central and reference targets are presented on a blue background test field, which saturates the S-cones (see Fig. 2). The wavelength of the blue background test field is 468 nm in the Densitometer.

One of the most important features of the Densitometer is that it has the option to adjust the flicker frequency. This enables the investigator to customize the optimal flicker frequency (OFF) for each subject, which results in a more discrete end point for the test, thus minimizing the variance between readings. The desired end point when using the Densitometer is a point of zero, or "null" flicker. Densitometer recordings are made under conditions of dimmed light (ambient illuminance: 4 lux, as measured with an Iso-Tech ILM 350 Lux Meter) at a viewing distance of 18.5 inches (47cm). As with the Maculometer, the log ratio of the foveal to parafoveal luminance values at null flicker gives a reading of MP optical density at the test locus.

Procedure

Measurement of each subject's MP was carried out first with the Maculometer and then with the Densitometer.

The Eyemet Maculometer

Prior to using the Maculometer, all subjects were shown explanatory Microsoft PowerPoint slides depicting the targets to be viewed, along with a verbal explanation of the method for recording minimum flicker matches. The subject was then allowed to make two or three practice minimum flicker matches prior to recording of results. Subjects were encouraged to make the matches quickly and reminded throughout the test that a point of no flicker would never be reached. The subject adjusted the luminance of the blue LED by turning a dial, and, once satisfied that they had reached the point of minimum flicker, the subject pressed the sample and hold button to record the result on the instrument readout. In between minimum flicker matches, the investigator moved the luminance control dial to an arbitrary, random position, so as to avoid the possibility of the subject learning how far to move the dial to achieve minimum flicker. At least four minimum flicker readings were recorded, as recommended in the standard operating procedure (SOP) for the Maculometer, first with the foveal target and then with the parafoveal target. Further readings were taken if the variance of the first four readings was >20%. Outliers, as identified using the bar graphs provided by the Maculometer software, were then discarded, leaving at least four minimum flicker radiance values for each target that had a variance <20%. If, after this procedure, the variance between readings remained >20%, the subject was excluded from the study analysis. All recordings were made under conditions of normal office light.

The Macular Metrics Densitometer

Prior to using the Densitometer, all subjects were shown an explanatory video describing the method for recording null flicker matches. The investigator then recorded the subject's CFF and OFF using an algorithm developed by Nolan and Stringham at Professor Max Snodderly's Vision Laboratory, Medical College of Georgia, Augusta, Georgia, USA. If a subject could not reach null flicker, the investigator increased the flicker frequency in increments of 1 Hz, until null flicker was perceived. Alternatively, if a subject exhibited a wide variation in null flicker readings (>10% of mean radiance at null flicker), the flicker frequency was decreased in increments of 1 Hz, until an acceptable null flicker range was achieved. An acceptable null flicker range was defined as one where the null flicker radiance values achieved by the subject were within 10% of the mean null flicker radiance at that test locus.

Subjects were required to perform at least three null flicker matches per target (foveal and parafoveal targets), as recommended in the SOP for the Densitometer . Further readings were taken if the variance of the first three

readings was >10%, and outliers were then removed, such that the variance of the remaining readings was <10%. All recordings were made under conditions of dimmed light.

Statistical Analysis

The statistical software package SPSS 14.0 for Windows was used for data analysis. The graphical software package Sigmaplot 8.0 for Windows was used for graphical presentation of results.

Relative ease of use of the two instruments was assessed by the McNemar chi-squared test. For subjects able to use both instruments, the paired *t*-test was used to analyze the differences in MP optical density measurements from the two instruments. Multiple regression analysis was used to investigate the relationship between this difference and a range of possible explanatory variables, namely age; MP optical density; sex; BMI; and family history of ARM.

RESULTS

Of the 121 subjects recruited for this study, 12 (9.92%) could not use the Maculometer and, of these, two (1.65%) could not use the Densitometer either, and this difference in the proportion of subjects unable to use each machine is statistically significant (McNemar chi-squared test, $p = 0.002$). Therefore, we performed our comparison analysis on the remaining 109 subjects who were able to use both instruments. The demographic and anthropometric data of our sample are summarized in Table 1.

TABLE 1 **Demographic and anthropometric data of 109 subjects**

Characteristic	N
Age	
Mean [years (±SD)]	46 (±11.13)
Range (years)	22–67
Sex	
Male	43 (39.4%)
Female	66 (60.6%)
Family History	
No family history of ARM	67 (61.5%)
Confirmed family history of ARM[a]	42 (38.5%)
Smoking status[b]	
Never	58 (53.2%)
Past	39 (35.8%)
Current	12 (11%)
BMI[c]	
Underweight (BMI <18.50)	1 (0.9%)
Desirable weight (BMI: 18.50-24.99)	52 (47.7%)
Overweight (BMI: 25-29.99)	39 (35.8%)
Obese (BMI > 30)	17 (15.6%)

[a]Family history of ARM was confirmed by letter from the individual's parent's health care provider.

[b]Never-smokers had smoked less than 100 cigarettes in their lifetime. Past smokers had smoked at least 100 cigarettes in their lifetime, but had not smoked for at least 1 year prior to investigation. Current smokers had smoked at least 100 cigarettes in their lifetime and had at least one cigarette in the year prior to investigation.

[c]BMI (body mass index) is defined as body weight in kilograms divided by height in meters squared (kg/m^2).

A scatterplot comparing the MP optical density values obtained with each instrument, with the line $y = x$ superimposed, is shown in Figure 3. A Bland-Altman plot for method comparison is shown in Figure 4, comparing the difference in MP optical density measured with each instrument against the mean MP optical density measured with both instruments. It is clear from this Bland-Altman plot that our data contains one outlier (difference >3SD below zero). When we reanalyzed our data excluding this subject, our conclusions were unaffected, therefore, all results that follow relate to our entire data set.

The mean (±SD) MP optical density measured at 0.5 degree eccentricity with the Maculometer was 0.394 (±0.170), and that with the Densitometer was 0.395 (±0.189). The mean difference (±SD) in MP optical density (Maculometer −Densitometer) was −0.001 (±0.088), with a 95% confidence interval for this mean difference of −0.018 to 0.016. As this interval contains 0, we conclude that there is, on average, no difference in measurements by the two instruments. For 50 of the 109 (45.9%) subjects included in this study, the Maculometer MP optical density measurements were lower than those of the Densitometer, for five (4.6%) subjects the readings were identical, and for 54 (49.5%) subjects the Maculometer readings were higher.

However, regression analysis, with the difference in the MP optical density readings obtained with each instrument (Maculometer reading − Densitometer reading) acting as the dependent variable, revealed that the difference in measurements was influenced by age and by MP optical density, reflected by the equation: $\text{diff} = 0.127 - 0.002\ (\text{age}) - 0.122\ (\text{average MP optical density})$, $R^2 = 0.10$. Sex, BMI, and presence or absence of family history of ARM were not found to be significant in this regression analysis at the 5% level. The finding that, in comparison with the Densitometer, the Maculometer tends to underestimate MP optical density in older individuals and in those with higher MP

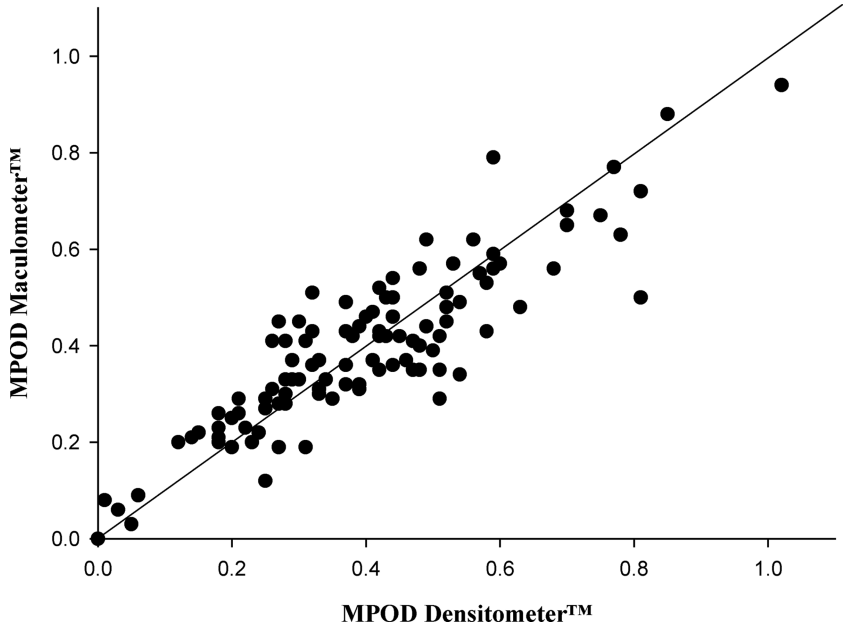

FIGURE 3 **Scatterplot comparing the MP optical density values obtained with each instrument, with the line y = x super imposed. MPOD, MP optical density.**

optical density values prompted us to compare readings on the two instruments for subgroups that might be affected by such bias.

In brief, we created three groups for comparison as follows: group 1, younger individuals (≤ the median age of 47 years) with a lower MP optical density (≤ the median MP optical density of 0.4); group 2, older individuals (> the median age of 47 years), or individuals with a higher MP optical density (> the median MP optical density of 0.4); and group 3, older individuals (> the median age of 47 years) with a higher MP optical density (> the median MP optical density of 0.4). The boxplot for the third of these groups (Fig. 5) clearly shows that the Maculometer tends to give a lower MP optical density reading in older individuals and in those with higher MP optical density levels ($p < 0.001$).

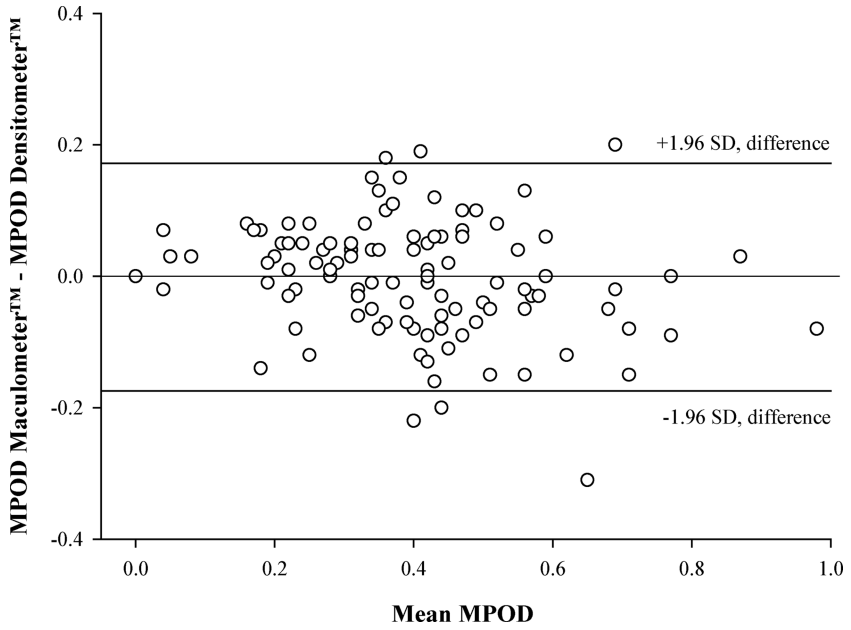

FIGURE 4 **Bland-Altman plot for method comparison, comparing the difference in MP optical density measured with each instrument against the mean MP optical density measured with both instruments.**

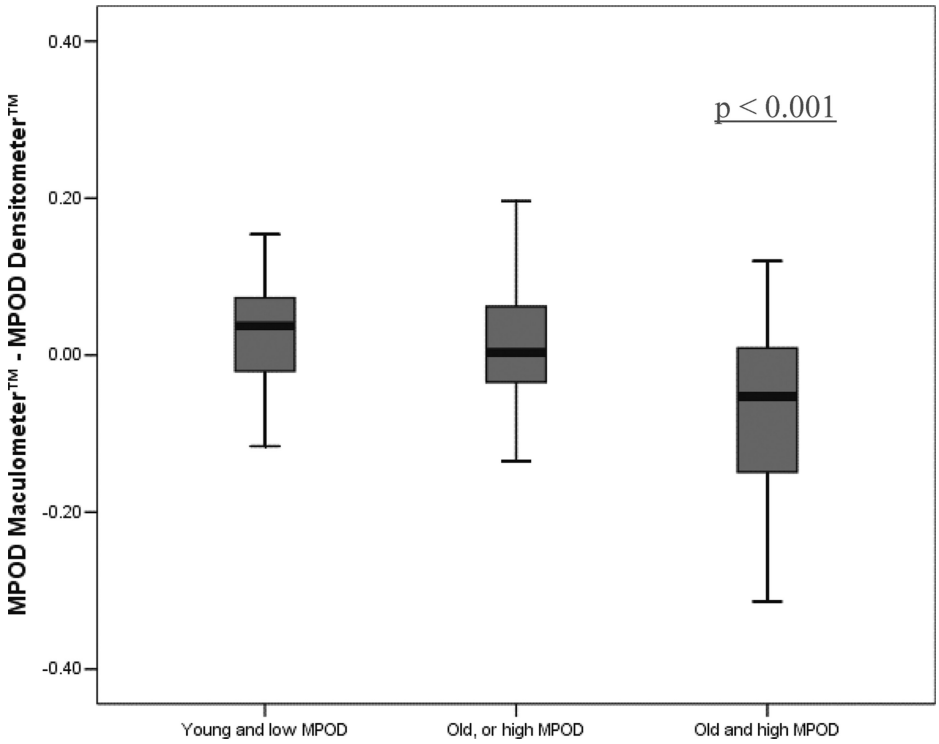

FIGURE 5 **Boxplots showing the difference in MP optical density measurements between the three groups, grouped according to age and MP optical density level. Group 1: younger individuals (≤ the median age of 47 years) with a lower MP optical density (≤ the median MP optical density of 0.4). Group 2: older individuals (> the median age of 47 years), or individuals with a higher MP optical density (> the median MP optical density of 0.4). Group 3: older individuals (> the median age of 47 years) with a higher MP optical density (> the median MP optical density of 0.4).**

In this sample, there was no statistically significant age-related decline in MP optical density values recorded with either instrument (Figs. 6 and 7).

DISCUSSION

In this study, we compared the MP optical density values obtained with two different HFP instruments in 109 healthy subjects aged between 22 and 67 years.

The major differences between the two instruments used in this study were as follows: the Densitometer uses a reference location of 7 degrees retinal eccentricity, whereas the Maculometer uses a reference location of 5.5 degrees; the Densitometer allows for adjustment of flicker frequency, whereas the Maculometer has a fixed flicker frequency; the light emitted from the blue LED in the Densitometer has a wavelength of 460 nm, which matches the peak absorption of MP, whereas the light emitted from the blue LED in the Maculometer has a wavelength of 470 nm; the luminance of the green and the blue flickering LEDs is varied in a yoked manner in the Densitometer, whereas the luminance is varied in an independent manner in the Maculometer.

Our first observation was that there was a higher proportion of subjects unable to use the Maculometer, due to an excessive variation in the luminance values chosen at the perceived end point with this instrument (coefficient of variation >20%). We attribute this limitation primarily to the fixed flicker frequency on this instrument, which contrasts with the adjustable flicker frequency of the Densitometer . Due to this inability to adjust the flicker frequency, it is not possible to customize the flicker frequency for each subject when using the Maculometer. As a result, some subjects may perceive a wider zone of minimum flicker when performing minimum flicker matches with this instrument. The end point of minimum flicker with the Maculometer is also a function of the independent manner in which the luminance of the green and blue LEDs is varied. This means that, even if the flicker frequency is optimal for a given subject, they will still perceive flicker due to the change in luminance between the green and blue LEDs. The resulting end point of minimum flicker is less discrete than an end point of null flicker, making the task of minimum flicker matching with the Maculometer more difficult than the task of finding null flicker with the Densitometer, with a consequential increase

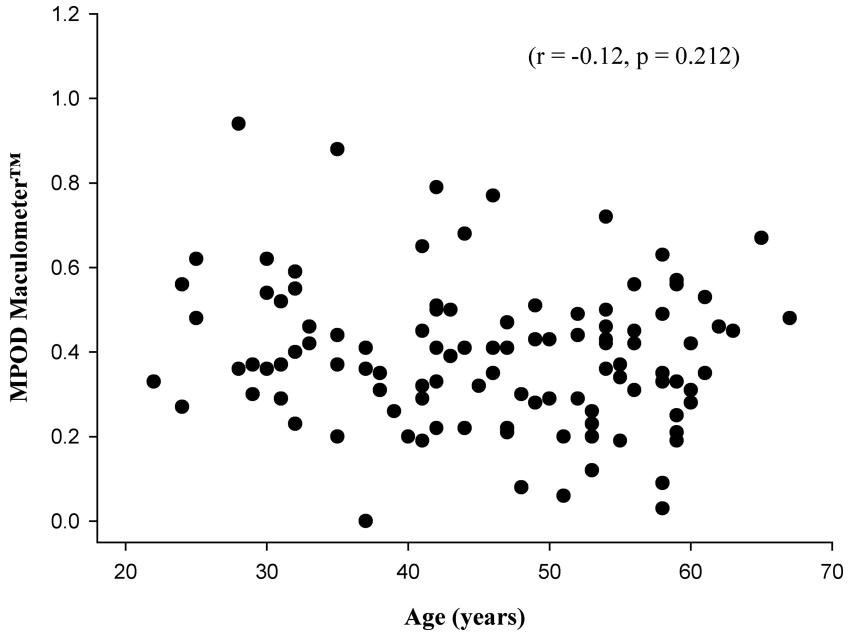

FIGURE 6 **The relationship between MP optical density and age, as measured by the Maculometer.**

in the variance of the readings obtained with the Maculometer. The proportion of subjects who can perform a given psychophysical task is an important determinant for instrument selection at the inception of any given study, and, therefore, our findings would favor the use of an instrument where flicker frequency can be adjusted for each individual and in which the luminance of the flickering LEDs is varied in a yoked manner.

The mean MP optical densities measured at 0.5 degree eccentricity were almost identical for the two instruments [0.394 (±0.170) with the Maculometer, versus 0.395 (±0.189) with the Densitometer], and these values fall within the range of 0.21 to 0.41 as reported in other previously published studies in which MP optical density was measured at 0.5 degree retinal eccentricity.[13,15–21] As the 95% confidence interval for the mean difference in MP optical density readings

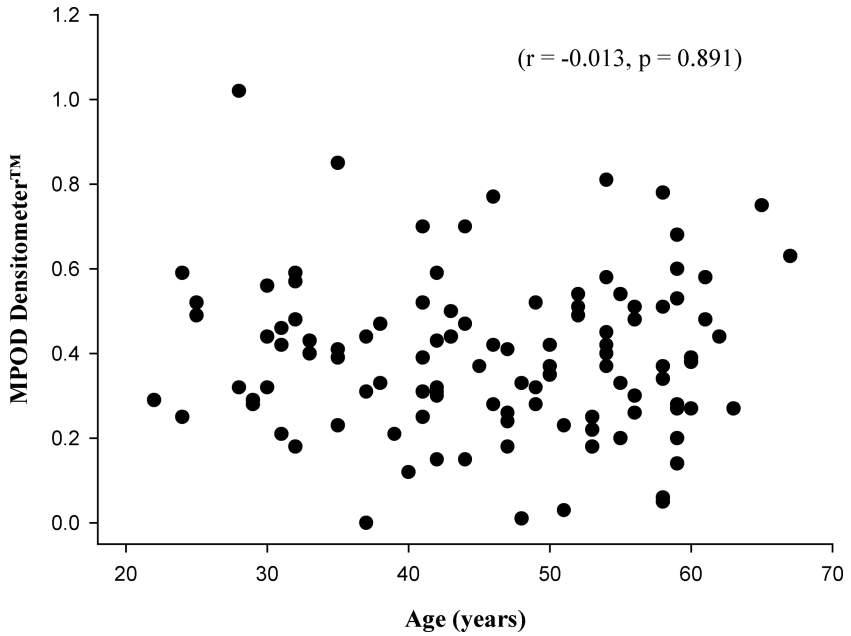

FIGURE 7 **The relationship between MP optical density and age, as measured by the Densitometer.**

between the two instruments used in this study contains zero, we can conclude that there is, on average, no difference in measurements provided by these two instruments.

However, it appears from the regression analysis that the Maculometer − Densitometer difference becomes more negative the older the subject and/or the greater their MP optical density. This suggests that the Maculometer tends to underestimate the "actual" MP optical density compared with the Densitometer in older subjects and in subjects who have higher levels of MP. Of note, sex, family history of ARM, or BMI did not have a significant influence on the difference between the MP optical density readings obtained with the two instruments.

The less eccentrically located reference point of the Maculometer probably explains the tendency of this instrument to underestimate MP optical density in older subjects and in subjects with higher levels of MP. Subjects with higher levels of MP optical density are known to have a wider spatial distribution of pigment within the retina.[9] Recently, Delori et al. reported on the spatial distribution of MP, as measured by two-wavelength autofluorescence, in 41 subjects.[22] They found that there was a broadening in the peripheral distribution of MP in older subjects, which was more marked in females than males. This increase in the lateral extent of MP in older subjects, also observed by Chen et al. using fundus reflectometry,[23] could result in an underestimation of MP optical density by HFP instruments using an insufficiently eccentric reference point, as we found with the Maculometer in this study. The cause of this age-related broadening in the distribution of MP within the retina is currently unknown. Cavallotti et al. have shown in postmortem histologic specimens that the mean retinal thickness decreases with increasing age.[24] Studies using optical coherence tomography have also demonstrated an age-related decrease in the thickness of the retinal nerve fiber layer.[25,26] It is plausible that these age-related changes in the retinal architecture may be accompanied by a redistribution of MP within the layer structure of the retina, accounting for the observed increase in the peripheral distribution of MP with increasing age. Further study of the spatial profile of MP, with an emphasis on any association with macular architecture and with age, is a prerequisite for an enhanced understanding of any such hypothesis.

Despite the fact that the two instruments used in this study use blue LEDs emitting light of different wavelengths, we did not find any systematic difference in the MP optical density values obtained with either instrument that could be attributable to this difference. The blue light emitted by the Densitometer peaks at 460 nm, which is the wavelength that is maximally absorbed by MP. The blue light emitted by the Maculometer, on the other hand, has a spectral power distribution that peaks at 470 nm, slightly above that maximally absorbed by MP. In theory, this difference should result in the Maculometer systematically underestimating MP optical density values compared with those obtained using the Densitometer; for example, for an average MP optical density level of 0.30, the difference would be expected to be approximately 0.02, and with a higher MP optical density (e.g., 0.6), this difference would be expected to increase to about 0.04–0.05.[27] However, we did not find any such systematic difference in this study.

Interestingly, an age-related decline in MP optical density was not observed with either instrument in this study. Despite many reports in the current literature as to the possible association between MP levels and age, it has been difficult to draw a firm conclusion on this issue to date. This is largely due to inconsistencies in the design and results of studies reporting on this association. However, in the largest study to date to report on the relationship between age and MP levels, Nolan et al. have reported a modest but significant age-related decline in MP optical density as measured by HFP.[28] This study was conducted on 828 subjects, which is more than double the number recruited in the next largest study using HFP to investigate the association between age and MP optical density, conducted by Ciulla et al.[21] Ciulla et al. did not demonstrate any age-effect in the 390 subjects included in their study, suggesting the need for large numbers of subjects to accurately demonstrate the modest age-related decline in MP optical density in a healthy population, as shown by Nolan et al.

In conclusion, a greater proportion of subjects can use a HFP instrument that has the option to adjust flicker frequency and in which the luminance of the green and blue flickering LEDs is varied in a yoked manner. These features increase the ease and accuracy with which measurements are made and should be present in all instruments selected for use in future large-scale studies. Overall, we observed good agreement between the MP optical density readings obtained with both instruments, but this deteriorated in older subjects and in those with higher MP optical density values. Therefore, when using HFP to measure MP optical density, the

significance of using a reference location at 7 degrees eccentricity or more is most obvious in such individuals, probably due to the increased peripheral distribution of pigment found in older subjects and those with higher MP levels. We recommend that, in future, all studies investigating MP optical density by HFP use an instrument in which the flicker frequency is adjustable, the luminance of the green and blue flickering LEDs is varied in a yoked manner, and a reference point of at least 7 degrees eccentricity is used.

ACKNOWLEDGMENTS

We would like to acknowledge Professor John Mellerio (School of Biosciences, University of Westminster, London, UK) for discussions regarding the Eyemet Maculometer, and Professor Billy Wooten (Department of Psychology, Brown University, Providence, Rhode Island, USA) for discussions regarding the Macular Metrics Densitometer.

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