**TITLE:** Reliability of Color Perimetry to Assess Macular Pigment Optical Density in Age-Related Macular Degeneration.

**Running head:** Reliability of color perimetry for MPOD measures.

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**ABSTRACT**

**Purpose:** The aim of this study was to determine the intra-session repeatability and inter-examiner reproducibility of the color perimetry technique when assessing in vivo macular pigment optical density (MPOD) in age-related macular degeneration (AMD) patients.

**Materials and Methods:** AMD patients were classified into 4 groups: early AMD (eAMD), intermediate AMD (iAMD), atrophic (aAMD) and neovascular (nAMD) after undergoing fundus photography (TRC 50DX type IA; Topcon Europe Medical B.V., Netherlands) and spectral-domain optical coherence tomography analysis (Topcon 3D-2000; Topcon Europe Medical B.V., Netherlands). Visual Acuity was over 20/80 and preservation of central fixation was confirmed in all patients using the MP-1 microperimeter (Nidek, Pavoda, Italia). To analyze repeatability, one examiner obtained three consecutive MPOD measures with MonCV3 device (Metrovision, Pérenchie, France). To study agreement between two observers, a second examiner performed another MPOD measurement in random order. Within-subject standard deviation (Sw), coefficient of variation (CVw), and intraclass correlation coefficient (ICC) data were obtained.

**Results:** Fifty two (32 females and 20 males) consecutive AMD patients having a mean age of 71.5 ± 8.2 years were recruited. Six had eAMD, 25 had iAMD, 10 had aAMD and 11 had nAMD. For repeatability, CVw values ranged from 22.3% (nAMD) to 41.0% (aAMD) and ICC values from 0.52 (iAMD) to 0.79 (nAMD). For agreement between two examiners, CVw values ranged from 20.1% (iAMD) to 37.8% (nAMD) and ICC values from 0.61 (nAMD) to 0.80 (aAMD).

**Conclusions:** The reliability (intra-session repeatability and inter-examiner reproducibility) of color perimetry technique to assess MPOD in AMD patients is only moderate. Thus, it cannot be recommended as a valid diagnostic test, when evaluating and monitoring MPOD in any AMD patient in research but also in the daily ophthalmological or optometric clinic.

**Keywords:** Age-related Macular Degeneration, macular pigment optical density, color perimetry technique, intra-sessionrepeatability, inter-examiner reproducibility.

**Introduction**

Age-related Macular Degeneration (AMD), a degenerative disorder of the central retina with a multifactorial etiology, is the leading cause of visual impairment in adults over 50 years of age in Europe.1 There has been different clinical classifications of the disease. The more recent one is based on fundus lesions assessed within 2 disc diameters of the fovea. Thus, disease progression criteria from early to late AMD are based on the presence of drusen, pigmentary abnormalities, geographic atrophy or choroidal neovascularization.2 The use of adequate protocols (i.e. treat-extend-stop) to provide anti-vascular endothelial growth factor (VEGF) therapy, makes feasible maintain or even improve vision from baseline treatment in neovascular AMD (nAMD) patients.3 However, in case of atrophic AMD there is no therapy able to restore the progressive anomaly observed in the retinal pigment epithelium (RPE) or photoreceptors. Thus, its management consists of vitamin supplements, and lifestyle recommendations like avoiding smoking or great alcohol consumption as well as body mass index reduction.4

Oxidative stress is associated with both the incidence and the progression of AMD.5 Initial Age-Related Eye Disease Study (AREDS) trial showed that the oral intake of antioxidants (Vitamins C and E, and beta carotenes) and zinc produced a reduction of the progression to advanced AMD.6 Later, the AREDS 2 trial showed that it was worth to replace the carotenoids form the original AREDS formula, and include lutein and zeaxanthin instead, specially for smokers and former smokers.7 Carotenoids can be found in orange and yellow fruits and green leafy vegetables, and lutein and zeaxanthin are ones of the most frequently eaten carotenoids. Besides, macular pigment (MP) is made up of the three carotenoids lutein, zeaxanthin, and meso-zeaxanthin (synthesized from lutein), and they are highly concentrated on the macular region (macula lutea), decreasing its concentration rapidly with eccentricity.8 It is thought that MP has a protective role from the damaging effects of free radicals produced by blue light, and it could also avoid development and progression of AMD Although low dietary and blood carotenoids were assumed to be modifiable risk factors for developing AMD, the protective effect of MPOD in the retina remains unclear. Consequently, in vivo evaluation of MP has become an important matter in the assessment and management of AMD patients.

In-vivo measurement of MP optical density (MPOD) can be performed using different techniques that could be based on physical methods like fundus autofluorescence, fundus reflectometry, resonance Raman spectroscopy and visual evoked potentials, or psychophysical ones like heterochromatic flicker photometry (HFP) and minimum motion photometry.9 Another psychophysical technique to evaluate MP is color perimetry. It is a simple non-invasive method that compares color sensitivity outcomes between two wavelengths (blue and red) differently absorbed by MP. Nonetheless, the reliability of the measurements obtained by any ophthalmic instrument should be determined prior to its widespread use to reduce the margin of error when classifying patients into a category (low, medium or high levels of MP) and to avoid erroneous supplementation treatment. Consequently, the aim of the present study was to estimate the intrasession repeatability and interobserver reproducibility of the color perimetry technique to assess MPOD in AMD patients.

**MATERIALS AND METHODS**

This prospective observational study was approved by the Valladolid East Area Ethics Committee (Valladolid, Spain) and complied with the Tenets of the Declaration of Helsinki. All candidates received detailed information about the nature of the investigation, and all provided their written consent.

**Participants.**

The study recruited consecutive caucasian patients with a diagnosis of AMD. Inclusion criteria for all participants were the following: age ≥ 55 years, best corrected visual acuity (BCVA) ≥20/80. Exclusion criteria included other ocular disease different from AMD, and any other physical or cognitive anomaly that could alter the performance of the clinical tests. To assure that AMD patients had central fixation, all patients underwent central fixation analysis using the MP-1 microperimeter (Nidek, Pavoda, Italia).

At the inclusion visit, the BCVA and the fixation analysis with MP-1 device were assessed. Then, fundus colour pictures and autofluorescence images (exciter filter of 500 to 610 nm and a barrier filter of 675 to 715 nm) were obtained (TRC 50DX type IA with a Kodak Megaplus 1.4i camera; Topcon Europe Medical B.V., Netherlands) within an area of 2 optic disc diameters centered in the fovea by using the device software (Topcon IMAGEnet i-base version 3.14.4). A spectral-domain optical coherence tomography (SD-OCT) was also obtained (Topcon 3D-2000; Topcon Europe Medical B.V., Netherlands) with the 3D Macula protocol. It consists of a raster-scan composed of 256×256 (vertical x horizontal) axial scans covering an area of 6×6 mm in the macular region. Consecutive volunteers were included in the following groups depending on the screening outcomes:2

* Early AMD (eAMD): Presence of drusen > 63 μm and ≤ 125 μm, without RPE alterations in the macular area.
* Intermediate AMD (iAMD): Presence of drusen > 125 μm and/or RPE alterations in the macular area.
* Advanced AMD subdivided into 2 groups:
	+ Atrophic AMD (aAMD): Presence of hipopigmented area of 175 μm with visible choroidal vessels within the studied area. Only patients with foveal sparing were included.
	+ Neovascular AMD (nAMD): Presence of signs of subretinal neovascularization secondary to AMD (presence of subretinal fluid, blood or thickening of the retina). Choroidal neovascularization was always type I, thus being under pigment epithelium. Macular area of included patients has to look as well preserved in fundus color and autofluorescence images. If patients had been previously treated with anti-VEGF drugs they had to be considered as controlled during the study, without signs of activity (presence of subretinal fluid, or increase in thickening of the retina, or blood, or decrease in vision).

**Color Perimetry Technique**

Color perimetry is a psychophysical technique that makes use of the spectral absorption properties and retinal location of MP in order to assess MPOD. We used the program MonCV3 and the MonPack systems (Metrovision, Pérenchie, France). The technique is based on the comparison of the thresholds obtained for blue and red light perception. Thresholds are measured in decibels (dB) and are estimated following bracketing strategies. A staircase 4-2-2-2 full-threshold strategy was used for the present study. The measurement procedure is based on the estimation of luminance differential thresholds for a blue and red stimulus. MP selectively absorbs blue incident light, with maximum absorption around 460nm and no absorption above 530 nm.10 Therefore, MPOD can be estimated as the difference in blue and red stimuli thresholds observed for macular and para-macular zones. In our study, stimuli were projected to the macula and to six different eccentric (10 degrees) locations, where MP is assumed to be negligible in comparison with the macular area. The stimulus had a Goldmann size III over a white background of 10 cd/m2.

Thus, the testing procedure was very similar to conventional automated perimetry evaluation. The eye not being tested was occluded. The patient had to focus in near distance, thus, optical correction was used to compensate for refractive errors and/or presbyopia if needed. Participants were initially instructed in how to perform the test. When macular sensitivity was evaluated, participants should stare at the centre of an empty black circle over a white background, and blue and red stimuli were presented in the middle. When para-macular sensitivity was assessed, a central dot was showed, and blue and red stimuli were presented at para-central locations (10 degrees). Volunteers were advised to press the bottom each time a stimulus was presented. Before starting, participants were instructed to locate their head in the forehead and to place their chin in the chin-rest, and the head location was adjusted. The device has a camera recording the patient´s eye, so that the clinician saw if the eye was properly located within the control area (rectangular) during the whole procedure. Also, the equipment has an automated ocular fixation control, so that eye movements could be recorded throughout the test. All volunteers received verbal instructions on how to perform the test and they had the desired time to make themselves familiar with the equipment and the procedure. Participants performed the test during the screening visit once inclusion and exclusion criteria were checked (and after signing the inform consent). This first test was a trial run and it was discarded, because we wanted to avoid learning effect and also allow familiarity with equipment and test.

**Statistical Analysis**

To estimate the intrasession repeatability, it is recommended to perform independent test using the same method on the same patient with the shortest time possible. Thus, participants underwent 3 consecutive MPOD evaluations performed by examiner 1. To estimate the intrasession repeatability, the within-subject standard deviation (Sw) of three consecutive measurements was calculated to obtain the intrasession coefficient of variation (CVw), which is defined as the ratio of the Sw over the mean (expressed as a percentage).11 In addition, the intrasession reliability was also estimated using the intraclass correlation coefficient (ICC),12 which was obtained after performing an one-way analysis of variance with repeated measures.

To estimate the agreement between examiners (inter-examiner reproducibility), a second examiner performed only one MPOD evaluation. The order of the procedures performed by each examiner was random to avoid bias. Also, MPOD assessment was performed within the shortest time to prevent fatigue bias. The first of the three MPOD evaluations performed by the first examiner was the one computed to establish agreement between examiners. The inter-examiner reproducibility was estimated by calculating first the inter-examiner Sw, and then, the inter-examiner CVw.11 Additionally, the interobserver ICC was also estimated. The paired t test was used to establish whether there was a significant systematic bias between examiners.

Data from the prospectively completed forms were entered into a database, and statistical calculations were performed by a PhD-licensed statistician (I.F.), using the R statistical package version 3.1.1 (R Core Team. Foundation for Statistical Computing, Vienna, Austria). Data distribution was evaluated using the Shapiro-Wilk test. The mean and the SD were calculated for normally distributed data. Spearman rank correlation coefficient was used to measure the association between the mean MPOD values and the SDw of the 3 measurements (intrasession repeatability) or 2 measurements (interobserver reproducibility) to confirm the assumption that the amplitude of variation was unrelated to the MPOD magnitude before proceeding with reliability analyses. An analysis of variance was performed to assess difference among the 3 MPOD measures obtained for each group. 2-tailed p≤0.05 was considered significant for all statistical tests.

**RESULTS**

A total of 52 consecutive AMD patients (32 females and 20 males) were recruited. Six were included in eAMD group, 25 in the iAMD group, 10 in the aAMD group and 11 in the nAMD group. The mean age of the control, eAMD, iAMD, aAMD and nAMD group was 67.4 ± 6.2, 67.8 ± 3.9, 73.3 ± 8.4, 70.0 ± 3.1 and 77.4 ± 8.7 years, respectively. There were not significant (p=0.07) differences in age among the groups.

**Intrasession Repeatability**

Table 1 shows the global mean, Sw and CVw observed in AMD groups for the intrasession repeatability estimations. There were not significant differences (p=0.66) among the mean MPOD values obtained for each study group. ICC value observed for the eAMD group was 0.72 (95% confidence interval (CI): 0.28, 1.00); for the iAMD group, 0.52 (95% CI: 0.28, 0.75); for the aAMD group, 0.68 (95% CI: 0.35, 1.00); and for the nAMD group, 0.79 (95% CI: 0.58, 1.00).

**Inter-examiner Reproducibility**

Mean differences in MPOD values between both examiners (Examiner 1 – examiner 2) were not significant for the iAMD group (0.18 dB (95% CI: -0.58, 0.93; p=0.63)); for the aAMD group (0.11 dB (95% CI: -1.42, 1.65; p=0.87)); nor for the nAMD group (0.31 dB (95% CI: -1.87, 2.48; p=0.76)). However, significant (p=0.01) mean differences were observed for the eAMD group (-1.50 dB (95% CI: -2.50, -0.49)).

 Table 2 shows the global mean, Sw and CVw observed in AMD groups for the interobserver reproducibility estimations. ICC values observed for the eAMD group, 0.72 (95% CI: 0.18, 1.00); for the iAMD group, 0.66 (95% CI: 0.42, 0.89); for the aAMD group, 0.80 (95% CI: 0.54,1.00); and for the nAMD group, 0.61 (95% CI: 0.17,1.00).

**DISCUSION**

MP carotenoids, lutein, zeaxanthin, and meso-zeaxanthin, have been assigned a putative protective role for AMD based on their ability to become absorbers of harmful light and antioxidants reacting with reactive oxygen species.13 The MP carotenoids can only be obtained from dietary sources by eating determined fruit and vegetables or supplements intake. Several studies have showed in different populations that diet plays an important role in the progression of AMD, because a diet rich in lutein or zeaxanthin decreases the risk of AMD.14-16 Consequently, assessing in vivo MP has become an important issue when dealing with AMD patients. Color perimetry is a psychophysical technique able to indirectly assess MPOD, and as any other evaluation test, must provide reliable measurements to avoid misleading when counseling AMD patients.

In our study, we evaluated the intra-session repeatability of the color perimetry technique in AMD patients, and we found CVw values were above 22% for all the AMD groups evaluated (Table 1). We did not find differences in mean MPOD values among the three consecutive measurements performed, however, a diagnostic test that might provide outcomes having variability above 22% cannot be considered adequate for clinical purposes. Regarding intra-session repeatability ICC values, all of them were below 0.8 (ICC for nAMD=0.79). The absence of a high ICC value (>0.90) indicates that most of the variability observed for the three MPOD measures is due to differences within the same AMD patient, instead of differences among all AMD patients recruited. Therefore, these outcomes show that color perimetry technique provides only moderate agreement between MPOD measurements obtained during the same session.

We decided to perform an inter-examiner reproducibility study because it has been already highlighted the importance of the data interpretation for MPOD results, at least when performing the HFP technique.17 In case of color perimetry technique, it can be initially thought that examiner might not play such an important role, however, it is psychophysical test like conventional automated perimetry. And for this later technique, it has been reported that threshold is affected by the way examiner delivers instructions, because conservative (*vs* liberal) instructions can cause patients to be more reluctant to respond.18 Consequently, it was worth to assess the agreement between two different examiners when performing the color perimetry technique. Similarly to the outcomes observed for intra-session repeatability analysis, inter-examiner reproducibility measurements yielded CVw values above 20% (Table 2) and ICC values ≤ 0.80. Inter-examiner reliability outcomes were not poorer than intra-examiner ones; however none of them is good enough to recommend color perimetry as a consistent tool to assess MPOD.

We were able to find in the literature only another study using color perimetry to assess MPOD. Demirel et al.19 evaluated the inter-session reproducibility of color perimetry after assessing healthy volunteers during three visits in three consecutive days. These authors obtained a wide range of ICC results (0.48 to 0.82) depending on the visits selected for the inter-session reproducibility estimation. These ICC values are within the range that we obtained in different AMD groups. These findings further support that the reliability of color perimetry to assess MPOD is low.

The reliability of other phsycophysical techniques to assess MOPD have been also estimated, despite different statistical variables has been used to estimate it. Several authors have evaluated the ability of HFP to provide consistent data. Loughman et al.20 obtained MOPD values during 3 different sessions using the HFP technique in healthy subjects. Based on the data they reported, the lowest inter-session reproducibility CVw value that could have been estimated (visit 2 *vs* visit 3) was around 28% for the MPS 9000 device (Tinsley Precision Instruments Ltd, Croyden, Essex, UK), and, close to 14% for the Macular Densitometer device (Macular Metrics II, Rehoboth, MA, USA). Likewise, Kinkelder et al.21 obtained MPOD values using two different devices (Macuscope; Macuvision Europe Ltd., Lapworth, Solihull, UK; and QuantifEye; MPS 9000 series), and estimated relative differences (defined as the differences of two values divided by their mean value) between two consecutive measurements. They obtained values of 32.2% and 18.1% for each instrument, respectively. Taking into account that the variability of MPOD as measured with the HFP technique was high, Howells et al.17 proposed a new improved protocol to assess MOPD using the MPS 9000 device. The protocol consisted of removal and adjustment of certain data with lower quality parameters. These authors evaluated healthy volunteers, and after upgrading the measurement procedure they estimated a better CVw value for intra-session and inter-session tests (around 19% and 12% based on the data reported, respectively).

Regarding the inter-rater agreement of the HFP technique, Bartlett et al.22 evaluated young normally sighted volunteers who underwent HFP assessment using the MPS 9000 instrument (also known as QuantifEye). They evaluated volunteers using two different observers during the same session (same day) on two occasions separated one week. Based on the data that they reported, the CVw they obtained was likely to be >35%. However, this study was performed prior to the upgrade of the measurement procedure later published by Howells et al.17

Random error estimated in the present study prevents from advising the use of color perimetry to monitor MP in AMD patients. We did not find significant differences between the 3 consecutive tests that we performed for estimating the intra-session repeatability. Therefore, it seems that there was not either fatigue or learning effect in our AMD patients, thought we cannot completely rule out its influence. The source of the random error that we found might be inherited from the psychophysical nature of the procedure. OCT and autofluorescence images were taken to be sure that the foveal area was well preserved in studied patients even thought they had advanced disease. Besides we performed a fixation test analysis with a microperimeter to ensure central fixation in our AMD patients, so that participants could stare at the fixation targets presented during the procedure. However, it does ensure that patient keeps their gaze properly align during the whole test. The device that we used to perform the color perimetry technique has got an eye-tracker, so that patient could be monitored throughout the whole procedure, and instructions could be provided in case excessive eye movement exists. Besides, the color perimetry technique requires also to project stimuli to para-central areas (10 degrees eccentricity) and we can not completely rule out that some of these explored points might be affected because of the own AMD disease in advanced cases. Consequently, reliability outcomes observed in our study are expected to be reduced in comparison with other studies that reported reliability data from other MPOD techniques recruiting only healthy young adult volunteers.

As previously mentioned, in-vivo MPOD measures can be also obtained using physical methods, and fundus reflectometry might be the most common. Thus, reliability studies have been also performed to assess its clinical validity. Dragostinoff et al.23 reported a CVw value of 6.2% and 8.0% in the healthy and AMD patients, respectively, after performing 5 evaluations in 5 consecutive days using a was measured with a custom-built densitometer. Moreover, Creuzot-Garcher et al.24 reported good ICC values (>0.80) when measuring MPOD with a commercial reflectometry technique (Visucam 200; Carl Zeiss Meditec AG, Jena, Germany), as long as the same examiner performed always the intra- and inter-session tests. However, they found that ICC values decreased to 0.73 when the evaluation was performed by two different examiners. Consequently, based on the reliability data reported by several authors, it seems that physical (objective) techniques should be used to evaluate MPOD instead of psychophysical (subjective) ones, to ensure more consistent data.

One limitation of the present study is the sample size. Taking into account the large prevalence of AMD in Occidental countries, we cannot ensure that our AMD sample fully represents the whole population of Caucasian AMD patients. Nonetheless, our aim was to estimate the reliability of the color perimetry technique to measure MPOD, and we recruited enough diverse AMD patients to provide valuable results for the daily clinic. Another limitation was that color perimetry evaluation follows a similar testing procedure as standard automated perimetry (i.e. monocular testing, pressing a bottom each time a stimulus is seen, bracketing techniques to obtain sensitivity thresholds, etc). And we did not take into consideration the possible experience on previous visual field testing of the recruited AMD patients. Nonetheless, we did not find MPOD differences among the outcomes obtained for intra-session repeatability analysis, in contrast to what should be expected in case there was learning effect.

In conclusion, the intra-session repeatability and inter-examiner reproducibility of color perimetry technique to evaluate MPOD in AMD patients is not enough good to advice the use of this psychophysical technique neither for research nor in clinical ophthalmological or optometric practice. It seems that objective techniques can provide better reliability outcomes than the subjective ones when evaluating the same AMD patients.25 Thus, instruments based on objective techniques should be recommended for clinical purposes when counseling AMD patients in their early-intermediate stages of the disease, taking into account that adequate diet supplementation produces short-term26 and long-term6-7 MP improvements.

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**DECLARATION OF INTEREST**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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