

## In Vivo Assessment of Retinal Carotenoids: Macular Pigment Detection Techniques and Their Impact on Monitoring Pigment Status<sup>1</sup>

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**ABSTRACT** Of the many carotenoids found within human tissue, only the carotenoids within the human retina can be assessed noninvasively at present. Such assessment should eventually provide a more complete understanding of the functional role of retinal lutein (L) and zeaxanthin (Z) (termed macular pigment, MP) in human vision. The emerging data allow for some initial observations. For example, there appears to be wide variation (>factor of 10) in the concentration of MP. Although MP levels have been recorded from nondetectable to 1.20 OD (optical density), the “average” levels, relative to what is possible, appear low. This may be due in part to the low average dietary intake of L and Z in the typical U.S. diet. Nonetheless, individual differences in MP may also be influenced by nondietary factors such as genetics, demographics and lifestyle characteristics. Some evidence indicates that the MP carotenoids may protect the retina and lens, and could improve vision through some optical mechanisms. Consequently, efforts to determine typical MP levels and the factors that influence individual differences in MP density should be continued. *J. Nutr.* 132: 535S–539S, 2002.

**KEY WORDS:** • *lutein* • *macular pigment* • *retinal carotenoids*

The macular pigment (MP) of the eye is composed primarily of three isomeric carotenoids, lutein (L), zeaxanthin (Z) and mesozeaxanthin (MZ). Lutein and Z are entirely of dietary origin, whereas MZ is hypothesized to arise from the conversion of L to MZ in the retina (1). Although these pigments are found throughout the tissues of the eye, they are concentrated in the macula lutea region of the retina, including the central retinal depression called the fovea in which the cone photoreceptors reach their maximal concentration. The fovea is a relatively small area within the macula, whose ganglion cells project onto a relatively large area of the visual cortex.

The fovea is particularly important for functional vision (e.g., acuity); legal blindness results when this area is lost to disease. For example, age-related macular degeneration (AMD) is characterized by pathologic changes in the retina, retinal pigment epithelium (RPE) and/or the choroid and preferentially affects the macular region of the retina. This is the leading cause of irreversible vision loss in the United

States among those  $\geq 65$  y old (2), and there is no treatment available for most patients (3). The loss of central vision results in the possible inability to recognize faces, to read or drive a car and therefore has a significant effect on an individual's ability to live independently (4). As summarized in a separate manuscript in this supplement (5), some epidemiologic evidence supports a role for dietary intake of the MP carotenoids L and Z in protection against age-related cataract and macular degeneration.

Consequently, there continues to be a critical need to assess MP in diverse populations and age groups (6), not only to determine the effects of intervention strategies such as dietary manipulations (7,8) and carotenoid supplement use effects (9,10) on MP status and disease risk (10) but also to compare different MP assessment techniques (6,10–14).

### Macular pigment assessment

Macular pigment has been assessed using a variety of ex vivo and in vivo techniques. These include but are not limited to autopsy analysis using chromatography (15,16) and microdensitometry (17), Raman detection (18), scanning laser ophthalmoscope (SLO) (19), color matching (20), reflectometry (6,10,19,21), autofluorescence spectrometry (6) and heterochromatic flicker photometry (HFP) using “Maxwellian” and “free-view” systems (12,13,22–24).

The ex vivo analysis of carotenoids within retinal tissue is most often done using HPLC (16) and microdensitometry (17). After the tissue has been prepared, HPLC separates the lipid-soluble carotenoids as they pass through a column of materials (usually silica or glass beads) so that different carotenoids elute at different times on the basis of their polarity.

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<sup>3</sup> Abbreviations used: AMD, age-related macular degeneration; BMI, body mass index; HFP, heterochromatic flicker photometry; L, lutein; MP, macular pigment; MZ, mesozeaxanthin; RPE, retinal pigment epithelium; SLO, scanning laser ophthalmoscope; Z, zeaxanthin.

High pressure is used to enhance the separation capacities of the system, and spectral absorption profiles can be obtained. Microdensitometry or microspectrophotometry is performed on excised retinas. In this procedure, the retina is extirpated from an enucleated eye and then fixed in a solution that will both preserve the tissue and help maintain its structure. Spectral absorbency of the carotenoids is then determined by the use of measuring beams directed at a small area of tissue. Contaminants from the fixative and degradation of the tissue before enucleation are limitations of this method.

In vivo analysis of L and Z can be done through a wide variety of techniques. One unique aspect in the study of retinal carotenoids is that, unlike other bodily tissues, L and Z can also be measured noninvasively. This is important because it allows larger numbers of subjects to be tested repeatedly (e.g., allowing active monitoring of interventions). It also bypasses some of the uncertainties inherent in using carotenoid blood samples as proxy indicators of the tissue status of the macular region. Although blood samples provide an indication of nutrient transport, they do not necessarily indicate retinal carotenoid deposition. MP optical density determinations may be more precise and indicative of long-term ocular nutrient status (13).

Photographic assessment of MP is accomplished by taking two photographs of the fundus (the inner basal surface of the eye), one obtained in short-wavelength (blue light, which is strongly absorbed by MP) and the other in middle-wavelength (green light, which is not absorbed by MP). These two photographs are then superposed and the densities are subtracted. The greatest difficulty with this method is the comparability of the photographs and optical interference due to anterior structures of the eye. These problems are exacerbated with age. Analysis of MP by the SLO and reflectometry methods is also typically based on a comparison of measures made using short- and long-wavelength light. With the SLO, MP is derived by subtracting measures obtained using a blue-green laser from measures obtained using a red laser. With reflectometry, monochromatic light is passed through the eye (after the photopigment has been bleached to minimize absorption) and is reflected off the back of the eye to a photodetector. The ratio of emitted and reflected light is used to derive absorption indices. Some authors have reported high reliability measuring MP with SLO and reflectometry (10).

Macular pigment can also be measured using Raman spectroscopy, which relies on the wavelength shifted scatter (i.e., resonance) of low energy laser light (18). These Raman signals, based on the vibrational or rotational motions of L and Z molecules, are stronger (within the waveband 440–550 nm) than most Raman signals from other materials and may therefore allow in vivo assessment. Although this method has not yet been validated, it is fast and direct.

Another direct method is based on the fluorescence of lipofuscin. By comparing the fluorescence of lipofuscin (metabolic debris located within the RPE) at 470 and 550 nm in the fovea where MP is the most dense and a parafoveal location where MP is optically immeasurable, a single-pass density measure can be derived. Both Raman detection and the fluorescence method are technically challenging and are therefore not feasible for many laboratories. Recent data suggest that the fluorescent method provides data that compare well with the more common psychophysical method of measuring MP on the basis of flicker photometry (5).

Psychophysical methodology using heterochromatic flicker photometry (HFP) takes advantage of the fact that MP is located anterior to the photoreceptors (25,26). The smaller the amount of light that is transmitted, the greater the OD of

the tissue. The density measured is the sum of the carotenoids present in the fovea (i.e., L, Z and MZ) (13). The basic measurement procedure involves presenting a small test stimulus that alternates between 460 and 550 nm in the fovea and parafovea. This test light appears to “flicker” and the subject is instructed to adjust the intensity of the 460-nm light until it matches the 550-nm light, which minimizes or eliminates the flicker. The amount of 460 nm light that is necessary to reach this minimum or null point is used to derive MP optical density.

A major limitation of this method is the fact that it is based on the subjects' successful completion of the task, which is often difficult for children or subjects with serious impairments in vision or cognition. Depending on the number of sites evaluated and the experience of the subject, assessment can take between 25 and 90 min. There might also be systematic differences between instruments used by different research groups (10). Training is required for designated personnel such as laboratory technicians or researchers to conduct the assessment, but the evaluation does not require the expertise or presence of medical personnel. Results are easy to calculate and interpret. The measurements do not require the use of pharmacologic agents, making repeat measures easy to perform. The method can be used on a large proportion of the population and may be more practically accessible to a larger number of laboratories (12). Because measurement of MP using flicker photometry appears to be valid, reliable and expeditious (27), this method is now being applied to larger populations and the effects of individual differences in MP can now begin to be considered.

#### **Macular pigment and its correlates as determined using HFP assessment**

The limited research on MP in vivo using HFP has been reported for ~840 subjects during the past 14 y (Table 1). Most HFP studies have typically measured MP at 0.50° retinal eccentricity, using a centrally fixated 1° test stimulus. The site measured in the fovea, combined with one parafoveal measurement is used to calculate MPOD (22,24,28). Values range from nondetectable to ~1.20 OD, with many studies indicating a mean MPOD between 0.10 and 0.40 OD when using a 1° central retinal stimulus. It is important to emphasize that these numbers represent MP density at one location within the retina and do not reflect the entire MP spatial distribution. Macular pigment has a sharp peak in the very center of the fovea, and then declines exponentially with increasing eccentricity (26). Based on relatively limited data (16,27), it is assumed that MP density approaches an asymptote between 4 and 6° of visual angle (~1–1.8 mm). This asymptote is used as the “zero” reference when making psychophysical measurements.

**Sex.** Several studies have suggested that females may have lower MP density than males (22,29,30). These differences range from 13 to 38%. Some studies, however, have not found sex differences in MP density (27,31)(Table 1). Differences between studies may be explained by the different characteristics of the samples. For example, the women in the study of Cuilla et al. (27) had significantly higher intakes of L and Z and higher total serum carotenoid levels compared with the men, making a direct comparison between the two groups difficult.

**Age.** Werner et al. (28) suggested that there may be a slight decline in MPOD as people age, but this decline was not found to be significant (Table 1). In his study, age accounted for ~4% of the variation in MPOD. Age has not been linked

TABLE 1

Summary of Studies on MPOD and Heterochromatic Flicker Photometry (30)

Study #*	#1	#2	#3	#4	#5	#6	#6	#6	#7	#8 <sup>^</sup>	#9
Groups**						A.	B.	C.			
Age range	21–39	10–90	19–22	19–83	21–63	22–36	60–84	22–84	17–19	21–81	18–51
Total # of subjects	27	50	20	88	32	10	27	37	217	46	280
Foveal Stimulus Size		1.00°	1.00°	1.00°	20 minutes	1.00°	1.00°	1.00°	1.00°	0.95°	1.00°
Site	0.67	0.50	0.50	0.50	1.00	0.50	0.50	0.50	0.50	0.475	0.50
Assessed: Degrees Retinal Eccentricity											
Mean MPOD	0.77	0.39	0.28	0.38: m 0.24: f	0.29	0.40	0.46	—	0.24: m 0.21: f	0.29	0.21: m 0.21: f
Age	—	ns	—	—	ns	—	p < 0.01 <sup>^^</sup>	ns	p < 0.02 <sup>^^</sup>	p < 0.0001 <sup>^^</sup>	ns
Sex	—	—	—	p < 0.001	—	—	—	—	p < 0.05	ns	ns

\* #1 (41), #2 (28), #3 (42), #4 (22), #5 (26), #6 (43), #7 (29), #8 (35), #9 (27).

\*\* Subgroups within study #6:

A = Young group of subjects

B = Old group of subjects

C = Combination of A and B

\*\*\* Significantly different from comparison group within the study reviewed.

<sup>^</sup> Method used to determine MPOD by HFP differed from all other investigators, thereby making comparison difficult. Results based on a subset of subjects identified as not at risk of AMD in England. All other studies are from subjects assessed in the United States.<sup>^^</sup> Macular pigment declined with age.

f = females.

m = males.

ns = not significant.

consistently to a decline in MP, although advanced age has (as the name implies) been strongly associated with the development of AMD (2,32–35). Beatty et al. (35) and Hammond et al. (36) suggested that there might be a trend toward a reduction in MP as individuals age, particularly in those >60 y old. This trend was relatively mild in the study of Hammond et al. of individuals (aged 22–36 and 60–84 y); there was about a 20% decline in MP associated with aging. This trend was more pronounced in Beatty's population (aged 21–81 y) in which the reported decline was close to 50%. Although these studies did not determine the reason for these declines, dietary changes may represent one possible explanation.

**Body mass index (BMI).** Because carotenoids are stored in body fat, it might be expected that MP density would be influenced by individual differences in body fat content. Consistent with this view, Hammond et al. (36) recently reported that an inverse relationship was found between MPOD and BMI ( $n = 680$  subjects,  $r = -0.12$ ,  $P < 0.0008$ ) and MPOD and percentage of body fat ( $n = 400$ ,  $r = -0.12$ ,  $P < 0.01$ ). These results were largely driven by the subjects with higher BMI (>29 kg/m<sup>2</sup>, 21% less MP) and a higher percentage of body fat (>27%, 16% less MP) compared with those individuals with lower BMI and percentage of body fat. Reported dietary intake and serum carotenoids were lower in those with higher BMI values in the subset of 280 subjects for whom dietary and serum values were available, suggesting that dietary differences might partially account for the lower MP values of the obese subjects. This interpretation is consistent with previous findings linking higher BMI with lower levels of circulating carotenoids (37,38).

**Dietary and serum L and Z values.** For many individuals, dietary and serum levels of L and Z are related to MPOD values (7,13,22). Hammond et al. (22) found that plasma L and Z were significantly related to dietary L/Z intake as reported by men ( $r = 0.70$ ,  $P < 0.0005$ ) and women ( $r = 0.56$ ,  $P < 0.005$ ). In addition, MPOD was also significantly related to plasma L and Z for men ( $r = 0.62$ ,  $P < 0.005$ ) and women ( $r = 0.30$ ,  $P < 0.05$ ) (21). Ciulla et al. (27) found that reported dietary L/Z intake accounted for 2.01% ( $P = 0.01$ ) of MPOD variance and that serum levels of L and Z predicted 7.05% ( $P < 0.0001$ ) of MPOD variance.

Hammond et al. (7) found that MPOD values could be increased in some, but not in all test subjects, after increased consumption of Z- and/or L-rich foods. Landrum et al. (9) found that two subjects given L supplements for 140 d experienced a mean increase in the peak MP density of 39 and 21%.

**Iris color.** Lighter iris color is related to low ocular melanin. Melanin and carotenoids may also protect the retina through similar mechanisms. Evidence (27,29,30,39) indicates that lower MP density is related to lighter iris color. This relationship may be the result of similar environmental pressures (e.g., light and oxygen) affecting the accumulation of both pigments and/or the depletion of MP that could result from the increased transmission of light through the sclera and iris of subjects with lighter colored irises. Increased transmission of light results in greater oxidative stress, which could theoretically deplete pigments acting as antioxidants.

**Smoking.** Smoking has been shown to deplete serum carotenoids and has consistently been identified as a risk factor for

AMD. Some evidence has indicated that MP is lower in current smokers (6,30). This effect appears to be particularly strong (e.g., dose dependent) when dietary factors are matched or controlled for (22). Nonetheless, because carotenoid supplementation has been shown to increase risk of lung cancer in heavy smokers, supplementation with L and Z for smokers with lower retinal levels must be approached with caution.

### Summary and future directions

The past 15 y have provided an important start for an interdisciplinary investigation on the significance of MP. Although correlates between MP and retinal health have emerged, there is much work yet to be done. For example, the functionality of MP is yet to be determined. Based on the available data representing a relatively small cross section of the U.S. population, a few conclusions are possible. First, MP variance is high, ranging from undetectable to 1.20 OD. Second, relative to what is physiologically possible, the mean MPOD appears to be low in many of the samples studied. This may be particularly significant when considered over decades. The outer fovea of a subject with low MP would suffer much higher exposure to actinic short-wave light transmission (near 100%) than a subject with very high MP (whose MP could block 97% of the transmission of this light). Finally, although MP appears to remain stable over time, it is not immutable. Intervention with diet and supplements appear to modify the MP density of most subjects.

Most of what is known about MP has been gathered by studying healthy individuals free of disease and at low risk of developing eye disease using the *in vivo* assessment techniques available. Although the methodology for assessing MP has been validated in normal nondiseased subjects, it is still unclear whether it is valid in subjects with retinal disease. This must be a priority in assessment development. Other important questions remain. For example, nearly all of the evidence supporting a protective (or optical) function for MP is indirect. If MP does protect the retina and/or improve visual performance, what level of MP is optimal? Is it feasible to supplement L to patients who already have retinal degeneration? Many patients are taking L supplements as researchers are beginning to study the effects of such interventions (8,30,40). Assessment techniques must be able to determine small changes in MP concentration and distribution after supplementation or changes in diet. Future studies will have to address whether the retinal degeneration of patients with higher levels of MP progress at the same rate as patients with similar characteristics (e.g., age, sex or iris color) but lower levels of MP. Does increasing MP improve visual function in patients with eye disease? If so, is this improvement mediated through an optical mechanism (e.g., by absorbing light scattered by a dense lens or reducing the effects of chromatic aberration) and/or by actually treating the underlying causes of their disease (e.g., by stabilizing cellular membranes)? Much more evidence is required to make informed decisions regarding the use of L and Z for optimal visual function. It is imperative that the *in vivo* assessment techniques continue to be used to gather data and the techniques evolve to be able to address the many questions remaining.

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