HPLC Measurement of Ocular Carotenoid Levels in Human Donor Eyes in the Lutein Supplementation Era

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Purpose. A substantial proportion of the population at risk for visual loss from age-related macular degeneration consumes supplements containing high doses of lutein, but clinical studies to date have shown only modest and variable increases in macular carotenoid pigments in response to supplementation. To determine whether lutein supplementation can indeed alter ocular carotenoid levels, the authors chemically measured levels of lutein, zeaxanthin, and their metabolites in the macula, peripheral retina, and lens of 228 eyes from 147 human donors and correlated these results with retrospective supplement histories from families of selected members of the study population.

Methods. Lenses and circular punches of macula (4-mm diameter) and equatorial peripheral retina (8-mm diameter) were dissected from donor eyes free of ocular disease procured from the local eye bank. The amounts of lutein, zeaxanthin, *meso*-zeaxanthin, and 3′-oxolutein were determined by HPLC with photodiode array and mass spectral detection.

Results. Eighteen percent of eyes from donors age 48 and older had unusually high levels (66.3 ± 15.1 ng) of macular carotenoids that were three times the rest of the older population's mean level (23.0 + 12.1 ng; $P < 0.001$). Carotenoid levels in these outliers were also unusually high in the lens and in the peripheral retina. Similar outliers were not present in donors younger than 48. Most of these outliers regularly consumed high-dose lutein supplements before death. Lutein supplementation was uncommon in older donors whose macular carotenoids were in the normal range.

Conclusions. The presence of unusually high levels of macular carotenoids in older donors who were regularly consuming high-dose lutein supplements supports the hypothesis that long-term lutein supplementation can raise levels of macular pigment. Elevated carotenoid levels in the peripheral retina and lens in these same donors could have important implications for understanding why some clinical methods of macular pigment measurement have had difficulty detecting robust and consistent responses in carotenoid supplementation trials. (*Invest Ophthalmol Vis Sci.* 2007;48:543–549) DOI:10.1167/iovs.06-0558

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In the past decade, a profound shift has occurred in the clinical management of patients at risk for visual loss from age-related macular degeneration (AMD).[1–4] AMD had long been considered to be in part a disorder related to chronic oxidative stress, but it was not until release of the results of the Age-Related Eye Disease Study (AREDS) in 2001 that definitive clinical recommendations could be made of the value of antioxidant supplementation in patients at high risk.[5] The AREDS formulation of high-dose zinc, vitamin C, vitamin E, and β-carotene was based on the best nutritional recommendations at the inception of the study in the 1980s, but subsequent studies indicated that other nutrients may be even more valuable in the prevention of severe visual loss from AMD.[6–8] The macular xanthophyll carotenoids, lutein and zeaxanthin, are of particular interest because, unlike β-carotene, they are specifically concentrated in the foveal region of the primate macula, where they can act as antioxidants and as optical filters for blue light, the most phototoxic region of the visible spectrum.[9–11] In the mid-1990s, publication of a series of studies from the Eye Disease Case-Control (EDCC) Study Group demonstrated that persons with high blood levels of lutein and zeaxanthin and high consumption of foods rich in these same carotenoids had significantly lower risk of exudative AMD,[12,13] and a number of subsequent independent epidemiologic studies and the AREDS study group have confirmed these findings.[13,14] Based on these results, it has become commonplace to add high doses of lutein (typically 6–20 mg/day as opposed to average dietary consumption of 1–2 mg/day) to supplements marketed to the elderly at risk for visual loss from AMD. Supplements containing high levels of zeaxanthin are also available, but their use is still uncommon. A follow-up study to AREDS (AREDS II), which will incorporate lutein and zeaxanthin supplementation, has been initiated to provide definitive prospective recommendations on the value of these supplements for AMD.

Carotenoid supplementation strategies for AMD rely on the assumption that consumption of these supplements will lead to clinically significant increases in the macular carotenoid pigment, but published and unpublished studies have reported unexpectedly modest and variable responses despite consistent increases in serum levels.[15–23] Possible explanations of these results include inadequate study length, saturation of ocular xanthophyll-binding proteins, and inherent limitations of the noninvasive measurement methods. The most commonly used noninvasive measurement methods, heterochromatic flicker photometry and autofluorescence imaging, operate under the premise that carotenoid levels at a peripheral retinal reference point are so low that they are optically undetectable.[24–29] This assumption has been well validated by studies on primate cadaver eyes that demonstrated the concentration of macular carotenoids per unit area measured by absorption spectroscopy, resonance Raman spectroscopy, and high-pressure liquid chromatography (HPLC) is 10 to 100 times higher in the macula than it is in the peripheral retina.[9–11,30–34] It should be noted, however, that this assumption cannot be simply extrapolated to the supplemented situation because these autopsy studies were conducted before the lutein supplementation era. It is possible that the pharmacologic doses of lutein currently consumed by many elderly persons could lead to

substantial increases in peripheral lutein levels that would artifactually alter noninvasively measured macular responses. To assess this possibility, we measured ocular carotenoid levels by HPLC in a large number of human donor eyes now that high-dose lutein supplementation has become a common practice among older adults in Utah.

METHODS

Chemicals

Standards of (3*R*,3′*R*,6′*R*)-lutein, zeaxanthins [(3*R*,3′*S-meso*)-zeaxanthin and dietary (3*R*,3′*R*)-zeaxanthin], and 3′-oxolutein were generous gifts from Kemin Health (Des Moines, IA), DSM (Schaffhausen, SH, Switzerland), and BASF (Ludwigshafen, Germany), respectively. They were individually dissolved in hexane containing 0.1% butylated hydroxytoluene (BHT) at concentrations of 1 μg/mL, stored at −70°C, and brought to room temperature before use. Composite working standard solutions were prepared by combining suitable aliquots of each individual standard stock solution and diluting them with HPLC mobile phase. Organic solvents were HPLC grade from Fisher Scientific (Hampton, NH).

Preparation of Macular, Retinal, and Lens Tissue Samples

Human donor eyes were obtained between January 2004 and April 2005 from the Utah Lions Eye Bank within 24 hours of death after corneas had been harvested for transplantation. Tissue procurement and distribution complied with the tenets of the Declaration of Helsinki. All eyecups were visually inspected to exclude the presence of obvious ocular disease. After carefully removing overlying vitreous, macular tissue was excised with a 4-mm–diameter circular trephine centered on the fovea, and peripheral equatorial retinal tissue was excised with an 8-mm–diameter circular trephine, making certain that the nearest edge of the punch was at least 10 mm from the fovea. Tissue samples were extracted three times with tetrahydrofuran (THF) containing 0.1% BHT by sonication at 5°C to 10°C for 30 minutes each time. Whole lenses were homogenized and then extracted in the same manner. Combined extracts were evaporated to dryness under vacuum at room temperature. The dried residue was re-dissolved in 1 mL HPLC mobile phase and centrifuged at approximately 2000*g* for 10 minutes to remove the minor amounts of insoluble solid particles.

HPLC Conditions

The extracts were analyzed on two HPLC systems on a gradient HPLC (Thermo Separations, San Jose, CA) equipped with a high-sensitivity UV6000 photodiode array detector.

System 1: Routine Carotenoid Separation. The mobile phase contained hexane/dichloromethane/methanol/*N*,*N*′-di-isopropylethylamine (80:19.2:0.7:0.1 vol/vol). HPLC separation was carried out at a flow rate of 1.0 mL/min on a cyano column (Microsorb, 25-cm length × 4.6-mm inner diameter [ID]; Rainin Instrument Co., Woburn, MA). The column was maintained at room temperature, and the HPLC detector was operated at 450 nm. Peak identities were confirmed by photodiode-array (PDA) spectra, by coelution with authentic standards, and by mass spectrometry. Calibration curves for peak quantitation were performed by injection of known amounts of carotenoid standards. We do not routinely include an internal standard because it can mask the presence of low abundance carotenoid metabolites, especially when samples are split and run on multiple HPLC columns.[35] We periodically perform quality control runs to confirm that our extraction and injection efficiencies exceed 95%.

System 2: Chiral Carotenoid Separation. The mobile phase contained hexane/isopropanol (95:5 vol/vol). HPLC separation was carried out at a flow rate of 0.7 mL/min on a chiral column (ChiralPak AD, 25-cm length × 4.6-mm ID; Chiral Technologies, Exton, PA) using the detection methods described.

Mass Spectrometry Equipment and Conditions

Levels of ocular carotenoids and their metabolites were sometimes so low that reliable quantitation by absorbance at 450 nm was not possible. In these situations, we used in-line mass spectrometry analysis because it is approximately 100 times more sensitive.[36] HPLC-MS was performed (Thermo Electron MSQ, San Jose, CA) with a single quadrupole mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) source after a 50% split of the column eluant. The molecular ions were initially acquired in full scan mode from 200 to 1000 Da with 0.2-step size and 2-ms dwell time. Selected ion monitoring (SIM) was performed using a dwell time of 200 ms for each channel. In SIM mode, the *m/z* channels 551 ± 1 ($M–H_2O^+$) and 569 ±1 (M^+) were used for lutein, 569 ±1 for zeaxanthin (M^+), and 567 ± 1 for 3′-oxolutein (M^+). Typical conditions were corona discharge current, 5 μA; RF lens bias voltage 0.1 V; cone voltage, 80 V; and heater temperature, 550°C. The ion source and tuning lens parameters were optimized by infusing standards through the built-in injector. For routine quantitative analysis, we used software (ThemoElectron MSQ Quan Browser; XCaliber Systems, Brazil, IN) that provided a peak integration of the ion intensity for each of the programmed mass ranges against standard curves generated from injection of authentic carotenoid standards. Automated analysis was followed by manual confirmation of in-source fragmentation patterns.

Statistical Analysis

Statistical analysis was performed (Origin version 6.0; Microcal, Northampton, MA). In most cases a two-population (independent) *t* test was performed with a significance level set at 0.05. All reported values are mean ± SD.

RESULTS

Total carotenoid levels were determined in 4-mm–diameter macular punches from 228 eyes from 147 donors ranging in age from 5 to 86 (Fig. 1A). Visual inspection of the data revealed that a cohort of donors 48 and older had unusually high levels (>50 ng) of total carotenoids. We designated this population as outliers for further analyses. These outliers comprised 13% of total eyes (29/228) and 18% of older eyes (29/160) and 14% of total donors (21/147) and 21% of older donors (21/99).

The average age for outlying donors was 65 ± 10 (range, 48 to 84; $n = 21$) years, and the "normal" older donor age was 64 ± 9 (range, 48 to 86; $n = 78$) years. The mean total macular carotenoid level of older outlier eyes was 66.3 ± 15.1 ng ($n = 29$), and the mean for the rest of the older donor eyes was 23.0 ± 12.1 ng ($n = 131$). These means were significantly different from each other ($P < 0.001$). A similar analysis in which all pairs of older donor eyes ($n = 45$ pairs) were averaged before statistical testing yielded a comparable statistically significant result ($P < 0.001$). Our nonoutlier macular carotenoid density of 1.79 ng/mm^2 for a 4-mm punch compares favorably with previously published values of 1.69 ng/mm^2 for 4.6-mm punches[11] and 1.39 ng/mm^2 for 8-mm punches.[10]

Both eyes were available for analysis in 15 of the 21 outlying donors. The concordance rate (>50 ng in both eyes) of these outliers was 53% (8 /15 pairs). The average level of carotenoids in the fellow eyes of discordant outliers (39.8 ± 4.4 ng; $n = 7$) was 45% less than that of the outlier eyes themselves (72.1 ± 25.2; $n = 7$; $P < 0.001$) but was significantly higher than the mean value of the rest of the "normal" older eyes (21.3 ± 11.3 ng; $n = 124$; $P < 0.001$).

Retrospective nutritional supplement and dietary histories were obtained from the families of 17 of the 21 outliers and

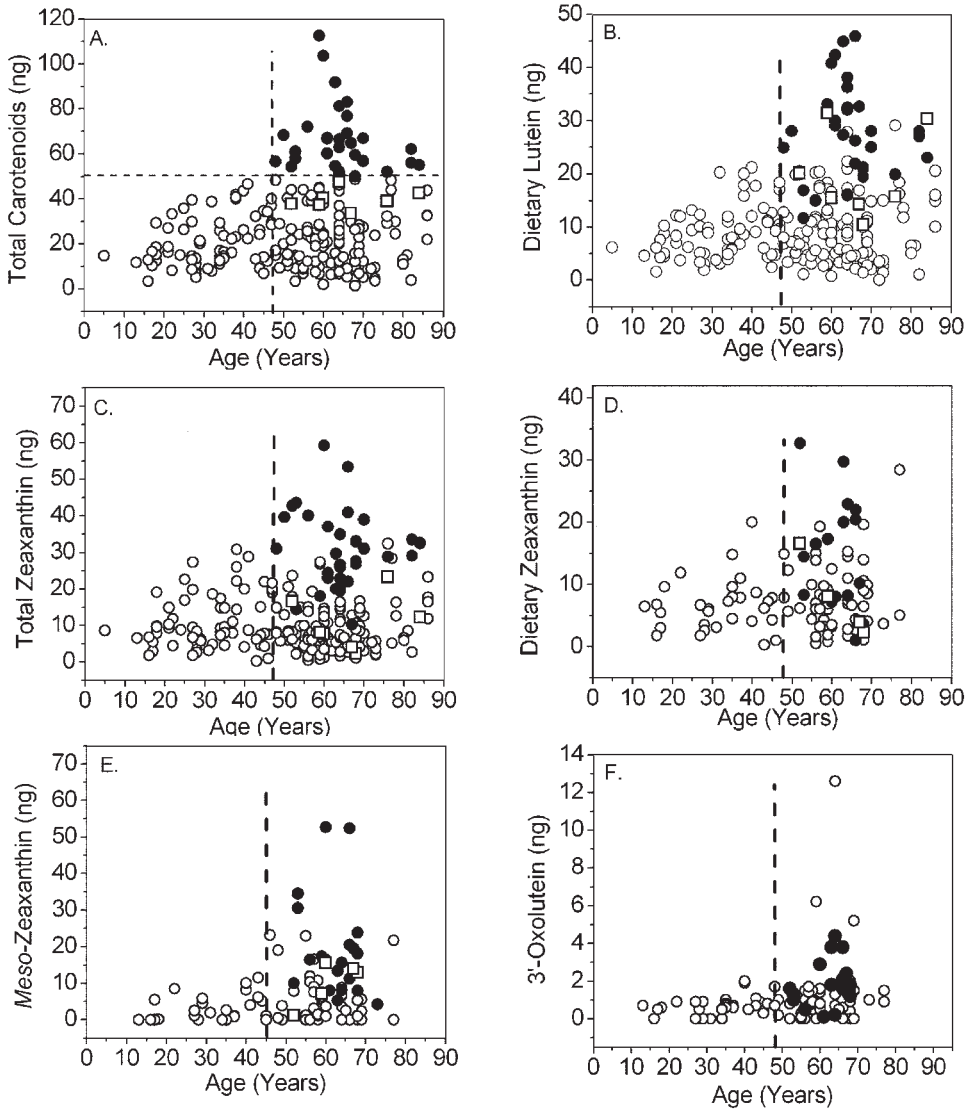

FIGURE 1. Age distribution of total carotenoids (**A**), dietary (3*R*, 3′*R*,6′*R*)-lutein (**B**), total zeaxanthin (**C**), dietary (3*R*, 3′*R*)-zeaxanthin (**D**), (3*R*,3′*S-meso*)-zeaxanthin (**E**), and 3′-oxolutein (**F**) in normal (*open circles*) and outlier (*filled circles*) eyes. Discordant fellow eyes of outliers are shown as *open squares*. Carotenoids were extracted from 4-mm–diameter macular punches ($n = 228$) and analyzed by HPLC-APCI/MS. All figures were partitioned to highlight the fact that outliers were 48 years of age or older and had unusually high levels (>50 ng) of total carotenoids.

from a random sample of 20 of the rest of the older donors. Eighty-two percent (14/17) of the outliers' families reported that the donor regularly consumed a high-dose (2.5–10 mg) lutein supplement daily, and the rest of the outliers' families reported that before death, the donor routinely consumed an unusually carotenoid-rich diet daily including spinach, corn, broccoli, and orange juice. None of the 20 nonoutliers' families surveyed reported regular lutein supplementation or consumption of a carotenoid-rich diet ($P < 0.001$).

None consumed supplements that contained high percentages of zeaxanthin, though it should be noted that commercial lutein supplements typically contain 6% zeaxanthin, which would supply 0.36 to 1.2 mg of zeaxanthin from a 6- to 20-mg lutein supplement. The family of a 52-year-old outlier reported a diet unusually rich in zeaxanthin (daily servings of corn), and this donor had the highest dietary zeaxanthin level in the macula of any eye analyzed (Fig. 1D).

We determined which carotenoids were responsible for the extraordinarily high levels in the outliers' maculae. The high levels of total macular carotenoids were driven primarily by lutein and, to a lesser extent, by its probable metabolite *meso*-zeaxanthin (Figures 1B-1E). The average lutein levels were 27.7 ± 8.9 ng in outlier eyes ($n = 29$) and 9.3 ± 5.8 ($n = 131$) in the rest of the older eyes ($P < 0.001$), and the average total zeaxanthin in outliers was 24.0 ± 10.9 ($n = 29$) compared with 10.1 ± 6.8 ($n = 131$) in the rest of the older eyes ($P < 0.001$). We observed that the mean values for dietary (3*R*, 3′*R*)-zeaxanthin were not statistically significant ($P = 0.38$) for outlier eyes (13.3 ± 6.9 ng; $n = 16$) than they were for older normal eyes (10.1 ± 7.1 ng; $n = 94$). However, significantly higher levels of nondietary (3*R*, 3′*S-meso*)-zeaxanthin were observed in the outlier eyes (19.4 ± 14.1 ng; $n = 16$) than in older normal eyes (7.4 ± 9.6 ng; $n = 107$; $P < 0.001$). Similarly, the ratio of (3*R*,3′*R*)-zeaxanthin to (3*R*,3′*S-meso*)-zeaxanthin (Z/M ratio) was significantly lower in outlier eyes than in control eyes (Table 1). 3′-Oxolutein was not significantly higher in outlier eyes than in age-matched normal eyes (1.9 ± 1.3 ng [$n = 16$] vs. 1.2 ± 1.8 ng [$n = 60$]; $P > 0.05$; Fig. 1F).

We next examined whether carotenoid levels were elevated in nonmacular tissues of the outliers' eyes (Fig. 2). Although data on extramacular tissue were available for only a limited number of the donor eyes, lutein levels were significantly elevated in the outliers in the peripheral retina ($P < 0.001$), but total zeaxanthin levels were not elevated ($P = 0.12$). Lutein levels in 8-mm punches of the peripheral retina were 8.4 ± 6.6 ng ($n = 9$) compared with 2.2 ± 1.4 ng in older normal retinas ($n = 32$; Table 1). Our nonoutlier peripheral carotenoid density of 0.070 ng/mm^2 for an 8-mm equatorial punch compares favorably with previously published values of

TABLE 1. Carotenoid Content of the Macula, Peripheral Retina, and Lens of Normal and Outlier Eyes

	Lutein (ng)	Zeaxanthin (ng)	Z/M Ratio	3′-Oxolutein (ng)	Total Carotenoids (ng)
Macula (4-mm-diameter punch)					
Young	9.4 ± 4.5 (n = 68)	10.8 ± 7.0 (n = 68)	1.70 ± 0.23 (n = 68)	0.6 ± 0.5 (n = 68)	21.4 ± 11.0 (n = 68)
Old	9.3 ± 5.8 (n = 131)	10.1 ± 6.8 (n = 131)	1.36 ± 0.31 (n = 94)	1.2 ± 1.8† (n = 60)	23.0 ± 12.1 (n = 131)
Outliers	27.7 ± 8.9* (n = 29)	24.0 ± 10.9* (n = 29)	0.68 ± 0.30* (n = 16)	1.9 ± 1.3 (n = 16)	66.3 ± 15.1* (n = 29)
Peripheral retina (8-mm-diameter punch)					
Young (n = 32)	2.5 ± 1.2	1.1 ± 0.7	ND	ND	3.6 ± 0.7
Old (n = 32)	2.2 ± 1.4	1.0 ± 1.1	ND	ND	3.4 ± 1.8
Outliers (n = 9)	8.4 ± 6.6*	0.7 ± 0.4	ND	ND	9.0 ± 5.6*
Lens (whole tissue)					
Young (n = 30)	0.5 ± 0.3	0.4 ± 0.2	ND	ND	0.9 ± 0.2
Old (n = 51)	0.7 ± 1.7	0.4 ± 0.2	ND	ND	1.5 ± 1.6
Outliers (n = 8)	4.9 ± 2.9*	0.9 ± 0.4*	ND	ND	5.8 ± 2.4*

All values are mean ± SD. Young donors were less than 48 years old. Old donors and all outliers were at least 48 years old. Zeaxanthin, combined dietary (3*R*,3′*R*)-zeaxanthin and non-dietary (3*R*,3′*S-meso*)-zeaxanthin; Z/M Ratio, the ratio of (3*R*,3′*R*)-zeaxanthin to (3*R*,3′*S-meso*)-zeaxanthin in macular tissues; ND, not determined.

* Statistically significant differences between the outliers and the age-matched normals ≥ 48 years old (all $P < 0.001$).

† There were no significant differences between young and old normals (all $P > 0.05$) except in the case of 3′-oxolutein ($P < 0.001$).

0.067 to 0.047 ng/mm^2 at 5.8 to 12.2 mm eccentricity.[11] On average, the peripheral lutein/zeaxanthin ratio in outlier eyes was highly unusual: 12.0:1 compared with that of older normal eyes, 2.2:1.

In the lens, outliers had significantly higher levels of lutein and total zeaxanthin ($P < 0.001$; Fig. 2). Lutein and zeaxanthin levels were 4.9 ± 2.9 ng/lens (n = 8) and 0.9 ± 0.4 ng/lens (n = 8), respectively, compared with a lutein level of 0.7 ± 1.7 ng/lens and a zeaxanthin level of 0.4 ± 0.2 ng/lens in older normal eyes (n = 51; Table 1). In addition, the ratio of lutein to total zeaxanthin in the outlier eyes was observed to be higher (5.4:1) than in older normal eyes (1.8:1).

DISCUSSION

Our finding that approximately 20% of older donors in Utah have extraordinarily high levels of macular carotenoids, three times the general population's average, is striking evidence of the impact of recent public health recommendations for anyone at risk for visual loss from AMD to increase antioxidant consumption. The outliers were always in the upper age range, consistent with product marketing campaigns for high-dose lutein supplements that primarily target the elderly, and we were able to confirm retrospectively that most of these outlying donors did indeed consume high-dose lutein supplements

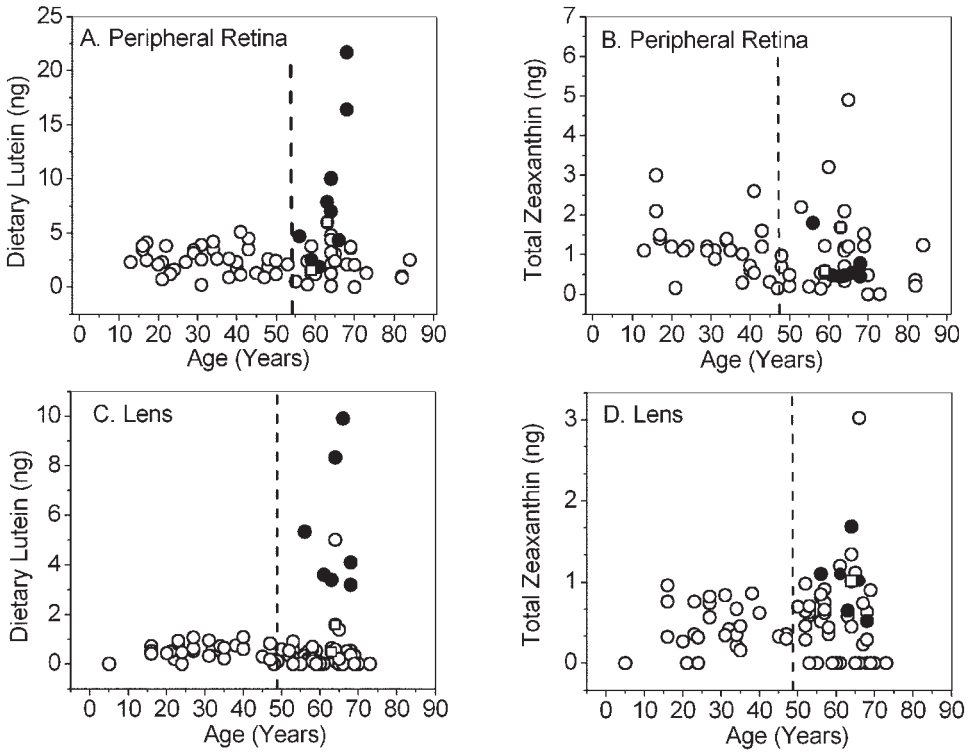

FIGURE 2. Age distribution of dietary lutein and total zeaxanthin from peripheral retina (8-mm–diameter punches) and whole lens in normal (*open circles*) and outlier (*filled circles*) eyes. Discordant fellow eyes of outliers are shown as *open squares*.

regularly before death. Increases in macular carotenoid levels were driven primarily by lutein and its probable metabolite *meso*-zeaxanthin. On the other hand, levels of dietary zeaxanthin and its possible metabolite 3′-oxolutein were not elevated, consistent with the current rarity of high-dose zeaxanthin supplementation in Utah.

We also found that, on average, donors with high levels of macular carotenoids had significantly elevated levels of carotenoids in lens and peripheral retina. Elevated lutein levels were consistently encountered in the lens (range, 2- to 10-fold above average), which may explain why some studies have implicated lutein as a protective nutritional factor against cataracts.[37,38] They were more variable, however, in the peripheral retina (range, 1- to 10-fold above average). Increased carotenoid concentrations in the lens and in the peripheral retina in response to supplementation could have profound effects on the various methods used to quantify macular pigment in living human eyes.

Heterochromatic flicker photometry (HFP) is the most commonly used method to measure macular pigment in clinical research.[17,18,24,26,27,39,40] A small spot of light projected onto the fovea rapidly alternates between blue, which is well absorbed by the macular carotenoids, and green, which is not. The subject varies the relative intensities of the two lights until the perception of flicker is minimized or eliminated, and the average energy of the blue light (B_{fov}) at minimal flicker from multiple tests is recorded. Because subjects vary markedly in the ratio of L and M cones that mediate color perception in HFP, the test must be repeated with an eccentric reference fixation point. To determine B_{ref}, macular pigment optical density (MPOD) is assumed to be zero. Typical reference points are at 4° to 9° eccentricity (1.2–2.6 mm at the retinal surface). The MPOD is then calculated with the equation MPOD = log (B_{fov}/B_{ref}). The zero MPOD assumption at approximately 7° is likely to be reasonable in an unsupplemented primate eye based on microspectrophotometry using absorbance and resonance Raman techniques in cadaver eyes,[32–34] but our data in this study raised questions as to whether this assumption remains valid in the supplemented state. In some subjects, substantial increases in carotenoid content (up to 10-fold) are exhibited in the equatorial region, very far beyond the traditional reference point of approximately 7°. We calculated that the average optical density attributable to xanthophyll carotenoids in the equatorial retina in one of these donors would be up to 0.01 at 460 nm. This would introduce a 3% underestimate of foveal MPOD for a subject with a typical foveal MPOD of 0.3, which is not particularly problematic if the equatorial periphery were used for B_{ref}. In HFP, however, B_{ref} is less than 2.6 mm from the center of the fovea. It is not unreasonable to predict that MPOD at approximately 7° might be in the 0.1 range or even higher in a supplemented subject because of a steep gradient of xanthophyll concentration from the periphery to the fovea, as evidenced in a previous human retinal HPLC microdissection study demonstrating that macular carotenoid levels at this eccentricity are typically more than 10 times higher than at the equatorial periphery.[11] This level of MPOD at B_{ref} would introduce a very large and potentially variable error in the foveal MPOD calculation (up to a 30% underestimate for a typical foveal MPOD of 0.3 or a 10% underestimate for a very high foveal MPOD of 1.0). The best way to avoid this problem would be to place B_{ref} as far as possible in the periphery. Unfortunately, reliable psychophysical performance of HFP much beyond 7° (i.e., >14°) is extraordinarily difficult.

Autofluorescence (AF) measurement of macular pigment is based on the measurement of attenuation of lipofuscin's inherent fluorescence by xanthophyll carotenoids.[25,28,29,41] In its most typical form, blue monochromatic light excites lipofuscin's main A2E fluorophore, which fluoresces at long wavelengths. Lutein and zeaxanthin are essentially nonfluorescent and absorb incident blue light, thereby attenuating A2E's fluorescence in the fovea. This method is amenable to high-resolution imaging with a digital camera or a scanning laser ophthalmoscope (SLO). If performed with a single wavelength, such as 488 nm, an assumption of reasonably uniform lipofuscin distribution and zero MPOD in the periphery must be made. The problems related to carotenoid supplementation discussed for HFP would therefore apply. Fortunately, a more peripheral reference point (i.e., >14°) could be chosen with this optical method. Sometimes the investigators make a second AF image with a green wavelength, such as 514 or 532 nm, that is not absorbed by carotenoids and that can then be used for digital subtraction from the 488-nm image to correct for inhomogeneities of lipofuscin distribution and various abnormalities. Zero MPOD assumption is still generally required.

Dual-wavelength reflectometry has also been performed using an SLO platform at 488 and 514 nm and a zero reference point of 14°.[22,42] This method's performance in a supplementation study would be expected to be comparable to AF imaging given that the assumptions are similar. Reflectometry can also be implemented in a quantitative nonimaging mode in which the fovea is illuminated with a series of narrow-bandpass filters, and a sophisticated optical model is used to calculate the average spectral contribution of the absorbance of the macular carotenoids. This method should be unaffected by peripheral changes in carotenoid content. On the other hand, changes in optical density of the lens in response to carotenoid deposition (approximately 0.01 optical density units) should be incorporated into the model or an overestimate of foveal MPOD will result.

Resonance Raman spectroscopy (RRS) has been used as a specific optical method to measure macular carotenoid pigments in the living human eye.[21,32,43] Light (488 nm) is briefly illuminated on a 1-mm–diameter region of the macula centered on the fovea, and wavelength-shifted light is analyzed on a Raman spectrometer. The intensity of the xanthophyll carotenoids' C = C vibration at 1525 cm^{-1} is linearly associated with macular carotenoid content. In the originally described integral mode, no peripheral correction is required; thus, values would be unaffected be any changes in the periphery, but substantial lens opacities or increases in lens carotenoid content could alter the values. Absorbance of the incident 488-nm laser light by carotenoids in the lens would mildly attenuate the observed Raman-shifted signal causing an underestimate of the foveal carotenoid content, but this would be counteracted by an opposing increase in the measured Raman signal from the lens carotenoids themselves. In the future, carotenoid supplementation studies using RRS should include a sufficient number of pseudophakic subjects to assess the magnitude of this potential problem in phakic subjects.

HPLC is sometimes referred to as the "gold standard" of ocular carotenoid measurement, but this technique has its own limitations, too. Although it is the most chemically specific, its sensitivity and spatial resolution cannot match any of the optical detection methods. In the past, we have consistently observed an age-related decline of macular pigment in our Utah clinic population with a variety of techniques, including RRS, AF imaging, and HFP,[21,40,42] but we did not observe such a decline in this HPLC study. Possible explanations include the fact that we used 4-mm tissue punches, which might blunt any age-related changes of foveal MPOD observed with methods with higher spatial resolution and which look at regions with diameters measuring 1 mm or less. In addition, all our studies in living humans specifically recruited clinic-based subjects who were not routinely consuming high-dose carotenoid sup-

plements, whereas the present study accepted all donor eyes regardless of supplementation history.

The findings reported here of elevated lenticular and retinal levels of ocular carotenoids apply only to lutein supplements because high-dose zeaxanthin supplementation is still too rare in our population. Zeaxanthin is more selectively concentrated in the fovea, where its binding protein, GSTP1, is found in very high concentrations.[44] Hence, peripheral retinal rises in response to supplementation may not be as high as they were with lutein. Lutein's binding protein in the human retina is still not identified, but we have partially purified one from human and quail liver (Bhosale P et al. *IOVS* 2005;46:ARVO E-Abstract 1756). This lutein-binding protein induces a large red shift of its carotenoid ligand's absorbance, which may also have important implications for the various noninvasive methods of carotenoid measurement.

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References

1. Schmidt-Erfurth U. Nutrition and retina. *Dev Ophthalmol*. 2005; 38:120–147.
2. West AL, Oren GA, Moroi SE. Evidence for the use of nutritional supplements and herbal medicines in common eye diseases. *Am J Ophthalmol*. 2006;141:157–166.
3. Hogg R, Chakravarthy U. AMD and micronutrient antioxidants. *Curr Eye Res*. 2004;29:387–401.
4. Bressler NM, Bressler SB, Congdon NG, et al. Potential public health impact of Age-Related Eye Disease Study results: AREDS report no. 11. *Arch Ophthalmol*. 2003;121:621–624.
5. Age-Related Eye Disease Study Research Group. A randomized, placebo-controlled, clinical trial of high-dose supplementation with vitamins C and E, beta carotene, and zinc for age-related macular degeneration and vision loss: AREDS report no. 8. *Arch Ophthalmol*. 2001;119:1417–1436.
6. Krinsky NI, Landrum JT, Bone RA. Biologic mechanisms of the protective role of lutein and zeaxanthin in the eye. *Annu Rev Nutr*. 2003;23:171–201.
7. Alves-Rodrigues A, Shao A. The science behind lutein. *Toxicol Lett*. 2004;150:57–83.
8. Zhao DY, Bhosale P, Bernstein PS. Carotenoids and ocular health. *Curr Topics Nutraceutical Res*. 2006;4:53–68.
9. Bernstein PS, Khachik F, Carvalho LS, Muir GJ, Zhao DY, Katz NB. Identification and quantitation of carotenoids and their metabolites in the tissues of the human eye. *Exp Eye Res*. 2001;72:215–223.
10. Handelman GJ, Dratz EA, Reay CC, van Kuijk FJGM. Carotenoids in the human macula and whole retina. *Invest Ophthalmol Vis Sci*. 1988;29:850–855.
11. Bone RA, Landrum JT, Fernandez L, Tarsis SL. Analysis of the macular pigment by HPLC: retinal distribution and age study. *Invest Ophthalmol Vis Sci*. 1988;29:843–849.
12. Seddon JM, Ajani UA, Sperduto RD, et al. Dietary carotenoids, vitamins A, C, and E, and advanced age-related macular degeneration: Eye Disease Case-Control Study Group. *JAMA*. 1994; 272:1413–1420.
13. Eye Disease Case-Control Study Group. Antioxidant status and neovascular age-related macular degeneration. *Arch Ophthalmol*. 1993;111:104–109.
14. Gale CR, Hall NF, Phillips DI, Martyn CN. Lutein and zeaxanthin status and risk of age-related macular degeneration. *Invest Ophthalmol Vis Sci*. 2003;44:2461–2465.
15. Bone RA, Landrum JT, Guerra LH, Ruiz CA. Lutein and zeaxanthin dietary supplements raise macular pigment density and serum concentrations of these carotenoids in humans. *J Nutr*. 2003;133: 992–998.
16. Richer S, Stiles W, Statkute L, et al. Double-masked, placebo-controlled, randomized trial of lutein and antioxidant supplementation in the intervention of atrophic age-related macular degeneration: the Veterans LAST study (Lutein Antioxidant Supplementation Trial). *Optometry*. 2004;75:216–230.
17. Landrum JT, Bone RA, Joa H, Kilburn MD, Moore LL, Sprague KE. A one year study of the macular pigment: the effect of 140 days of a lutein supplement. *Exp Eye Res*. 1997;65:57–62.
18. Hammond BR Jr, Johnson EJ, Russell RM, et al. Dietary modification of human macular pigment density. *Invest Ophthalmol Vis Sci*. 1997;38:1795–1801.
19. Koh HH, Murray IJ, Nolan D, Carden D, Feather J, Beatty S. Plasma and macular responses to lutein supplement in subjects with and without age-related maculopathy: a pilot study. *Exp Eye Res*. 2004; 79:21–27.
20. Duncan JL, Aleman TS, Gardner LM, et al. Macular pigment and lutein supplementation in choroideremia. *Exp Eye Res*. 2002;74: 371–381.
21. Bernstein PS, Zhao DY, Sharifzadeh M, Ermakov IV, Gellermann W. Resonance Raman measurement of macular carotenoids in the living human eye. *Arch Biochem Biophys*. 2004;430:163–169.
22. Berendschot TT, Goldbohm RA, Klopping WA, van de Kraats J, van Norel J, van Norren D. Influence of lutein supplementation on macular pigment, assessed with two objective techniques. *Invest Ophthalmol Vis Sci*. 2000;41:3322–3326.
23. Franciose JL, Askew WE, Lang JC, Bernstein PS. Serum and macular responses to antioxidant supplementation versus a carotenoid-rich dietary intervention in the elderly. *Curr Topics Nutraceutical Res*. 2006;4:69–76.
24. Snodderly DM, Mares JA, Wooten BR, et al. Macular pigment measurement by heterochromatic flicker photometry in older subjects: the Carotenoids and Age-Related Eye Disease Study. *Invest Ophthalmol Vis Sci*. 2004;45:531–538.
25. Smith RT, Koniarek JP, Chan J, Nagasaki T, Sparrow JR, Langton K. Autofluorescence characteristics of normal foveas and reconstruction of foveal autofluorescence from limited data subsets. *Invest Ophthalmol Vis Sci*. 2005;46:2940–2946.
26. Bone RA, Landrum JT. Heterochromatic flicker photometry. *Arch Biochem Biophys*. 2004;430:137–142.
27. Wooten BR, Hammond BR Jr. Spectral absorbance and spatial distribution of macular pigment using heterochromatic flicker photometry. *Optom Vis Sci*. 2005;82:378–386.
28. Trieschmann M, Spital G, Lommatzsch A, et al. Macular pigment: quantitative analysis on autofluorescence images. *Graefes Arch Clin Exp Ophthalmol*. 2003;241:1006–1012.
29. Delori FC. Autofluorescence method to measure macular pigment optical densities fluorometry and autofluorescence imaging. *Arch Biochem Biophys*. 2004;430:156–162.
30. Khachik F, de Moura FF, Zhao DY, Aebischer CP, Bernstein PS. Transformations of selected carotenoids in plasma, liver, and ocular tissues of humans and in nonprimate animal models. *Invest Ophthalmol Vis Sci*. 2002;43:3383–3392.
31. Bone RA, Landrum JT, Friedes LM, et al. Distribution of lutein and zeaxanthin stereoisomers in the human retina. *Exp Eye Res*. 1997; 64:211–218.
32. Bernstein PS, Yoshida MD, Katz NB, McClane RW, Gellermann W. Raman detection of macular carotenoid pigments in intact human retina. *Invest Ophthalmol Vis Sci*. 1998;39:2003–2011.
33. Snodderly DM, Handelman GJ, Adler AJ. Distribution of individual macular pigment carotenoids in central retina of macaque and squirrel monkeys. *Invest Ophthalmol Vis Sci*. 1991;32:268–279.
34. Handelman GJ, Snodderly DM, Krinsky NI, Russett MD, Adler AJ. Biological control of primate macular pigment: biochemical and densitometric studies. *Invest Ophthalmol Vis Sci*. 1991;32:257–267.
35. Bhosale P, Zhao DY, Serban B, Bernstein PS. Identification of 3-methoxyzeaxanthin as a novel age-related carotenoid metabolite in the human macula. *Invest Ophthalmol Vis Sci.* In press.
36. Bhosale P, Bernstein PS. Quantitative measurement of 3′-oxolutein from human retina by normal-phase high-performance liquid chromatography coupled to atmospheric pressure chemical ionization mass spectrometry. *Anal Biochem*. 2005;345:296–301.
37. Berendschot TT, Broekmans WM, Klopping-Ketelaars IA, et al. Lens aging in relation to nutritional determinants and possible risk factors for age-related cataract. *Arch Ophthalmol*. 2002;120:1732–1737.

38. Meyer CH, Sekundo W. Nutritional supplementation to prevent cataract formation. *Dev Ophthalmol*. 2005;38:103–119.
39. Neelam K, O'Gorman N, Nolan J, et al. Measurement of macular pigment: Raman spectroscopy versus heterochromatic flicker photometry. *Invest Ophthalmol Vis Sci*. 2005;46:1023–1032.
40. Wooten BR, Hammond BR Jr. Spectral absorbance and spatial distribution of macular pigment using heterochromatic flicker photometry. *Optom Vis Sci*. 2005;82:378–386.
41. Sharifzadeh M, Bernstein PS, Gellermann W. Non-mydriatic fluorescence-based imaging of human macular pigment distributions. *J Opt Soc Am A*. 2006;23:2373–2387.
42. Berendschot TT, van Norren D. Objective determination of the macular pigment optical density using fundus reflectance spectroscopy. *Arch Biochem Biophys*. 2004;430:149–155.
43. Gellermann W, Ermakov IV, Ermakova MR, McClane RW, Zhao DY, Bernstein PS. In vivo resonant Raman measurement of macular carotenoid pigments in the young and the aging human retina. *J Opt Soc Am A*. 2002;19:1172–1186.
44. Bhosale P, Larson AJ, Frederick JM, Southwick K, Thulin CD, Bernstein PS. Identification and characterization of a Pi isoform of glutathione S-transferase (GSTP1) as a zeaxanthin-binding protein in the macula of the human eye. *J Biol Chem*. 2004;279:49447–49454.