Macular Pigment in Donor Eyes with and without AMD: A Case-Control Study

Richard A. Bone,[1] *John T. Landrum,*[2] *Susan T. Mayne,*[3] *Christina M. Gomez,*[1] *Susan E. Tibor,*[2] *and Ewa E. Twaroska*[1]

Purpose. To determine whether there is an association between the density of macular pigment in the human retina and the risk of age-related macular degeneration (AMD).

Methods. Retinas from 56 donors with AMD and 56 controls were cut into three concentric regions centered on the fovea. The inner, medial, and outer regions covered the visual angles 0° to 5°, 5° to 19°, and 19° to 38°, respectively. The amounts of lutein (L) and zeaxanthin (Z) extracted from each tissue sample were determined by high-performance liquid chromatography.

Results. L and Z levels in all three concentric regions were less, on average, for the AMD donors than for the controls. The differences decreased in magnitude from the inner to medial to outer regions. The lower levels found in the inner and medial regions for AMD donors may be attributable, in part, to the disease. Comparisons between AMD donors and controls using the outer (peripheral) region were considered more reliable. For this region, logistic regression analysis indicated that those in the highest quartile of L and Z level had an 82% lower risk for AMD compared with those in the lowest quartile (age- and sex-adjusted odds ratio = 0.18, 95% confidence interval = 0.05–0.64).

Conclusions. The results are consistent with a theoretical model that proposes an inverse association between risk of AMD and the amounts of L and Z in the retina. The results are inconsistent with a model that attributes a loss of L and Z in the retina to the destructive effects of AMD. (*Invest Ophthalmol Vis Sci.* 2001;42:235–240)

Dietary supplements containing lutein are currently being promoted by a number of nutraceutical manufacturers for their supposed beneficial effects on the health of the eye. Such claims, which are not yet proven, are based on circumstantial evidence emerging from a number of studies. Lutein (L) and zeaxanthin (Z) are the two carotenoids that form the macular pigment (MP) of the primate retina.[1] The Eye Disease Case-Control Study Group has reported inverse associations between risk of advanced, neovascular, age-related macular degeneration (AMD) and levels of L and Z in the subjects' diet and serum.[2,3] Other studies, however, have reported no such associations.[4,5] Associations have also been reported between MP density and a number of risk factors for AMD. Thus subjects who were smokers, who were female, or who had light-colored irises tended to have, on average, lower MP densities than smokers, males, or those having dark irises, respectively.[6,7,8] The prevalence of AMD tends to be greater among individuals with the three former characteristics.[9–12] In the advanced form of AMD known as geographic atrophy, the foveal center, where MP density peaks, tends to be spared until the disease is well-advanced.[13,14] This region is similarly spared in annular macular degeneration, and the possible protective influence of the macular pigment has been discussed by Weiter et al.[15] Comprehensive reviews have been published by Snodderly,[16] Schalch et al.,[17] and Beatty et al.[18] exploring the evidence for a protective function by the macular pigment against AMD and the mechanisms by which it might act.

As far as we are aware, no studies have attempted to evaluate the possible association between L and Z concentrations in the retina and risk of AMD. There are two general approaches for measuring L and Z in the retina: direct analytical measurements on autopsy eyes, and indirect MP optical density measurements using, for example, heterochromatic flicker photometry.[6,7,8] For subjects who have advanced forms of AMD, flicker photometry may prove difficult, if not impossible, and could lead to ambiguous results. To our knowledge, the technique has been validated only in subjects without retinal disease.[19] We therefore adopted an analytical approach, using high-performance liquid chromatography (HPLC), to quantify the distribution of L and Z in human autopsy retinas. The study was designed to compare the amounts of L and Z in donors diagnosed with AMD to those in a control group without the disease. The purpose of the analysis was to evaluate a possible association between the amounts of L and Z found in the retina and the risk of AMD.

Methods

Tissue Samples

Human eyes from 56 donors with AMD and 56 controls without the disease were obtained from the National Disease Research Interchange (NDRI). Procurement methods for the tissues were humane, including proper consent and approval, and complied with the tenets of the Declaration of Helsinki. Donor information sheets generally provided each donor's age, sex, ethnicity, and medical condition including any diagnosis of AMD. A summary of donor characteristics is presented in Table 1. In the majority of cases, the NDRI was unable to provide specific information as to the type of AMD, severity of the condition, or the diagnostic history. In a few cases, they were able to inform us that the AMD was "wet" (6 cases) or "dry" (9 cases). No eyes were excluded from this study based solely on the donor's cause of death. However, no eyes were accepted from diabetic donors, because of the possibility of diabetic retinopathy, or from those donors diagnosed with an eye disease other than AMD.

Eyes were enucleated within 6 hours of death, fixed in formalin, and shipped on wet ice to our laboratory where they were refrigerated at 0°C before dissection. The eyes were dissected in 0.9% saline solution, generally on the same day they were received, but always within 5 days. The use of formalin not only reduces postmortem degradation, but considerably eases the task of removing the retina without tearing, and does not result in any measurable leaching of

From the Departments of [1]Physics and [2]Chemistry, Florida International University, Miami, Florida; and the [3]Department of Epidemiology and Public Health, Yale University School of Medicine, New Haven, Connecticut.

Supported by National Institutes of Health Grant GM08205.

Submitted for publication May 30, 2000; revised August 17, 2000; accepted September 22, 2000.

Commercial relationships policy: N.

Corresponding author: Richard A. Bone, Department of Physics, Florida International University, 11200 SW 8th Street, Miami, FL 33199. bone@fiu.edu

TABLE 1. Demographic Characteristics of Study Subjects, by Case-Control Status

	AMD Cases	Controls	*P* Value
	$n = 56$	$n = 56$	
Age (Mean ± SD)	82 ± 8	77 ± 8	0.0018
Range	65–98	58–98	
Sex			
Males	21	24	0.36
Females	34	27	
Missing	1	5	
Race			
Caucasian	48	49	0.23
Hispanic/Black	1	2	
Missing	7	5	

carotenoids from the tissue. Even so, tearing was encountered in some instances, and any loss of tissue was grounds for rejecting the entire retina. Each intact retina was divided into three concentric tissue samples centered on the fovea using a cutting device that has been described elsewhere.[20] Briefly, the retina was draped on a 1-inch Lucite sphere. Three guided trephines of 3-, 11-, and 21-mm diameter were brought to bear on the retina. The central portion, referred to as inner, was a 3-mm-diameter disc of tissue containing the yellow spot. The disc corresponded to a range of visual angles, ~ 0° to 5°, and had an area of 7.1 mm^2. The medial and outer portions were concentric annuli, covering the ranges 5° to 19° (area 93 mm^2), and 19° to 38° (area 343 mm^2). The positions of the two smaller trephines relative to the retina's architecture are shown in Figure 1.

To extract the carotenoids, each tissue sample was homogenized in a glass tissue-grinder containing 2 ml of ethanol/water (1:1) and 0.5 ml of an ethanol solution of ~10 ng of lutein monopropyl ether as an internal standard. The homogenate was transferred to a large culture tube and the tissue-grinder rinsed with three 2 ml aliquots of ethanol/water and two 5 ml aliquots of hexane, the rinses being added to the culture tube. Thorough extraction of the carotenoids was accomplished by placing the culture tube first in an ultrasonic bath for 1 minute, then on a vortex mixer for 1 minute. The carotenoid-containing hexane layer was separated by centrifuging for 3 minutes at ~ 1400*g*. The hexane layer was then transferred to a pear-shaped flask and dried under a stream of N_2.

HPLC Analysis

Quantification of the L and Z content in each sample was accomplished by reversed-phase HPLC using a 250×2 mm Ultracarb ODS 3 μm column (Phenomenex, Torrance, CA). The mobile phase was acetonitrile/methanol (85:15) with the addition of 0.1% triethylamine to inhibit degradation of carotenoids. The flow rate was 0.2 ml/min and detection was at 451 nm. This wavelength was chosen because the extinction coefficients of L, Z, and the internal standard at 451 nm were determined to be equal for the particular solvent mix that was used. Therefore, the amounts of L and Z in a sample could be determined by comparing their chromatogram peak areas with that of the internal standard.

Statistical Analysis

Demographic characteristics of the cases and controls were compared using Students' *t*-test or χ^2 analysis, as appropriate. All significance tests were 2-sided, with a *P* value of 0.05 or less considered statistically significant. The cases were significantly older than controls and had a greater proportion of women, so these variables were considered in all multivariate analyses. Mean levels of L and Z were calculated in the inner, medial, and outer regions, with case-control differences evaluated using Students' *t*-test. For each concentric region, L and Z levels were categorized into quartiles, based on the control distribution for that region. Logistic regression analysis was then used to estimate the odds ratios and 95% confidence intervals, with adjustment for age (continuous) and sex. The lowest quartile was set as the referent category. The test for linear trend was evaluated by modeling the quartile values as a continuous variable in a logistic regression model, again adjusting for age and sex.

RESULTS

A total of 224 eyes, from 56 donors reported to have AMD and 56 non-AMD controls, were obtained. The demographic characteristics of the donors are shown in Table 1. For 63% of the donors, analyses for both the left and right eyes were completed, and the average L and Z content was calculated for the inner, medial, and outer regions. For the remaining 37% (20 controls, 21 cases), analysis was completed for one eye only, and the L and Z content in this eye was assumed to be representative of the average for the subject. Data for the fellow eye were not obtained for several reasons including torn or incomplete tissue samples, handling errors, and equipment malfunctions (e.g., electrical interruptions). Where data were available from both eyes, correlation analysis was used to determine whether the total L + Z levels in the combined inner, medial, and outer regions in both eyes were correlated. For the AMD cases, the linear correlation coefficient, *r* was 0.90 ($P < 0.0001$), and for the controls, *r* was 0.79 ($P < 0.0001$). These values provide justification for averaging the data obtained from the donor's left and right eyes.

In the inner region, the AMD cases were found to have, on average, 62% of the L and Z found in the controls. Based on a 2-sided *t*-test, the difference was significant at $P = 0.0002$. In the medial and outer regions, the figures were 73% ($P = 0.014$), and 79% ($P = 0.05$), respectively (Fig. 2). In gender-specific analyses, mean differences were more notable for females (case-control differences statistically significant for inner, medial and outer L + Z) compared with males (case-control differences significant only for inner L + Z).

As can be seen from Figure 2, there was a trend of decreasing difference between the cases and controls with increasing eccentricity from the fovea. This observation raises the possi-

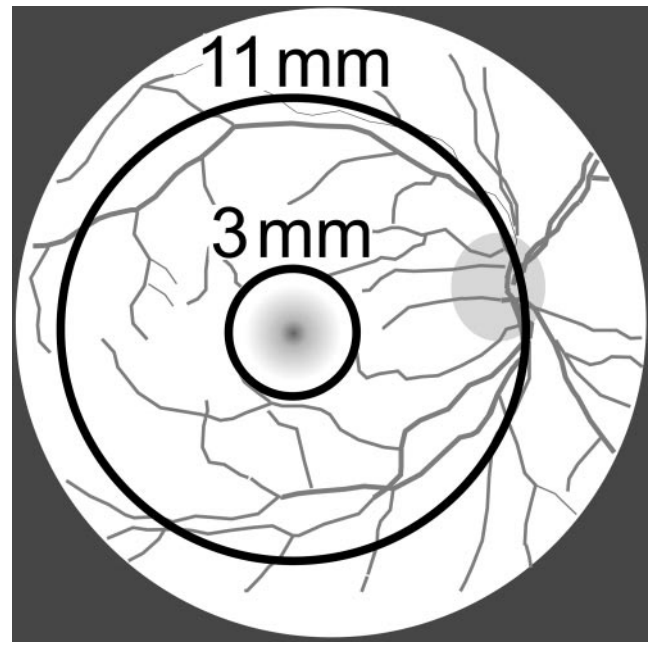

FIGURE 1. Schematic view of the human fundus showing the placement of the 3-mm and 11-mm-diameter trephines. These, together with a 21-mm trephine, were used to obtain three concentric tissue samples centered on the fovea.

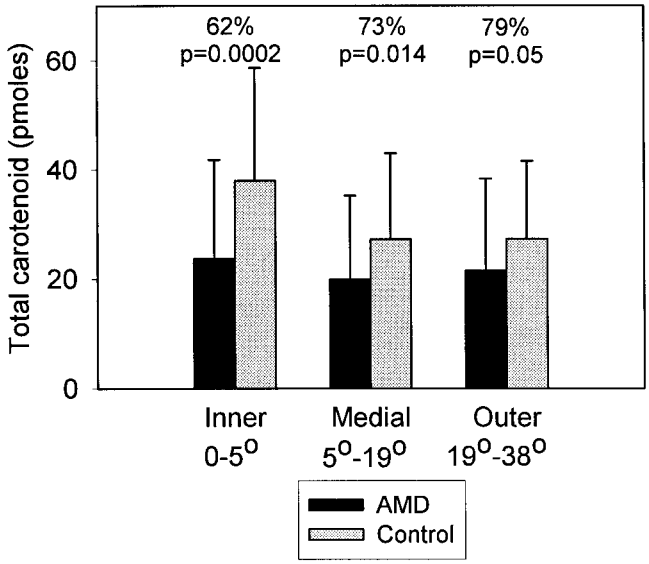

FIGURE 2. Comparison of the mean total amounts (± SD) of combined L and Z in the inner, medial, and outer regions of the retinas of AMD and control donors. In the inner (0° to 5° visual angle), medial (5° to 19°), and outer (19° to 38°) regions, the AMD group had 62%, 73%, and 79%, respectively, of the L and Z found in the controls. The differences, based on a 2-sided *t*-test, were significant at $P = 0.0002$, 0.014, and 0.05, respectively.

bility that the differences in L- and Z- content are a consequence of AMD, which causes more damage in the macula than the periphery. For this reason, particular emphasis was given to statistical analysis of the outer annulus where the potential loss of carotenoid-containing tissue due to AMD is expected to be least.

The amounts of carotenoid per unit area in the outer annulus for cases and controls were divided into quartiles according to the distribution of these amounts among control subjects only. Table 2 displays the median value for each quartile, together with the odds ratios for AMD, adjusted for age and sex. Corresponding results for the inner disc and medial annulus are presented for comparison. In Figure 3, the odds ratios for the outer annulus are plotted as a function of the median L and Z per unit area for each quartile. There was a significant trend for reduction in risk for AMD with increasing amount of carotenoid in the outer annulus. Those in the highest quartile of L and Z per unit area had an 82% lower risk for AMD, compared with those in the lowest quartile (OR = 0.18, 95% CI = 0.05 to 0.64, P for linear trend = 0.027).

The primary analytic approach we used to summarize the data in this case-control study is the logistic model, which was used to calculate odds ratios as the measure of association between L plus Z and case-control status. The logistic model makes no assumptions about normally distributed data; transformation is not required. Nonetheless, we examined the normality of the distributions of the variables inner, medial, and outer regions, separately in cases and controls. For some, there is no statistical evidence of non-normally distributed data (e.g., controls, outer region levels). For others, the distribution is slightly skewed toward the right. However, it is clear that outliers are not driving these results in that the entire distribution is shifted in the cases compared with the controls.

It is unlikely that high levels of L and Z in the periphery could have a protective effect against a disease that characteristically affects the macula. However, a high level of L and Z in the periphery has been found to be indicative of a correspondingly high level in the macula,[21] where it might have the potential to be protective. With the current data, we performed a linear correlation analysis between the amounts of L and Z in the inner disc and outer annulus, using control eyes only. The results shown in Figure 4 (open circles, solid line) indicate a positive correlation between the two quantities ($r = 0.69$, $P < 0.0001$). Therefore, AMD subjects in the lowest quartile of L and Z in the outer annulus would generally be among the lowest for L and Z in the macula, disregarding any possible loss of L and Z due to the disease. For comparison purposes, Figure 4 also displays the corresponding results for the AMD subjects (filled circles, dashed line, $r = 0.69$, $P < 0.0001$). The majority of the data points, together with their regression line, are seen to lie below the regression line for the control subjects, consistent with the possibility that AMD may be causing some loss of L and Z in the inner region. As another way to examine this, the inner-to-outer region ratio of L and Z was compared in cases versus controls. The mean ratio was less in cases than controls (mean 64.0 vs. 75.5), although the means were not significantly different ($P = 0.13$).

No significant differences were observed between the L and Z levels in the nine AMD cases reported to be dry and the six reported to be wet for either the inner, medial, or outer regions. We also examined the effect of age on the L and Z levels. For the AMD cases, there was no significant increase or decrease with age in either the inner, medial, or outer regions. For the control group, the L + Z levels showed a slight tendency to increase with age in all three regions, being sig-

TABLE 2. Odds Ratios for Age-Related Macular Degeneration Based on L + Z per Unit Area (pmol/mm^2) in Different Parts of the Retina and Derived from Logistic Regression

	Quartile				*P* for Trend (Adjusted)
	1	2	3	4	
Inner					
Median L + Z	2.05	4.16	5.58	9.08	
Odds Ratio	1.0	0.32	0.14	0.07	0.0005
95% C.I.		0.10–0.96	0.04–0.49	0.02–0.33	
Medial					
Median L + Z	0.097	0.203	0.320	0.497	
Odds Ratio	1.0	0.29	0.29	0.22	0.019
95% C.I.		0.10–0.90	0.08–0.99	0.06–0.80	
Outer					
Median L + Z	0.031	0.063	0.087	0.135	
Odds Ratio	1.0	0.30	0.17	0.18	0.027
95% C.I.		0.09–0.99	0.05–0.58	0.05–0.64	

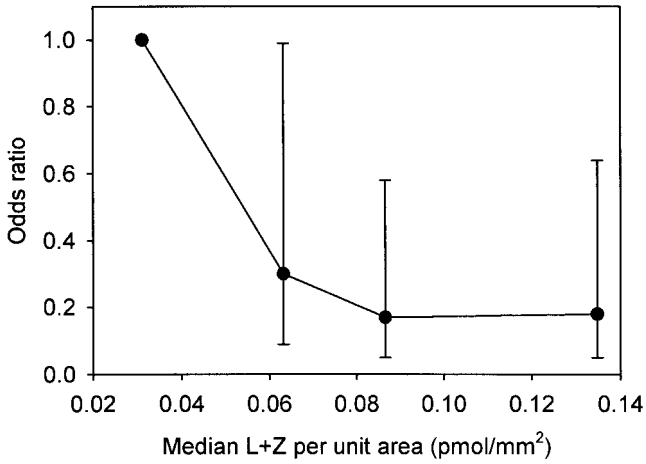

FIGURE 3. For the outer region of the retina (19° to 38°), the odds ratios for AMD for the four quartiles (lowest quartile set as referent category) show an approximately inverse association with the median L + Z values. The 95% confidence intervals are indicated by the *vertical bars*.

nificant in the outer region only [1.8 ± 0.7 (fmol/mm^2)/year; $r = 0.34$, $P = 0.01$].

DISCUSSION

AMD is often referred to as a multifactorial disease with age, female sex, light iris color, and smoking as recognized risk factors.[9–12] In addition, epidemiologic studies link low levels of L and Z in the diet and serum with an increased risk of neovascular AMD.[2,3] There is evidence that individuals having these low levels would tend toward low levels of MP.[21] The results of the present study reinforce this concept and suggest that a low level of MP should be considered as a potential risk factor for AMD.

The differences that we have observed between the amounts of L and Z in the retinas of donors with and without AMD are possibly underrepresented in this study as a result of the paucity of donor information that the NDRI was able to provide. For example, the appearance of drusen in a patient's retina may have prompted an incorrect or premature diagnosis of AMD. Similarly, an undiagnosed individual who served as a control subject, may have had the disease. Thus it is likely that some of the AMD cases should have been assigned to the control group, and vice versa. If AMD is indeed associated with lower-than-average levels of L and Z in the retina, such misclassification of disease status would have the effect of decreasing the differences between the case and control groups. It must also be noted that the L and Z levels were measured at the time of the donor's death and may not be representative of the levels earlier in life when their postulated protective influence would be relevant. Given the magnitude of the observed odds ratios in Table 2, however, neither of these considerations is likely to be operating to any significant extent.

There is a crucial question to consider: Do our results indicate that individuals with low amounts of L and Z in their retinas are at greater risk of acquiring AMD, or that low amounts of L and Z are merely a consequence of the disease? To help resolve this question, we have developed two theoretical models that generate odds ratios corresponding to each of these possibilities. For the first model, we began with a set of 1000 random numbers having a pseudonormal distribution covering the range of carotenoid levels that we observed in the outer annulus (~ 3–60 pmol). The set was characterized by approximately the same mean and SD that characterized the experimental data. It was obtained by removing equal, small numbers of numbers from either end of an appropriate normal distribution. This set was assumed to represent a sample of carotenoid levels in the human population. The numbers were ranked and divided into quartiles, and the median for each quartile was determined. We then assigned a level of risk for AMD for each quartile that was inversely proportional to the median, and used this number as a P value to generate a corresponding set of 250 Bernoulli random variables. (These variables are obtained from a set of random numbers with a uniform distribution in the range 0 to 1. If the number is less than or equal to P, the variable is assigned the value 1; otherwise, it is assigned the value 0.) In our model, the "subject" acquired AMD if the variable was equal to 1; if equal to 0, the "subject" did not. To generate odds ratios, new quartiles were established according to the distribution of carotenoid levels among the non-AMD "control subjects" (as was done with the experimental data). Figure 5 (open circles) shows the odds ratios plotted against the median values for these quartiles. As the risk for AMD in this model is inversely proportional to the median carotenoid level, the characteristic hyperbolic shape is to be anticipated.

To model the second possibility, that low carotenoid levels are a consequence of AMD, we generated two columns of 1000 random numbers each. Each column spanned the same range (~ 3–60 pmol) and was characterized by the same mean value and SD. The first of these columns represented the carotenoid levels among "controls", and the second represented carotenoid levels among "AMD cases" before the destructive effects of AMD. The interpretation of our experimental results (Fig. 2) that is consistent with this model, is that AMD is responsible for an approximately 20% loss of L and Z in the periphery. We therefore generated a third column of 1000 random numbers, with a pseudonormal distribution having a mean value of 0.8, to represent the fraction of carotenoid surviving the destructive effects of AMD. Reduced carotenoid levels among "AMD cases" were obtained by multiplying this column by the second column. Odds ratios, shown in Figure 5 (filled circles), could then be calculated from an analysis of these numbers and the "control" numbers in the first column.

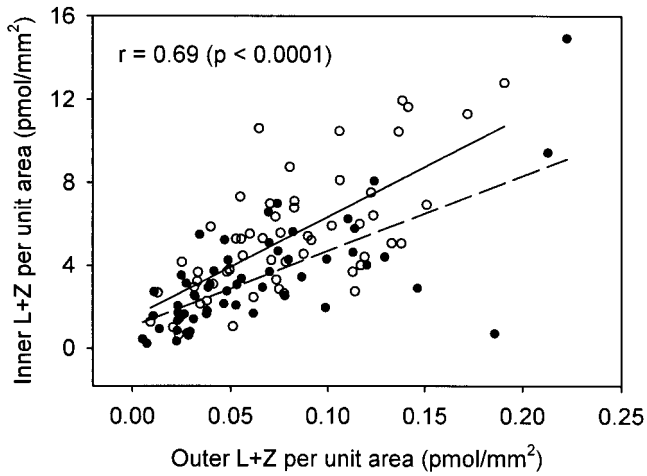

FIGURE 4. Scatterplot of L + Z per unit area in the outer region (19° to 38°) of the retina versus the inner region (0° to 5°). The upper, solid regression line is based on control donors only. Each data point (*open circles*) represents, when available, the average of the donor's left and right eyes. The majority of the corresponding data points for the AMD donors (*filled circles*), together with their regression line (*dashed line*), lie below the regression line for the controls. (For both regression lines, $r = 0.69$, $P < 0.0001$.)

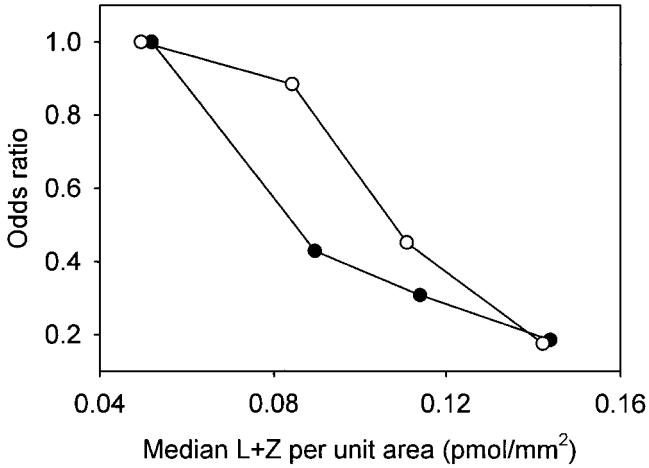

FIGURE 5. Quartile odds ratios for AMD predicted by two theoretical models produce markedly different curvatures when plotted as functions of the median L + Z per unit area. The *filled circles* are obtained when the risk for AMD is inversely proportional to the median L + Z per unit area of retina. The *open circles* are generated in a situation where AMD leads to lower levels of L and Z in the retina.

The two models produce distinctly different trends. If prevalence of AMD is inversely associated with L and Z levels, the biggest jump in odds ratios occurs between the lowest and second lowest quartiles. Conversely, if AMD causes loss of L and Z, the smallest jump in odds ratios occurs between these two quartiles. This is a general result and not sensitive to the specific choice of parameters in the two models. For example, using uniform, rather than pseudonormal, distributions of random numbers in the two models did not produce a substantially different result. The experimentally determined odds ratios in Figure 3 follow a similar trend to the lower curve in Figure 5, consistent with the proposal that individuals with low amounts of L and Z in their retinas are at greater risk of acquiring AMD. Our results are inconsistent with the hypothesis that AMD causes loss of L and Z in the peripheral retina between 19° and 38° eccentricity. Although emphasis has been placed on the outer region, for reasons given previously, Table 2 indicates that the odds ratios for all three regions behaved in a similar fashion. Thus according to our model, loss of L and Z due to AMD was not the major factor anywhere in the retina.

Is there any evidence suggesting that AMD does cause loss of carotenoid-containing tissue in the peripheral part of the retina? In transverse sections through the central macula, the carotenoids are visible and have been mapped by microspectrophotometry.[22] They are found in abundance in the receptor axon layer and inner plexiform layer, but their localization in specific cells has not been established. In the peripheral retina, concentrations of carotenoids are very low, precluding this technique. However, a recent study by Sommerburg et al., involving HPLC analysis of isolated rod outer segments (ROS), has revealed the presence of L and Z in these structures.[23] Even more recently, Rapp et al. have confirmed this observation in both the perifoveal and peripheral retina but have found that the total mass of L and Z in ROS is less than in the residual (ROS-depleted) retinal membranes.[24] In the parafoveal retina, between approximately 1.5° and 10° eccentricity from the foveal center, there is an increasing loss of rods with age.[25] This loss has been estimated to be undetectable 8 mm (~ 28°) from the foveal center. The loss of rods in the parafoveal retina is compensated to some extent by the remaining rods that expand to fill the space vacated by the dying rods. Possibly, as a result, there is no net loss of the fraction of L and Z that is associated with parafoveal rods. This suggestion is supported by our observation here and elsewhere[20] that there is no age-related decline in L and Z levels anywhere in the retina. In another study, photoreceptor losses were compared in the eyes of AMD donors and controls.[26] In nonexudative AMD, receptor densities were normal at eccentricities greater than 10°. In exudative AMD, preferential rod loss (as opposed to cone loss) was reported up to 2.5 mm from the margin of the disciform scar. However, no data were available for the peripheral retina. The two studies by Sommerburg et al.[23] and Rapp et al.[24] also reported the presence of low concentrations of L and Z in the retinal pigment epithelium (RPE). In both the macular and peripheral RPE, the amounts of L and Z were approximately 15% of those found in the adjacent regions of the retina.[23] Histopathologic changes occur with age in the peripheral RPE, as well as Bruch's membrane and the choriocapillaris, but are not correlated with the presence or absence of AMD.[27] Similarly peripheral retinal function (>15°) is affected by age, but has been reported to be no worse in the majority of AMD cases.[27,28] For these reasons, the hypothesis, that AMD does not cause loss of L- and Z-containing tissues in the peripheral retina, remains tenable, if unproven.

The strength of this study is that it is the first, to our knowledge, to estimate the association between low levels of L and Z concentrations in human retinas and risk of AMD. Although our results show an inverse association between the risk of AMD and MP density, they do not, by themselves, imply a causal association. It is quite possible that some other factor both increases the likelihood of a person developing AMD, and leads to low levels of L and Z in the retina. As we were unable to obtain data from these donors on some important potential confounding variables, such as tobacco use, we cannot adjust our estimates for this type of confounding. Future studies will need to replicate these findings, with better evaluation of possible confounding, particularly by smoking. Such studies would be greatly strengthened by including detailed pathologic analysis in the protocol to provide assessment and classification of the donors' eye disease.

Our results reinforce those of earlier epidemiologic studies that show associations between low levels of L and Z in the diet or serum and increased risk of neovascular AMD, even after adjustment for smoking.[2,3] The assumption would be that, in general, individuals having these low levels of L and Z in the diet or serum would have correspondingly low levels in their retinas. But this may not always be the case, and could be the reason for the insignificant associations that have been observed in other studies.[4,5] Future epidemiologic studies would be strengthened by measuring L and Z levels directly in the retina rather than by proxy in the diet or serum.

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