

Supplementary Material S1

METHODS

Study Sample

The Women's Health Initiative – Observational Study (WHI-OS) is a prospective cohort study assessing the most common causes of morbidity and mortality in over 93,000 postmenopausal women (aged 50-79 years) recruited from 40 sites throughout the United States in 1994-1998. CAREDS is an ancillary study to the WHI-OS examining associations between intakes of lutein and zeaxanthin (from foods and supplements) and the prevalence of age-related eye disease, including macular degeneration. WHI-OS participants with baseline carotenoid intake >78th or <28th percentiles were eligible for CAREDS and were recruited (2001-2004) from three WHI-OS centers in Madison, WI, Iowa City, IA, and Portland, OR (N=3,140). Women at these extreme levels of lutein intake, combined, did not differ significantly in age, education, body mass index, smoking status, use of supplements or hormone therapy, or history of diabetes or cardiovascular disease compared to women between the 28th and 78th percentiles.¹

Among the 3,140 women eligible for enrollment in CAREDS, 93 died or were lost to follow-up between selection in year 2000 and enrollment in the CAREDS in 2001-2004. The remaining women were sent letters inviting them to participate in the CAREDS. A total of 1,042 women declined participation and 2,005 were enrolled (64%). Compared to those who declined participation, participating women had diets higher in lutein and zeaxanthin (median 1.03 versus 0.95 mg/day), were more likely to use nutrient supplements (60% versus 55%) and hormone therapy (54% versus 43%). Participants were also more likely to have graduated high school (77% versus 66%), less likely to smoke (4% versus 7%) or have diabetes (4% versus 6%), and had lower median waist circumference (82 cm versus 86 cm).

Of the 2,005 enrolled, DNA was requested from 1,787 women who also had data on age-related macular degeneration (AMD). Of those, 1,772 approved use of their stored DNA

and had enough DNA sample available to be sent for genotyping. 1,697 of these women had sufficient DNA for genotyping. All procedures conformed to the Declaration of Helsinki, informed consent was obtained from all participants, and approval was granted by the Institutional Review Board at each University. As described in the “*Genotyping*” section below, 1,585 of these 1,697 women also had macular pigment optical density measured.

Dietary lutein and zeaxanthin estimates

Diet was assessed at WHI baseline (1994-1998; as selection criteria) and at CAREDS baseline (2001-2004; to study in relation to MPOD) by using a semi-quantitative food-frequency questionnaire that was based on the instrument used in the Women’s Health Trial Feasibility Study². Nutrient estimates in WHI and CAREDS were computed from responses to food-frequency questionnaires at the Fred Hutchinson Cancer Research Center. Estimates of lutein and zeaxanthin intakes at both time periods were modified by response to a query about the proportion of time that dark greens were consumed in salads compared to light greens at the time of CAREDS and in an earlier time period (1986-1988). Average daily intake of lutein and zeaxanthin from supplements was computed from response to vitamin supplement questionnaires collected at CAREDS. The questionnaire queried the dose, frequency, and duration of specific supplement intake. Intake from food and supplemental sources were summed in analyses.

Macular Pigment Optical Density (MPOD) measurements

Macular pigment optical density (MPOD) is a relative measure of lutein and zeaxanthin in the retina. MPOD was determined at CAREDS baseline (2001-2004) examinations using a standardized protocol by the psychophysical method of customized heterochromatic flicker photometry. This protocol had high test-retest reliability ($r=0.90$) and has been described in detail previously.³ Briefly, the participants were fitted with trial frames and appropriate lenses for

testing after refraction. The best flicker frequency for heterochromatic flicker photometry was then established for each participant. The participant made four separate determinations for each target at several retinal locations. The participant was instructed to fixate at the center of the following targets: 0.25, 0.50, 1.00, and 1.75 degrees from the foveal center so that MPOD at different eccentricities was measured in reference to a target at 7 degrees from the center. Measurements were also made with the left eye at 0.5 and 1.0 degree from the foveal center. For this analysis, SNP associations were tested with MPOD at all eccentricities but will be primarily reported for 0.5 degrees eccentricity in the right eye only, as this is the measure with the lowest within participant to between participant variability.³ Previously, determinants of MPOD at different eccentricities did not substantially differ in this sample.¹

Serum analyses of lutein and zeaxanthin

Serum samples, which were obtained from participants in WHI baseline examinations and stored at -80°C, were analyzed for concentrations of *trans*-lutein and *trans*-zeaxanthin at Tufts University by a reverse phase HPLC analysis.⁴ Blind duplicates were analyzed in each batch of serum analyses (total of 57 participants). Mean coefficient of variation was 7.0% for all *trans*-lutein and 9.6% for all *trans*-zeaxanthin.

Covariates

During CAREDS study visits, weight (with a calibrated scale), height (with a stadiometer), and waist and hip circumferences (with a tape measure 1 inch above navel and at widest spot between waist and hips) were measured. The presence of physician diagnosed diabetes, family history of cataract and macular degeneration, use of cholesterol lowering medications, smoking status, use of hormone therapy, alcohol intake, and ocular sunlight exposure was obtained from questionnaires completed at WHI entry and updated at time of CAREDS study visits as previously described.¹ Additional variables evaluated for their

relationship to MPOD including blood levels of high sensitivity C-reactive protein (hs CRP) and 25-hydroxyvitamin D (25(OH)D) levels⁵ and composite dietary measures including the modified 2005 Healthy Eating Index and Healthy Lifestyle Score⁶.

Genotyping

For the present analysis, we evaluated 473 SNPs from 26 candidate genes for carotenoid status and 190 SNPs were ancestry informative markers (AIMs) for discerning northwest-southeast European ancestry and southeastern-Ashkenazi Jewish ancestry clines.⁷

The genes considered as candidates for carotenoid status included genes encoding proteins that 1) specifically bind carotenoids in the retina (glutathione S-transferase pi 1 (*GSTP1*) and StAR-related lipid transfer domain containing 3 (*STARD3*)), 2) cleave carotenoids (beta-carotene 15,15'-monooxygenase 1 (*BCMO1*) and beta-carotene oxygenase 2 (*BCO2*)) or are involved in retinoid trafficking (RAR-related orphan receptor A (*RORA*) and retinal pigment epithelium-specific protein 65kDa (*RPE65*)), 3) bind and facilitate lipid and carotenoid absorption, transport, and uptake into tissues (CD36 molecule (thrombospondin receptor) (*CD36*)), scavenger receptor class B, member 1 (*SCARB1*) and scavenger receptor class B, member 2 (*SCARB2*)), 4) are related to high density lipoprotein levels, transport or status (ATP-binding cassette, sub-family A, member 1 (*ABCA1*), ATP-binding cassette, sub-family G, member 5 (*ABCG5*) and ATP-binding cassette, sub-family G, member 8 (*ABCG8*), hepatic lipase (*LIPC*), cholesteryl ester transfer protein, plasma (*CETP*), apolipoprotein E (*APOE*) and *SCARB1*)), 5) are related to long-chain omega-3 fatty acid status in the blood or retina (elongation of very long chain fatty acid protein 2 (*ELOVL2*), elongation of very long chain fatty acid protein 4 (*ELOVL4*) and elongation of very long chain fatty acid protein 5 (*ELOVL5*)), and 6) have been related age-related macular degeneration or other retinopathies and also related to carotenoid or retinoid levels or metabolism (complement factor H (*CFH*), age-related maculopathy susceptibility 2 (*ARMS2*), aldehyde dehydrogenase 3 family, member A2

(*ALDH3A2*), and retinal pigment epithelium-specific protein 65kDa (*RPE65*). SNPs within candidate genes were chosen based on previous literature or as tag SNPs for their respective gene. Tagging was conducted using the HapMap Genome Browser Release #27 (<http://hapmap.ncbi.nlm.nih.gov/>) CEU reference population and filtering for a minor allele frequency ≥ 0.05 and an $r^2 \geq 0.80$.

DNA was extracted from the buffy coats of blood obtained at WHI-OS baseline examinations (1994-1998) that have been stored frozen at -80°C . SNPs were genotyped at Case Western Reserve University (Cleveland, OH) using an Illumina Custom GoldenGate Assay. Genotype calls were made using Illumina Genome Studio. Twenty-four SNPs not designable to the custom Illumina assay were instead genotyped using KASP Assay at LCG Genomics (formerly KBiosciences, Teddington, Middlesex, UK) and called via the KASP SNP Genotyping System.

For the 473 SNPs, 20 failed genotyping and 13 had Hardy-Weinberg equilibrium (HWE) χ^2 P -values $< 1.0 \times 10^{-6}$. This resulted in 440 SNPs for association testing with MPOD, all with call rates $> 98\%$. See **Supplementary Material S2** for list of all SNPs tested for association with MPOD. Using the same QC thresholds, 176 of the 190 AIMS were used for principal component analysis.

Of the 1,697 individuals included for genotyping, women were removed from analysis if their individual genotyping call-rate was $< 90\%$ ($n=21$), overall heterozygosity $> 44.5\%$ ($n=12$), or genotype concordance between individuals $> 95\%$ ($n=6$). These were not mutually exclusive filters and resulted in a total of 1,663 CAREDS participants (98%) passing QC tests, of which 1,585 also had MPOD measures and relevant covariate data. CAREDS women excluded from this particular study ($n=420$) due to missing genotype or MPOD data were compared relative to the women included ($n=1,585$). Women excluded were slightly older (mean age 70.7 versus 69.4, $P < 0.01$) and had higher serum lutein and zeaxanthin, after adjusting for age differences (0.33 versus 0.31 $\mu\text{mol/L}$, $P=0.01$). Distributions of MPOD and predictors of MPOD, such as

dietary lutein and zeaxanthin, diabetes, waist circumference, dietary fiber and polyunsaturated fat, were similar between the two groups ($P \geq 0.20$).

Overall, there were 440 candidate carotenoid SNPs and 176 AIMS available for analysis in 1,585 CAREDS women who also had MPOD data.

Statistical analysis

Data management and standard statistical analyses were performed using SAS software version 9.2 (SAS Institute Inc, Cary NC) and genetic association analyses were performed using PLINK version 1.07 (<http://pngu.mgh.harvard.edu/purcell/plink/>).⁸

The correlations between MPOD and other dietary, lifestyle, serum, physical, and medical attributes were determined by computing Pearson correlation coefficients after adjustment for dietary lutein and zeaxanthin. Mean MPOD by levels of these characteristics were computed adjusting for lutein and zeaxanthin intake using least-squares linear regression modeling. A multivariable model of environmental predictors of MPOD was built using forward selection to both confirm our previous results¹ and consider new possible predictors of MPOD such as serum measures of C-reactive protein and 25(OH)D or composite dietary and lifestyle scores including the Healthy Eating Index and Healthy Lifestyle Score.

The CAREDS population is over 97% self-reported Caucasian ancestry. However, to minimize the risk of residual confounding due to population stratification within a sample of European ancestry, we used 176 AIMS designed to capture European ancestry clines to conduct principal components analysis (PCA) using the SmartPCA program in EIGENSOFT (http://genetics.med.harvard.edu/reich/Reich_Lab/Software.html).^{9,10} The first two principal components accounted for 3.1% and 1.3% of the genotype variability, respectively, and were used in adjustment of ancestry in SNP association models.

SNPs from carotenoid candidate genes were tested for association with MPOD using least-squares linear regression, assuming a one-degree-of-freedom additive genetic model. In

the circumstance where less than 10 people were homozygous for the minor allele, the one-degree-of-freedom dominant genetic model was tested instead. SNPs were first tested using a minimal adjustment model including intake of lutein and zeaxanthin (supplements and dietary) and ancestry (via first two principal components). SNPs with marginal statistical significance ($P \leq 0.05$) in the minimally adjusted model were further tested to see if the association was independent from other predictors of MPOD in CAREDS¹ including waist circumference, diabetes, and dietary fiber. Finally, serum lutein and zeaxanthin was included in the model to understand if variation in the SNP was associated with MPOD independently, or mediated via serum concentrations of lutein and zeaxanthin. If multiple SNPs within a gene were associated with MPOD, conditional modeling within each gene was done using forward selection and beginning with the most significant SNP to ascertain independence of SNP effects. Linkage disequilibrium (r^2 and D') between pairs of SNPs was estimated and haplotype blocks were determined in Haploview version 4.2.^{11,12}

SNPs associated with MPOD were tested for interactions with lutein and zeaxanthin intake and waist circumference, two strong, modifiable predictors of MPOD. Statistical interactions were tested using an interaction term in the linear regression model where the SNP is coded additively and the environmental factor continuous. When the one-degree-of-freedom test for interaction was suggestive ($P < 0.20$), SNP-MPOD associations were stratified by tertile of environmental variable to qualitatively understand dependency of SNP effects by waist or dietary intake.

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