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Smoking, Dietary Betaine, Methionine, and Vitamin D in Monozygotic Twins with Discordant Macular Degeneration: Epigenetic Implications

Johanna M. Seddon, MD, ScM[1,2,*], **Robyn Reynolds, MPH**[1,*], **Heeral R. Shah, MD**[2], and **Bernard Rosner, PhD**[3]

[1]Ophthalmic Epidemiology and Genetics Service, New England Eye Center, Tufts Medical Center, Boston, Massachusetts

[2]Department of Ophthalmology, Tufts Medical Center, and Tufts University School of Medicine, Boston, Massachusetts

[3]Channing Laboratory, Boston, Massachusetts

Abstract

Objective—We evaluated monozygotic twin pairs with discordant age-related macular degeneration (AMD) phenotypes to assess differences in behavioral and nutritional factors.

Design—Case series.

Participants—Caucasian male twin pairs from the United States Twin Study of Macular Degeneration.

Methods—Twin pairs were genotyped to confirm monozygosity. Ocular characteristics were evaluated based on fundus photographs using the Wisconsin Grading System and a 5-grade Clinical Age-Related Maculopathy Staging System. We selected twin pairs discordant in each of the following phenotypic categories: Stage of AMD (n = 28), drusen area (n = 60), drusen size (n = 40), and increased pigment area (n = 56). The Wilcoxon signed-rank test and linear regression were used to assess associations between behavioral and nutritional characteristics and each phenotype within discordant twin pairs.

Main Outcome Measures—Differences in smoking and dietary factors within twin pairs discordant for stage of AMD, drusen area, drusen size, and pigment area.

Results—Representative fundus photographs depict the discordant phenotypes. Pack-years of smoking were higher for the twin with the more advanced stage of AMD ($P = 0.05$). Higher dietary intake of vitamin D was present in the twins with less severe AMD ($P = 0.01$) and smaller drusen size ($P = 0.05$) compared with co-twins, adjusted for smoking and age. Dietary intakes of betaine and methionine were significantly higher in the twin with lower stage of AMD ($P = 0.009$) and smaller drusen area ($P = 0.03$), respectively.

Correspondence: Johanna M. Seddon, MD, Ophthalmic Epidemiology and Genetics Service, Tufts Medical Center, 800 Washington St. #450, Boston, MA 02111. jseddon@tuftsmedicalcenter.org.
*J.M.S. and R.R. contributed equally to this work.

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Conclusions—The twin with the more advanced stage of AMD, larger drusen area, drusen size, and pigment area tended to be the heavier smoker. The twin with the earlier stage of AMD, smaller drusen size and area, and less pigment tended to have higher dietary vitamin D, betaine, or methionine intake. Results suggest that behavioral and nutritional factors associated with epigenetic mechanisms are involved in the etiology of AMD, in addition to genetic susceptibility.

Genetic and environmental risk factors, including diet, smoking, and body mass index, are associated with age-related macular degeneration (AMD).[1–12] The US Twin Study of Age-Related Macular Degeneration, a population-based study of monozygotic (MZ) and dizygotic (DZ) twin pairs who were concordant and discordant for AMD, was established to evaluate the relative effects of genetics and the environment on AMD.[1] Based on the results of this study, genetic factors explain 46% to 71% of disease severity and environmental factors explain 19% to 37%[1] (Seddon JM, Samelson LJ, Page WF, Neale MC. Twin study of macular degeneration: methodology and application to genetic epidemiologic studies [ARVO abstract]. Invest Ophthalmol Vis Sci 1997;38:S676). The US Twin Study also evaluated drusen and pigment subphenotypes and heritability estimates ranged from 0.26 to 0.71.[1] Because genetic factors are strong determinants for development and progression of AMD, MZ twin pairs who have the same genetic makeup but have different stages of AMD provide a unique population to assess behavioral and nutritional risk factors associated with AMD.

We previously reported associations between AMD and cigarette smoking, fish consumption, and omega-3 and linoleic acid intake among MZ and DZ twins in this study population.[2] We sought to expand these findings and determine how these risk factors are associated in MZ twins with discordant stages of AMD and discordant macular phenotypes associated with AMD.

Because MZ twins are genetically identical, they are considered ideal experimental models for studying the role of environmental factors as determinants of complex diseases and phenotypes. We used the common genetic makeup of the MZ twin population to explore novel dietary factors associated with anti-inflammatory mechanisms. Vitamin D was found to reduce the risk of early AMD[13] and has anti-inflammatory properties. Several genes involved in inflammatory immune responses are associated with AMD.[14–23] Fish is a source of vitamin D as well as omega-3 fatty acids, and increased fish intake may reduce risk of AMD.[2,9,24,25]

Epigenetics is a change in DNA and chromatins, which regulates genomic functions that occur without a change in DNA sequence.[26,27] This mechanism could help to explain how environmental factors influence phenotypic variation of chronic, complex, non-Mendelian diseases in MZ twins.[26,27] Cancer and several diseases associated with inflammation, such as atherosclerosis, osteoarthritis, and lupus erythematosus, are thought to be associated with epigenetic changes.[27] Given that AMD is a complex disease, is associated with inflammation, and has many of the same risk factors as cardiovascular disease, we investigated smoking and dietary nutrients reported to be associated with epigenetic changes, including choline, betaine (a choline derivative), and methionine.[27–29] We evaluated these behavioral and nutritional factors among MZ twin pairs with discordant stages and signs of AMD to better understand the potential influence of environmental and epigenetic related factors on AMD.

Methods

Study Population

The study population was derived from the National Academy of Sciences—National Research Council World War II Veterans Twin Registry, which includes medical information for 15 924 white male twin pairs born between 1917 and 1927.[30] Details of this study population and the design of the twin study have been previously described[1] (Seddon JM, Samelson LJ, Page WF, Neale MC. Twin study of macular degeneration: methodology and application to genetic epidemiologic studies [ARVO abstract]. Invest Ophthalmol Vis Sci 1997;38:S676). Briefly, 12 126 individuals were surveyed for a prior diagnosis of AMD. For those twins who responded yes to having been diagnosed with AMD (n = 684), their medical records were reviewed to confirm AMD status and eligibility. After medical record review, 340 twin pairs in which 1 or both twins were diagnosed with AMD agreed to participate in the study. An additional 51 pairs from the oldest age group, in which neither twin had a diagnosis of AMD, were examined and enrolled as controls. Fifty-eight singletons were also enrolled (co-twin refused, was too ill, or was deceased). A total of 840 individual twins were enrolled in the US Twin Study of Age-Related Macular Degeneration including 440 MZ and 400 DZ individuals.[1]

For this study, we evaluated last known stage of AMD, and selected 3 macular phenotypes related to AMD, namely, drusen size, drusen area, and area of increased pigment. The MZ twin pairs that were discordant within each category were identified. We defined discordant pairs by their worse eye phenotype as follows: stage of AMD (n = 28)—each co-twin had a different grade based on the Clinical Age-Related Maculopathy Staging System (CARMS)[31] at last known grade, with 1 twin being affected with advanced AMD (either geographic atrophy [GA] or neovascular [NV] disease), and the other twin without AMD or with an earlier stage; drusen size (n = 40)—each co-twin had different drusen size at baseline, and 1 twin had drusen 63 μm in diameter or larger; drusen area within the grid (n = 60)—each co-twin had a different drusen area at baseline, and the more advanced twin had an area with diameter ≥125 μm; or area of increased pigment within the grid (n = 56)—each co-twin had a different area of increased pigment at baseline, and the twin with the worse grade had an area with diameter at least 63 μm. This research complied with the tenets of the Declaration of Helsinki and institutional review board approval was obtained.

Zygosity Status and Genotyping

In 90% of twin pairs, zygosity status was originally determined using questionnaire data from the National Academy of Sciences—National Research Council World War II Veterans Twin Registry, and there was 90% agreement with blood typing.[30,32,33] Of the 10% with unknown zygosity, DNA was evaluated and zygosity was determined by polymerase chain reaction and microsatellite typing using multiplex analyses of 8 microsatellite loci from 8 different chromosomes with polymorphic information content of ≥0.8.[30] For this study, DNA samples from the twins were assessed for 8 single nucleotide polymorphisms in genes demonstrated to be related to AMD: (1) complement factor H (*CFH*) *Y402H* (rs1061170); (2) *CFH* rs1410996[14–19]; (3) *ARMS2/HTRA1* rs10490924[14,34–37]; (4) complement component 2 or *C2* E318D (rs9332739)[14,20]; (5) complement factor B or *CFB* R32Q (rs641153)[14,20]; (6) complement component 3 or *C3* R102G (rs2230199)[21,22]; (7) complement factor I or *CFI* (rs10033900)[23]; and (8) hepatic lipase C or *LIPC* (rs10468017).[38] Genotyping was performed using primer mass extension and MALDI-TOF MS analysis (MassEXTEND methodology of Sequenom, San Diego, CA) at the Broad Institute Center for Genotyping and Analysis (Cambridge, MA). Co-twins within each MZ pair had identical genotypes.

Macular Characteristics and Phenotypes

All twins underwent a dilated fundus examination and fundus photography. Ophthalmologists were unaware of the twin's zygosity or disease status of the co-twin. Refraction, best-corrected visual acuity, intraocular pressure, and iris color were determined. Fundus examination was conducted to determine the stage of AMD. Fundus photography was performed according to a standard protocol in which stereo pair 30° fundus photographs centered on the disc, fovea, and temporal to the fovea of each eye were taken. Photographs were evaluated for signs of AMD by using a grid with a 3000-micron radius centered on the fovea using 2 systems.[1] Study examination data and photographs were evaluated by J.M.S. and were assigned an AMD grade by the CARMS grading system (grades 1–5).[31] Unlike the Age-Related Eye Disease Study system, level of visual acuity was not considered in this classification system, drusenoid retinal pigment epithelial detachment was graded separately, GA included noncentral as well as central atrophy, noncentral atrophy was categorized as advanced disease, and NV disease was classified separately. Fundus photographs were also assessed by the Wisconsin Reading Center using the Wisconsin AMD Grading System in which drusen and pigment area were estimated using standard circles of specified diameter: 63, 125, 250, 362, 650, and 1061 μm.[39] The grader was masked to clinical diagnosis and zygosity status.

Smoking and Dietary Characteristics

Smoking history was collected using a standardized risk factor questionnaire administered at baseline. Pack-years were calculated by multiplying average number of cigarettes per day by years smoked divided by 20 (the number of cigarettes in 1 pack). For the assessment of nutritional factors, food frequency questionnaires were administered at baseline, and the details of data collection and analysis of the dietary nutrients are described elsewhere.[2,9,24,40] Dietary variables were log transformed and adjusted for caloric intake. We determined whether twins lived north or south of the 37th parallel to control for any large differences in vitamin D exposure from sunlight.

Statistical Analysis

The AMD phenotypes for each twin were categorized based on the worse eye and given an ordinal rank as follows: stage of AMD—1 (no AMD), 2 (early AMD), 3 (intermediate AMD), 4 (GA), 5 (NV AMD); drusen size—0 (none), 1 (>0, <63 μm), 2 (≥63 μm, <125 μm), 3 (≥125 μm, <250 μm), 4 (≥250 μm); drusen area—0 (<63 μm), 1 (≥63 μm, <125 μm), 2 (≥125 μm, <250 μm), 3 (≥250 μm, <362 μm), 4 (≥362 μm, <650 μm), 5 (≥650 μm, <1061 μm), 6 (≥1061 μm); and pigment area—0 (none), 1 (>0, <63 μm), 2 (≥63 μm, <125 μm), 3 (≥125μm, <250 μm), 4 (≥250 μm, <650 μm), 5 (≥650 μm).

The twin with the higher ranked phenotype was designated TW (higher) and the co-twin, with the lower ranked phenotype, was designated TW (lower) for all tables and statistical analyses. Signed-rank tests were used to calculate *P* values for the unadjusted difference between continuous behavioral and nutritional characteristics of TW (higher) and TW (lower). Linear regression was used to calculate parameter estimates for the regression of difference in a risk factor on the difference between TW (higher) and TW (lower) phenotype score for each risk factor, adjusting for the difference in age between TW (higher) and TW (lower) at the time of phenotype ascertainment. For ordinal ranked macular characteristics, we selected twin pairs who had a score difference of ≥2, signifying a more distinct difference in phenotype. Nutritional factors were also adjusted for the difference in pack-years of smoking between TW (higher) and TW (lower). Parameter estimates for nutrients were based on log transformation. *P* values ≤0.05 were considered significant. We used SAS (SAS Inc., Cary, NC) version 9.2 for all statistical analyses.

Results

Figure 1 displays fundus photographs and ocular coherence tomography scans that demonstrate the differences in phenotypes between co-twins for a representative twin pair, and the difference in progression of the twins over the course of 10 years. This figure highlights and documents some of the macular phenotypic differences between MZ twins.

Table 1 shows the distribution of discordant AMD phenotypes among twin pairs. Among 28 twin pairs discordant for stage of AMD, there are 4 pairs for whom 1 twin has either GA or NV and the co-twin is unaffected. The largest number of discordant pairs is in the category where TW (higher) has NV disease and TW (lower) has early AMD. In most of the twin pairs discordant for drusen size, drusen area, and increased pigment area, twin (lower) had no sign of drusen or pigment, while twin (higher) and some sign.

As shown in Table 2, we determined if mean values of behavioral and nutritional variables differed between TW (higher) and TW (lower) among AMD phenotypes. For twins discordant for AMD grade, TW (higher) was a significantly heavier smoker than TW (lower) ($P = 0.048$), and for each 1 unit difference in grade, there is a 4.48-year increase in pack-years. Figure 2 displays the mean difference in pack-years of smoking according to differences in grade within a twin pair. There is a greater difference in pack-years within the twin pairs when the degree of discordance is greater. For other macular phenotypes, TW (higher) also tended to be a heavier smoker than TW (lower), although this difference was not statistically significant.

Fish and omega-3 fatty acid intakes were lower for TW (higher) for all AMD phenotypes, and linoleic acid intake was increased for TW (higher) for most AMD phenotypes, although these differences were not statistically significant after adjustment for age and pack-years. Dietary vitamin D was significantly higher for TW (lower) for both AMD grade ($P = 0.01$) and drusen size ($P = 0.05$), when adjusting for age and pack-years. This trend persisted for pigment area, although it was not significant. Figure 3 displays the mean differences in dietary vitamin D levels according to differences in grade within twin pairs. The difference in dietary vitamin D intake is larger when the degree of discordance is greater. In a separate analysis of AMD grade differences, vitamin D with supplements was evaluated and results were similar ($P = 0.04$).

Both unadjusted and adjusted dietary betaine levels were significantly lower for the higher grade ($P = 0.022$ and $P = 0.009$, respectively). This trend in the same direction was seen for drusen size and increased pigment area (not statistically significant). Adjusted methionine intake was significantly higher ($P = 0.03$) for TW (lower) with respect to drusen area. There was also a nonsignificant trend for methionine to be higher in TW (lower) for AMD grade, drusen size, and pigment area. No significant differences were seen for dietary choline.

Discussion

To our knowledge, this is the first study to evaluate environmental and epigenetic factors, novel nutritional factors, and early and advanced AMD phenotypes in a population composed solely of elderly discordant MZ twins with the same genetic susceptibility (Shah HR, Reynolds R, Seddon JM. Discordant Age-Related Macular Degeneration Characteristics in Monozygotic Twin Pairs. Invest Ophthalmol Vis Sci 2010;52:ARVO E-abstract 4536). Age-related macular degeneration has a high heritability of up to 0.71,[1] and genetic variants are associated with development, progression, and ocular characteristics of the disease.[1,3,4,14–23,41–43] Utilizing a population where genotype is inherently controlled for, we were able to further assess differences in environmental and macular phenotypes associated with AMD. When we considered how behavioral factors might contribute to the

differences in phenotype, among genetically indentical individuals, we found that the twin with the more advanced AMD was a heavier smoker. The twin with the larger drusen size, drusen area, and pigment area also tended to smoke a larger amount for a longer duration.

Smoking and diet are documented modifiable environmental factors associated with the development of AMD.[2,6–12] We confirmed that smoking is a risk factor in our study with a novel design. A diet high in fish consumption has been associated with decreased risk of AMD.[2,9,24,25] The twin with the earlier stage of AMD tended to eat more fish than the twin with the later stage of AMD, although this difference was not significant in this study. In a previous study that assessed omega-3 fatty acids and fish intake in the total population of twins from which these MZ twins were derived, a significant inverse association was found between fish intake and AMD.[2]

We also used this unique population to explore other dietary associations with AMD. We found a significant inverse association between AMD and drusen size and intake of dietary vitamin D, controlling for residence north or south of the 37th parallel, to account for sunlight exposure to some extent. Vitamin D may reduce risk of AMD because it has anti-inflammatory or antiangiogenic properties.[44] In the third National Health and Nutrition Examination Survey, Parekh et al[13] found an inverse association between prevalence of early AMD and soft drusen for foods rich in vitamin D and serum levels of vitamin D. Further studies are needed to confirm the inverse association with dietary vitamin D found in our study, as well as to evaluate sunlight exposure as another source of vitamin D.

We also found inverse associations between (a) AMD and higher intake of betaine and (b) drusen area and higher intake of methionine. Differences in epigenetic regulation of genes and their expression levels may influence phenotypes in MZ twins.[26] These changes may be inherited, can arise during the lifetime of MZ twins, and can be associated with environmental factors such as diet, physical activity, and smoking.[26–29] Epigenetic changes are characterized by chemical modifications such as DNA methylation or histone acetylation.[27] Dietary methionine, choline, and betaine (derived from choline) have been shown to have an effect on epigenetic mechanisms and DNA methylation.[27,29] The methylation of homocysteine involves betaine, choline, methionine, and folate interactions, which lowers homocysteine levels, and the capacity to methylate homocysteine leads to elevated levels of homocysteine.[29] Of note, a few articles have shown that increased levels of homocysteine can increase the risk of AMD.[45,46] Betaine is found mainly in fish, grain products, and spinach.[47,48] Sources of methionine include poultry, fish, and dairy products.[49]

Previously in our study population, we calculated heritability estimates for macular phenotypes including drusen area with diameter ≥175 μm (0.71), maximum drusen size of ≥125 μm (0.55), presence of soft drusen (0.54), increased pigment (0.43), and retinal pigment epithelial de-pigmentation (0.35).[1] Another twin study (n = 506) of younger female MZ and DZ pairs found that large soft drusen and presence of >20 hard drusen were heritable in early AMD.[50] In another cohort of 220 MZ and DZ twins, having >20 drusen per eye was confirmed to be highly heritable.[51] A twin study of 150 female MZ and DZ pairs estimated the heritability of macular pigment to be 0.67 using heterochromatic flicker photometry imaging technique, and 0.85 using fundus autofluoresence.[52] These results of twin studies together with recent discovery of AMD genes indicate that genetic factors play a relatively greater role than environmental factors in the etiology of the disease.[11] However, other factors such as environmental or epigenetic mechanisms must be involved, because not all MZ twins have the same AMD phenotype.

Our study population consists of a unique sample of elderly MZ twins who are discordant for early as well as advanced stages of AMD. Other advantages include the clinical examination of all twins according to standard protocols, standard grading of fundus photographs, follow-up to determine most recent grade, and collection of environmental and dietary risk factor data. Twins were confirmed to be MZ through the National Academy of Sciences—National Research Council World War II Veterans Twin Registry blood typing as well as through direct genotyping. Unlike other twin studies, we assessed specific dietary variables from a food frequency questionnaire, and we found 3 that had a significant inverse relationship with grade of AMD, drusen size, and drusen area. Another sample of elderly MZ twins discordant for early, intermediate, and advanced AMD with information on diet and other factors would be useful for a combined meta-analysis to increase statistical power.

In summary, among MZ twins with identical genes and different AMD phenotypes, heavier smoking was significantly related to more advanced AMD. Dietary intakes of vitamin D, methionine, and betaine were inversely associated with AMD, drusen size, and drusen area. Vitamin D has anti-inflammatory and anti-angiogenic properties, which may have a protective effect on AMD development.[13,44] Smoking and some nutritional factors may induce epigenetic changes that modify gene expression, leading to different phenotypes. These findings and the discordant phenotypes seen in some pairs of MZ twins implicate that factors other than DNA sequence are involved in the etiology of AMD. Results of this study are biologically plausible, generate hypotheses, and warrant further investigation.

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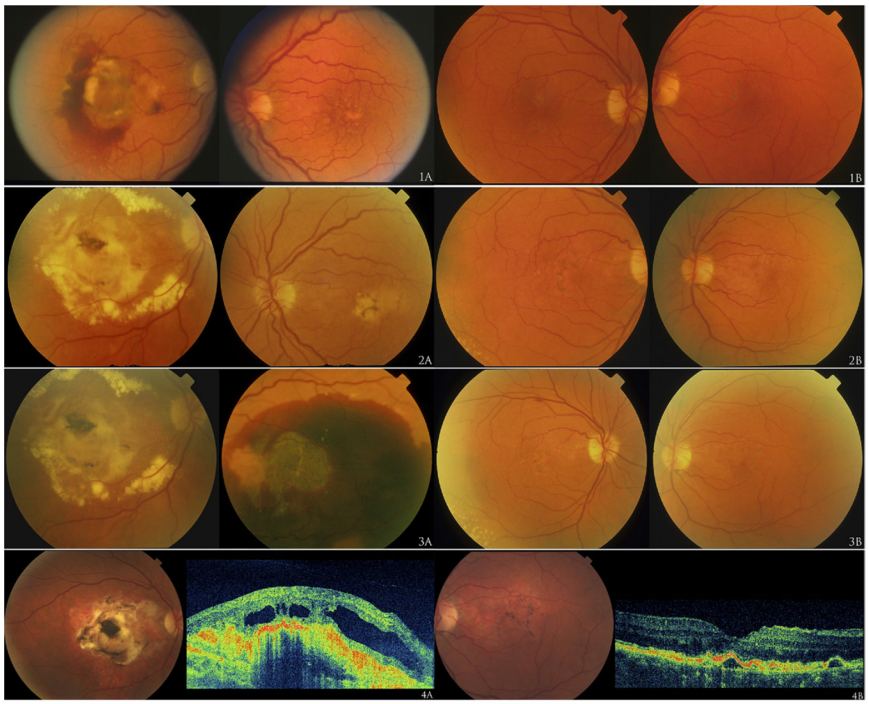

Figure 1.

Discordant macular phenotypes within 1 monozygotic twin pair. The fundus appearance of twin A is represented in panels 1A to 4A, and that of twin B is depicted is panels 1B to 4B. Photos were taken at clinic visits when twins were age 64 (March 1997), 68 (April 2001), 68½ (July 2001), and 74 (August 2007). Panels 4A and 4B are representative ultra– high resolution optical coherence tomography scans. Twin A had advanced neovascular disease and twin B had drusen and retinal pigment epithelial irregularities.

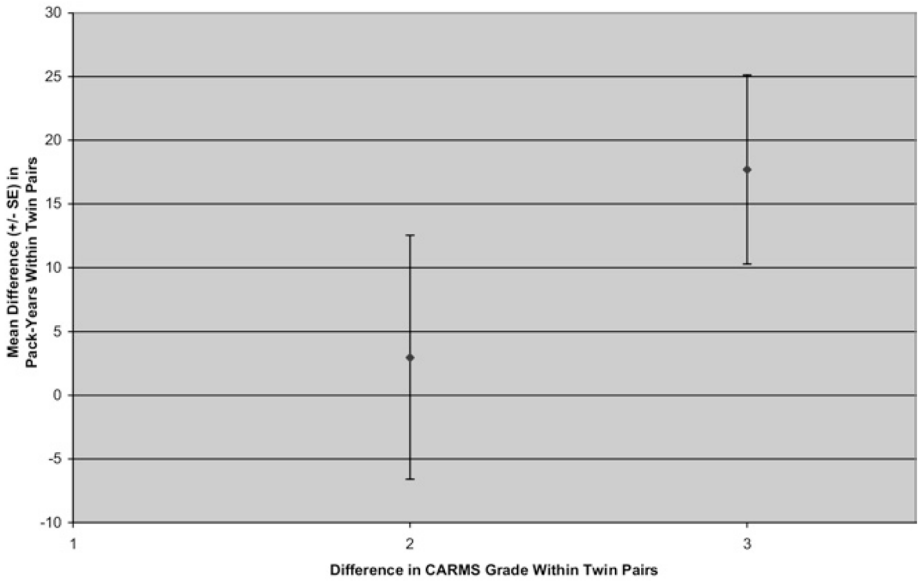

Figure 2.
Mean difference (± standard error [SE]) in pack-years according to difference in Clinical Age-Related Maculopathy Staging System (CARMS) grade of age-related macular degeneration within twin pairs.

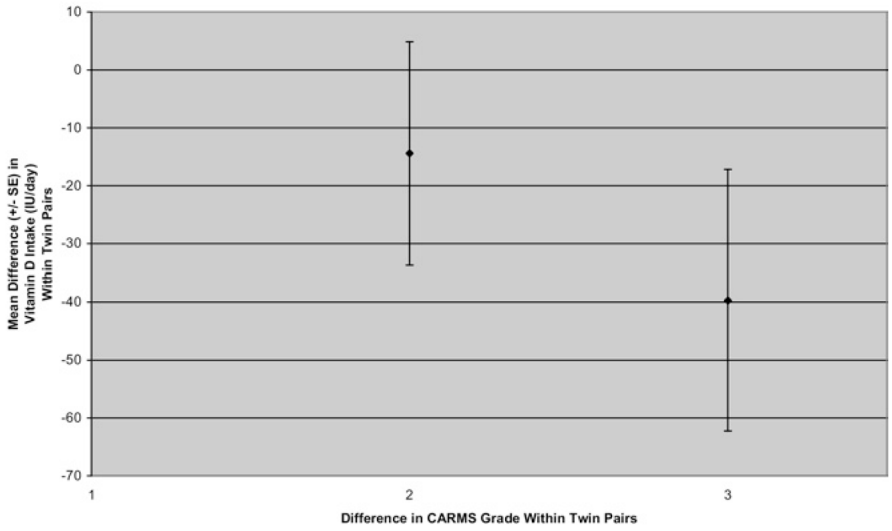

Figure 3.
Mean difference (± standard error [SE]) in vitamin D intake according to differences in Clinical Age-Related Maculopathy Staging System (CARMS) grade of age-related macular degeneration within twin pairs.

Table 1

Distribution of Discordant Macular Phenotypes in Monozygotic Twin Pairs

Twin (higher)* phenotype	**Twin (lower)† Phenotype**					
CARMS‡ grade	1 (no AMD)	2 (early AMD)	3 (intermediate AMD)	Total number of pairs		
4 (geographic atrophy)	1	2	0	3		
5 (neovascular)	3	13	9	25		
Total number of pairs	4	15	9	28		
Drusen size§	0 (None)	1 (<63μm)	2 (≥63 μm, <125μm)	Total Number of pairs		
2 (≥63 μm, <125 μm)	15	0	0	15		
3 (≥125 μm, <250 μm)	7	11	0	18		
4 (≥250 μm)	2	2	3	7		
Total number of pairs	24	13	3	40		
Drusen area§	0 (<63 μm)	1 (≥63 μm, <125 μm)	2 (≥125 μm, <250 μm)	3 (≥250 μm, <362 μm)	4 (≥362 μm, <650 μm)	Total number of pairs
2 (≥125 μm, <250 μm)	16	0	0	0	0	16
3 (≥250 μm, <362 μm)	7	3	0	0	0	10
4 (≥362 μm, <650 μm)	6	2	8	0	0	16
5 (≥650 μm, <1061 μm)	2	5	1	1	0	9
6 (≥1061 μm)	1	1	1	3	3	9
Total number of pairs	32	11	10	4	3	60
Pigment area§	0 (None)	1 (<63 μm)	2 (≥63 μm, <125 μm)	Total number of pairs		
2 (≥63 μm, <125 μm)	19	0	0	19		
3 (≥125 μm, <250 μm)	12	0	0	12		
4 (≥250 μm, <650 μm)	11	0	6	17		
5 (≥650 μm)	6	0	2	8		
Total number of pairs	48	0	8	56		

*Twin with the “higher” ranked phenotype (Clinical Age-Related Maculopathy Staging System (CARMS) grade, drusen size, drusen area, area of pigment).

†Twin with the “lower” ranked phenotype (CARMS grade, drusen size, drusen area, area of pigment).

‡Last known grade.

§Phenotype within the grid. Grid is composed of 3 circles concentric with the macula. The radius of outer circle is 3000 μm. Diameters of the standard circles are shown for drusen area and pigment area.

Table 2

Differences in Risk Factors Among Monozygotic Twins With Discordant Macular Phenotypes

	Risk Factor							
Macular Phenotype	***Smoking (pack-years)***	***Fish Intake (servings/day)***	***Omega-3 (g/d)*** **	***Linoleic Acid (g/d)*** **	***Vitamin D (IU/d)*** **	***Betaine (mg/d)*** **	***Methionine (g/d)*** **	***Choline (mg/d)*** **
CARMS * grade (n = 28 discordant pairs)								
Twin (higher) † (mean ± SE)	36.47 ± 7.47	0.22 ± 0.029	0.18 ± 0.019	10.59 ± 0.42	170.85 ± 16.87	105.30 ± 6.18	1.59 ± 0.052	321.48 ± 13.57
Twin (lower) ‡ (mean ± SE)	24.56 ± 6.42	0.29 ± 0.045	0.24 ± 0.030	10.54 ± 0.62	200.27 ± 18.52	140.89 ± 14.62	1.71 ± 0.055	325.89 ± 9.76
Mean difference (twin higher − twin lower) ± SE	11.91 ± 5.92	−0.069 ± 0.050	−0.055 ± 0.032	0.050 ± 0.84	−29.42 ± 15.42	−35.59 ± 15.096	−0.12 ± 0.068	−4.41 ± 12.13
Raw *P* value §	0.077	0.50	0.22	0.87	0.071	0.022	0.067	0.17
Within-pair $\beta \pm$ SE ‖	4.48 ± 2.16	−0.013 ± 0.019	−0.047 ± 0.062	0.012 ± 0.031	−0.105 ± 0.038	−0.105 ± 0.037	−0.030 ± 0.016	−0.012 ± 0.014
Within-pair *P* value	0.048	0.52	0.46	0.69	0.01	0.009	0.074	0.41
Drusen size ¶ (n = 40 discordant pairs)								
Twin (higher) † (mean ± SE)	37.11 ± 6.97	0.23 ± 0.032	0.18 ± 0.025	11.00 ± 0.57	203.84 ± 19.15	127.27 ± 11.97	1.66 ± 0.057	323.72 ± 9.46
Twin (lower) ‡ (mean ± SE)	27.45 ± 3.96	0.28 ± 0.031	0.29 ± 0.051	9.85 ± 0.43	229.64 ± 17.75	136.70 ± 9.30	1.74 ± 0.042	337.011 ± 6.82
Mean difference (twin higher − twin lower) ± SE	9.67 ± 6.46	−0.049 ± 0.042	−0.106 ± 0.057	1.16 ± 0.65	−25.80 ± 21.56	−9.42 ± 15.64	−0.073 ± 0.065	−13.29 ± 9.97
Raw *P* value §	0.18	0.10	0.1	0.100	0.69	0.14	0.28	0.16
Within-pair $\beta \pm$ SE ‖	4.75 ± 2.70	−0.020 ± 0.017	−0.13 ± 0.075	0.033 ± 0.028	−0.099 ± 0.048	−0.039 ± 0.039	−0.022 ± 0.017	−0.020 ± 0.014
Within-pair *P* value	0.087	0.25	0.089	0.25	0.05	0.33	0.22	0.18
Drusen area ¶ (n = 60 discordant pairs)								
Twin (higher) † (mean ± SE)	30.38 ± 5.00	0.20 ± 0.022	0.20 ± 0.031	10.25 ± 0.41	201.61 ± 15.64	130.46 ± 9.72	1.65 ± 0.044	326.69 ± 7.92
Twin (lower) ‡ (mean ± SE)	26.79 ± 4.032	0.25 ± 0.022	0.21 ± 0.026	9.95 ± 0.35	198.30 ± 11.56	129.13 ± 8.59	1.74 ± 0.039	334.85 ± 6.13

	Risk Factor							
Macular Phenotype	***Smoking (pack-years)***	***Fish Intake (servings/day)***	***Omega-3 (g/d)*** **	***Linoleic Acid (g/d)*** **	***Vitamin D (IU/d)*** **	***Betaine (mg/d)*** **	***Methionine (g/d)*** **	***Choline (mg/d)*** **
Mean difference (twin higher − twin lower) ± SE	3.59 ± 5.34	−0.049 ± 0.030	−0.0074 ± 0.037	0.301 ± 0.501	3.303 ± 14.77	1.33 ± 12.84	−0.086 ± 0.051	−8.16 ± 7.95
Raw *P* value§	0.42	0.052	0.24	0.47	0.61	0.65	0.12	0.31
Within-pair *β* ± SE‖	2.78 ± 1.78	−0.017 ± 0.010	−0.061 ± 0.039	0.0013 ± 0.017	−0.030 ± 0.026	−0.017 ± 0.026	−0.022 ± 0.010	−0.014 ± 0.0087
Within-pair *P* value	0.12	0.10	0.13	0.94	0.26	0.51	0.033	0.107
Pigment area¶ (n = 56 discordant pairs)								
Twin (higher)† (mean ± SE)	26.37 ± 3.99	0.20 ± 0.022	0.15 ± 0.013	10.07 ± 0.32	183.35 ± 12.73	119.51 ± 9.23	1.67 ± 0.040	333.61 ± 8.12
Twin (lower)‡ (mean ± SE)	19.35 ± 2.96	0.23 ± 0.019	0.21 ± 0.027	10.88 ± 0.39	203.18 ± 14.85	128.16 ± 9.03	1.75 ± 0.050	332.92 ± 8.44
Mean difference (twin higher − twin lower) ± SE	7.02 ± 3.71	−0.030 ± 0.029	−0.055 ± 0.028	−0.81 ± 0.502	−19.83 ± 14.40	−8.65 ± 12.51	−0.077 ± 0.055	0.69 ± 10.025
Raw *P* value§	0.083	0.12	0.082	0.16	0.21	0.17	0.19	0.99
Within-pair *β* ± SE‖	2.32 ± 1.20	−0.0068 ± 0.0095	−0.067 ± 0.036	−0.022 ± 0.016	−0.040 ± 0.026	−0.035 ± 0.024	−0.010 ± 0.011	−0.00057 ± 0.010
Within-pair *P* value	0.057	0.47	0.067	0.18	0.14	0.15	0.37	0.96

SE = standard error.

*Clinical Age-Related Maculopathy Staging System (CARMS) last known grade.

†Twin with “higher” ranked phenotype (CARMS grade, drusen size, drusen area, area of pigment).

‡Twin with “lower” ranked phenotype (CARMS grade, drusen size, drusen area, area of pigment).

§Signed-rank test.

‖Parameter estimate calculated using linear regression; *β* represents the expected difference in a risk factor (or log value for dietary risk factors) for each 1 unit increase in phenotype (minimum = 2). Smoking analyses were adjusted for age differences between twins at time of AMD grade assignment. All nutritional factors were adjusted for age and smoking (pack-years) differences between TW (higher) and TW (lower) in a twin pair. Vitamin D adjusted for north/south geographical location.

¶Phenotype within the grid. The grid is composed of 3 circles concentric with the macula. The radius of outer circle is 3000 μm.

**Adjusted for caloric intake.