Association between adherence to the Mediterranean diet and oxidative stress

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ABSTRACT

Background: The cardioprotective property of the Mediterranean diet has been attributed to its antioxidant capacity, but direct investigation of this mechanism has been limited.

Objective: We examined the association between the Mediterranean diet and an established plasma marker of oxidative stress, the ratio of reduced to oxidized glutathione (GSH/GSSG), in a well-controlled study of twins.

Design: We administered the Willett food-frequency questionnaire to 138 monozygotic and dizygotic twin pairs and to 21 unpaired twins and derived a score measuring adherence to the Mediterranean diet. Fasting plasma GSH and GSSG concentrations were measured to calculate the GSH/GSSG ratio. The higher the ratio, the lower the oxidative stress. Mixed-effect regression analysis was used to partition the association into between- and within-twin pair differences. When within-pair effects are examined, twins are matched for sociodemographic and familial factors.

Results: A one-unit increment in the diet score was associated with a 7% higher GSH/GSSG ratio ($P = 0.03$) after adjustment for energy intake, other nutritional factors, cardiovascular disease risk factors, and medication use. The association persisted within twin pairs: a one-unit within-pair absolute difference in the diet score was associated with a 10% (95% CI: 2.7, 18.0) higher GSH/GSSG ratio in the twin with the higher score than in the co-twin with the lower score ($P = 0.007$). Results were similar in monozygotic and dizygotic twin pairs.

Conclusions: The association between the Mediterranean diet and plasma oxidative stress is robust and is not confounded by genetic or shared environmental factors. Decreased oxidative stress is a plausible mechanism linking the Mediterranean diet to reduced cardiovascular disease risk. Am J Clin Nutr 2008;88:1364–70.

INTRODUCTION

Greater adherence to the Mediterranean diet is associated with a lower risk of cardiovascular disease (1, 2). The leading hypothesis on the mechanism of this association is a decrease in oxidative stress due to the antioxidant capacity of the diet (3). However, although the antioxidant properties of the Mediterranean diet are known, direct investigation of whether this diet is associated with lower oxidative stress is limited. Furthermore, because dietary habits in adult life are influenced by the habits acquired while growing up (4–6), they may be confounded by other exposures or behaviors shared by members of the same family. Previous studies have not been able to adequately control for familial factors when investigating the association between the Mediterranean diet and oxidative stress.

Oxidative stress is a pathophysiological pathway thought to influence all aspects of atherosclerosis development and cardiovascular disease risk (7). Glutathione (GSH, reduced form) and glutathione disulfide (GSSG, oxidized form) in plasma are transported from tissues by concentration-dependent transport systems (8). Results from animal experiments have shown that the ratio of GSH to GSSG (GSH/GSSG) in plasma decreases in response to tissue oxidative stress (9). Oxidative stress is conceptualized as a disruption of redox signaling and control (10). Therefore, GSH/GSSG may be preferable to either GSH or GSSG alone as an overall indicator of redox status and, thus, is used as a marker of oxidative stress (11). Biochemically, GSH/GSSG redox decreases lipid hydroperoxides by reducing these peroxides into alcohols and suppressing their generation (12, 13). Decreased lipid hydroperoxides, in turn, lower oxidized low-density lipoproteins and thus can inhibit atherosclerosis (12, 13). Additionally, a lower GSH/GSSG may result in protein glutathionylation and oxidatively altered GSH-GSSG redox signaling (11) and associated gene expression and apoptosis, which may contribute to atherosclerosis. Clinically, an unfavorable GSH/GSSG was found in patients with acute myocardial infarction.

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compared with controls (14) and was related to the progression of atherosclerotic lesions after percutaneous coronary intervention (15).

Using a sample of monozygotic and dizygotic middle-aged male twins raised in the same family, we examined the association between degree of adherence to the Mediterranean diet and plasma GSH/GSSG. Twins are naturally matched for demographic, familial, and other environmental influences while growing up. Monozygotic pairs are also 100% matched for genetic factors, whereas dizygotic pairs share on average 50% of their genes. In pairs whose members differed in level of adherence to the Mediterranean diet, we examined whether the association persisted when comparing each twin with his co-twin. If the association is found within twin pairs, it is not confounded by early environmental or familial factors. If the association is observed within monozygotic pairs, it is also independent of genetic factors.

SUBJECTS AND METHODS

Participants

The Twins Heart Study (THS) is an investigation of psychological, behavioral and biological risk factors for subclinical cardiovascular disease. The THS includes 180 pairs of monozygotic and dizygotic male twins from the Vietnam Era Twin Registry—a registry of male-male twin pairs, both of whom served in the US military during the Vietnam era (16). Twins selected for inclusion in the THS were born between 1946 and 1956, which represented >90% of the twins in the Vietnam Era Twin Registry. On the basis of self-reported data from 1990, eligible twins were free of symptomatic cardiovascular diseases (17). Zygosity information determined by DNA analysis was available from all but 11 twin pairs. The zygosity of these 11 pairs was assessed by using questionnaires supplemented with blood group typing data abstracted from military records (18), which in our sample had an accuracy of 94%.

For the THS, random samples of twins in 2 strata were selected from the registry: 1 stratum included twins discordant for a lifetime history of major depression, and the other stratum included twins with no history of depression. All twin pairs were examined at the Emory University General Clinical Research Center between March 2002 and March 2006. The assessment included a comprehensive history, physical exam, and biochemical measures; we also obtained updated information about symptomatic cardiovascular disease. Because the measurement of plasma GSH and GSSG was not available for the first 11 twin pairs, the available sample for this study was 169 twin pairs. We further excluded 41 participants, including 1 with no dietary data, 4 with an implausible energy intake (≥6000 or <500 kcal/d) (19), 34 with previous cardiovascular disease as assessed at our clinic visit, and 2 with missing GSH/GSSG data. A number of unpaired twins resulted from these exclusions, which were retained in the analyses. Inclusion of unpaired twins is common practice in twin modeling because it allows full use of all available data (20). Therefore, our analyses were based on 297 twins, including 81 monozygotic and 57 dizygotic twin pairs, and 9 monozygotic and 12 dizygotic unpaired twins. The study protocol was approved by the Institutional Review Board of Emory University, and informed consent was obtained from all subjects.

Diet assessment

We used the Willett self-administered semiquantitative food-frequency questionnaire (21) to collect dietary data over the previous 12 mo. The questionnaire classifies average food intake according to 9 frequency categories ranging from “almost never or less than once per month” to “≥6 times/d” by using standardized portion sizes for each dietary item, including beverages and nutritional supplements. Questionnaires were scored by the Nutrition Questionnaire Service Center, Channing Laboratory, Harvard University, and nutrient intake data were derived by using the nutrient database of the US Department of Agriculture (21). Daily food intake in grams was calculated from food intake frequency and portion sizes.

Mediterranean diet score

The Mediterranean diet is characterized by a high intake of fruit, vegetables, bread, other forms of cereals, beans, nuts, and seeds; a low-to-moderate intake of dairy products, fish, poultry and wine; a low intake of red meat; egg consumption ≤4 times/wk; and olive oil as an important fat source (22). We measured adherence to the Mediterranean diet using a Mediterranean diet score (MDS) described by Trichopoulou et al (1), which is based on a priori assumptions about 9 desirable or undesirable dietary components for health (See Appendix Table 1 under “Supplemental data” in the online issue). The 7 desirable components include cereals, vegetables, fruit and nuts, legumes, fish, a high dietary ratio of monounsaturated to saturated fatty acids (as reflected by high olive oil consumption), and moderate alcohol consumption; the 2 undesirable components are meat and dairy food products. To conduct analyses stratified by zygosity in our all-male sample, we constructed the score using zygosity-specific, rather than sex-specific, medians of food intakes (adjusted to 2500 kcal). A value of 1 was assigned to a high intake (≥median) of each desirable component or a low intake (<median) of each undesirable food. All other intakes received a value of 0 (1, 23). For alcohol, a value of 1 was assigned to moderate consumption, that is, an intake above the zygosity-specific median (1.91 g/d for monozygotic or dizygotic) and ≤33 g/d. The latter is the upper limit of daily alcohol intake considered to be “moderate” among American men and is equivalent to ≈2 alcoholic drinks/d (24, 25). The MDS was the sum of all values from the 9 components, ranging from 0 to 9; the higher the score, the greater the adherence to the Mediterranean diet.

We also devised 4 slight variations of the MDS to evaluate the robustness of our findings. First, we followed an earlier method published by Trichopoulou et al (23) to calculate a score, MDS1, ranging from 0 to 8, in which fish was included in the meat group (26) and was not considered a desirable dietary component; potatoes were included with cereals; and eggs were included with meats (23). In a second variant of the score, MDS2, ranging from 0 to 8, fish was excluded from meat, and its intake was forced as a covariate into the models. In a third variant of the score, MDS3, ranging from 0 to 8, fish and eggs were excluded from the meat group and included as a separate desirable component; potatoes were included with cereals; and eggs were included with meats.
Assessment of known cardiovascular disease risk factors

We assessed smoking, education, and marital status using standardized questionnaires. Physical activity was evaluated with the validated Baecke questionnaire (27). Waist and hip circumferences were measured and used to calculate the waist-to-hip ratio. Systolic and diastolic blood pressures were measured with a mercury sphygmomanometer according to a standard protocol (28). Hypertension was defined as a systolic blood pressure ≥ 140 and/or a diastolic blood pressure ≥ 90 mm Hg or current use of antihypertensive medications. Diabetes was defined as a fasting plasma glucose concentration ≥ 126 mg/dL (29) or current treatment with insulin or oral antihyperglycemic agents. Depressive symptoms were measured with the Beck Depression Inventory, which yielded a continuous score (30). Current use of aspirin and statins was also recorded. Serum creatinine concentration was measured with a kinetic alkaline picrate method and used to calculate estimated glomerular filtration rate (eGFR) based on the following formula (31):

\[
eeGFR = 186 \times (\text{serum creatinine concentrations}) - 1.154 \times (\text{age}) - 0.203 \times (0.742 \text{ if female}) \times (1.210 \text{ if African American}) (I)
\]

Biochemical analysis

Blood samples for GSH and GSSG assays were assayed according to established procedures with twin pair samples assessed in the same analytic run (11, 32). A 9-h overnight fasting blood sample was collected into tubes containing 100 mmol/L sodium heparin, 1 mg bathophenanthroline disulfonate, and 2 mg iodoacetic acid to inhibit GSH autoxidation and degradation by γ-glutamyltranspeptidase. After centrifugation, plasma was transferred to a microcentrifuge tube with 200 μL of a 10% (wt:vol) perchloric acid solution containing 0.2 mol boric acid/L and 10 μmol/L γ-glutathione-glutathione and stored at −80 °C until analyzed. GSH and GSSG concentrations were measured in a single run with the use of a validated HPLC assay (11, 32). The method for plasma GSH and GSSG assays included procedures to avoid hemolysis (32); samples with evidence of hemolysis were discarded before analysis. The inter- and intraassay variabilities for all assays were < 10%. GSH and GSSG concentrations were used to calculate GSH/GSSG. We also used the Nernst equation to calculate the glutathione redox potential for the GSH/GSSG couple (E0, GSH/GSSG) (11). Because all values for E0 GSH/GSSG were negative, the absolute value, or aE0, GSH/ GSSG, was used in the analysis. The higher the GSH/GSSG value or the aE0, GSH/GSSG, the lower the oxidative stress. Fasting plasma glucose, triacylglycerols, and total, low-, and HDL cholesterol were measured by using standard methods.

Statistical analysis

We defined “within-pair absolute differences” as differences between a twin with a higher MDS score and his twin brother with a lower score. The right skewed oxidative stress biomarkers were log-transformed to improve normality. The association between the MDS and oxidative stress biomarkers was assessed by fitting linear regression models adapted for twin studies and examined at 2 levels (20): between-subject and within-pair. Because dependent variables were log-transformed, the results were expressed as percentage differences in the nontransformed values using the following formula:

\[
\frac{(\exp(\beta) - 1) \times 100}{\text{percent}}
\]

where \( \beta \) is the regression coefficient, and \( \exp(\beta) \) returns the exponential value of the variable.

We first treated twins as individuals while accounting for twin pair clustering (Table 1). The association at this level is the weighted average of within-pair and between-pair information (20). The MDS was analyzed primarily as a continuous variable and secondarily as a categorical variable according to quartiles (0 to 3, 4, 5, and 6 to 9). Category midpoints were used for analysis. Next, we performed within-pair analyses to examine differences in biomarkers between co-twins in each pair (Table 2). The within-pair effects were inherently controlled for shared demographic, familial, and, in monozygotic pairs, 100% genetic influences; additionally, environmental factors during the day of testing were controlled for because co-twins were examined at the same time. We fitted mixed models for twins (20), which allowed for partitioning within- and between-pair differences in the dependent variable as a function of the independent variables. In these models the within-pair \( \beta \) coefficient describes the individual twin variation from the twin pair average, and it is identical to the \( \beta \) coefficient from a model that fits the absolute difference between the co-twins (20). Thus, the percentage difference calculated from this variable represents the percentage increment/decrement in biomarker concentrations per one-unit absolute difference in the MDS between twin brothers, comparing a twin with a one-unit higher MDS to his twin brother with a lower MDS. The “base model” was adjusted for nutritional factors, including total energy intake, potato and egg consumption (1), dietary supplements (fish oil, vitamins, and minerals such as zinc, selenium, and iron), and other relevant nutritional habits (extent of habitual removal of visible fat on meat; consumption of punches, fruit drinks, and fried foods; and types of cooking oils). To this model we added sociodemographic factors (age, years of education, and current marital status), lifestyle factors (current smoking, waist-to-hip ratio, and physical activity), and cardiovascular disease risk factors (depressive symptom scores, fasting glucose, systolic blood pressure, LDL cholesterol, and the ratio of HDL cholesterol to triacylglycerols), and use of aspirin and statins. To rule out model overfitting, we fitted parsimonious models after backward elimination. However, our associations of interest were similar in the parsimonious models and in the full models. All analyses were conducted by using SAS software version 9.1 (SAS Institute, Cary, NC). Significance levels were set at 0.05 (2-sided).

RESULTS

Sample characteristics

Men with a higher MDS were more educated, more physically active, less likely to smoke, had lower depressive symptom scores, and were more likely to use fish-oil supplements (Table 3). The sample was 94% non-Hispanic white, 3% African American, and 3% other race/ethnic groups. This distribution reflected the racial distribution of the Vietnam Era Twin Registry from which it was sampled. Except for GSH, intraclass coefficients for MDS, GSSG, GSH/GSSG, and aE0, GSH/GSSG were greater...
among monozygotic than among dizygotic twins, which suggests that genetic factors contribute to these traits (See Appendix Table 2 under “Supplemental data” in the online issue).

**Overall associations**

When the MDS was treated as a continuous variable, for each 1-unit increment in the MDS, GSH was 0.9% higher (P = 0.71), GSSG concentrations were 6.4% lower (P = 0.07), GSH/GSSG was 8.0% higher (P = 0.008), and aEh GSH/GSSG was 0.8% lower (P = 0.03) after nutritional factors not included in the MDS were adjusted for (model 1 in Table 1). After further adjustment for traditional cardiovascular risk factors and medication use, the association of the MDS, either as a continuous or as a categorical variable, with the GSH/GSSG ratio remained statistically significant (model 2 in Table 1). In the fully adjusted model, twins in the highest MDS quartile had a GSH/GSSG 31% higher (95% CI: 27.7, 35.6) compared to those in the lowest quartile.

**Within-pair results**

The mean within-pair absolute difference in the MDS was 1.6% (range: 6) in monozygotic and 1.9% (range: 7) in dizygotic twins. The association between within-pair difference in the MDS and biomarkers were not different by zygosity (all P > 0.4 for the interactions) (See Appendix Table 3 under “Supplemental data” in the online issue), which suggests that genetic factors do not play a substantial role in these associations. In all of the subsequent analyses, we therefore pooled monozygotic and dizygotic pairs (Table 2). In the combined sample of monozygotic and dizygotic pairs, within-pair associations of the MDS with GSSG concentrations and GSH/GSSG were statistically significant in all models, whereas the association with aEh GSH/GSSG was nearly statistically significant (Table 2). In the fully adjusted model (model 2 in Table 2), a one-unit within-pair absolute difference in the MDS was associated with a 10% lower GSSG concentration (P = 0.02) and a 10% higher GSH/GSSG (P = 0.007).

Similar results were obtained by using a 3-level smoking variable (never smoked, current smoker, and past smoker), excluding 4 subjects with elevated concentrations of inflammatory biomarkers (3 subjects with high sensitive C-reactive protein > 30 mg/L and one subject with tumor necrosis factor-α > 200 pg/mL), further controlling for interleukin-6 and C-reactive protein concentrations, further controlling for diabetes mellitus and renal function measured by using eGFR, and further controlling for antihypertensive and antihyperglycemic medications. Furthermore, when we repeated the analyses using published variations of the Mediterranean score (MDS1, MDS2, MDS3, and MDS4), the results remained similar.

**DISCUSSION**

We found a robust inverse association between adherence to the Mediterranean diet and oxidative stress as measured by GSH/GSSG, primarily because of lower GSSG concentrations, independent of a wide range of known cardiovascular disease risk factors. This finding persisted when co-twins within pairs were compared, either monozygotic or dizygotic, which suggests that shared familial and genetic factors do not confound the association between adherence to the Mediterranean diet and oxidative stress as measured by GSH/GSSG. Similar, but borderline significant, trends, were found for aEh GSH/GSSG. The results were robust to slight variations in the Mediterranean diet score.
Our results are important, particularly in view of the lack of randomized controlled trials assessing the effects of the Mediterranean diet on glutathione redox pathways in the general population. A few small trials (33–36) and one large trial (37), however, examined short- or intermediate-term effects of the Mediterranean diet on other circulating markers of oxidative stress, including urinary F2-isoprostanes (33), plasma malondialdehyde (34), oxidized LDL, and others (35–37). In the largest of these trials, subjects assigned to the Mediterranean diet had lower oxidized LDL than did those following the control diet (37). Other trials, however, yielded mixed results. One of the reasons for these inconsistencies may be the different biomarkers measured. Individual markers may signal different metabolic pathways (10), and some pathways but not others may be influenced by diet. Another reason for the discrepant results is the potential for unmeasured confounding, such as that by genetic background and other familial factors. Our twin study clearly overcame this potential limitation, which indicated an association in twin pairs matched for familial and (among monozygotic) genetic factors.

Our results are consistent with animal experiments showing that polyphenols naturally present in Mediterranean foods decrease the GSSG/GSH ratio and GSSG concentrations without affecting GSH concentrations (38, 39). Our results also expand our recent finding, in this same population, of an inverse association between Mediterranean diet and inflammation (40), since inflammation and oxidative stress are tightly inter-dependent.

Several possible mechanisms explain an increased plasma GSH/GSSG with a Mediterranean diet, primarily through decreased GSSG concentrations. First, GSH is oxidized into GSSG by the enzyme glutathione peroxidase (41); in this process, GSH quenches peroxides. GSSG reverts to GSH via glutathione reductase with concomitant oxidation of NADPH (42). Diverse nutrients and biofactors in foods characteristic of the Mediterranean diet may provide higher NADPH (41) and up-regulate glutathione reductase activity (43), which leads to a decrease in GSSG and a resulting higher GSH/GSSG. Second, a “sparring effect” on the GSH/GSSG redox cycle may also contribute to the increased GSH/GSSG. By providing other antioxidants (such as vitamin C, vitamin E, carotenoids, polyphenols, zinc, and selenium) and ensuring adequate activity and efficiency of antioxidant enzymes (12), a diet approximating the Mediterranean diet may decrease the utilization of the GSH/GSSG antioxidant pathway, which may “spare” the GSH/GSSG recycle (44). Furthermore, the low content of prooxidants in the diet may also contribute to a higher GSH/GSSG (45). Although there was a robust association with GSH/GSSG, the Mediterranean diet was weakly associated with the glutathione redox potential. Because glutathione redox potential is more sensitive to a concentration change of GSH than of GSSG (32) and adherence to the Mediterranean diet was not significantly associated with GSH concentrations, the observed results were not unexpected.

Our study had some limitations. The sample was restricted to males; therefore, our results may not be generalizable to females. Because our study was cross-sectional and observational, unmeasured confounding was possible. Our study addressed a pattern of diet that contains elements of, but is not necessarily equal to, the originally defined Mediterranean diet (46), because the latter is rare in Western countries today. However, sufficient
MEDITERRANEAN DIET AND OXIDATIVE STRESS

Characteristics according to the Mediterranean diet score in the entire sample

<table>
<thead>
<tr>
<th>Variable</th>
<th>(0–3) (n = 88)</th>
<th>(4) (n = 63)</th>
<th>(5) (n = 66)</th>
<th>(6–9) (n = 80)</th>
<th>P for trend&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>54.0 (51.5, 56.0)&lt;sup&gt;7&lt;/sup&gt;</td>
<td>55.0 (53.0, 57.0)</td>
<td>55.5 (53.0, 57.0)</td>
<td>55.0 (54.0, 57.0)</td>
<td>0.63</td>
</tr>
<tr>
<td>Education (y)</td>
<td>13.0 (12.0, 14.5)</td>
<td>14.0 (13.0, 16.0)</td>
<td>15.0 (13.0, 16.0)</td>
<td>14.5 (13.0, 16.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Married [n (%)]</td>
<td>71 (76.3)</td>
<td>50 (73.5)</td>
<td>56 (78.9)</td>
<td>73 (83.9)</td>
<td>0.37</td>
</tr>
<tr>
<td>Current smoker [n (%)]</td>
<td>26 (28.0)</td>
<td>14 (20.6)</td>
<td>7 (9.9)</td>
<td>10 (11.5)</td>
<td>0.002</td>
</tr>
<tr>
<td>Ethanol intake (g/d)</td>
<td>0 (0, 10.9)</td>
<td>1.1 (0, 13.6)</td>
<td>2.5 (0, 9.9)</td>
<td>4.0 (0.5, 10.9)</td>
<td>0.013</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>29.6 ± 5.4&lt;sup&gt;4&lt;/sup&gt;</td>
<td>29.2 ± 5.0</td>
<td>29.3 ± 4.1</td>
<td>28.4 ± 3.5</td>
<td>0.16</td>
</tr>
<tr>
<td>Waist-to-hip ratio</td>
<td>0.94 ± 0.06</td>
<td>0.95 ± 0.07</td>
<td>0.93 ± 0.06</td>
<td>0.94 ± 0.06</td>
<td>0.83</td>
</tr>
<tr>
<td>Physical activity (unit)</td>
<td>7.20 (5.93, 8.32)</td>
<td>7.41 (6.59, 8.38)</td>
<td>7.82 (6.93, 8.69)</td>
<td>7.63 (7.07, 8.34)</td>
<td>0.02</td>
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</table>

Clinical and biochemical features

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mediterranean diet score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Depressive symptoms (BDI score)</td>
<td>4.0 (1.0, 8.5)</td>
</tr>
<tr>
<td>Plasma glucose concentration (mg/dL)&lt;sup&gt;6&lt;/sup&gt;</td>
<td>97 (90, 104)</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>131 ± 15</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>81 ± 11</td>
</tr>
<tr>
<td>Total triacylglycerol (mg/dL)&lt;sup&gt;4&lt;/sup&gt;</td>
<td>172 (124, 231)</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)&lt;sup&gt;4&lt;/sup&gt;</td>
<td>188 ± 33</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)&lt;sup&gt;4&lt;/sup&gt;</td>
<td>37 (31, 45)</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dL)&lt;sup&gt;4&lt;/sup&gt;</td>
<td>125 ± 32</td>
</tr>
<tr>
<td>Fish-oil supplement use [n (%)]</td>
<td>2 (2.2)</td>
</tr>
<tr>
<td>Statin use [n (%)]</td>
<td>21 (22.6)</td>
</tr>
<tr>
<td>Aspirin use [n (%)]</td>
<td>19 (20.4)</td>
</tr>
<tr>
<td>Antihypertensive use [n (%)]</td>
<td>16 (18.2)</td>
</tr>
<tr>
<td>Antihyperglycemic use [n (%)]</td>
<td>6 (6.5)</td>
</tr>
</tbody>
</table>

Plasma oxidative stress biomarkers

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mediterranean diet score</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH (µmol/L)</td>
<td>1.41 (0.86, 2.02)</td>
</tr>
<tr>
<td>GSSG (µmol/L)</td>
<td>0.07 (0.02, 0.10)</td>
</tr>
<tr>
<td>GSH/GSSG</td>
<td>24.2 (14.5, 47.0)</td>
</tr>
<tr>
<td>aE&lt;sub&gt;6&lt;/sub&gt;GSH/GSSG (mV)</td>
<td>130 (122, 140)</td>
</tr>
</tbody>
</table>

<sup>1</sup> BDI, Beck Depression Inventory; GSH, glutathione; GSSG, glutathione disulfide; GSH/GSSG, ratio of GSH to GSSG; aE<sub>6</sub>GSH/GSSG, absolute value of the redox potential for the GSH/GSSG couple.
<sup>2</sup> Test for trend across diet score groups. All P values were corrected for clustering within a twin pair according to the twin type by using linear mixed models for continuous variables and generalized estimated equation logistic models for dichotomous variables. Means and medians presented are raw values.
<sup>3</sup> Median; 25th and 75th percentile in parentheses (all such values).
<sup>4</sup> x ± SD (all such values).
<sup>5</sup> Test to convert values to mmol/L, multiply by 0.0555.
<sup>6</sup> Test to convert values to mmol/L, multiply by 0.0113.
<sup>7</sup> Test to convert values to mmol/L, multiply by 0.0259.

Variations in diet across individuals allowed us to rank our subjects on the basis of the similarity of their diet to the Mediterranean diet (46). The Willett food-frequency questionnaire may not accurately estimate absolute food and nutrient intakes, but it is appropriate in our investigation of the diet-outcome relations after energy adjustment (47). As in other common food-frequency questionnaires used across the United States, combined food items containing 2 or more components of the MDS can misclassify individual MDS components. However, we carefully decomposed combined items into individual ingredients using appropriate recipes, which minimized the chance of misclassification.

On the other hand, a major strength of our study was the use of a twin sample. Twins are a powerful resource to dissect complex associations, because they allow us to control for unmeasured and unknown confounding, such as genetic factors and socioeconomic, behavioral, and lifestyle characteristics acquired when growing up in the same family. This is particularly important for dietary habits, which are likely to be confounded by other lifestyle behaviors learned by individuals raised in the same family. By comparing each twin with his co-twin brother, we were able to control for these unmeasured confounders.

In conclusion, we showed a robust association between adherence to the Mediterranean diet and lower oxidative stress as indicated by the plasma GSH/GSSG. The association was not confounded by conventional risk factors, familial influences, or genetic factors. Our findings support the hypothesis that the Mediterranean diet has cardioprotective effects through the lowering of oxidative stress.

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