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Protective mechanisms of the Mediterranean diet on cardiovascular disease: a twin study

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ABSTRACT

The Mediterranean diet is cardioprotective, however underlying mechanisms are unknown. We examined the association between the Mediterranean diet and inflammation/oxidative stress, and between alpha-linolenic acid (ALA) and inflammation related to cellular response to interleukin-6 (IL-6), evaluating whether the associations persisted after further controlling for familial factors. We used data from raised-together middle-aged male twins, including monozygotic and dizygotic twins, in the Twins Heart Study (THS) at Emory University. Dietary data over the previous one year was collected with the Willett Food Frequency Questionnaire. We derived a diet score measuring adherence to the Mediterranean diet following a published algorithm. Mixed-effect regression analysis was used to partition the association into between- and within-twin pair differences. When examining within-pair effects, twins were matched for sociodemographic and familial factors. After adjusting for energy intake, other nutritional factors, known cardiovascular risk factors, and medication use, a one-unit increment in the diet score was associated with 5% lower interleukin-6 (IL-6) levels (P=0.008) and a 7% higher ratio of reduced (GSH) to oxidized glutathione (GSSG) (P=0.03), but not C-reactive protein (CRP) levels. A one-gram increment in habitual dietary ALA intake was associated with 11.4% lower levels of soluble IL-6 receptor (sIL-6R) (P=0.009). The association persisted within twin pairs: a one-unit within-pair absolute difference in the diet score was associated with 9.2% lower IL-6 levels (P <0.0001) and a 10% higher GSH/GSSG ratio (P=0.007); a one-gram within-pair absolute difference in dietary ALA intake was associated with 12.2% lower sIL-6R levels (P=0.005). In conclusion, the association between the Mediterranean diet and systemic inflammation/oxidative stress,

and that between habitual dietary ALA intake and sIL-6R is not confounded by genetic and shared environmental factors. Decreased inflammation and /or oxidative stress are plausible mechanisms linking Mediterranean diet to reduced cardiovascular risk, and habitual dietary ALA may contribute to the cardioprotective properties of the Mediterranean diet.

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PROTECTIVE MECHANISMS OF THE MEDITERRANEAN DIET ON CARDIOVASCULAR DISEASE: A TWIN STUDY

By

Jun Dai

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CHAPTER I

INTRODUCTION

The term Mediterranean diet refers to a dietary pattern typical of many regions in Greece and southern Italy in the early 1960s, including a high intake of fruits, vegetables, bread, other forms of cereals, potatoes, beans, nuts and seeds; low to moderate amounts of dairy products, fish, poultry and wine; low amounts of red meat; eggs consumed no more than four times weekly; and olive oil as an important fat source.[1] The term, the Mediterranean diet, is used in this context to emphasize the overall, whole function of the diet, a combination of a variety of foods containing numerous known nutrients and biofactors and unknown factors. Since diet is heterogeneous across individuals, and patterns of diet can be close to, although it is not necessarily equal to, the 1960's Mediterranean diet.[1] In observational epidemiological studies, the Mediterranean diet becomes a conceptual term to describe any diet that has elements of the originally described diet.

The Mediterranean diet is cardioprotective with clinically substantial magnitude. For example, American people with the greatest adherence to the Mediterranean diet were at 20-24% lower risk to die from cardiovascular disease than those with the lowest adherence.[2] Large-size randomized controlled trials suggested that patients on the Mediterranean diet had a 33% to 50% lower risk for the recurrence of adverse cardiac events than those on either the step I National Cholesterol Education Program (NCEP) prudent diet[3] or the prudent Western-type diet.[4] This cardioprotective role of the

Mediterranean diet against a recurrence of myocardial infarction after the first myocardial infarction lasted up to 4 years.[4]

At the nutrient level, since the Mediterranean diet is rich in plant foods, it is suggested that alpha-linolenic acid (ALA) contributes to the cardioprotective properties of this diet.[5] ALA, a plant 18-carbon ω-3 long-chain polyunsaturated fatty acid (18:3ω-3), is essential for humans because it cannot be synthesized *in vivo* due to lack of Δ12 and Δ15 desaturases. Humans can synthesize ω-3 long-chain polyunsaturated fatty acid derivatives with 20 or more carbons (ω-3 ≥ 20C-PUFA) from ALA, including mostly eicosapentaenoic (20:5ω-3) and docosapentaenoic acid (22:5ω-3), but also a limited amount of docosahexaenoic acid (22:6ω-3). Dietary alpha-linolenic acid (ALA) has been suggested to be protective against cardiovascular diseases.[6]

The precise mechanisms of the cardioprotective properties of the Mediterranean diet are unclear. It is well known that diet is closely associated with immunity,[7] and diet utilization *in vivo* involves intensively oxidative reactions.[5] Although dietary deficiency of essential fatty acids, either linoleic (18:3ω-6) or linolenic acid, is rare in the US general population, greater intake of linoleic acid relative to ALA results in an imbalance of ω-6 to ω-3 fatty acids, which may create a pro-inflammatory milieu.[8] Therefore, it is likely that the Mediterranean diet influences systemic inflammation and oxidative stress, which are established pathways in the pathogenesis of atherosclerosis, and also that habitual dietary ALA intake affects inflammation.

The association between the Mediterranean diet and systemic inflammation[9-11] and oxidative stress[12-15] were not consistently found in randomized controlled trials. Previous

studies of the association of dietary ALA with inflammation have also yielded inconsistent results.[16-21] A possible reason for the inconsistent findings may be due to genetic differences in the response to diet.

In addition, since dietary habits are acquired while growing up,[22-24] they are likely to be associated with other environmental conditions shared by members of the same family, such as unmeasured socioeconomic and lifestyle factors, which can also influence inflammation and oxidative stress, and thus act as confounders. However, no prior study has controlled for the influence of familial factors (either genetic, common environmental factors or both) in the association between the Mediterranean diet and inflammation.

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 by twins growing up in the same family. Since monozygotic (MZ) twins share 100% of their genes while dizygotic (DZ) twins share on the average 50% of their genes, if genetic factors contribute to a trait, MZ twins should be more similar on this trait than DZ twins. By comparing each twin with the co-twin in a sample of MZ and DZ middle-aged male twins raised in the same family, we can determine if the association persists after accounting for common genetic and environmental factors. If the association is found within twin pairs, it is not confounded by early environmental/familial factors. If the association is observed within MZ pairs, it is also independent of genetic factors.

In chapter III, we explore the association between adherence to the Mediterranean diet and systemic inflammation, and whether this association persists after further controlling for common genes and environment. Following the same strategy, in chapter

IV, we explore the association between adherence to the Mediterranean diet and oxidative stress, and in chapter V explore the association between habitual intake of alpha-linolenic acid and inflammation related to cellular response to interleukin-6.

The current study greatly contributes to epidemiological research and public health promotion. Disentangling the genetic influence on the diet-disease association will sharpen insights into the nutritional plausibility of cardioprotective properties of the Mediterranean diet, and substantiate the protective role of the Mediterranean diet *per se* against cardiovascular risk. The facts are that forty-three percent of all deaths in the United States each year are related to cardiovascular disease (CVD);[25] the cost for cardiovascular disease is estimated at 403.1 billion dollars for 2006;[26] and diet is a modifiable life-long factor, while genetic factors are not, consuming a healthier diet, such as the Mediterranean diet, to prevent from cardiovascular disease, is feasible in the USA.[27] Our findings will strongly support the benefits of the Mediterranean diet in preventing cardiovascular disease.

CHAPTER II

LITERATURE REVIEW

I. Pathophysiology in inflammation, oxidative stress and atherosclerotic cardiovascular disease

Atherosclerosis is thought to be an inflammatory process.[28] Innate and acquired immune responses as well as cytokine production work in concert in inflammation.[28, 29] In the early stage of atherosclerosis, two significant innate immune events involved in inflammation occur: the migration of monocytes from blood into vascular intima (emigration); and the subsequent transformation of monocytes into macrophages. Emigration of leukocytes including monocytes is mediated via cellular adhesion molecules (CAMs).[30] This migration includes three successive steps in the movement of circulating leukocytes toward the vascular wall: rolling of the leukocytes, adhering to the endothelial lining of the vascular wall endothelial cells (stopping), and then escaping into the vessel wall through the tight joint-gap between endothelial cells.[30, 31] The selectin family of CAMs, including sialyl-Lewis^x, P-selectin, E-selectin and L-selectin, mediates the weak adhesive interaction between leukocytes and endothelial cells. This interaction slows down the movement of leukocytes in the blood flow and makes them roll on the venular endothelium (rolling).[30, 31] The immunoglobin superfamily of CAMs, such as intercellular cell adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1), further firms adherence of leukocytes to the endothelium, so called "stopping".[30, 31] Then, the concentration gradient of chemoattractants directs leukocytes

to move into the intima.[32] For example, monocyte chemoattractant protein-1 (MCP-1)[33] is crucial for the recruitment, migration, and accumulation of monocytes in the intima, where they are transformed into macrophages. In the preceding step, monocyte-derived macrophages take up and accumulate lipids and become foam cells, the early hallmark of atherosclerosis.[28]

In acquired immune responses, B and T cells are activated after being exposed to antigens.[29] Activated B cells produce specific autoantibodies against oxidized low-density lipoproteins (ox-LDL), prime autoantigens, that are thought to play a role in the pathogenesis of atherosclerosis.[34] Activated pro-inflammatory T help-1 cells generate pro-inflammatory cytokines including tumor necrotic factor-alpha (TNF-α) and interleukin (IL)-1, which increase the secretion of IL-6.[29] On the other hand, activated T cells along with T helper-2 cells can release anti-inflammatory cytokines, including IL-4, IL-10, and IL-11, and hamper the atherosclerotic formation.[29, 35] Cytokines, in turn, further regulate the activity of cells involved in innate and acquired immunity via corresponding receptors.[36]

Cytokines also regulate other non-immune cells including vascular endothelial cells. These non-immune cells and/or their products participate in the development of atherosclerosis. IL-6 increases the endothelial expression of CAMs[37] resulting in the localized endothelial dysfunction, a very early feature in atherosclerosis.[38] IL-6 accelerates coagulation without affecting fibrinolysis[39] and thus promotes advanced atherosclerosis. Although TNF-α, IL-1 and IL-6 regulate the hepatic production of acute phase proteins such as C-reactive protein (CRP) and fibrinogen,[40, 41] IL-6 is the dominant stimulator in humans.[42] The IL-6 hepatic product CRP, an activator of innate immunity

and a modulator of adaptive immune responses,[43] binds to ox-LDL,[44] causes the aggregation of ox-LDL,[45] and enhances binding of ox-LDL to monocytic/macrophage-like cells via Fcgamma receptors.[46] Consequently, CRP promotes the uptake of ox-LDL by monocyte-derived macrophages through a CD_{32}-independent pathway,[45] and therefore contributes to the foam cell formation. These evidences imply that CRP may not only be a risk marker, but may also play a role in atherogenesis.[44] Fibrinogen participates in the development and progression of atherosclerotic plaques[47] via the enhancement of blood coagulation, blood rheology and platelet aggregation as well as direct effects on the vascular wall.[48] In addition, pro-inflammatory cytokines act on arterial smooth muscle cells and thus accelerate atherosclerosis. Influenced by these cytokines, arterial smooth muscle cells undergo a phenotype change (from physiological contractile to secretory phenotype), proliferate, and migrate from arterial media into the intima,[38] the feature of the intermediate lesions of atherosclerosis.

Innate and acquired immunity as well as cytokines also affect the vulnerability of atherosclerostic plaques.[49] Inflammation, cytokines and inflammatory mediators inhibit collagen synthesis and increase collagenolysis, leading to the unstable plaques. The rupture of the unstable plaques including a fibrous cap causes the acute adverse cardiac events.[49]

Oxidative stress also contributes to atherosclerosis, in concert with inflammation.[50] Oxidative stress is conceptualized as a disruption of redox signaling and control.[51] Reactive oxygen species (ROS) are involved in this process. ROS include free radicals and non-free radicals. Free radicals have an unpaired electron such as superoxide anion ($O^{\bullet 2-}$), hydroxyl radical ($^{\bullet}OH$), and nitric oxide ($^{\bullet}NO$). In contrast, non-free radicals,

such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$),[52] do not have an unpaired electrons. The major cellular thiol/disulfide systems, including reduced (GSH)/oxidized glutathione (GSSG), thioredoxin-1 (-SH2/-SS-), and cysteine/cystine (Cys/CySS), also play pivotal roles in redox signaling and control.[51]

Oxidative stress involves virtually all aspects of atherosclerosis including endothelial dysfunction, ox-LDL formation, and vascular remodeling. For example, ROS impair NO-dependent vasodilation through reducing nitric oxide (NO) bioavailability,[53, 54] and thus result in endothelial dysfunction, the very early characteristic of atherosclerosis. ROS oxidize LDL into ox-LDL, the major trigger for macrophages to take up lipids.[55, 56] ROS are also involved in the remodeling of vascular tissue.[29] The inflammatory process is accompanied by the ROS burst, in turn, ROS and their products regulate cytokines and immune signaling by activating NF-kappa B through the toll-like receptor 4.[57, 58] The activation of NF-kappaB regulates the gene expression of cytokines and other immune response genes. This vicious cycle between inflammation and oxidative stress exaggerates atherosclerosis.[50]

Another example is how the GSH/GSSG modulates atherosclerosis. The GSH/GSSG redox directly decreases lipid hydroperoxides by reducing these peroxides into alcohols.[52, 59] The GSH/GSSG redox also decreases the production of hydrogen peroxide and thus indirectly inhibits the generation of the lipid hydroperoxides.[52, 59] Decreased lipid hydroperoxides, in turn, decrease oxidized low-density lipoproteins and thus may inhibit atherosclerosis.[52, 59] Additionally, a lower GSH/GSSG ratio may result in: 1) protein glutathionylation, 2) oxidatively altered GSH-GSSG redox signaling,[60] 3)

modulated gene expression, and 4) apoptosis. All these consequences may contribute to atherosclerosis.

In summary, inflammation and oxidative stress, work in concert, leading to endothelial dysfunction and atherosclerotic lesions,[28] the instability of atherosclerotic plaque, and the formation of thrombosis. These pathologically vascular alterations lead to ischemic CVD[61-68] because of reduced or blocked blood flow.

Biomarkers of low-grade systemic inflammation

Inflammatory biomarkers include acute phase proteins, cytokines (such as TNF-alpha, IL-1, and IL-6) and their soluble receptors, and inflammatory cells (such as white blood cells). Acute phase proteins are defined as any protein whose plasma concentration increases or decreases by 25% or more during certain inflammatory disorders.[69] The acute phase proteins include C-reactive protein (CRP), serum amyloid A (SAA), fibrinogen, von Willebrand factor, and others.[69] Since the concentration of CRP, SAA, fibrinogen, or von Willebrand factor rises in inflammation, they are also called positive acute-phase proteins.[69] IL-1 can stimulate the synthesis of TNF-alpha in different cells such as monocytes, endothelial cells and fibroblasts.[70] IL-6, IL-1 alpha and TNF-alpha in various combinations are all capable of influencing synthesis of SAA in the human hepatoma cells whereas only IL-6 and IL-1 alpha can influence CRP synthesis.[71] Compared with IL-1, IL-6 has a greater capacity to stimulate the hepatic synthesis of CRP in humans.[40]

Soluble IL-6 receptor (sIL-6R) enhances and broadens IL-6 actions on target cells, and thus sIL-6R and IL-6 work in concert in systemic inflammation related to cellular

response to IL-6 which has been linked to atherosclerosis.[72] Through the formation of the IL-6/sIL-6R complex, sIL-6R prolongs the IL-6 half-life, amplifies responses of cells with membrane-bound-IL-6 receptors (mIL-6R) to IL-6, enables mIL-6R-deficient cells responsive to IL-6,[73] and augments IL-6 actions on many aspects of atherosclerosis.[29] Both IL-6 and sIL-6R have been related to increased susceptibility to cardiovascular diseases.[66, 68]

Not all inflammatory biomarkers have a clear cutoff point for defining a clinical normal value. The circulating CRP concentration in most healthy subjects is usually 1 mg/L with a normal value being defined as <10 mg/L regarding clinically active infection and inflammation.[74] On the contrary, there is no clinical cutoff for the normal plasma concentration of IL-6. Data from apparently healthy men participating in the Physicians' Health Study in the US showed that IL-6 levels ranged between 0.015 and 10.01 pg/mL, virtually identical to that expected from fresh blood samples obtained in otherwise healthy populations (0.01 to 11.5 pg/mL; data on file, R&D Systems, Minneapolis, Minn).[66] Some factors may affect the concentrations of inflammatory biomarkers. For instance, age-related elevations in the levels of circulating cytokines and acute-phase proteins have been reported.[75]

Systemic low-grade inflammation is a very loose term to describe a subtle and persistent alteration in the immune response. Systemic low-grade inflammation may be suggested as a status, in which plasma concentrations of cytokines and acute phase proteins rise by the two to three fold;[75] however, there is no consensus on the cutoff to define low-grade systemic inflammation. For example, CRP level less than 3 mg/L has been used as a cutoff for low-grade systemic inflammation.[76] A common approach in

epidemiological research is to compare people in the highest category generated by tertile, quartile, quintile, decile or centile with those in the lowest group based on concentrations of inflammatory biomarkers among the study population.[77]

Biomarkers of oxidative stress

Oxidative stress biomarkers that reflect oxidative-antioxidant status *in vivo* are numerous and can be categorized into several groups. Each group of oxidative stress biomarkers may signal a specific oxidative stress pathway.

The first group includes circulating concentrations of ox-LDL and autoantibodies against ox-LDL.[78] The second group contains lipid peroxidation products including malondialdehyde (MDA), thiobarbituric acid reactive substances (TBARS), and a family of isoprostanes. Among a variety of peroxides, MDA or TBARS have been widely used as biomarkers for oxidative stress for a long time. MDA is one of the terminal aldehyde products of lipid peroxidation, while TBARS mainly includes MDA. In the family of isoprostanes, F_2-isoprostanes are a series of prostaglandin F_2-like compounds produced through the radical-catalyzed lipid peroxidation *in vivo*, independent of cyclooxygenase. F_2-isoprostanes are derived mainly through the arachidonic acid pathway.[79] One of the most abundant F_2-isoprostane in humans is 8-epi-PGF_2alpha (8-isoprostane). Urinary or circulating concentrations of 8-isoprostane is used as the biomarker for systemic oxidative stress in the recent years.[80, 81] Fluorescent oxidation products of lipid peroxidation is the most recent biomarker for oxidative stress.[82]

The concentration and/or the activity of antioxidant or oxidative enzymes are used as the third group of biomarkers to indicate oxidative stress. Common antioxidant enzymes include superoxide dismutase (SOD), glutathione peroxidase (GPx), and

catalase (CAT). Myeloperoxidase is an oxidative enzyme used as a biomarker for oxidative stress and atherosclerosis.[83]

The fourth group of oxidative stress biomarkers is the antioxidant levels in different biological specimens. Plasma concentrations of vitamin C, alpha-tocopherol, carotenes, carotenoids, and uric acid are used to reflect the anti-oxidative defense. Reduced glutathione (GSH), the smallest intracellular thio (-SH), is a very important antioxidant molecule. In the reduction-oxidation reaction, GSH is converted to oxidized glutathione, glutathione sulfide (GSSG). The ratio of GSH to GSSG and the glutathione redox potential for the GSH-GSSG couple (E_h GSH/GSSG) are measures of oxidative stress. Both glutathione indices may be preferable to either GSH or GSSG alone as an overall indicator of redox status, because they quantify the dynamic balance between oxidants and antioxidants.[60, 84] The calculation of the ratio of GSH to GSSG concentrations is simple, while the E_h GSH/GSSG has to be calculated based on the Nernst equation.[60]. E_h GSH/GSSG is proportional to $[GSH]^2/[GSSG]$ because 2 GSH molecules are oxidized per 1 molecule of GSSG formed, therefore the E_h GSH/GSSG is more sensitive to the GSH concentration.[60, 84] The higher the GSH/GSSG ratio or the lower E_h GSH/GSSG, the lower the oxidative stress.

The fifth group of oxidative stress biomarkers includes oxidative adducts of biological macromolecules, such as DNA, due to the oxidative damage. For example, the 8-OH-dG concentration is commonly used to represent DNA oxidative damage.[85]

Antioxidant capacity, the sixth group of oxidative stress biomarkers, is measured using different methods. The susceptibility of LDL to metal-induced oxidation, measured as the lag time, is a biomarker for antioxidant capacity.[86] The longer the lag time, the

greater is the antioxidant capacity of LDL.[86] Another biomarker for antioxidant capacity is total antioxidant capacity (TAC). Several methods have been used to measure TAC including the oxygen radical absorbance capacity (ORAC) assay,[87] the Randox Trolox-equivalent antioxidant capacity (Randox-TEAC) assay,[88] the ferric reducing ability (FRAP) assay,[88] and the detection of reactive oxygen metabolite (d-ROMS). However, FRAP may not be suitable for serum and plasma samples, since the FRAP test is more suitable for non-protein samples.[89]

Finally, the ROS concentrations can be directly measured and used to indicate oxidative stress. For example, the free oxygen radical test (FORT) examines levels of organic hydroperoxides.[90]

Biomarkers of inflammation and oxidative stress in the epidemiological study

Not all biomarkers have been thoroughly investigated for their applications in the epidemiological study regarding their reproducibility and their capacity in ranking individuals.

The reproducibility of biomarker levels over a given time period is important in epidemiological studies in order to demonstrate the usual levels of inflammation or oxidative stress. The majority of the biomarkers of inflammation and oxidative stress is stable over the given time periods.[82, 91, 92] (**Figure 1**) For example, among men, plasma CRP, reactive oxygen metabolites (ROM), and free radical antioxidant potency (FRAP) were measured using samples approximately 5 years apart,[91] and their reproducibility was similar to that of cholesterol measured as intraclass correlation coefficients (ICC) (ICC for CRP, ROM, FRAP and cholesterol was 0.61, 0.43, 0.8, and 0.58, respectively).[91]

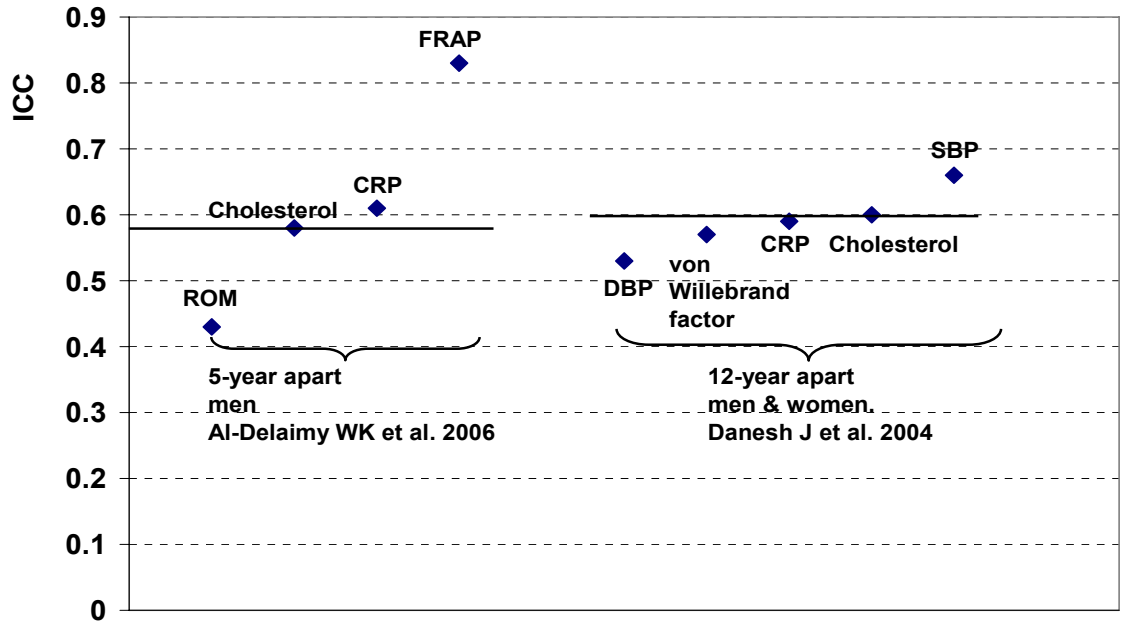

Figure 1. Reproducibility of some biomarkers of inflammation or oxidative stress

In addition, ranking individuals in the population is another critical issue in the epidemiological study. In term of ranking individuals, fasting plasma ox-LDL levels over 1 week are similar to that of cholesterol over 6 months.[93] Discrimination ratio and index of individuality are used to reflect the degree of ranking individuals of a biomarker. For instance, discrimination ratio (a ratio of the between-subject variation (SD_B) to the within-subject variation (SD_W)) for the circulating IL-6 levels, measured 6-month apart, is better than that for CRP, fibrinogen, SAA, or TNF-alpha (**Figure 2**),[94] indicating that IL-6 level is the best inflammatory biomarker in ranking individuals. Another example is that CRP levels and cholesterol concentration over 6 months are comparable in ranking individuals reflected by index of individuality (within-subject variation coefficient divided by between-subject variation coefficient).[95, 96] Prior evidences do not support the

usage of fibrinogen levels in the epidemiological study due to the poor ability to rank individuals.[94, 95]

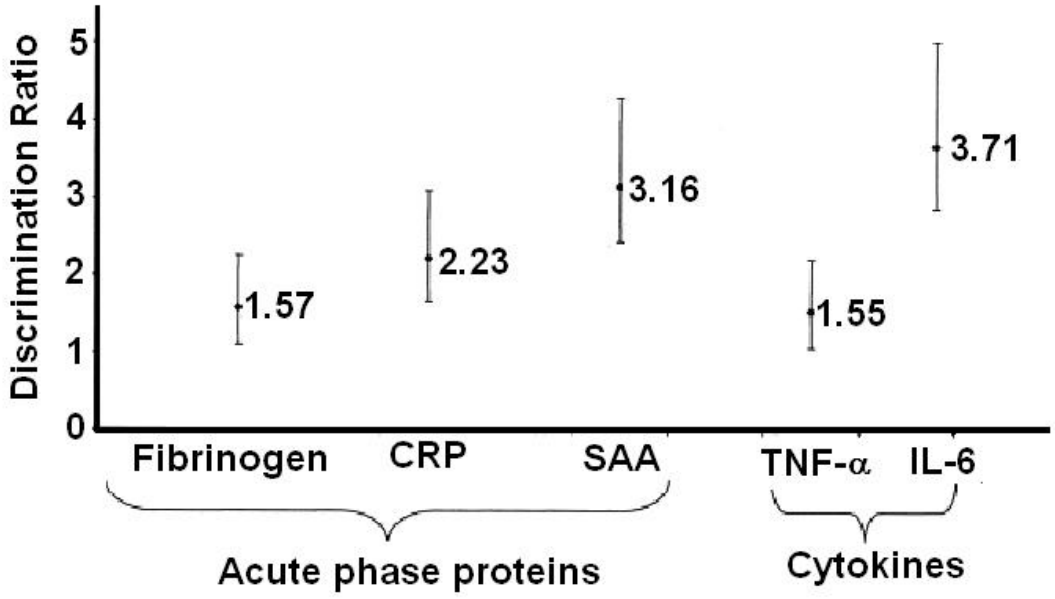

Figure 2. Discrimination ratio for each inflammatory marker (error bars represent 95% confidence interval). Adapted from Browning LM et al. Metabolism. 2004[94].

II. The Mediterranean diet

The term Mediterranean diet refers to a dietary pattern typical of many regions in Greece and southern Italy in the early 1960s. It includes a high intake of fruits, vegetables, bread, other forms of cereals, potatoes, beans, nuts and seeds; low to moderate amounts of dairy products, fish, poultry and wine; low amounts of red meat; eggs consumed no more than four times weekly; and olive oil as an important fat source.[1, 97] In the randomized trial, this diet can be accurately prescribed and prepared, but in the general population,

this diet is rare in Western countries. In the general population, diet is heterogeneous across individuals, and a pattern of individual's diet can be close to, although not necessarily equal to, the original definition of the Mediterranean diet.[1, 97] Therefore, in observational epidemiological studies, the Mediterranean diet becomes a conceptual term to describe any diet that has the elements of this diet. The term, "adherence to the Mediterranean diet", has also been used to quantitatively describe the similarity of individuals' diet to the Mediterranean diet in comparison to other people within their population.[97]

Quantitative methods to evaluate dietary patterns

Diets are diverse because they are influenced by the culture, tradition, economics, and food supply including food resources, food accessibility, food storage, food processing and food acceptability. Despite this diversity, people in a geographical area share common dietary features that can be qualitatively and/or quantitatively described as a dietary pattern such as the Mediterranean diet.[97] A dietary pattern can be described using food items/groups, nutrient intakes, or both. The terms food pattern,[98] food consumption pattern,[99] or eating pattern,[100] are alternative terms for describing a dietary pattern based on food items or groups.[98-100] At the individual level, a dietary pattern characterizes the usual or habitual diet of a person.

The rationale to focus on the whole diet (dietary pattern) when examining effects on health, rather than nutrients, has been reviewed by Hu FB.[101] Briefly, it is based on four conceptual and methodological facts: 1) human beings are exposed to a combination of various foods in their diet, containing diverse nutrients; 2) there are cumulative effects

of foods on humans; 3) there are extensive interactions among nutrients and non-nutrient biofactors during absorption, metabolism and physiological function *in vivo*; and 4) the whole diet analysis can avoid the potential confounding effects in the 'single nutrient/food'-disease relationship caused by the association between the intake of nutrients/foods and a certain diet. Such confounding effects can not be totally removed using adjustment in multivariate analysis.

A quantitative method is often used to describe a dietary pattern in epidemiological research on the diet-disease relation. There is no one unique method to obtain a dietary pattern that fits all research interests, because both diet and research objectives are diverse. The construction of a diet score is a common approach to quantitatively describe the similarity of an individual's diet to a defined diet model like the Mediterranean diet. Either descriptive or advanced statistical approaches have been used to derive a diet score. The dietary pattern derived using descriptive statistical approach is called a theoretically derived dietary patterns.[102] On the contrary, the dietary pattern derived using advanced statistical approaches, is called empirical dietary patterns.[102] Both dietary patterns can be derived from the same existing dietary data from a population. However, it is simpler and more convenient to obtain the theoretically derived dietary patterns because of only using descriptive statistics.

Theoretically derived dietary pattern An example of a theoretically derived dietary pattern was the Mediterranean diet. This dietary pattern has been described[1, 97] and quantitatively illustrated in the national dietary guideline in the Mediterranean areas such as Greece dietary guideline.[103] Trichopoulou's team proposed an algorithm to obtain a Mediterranean diet score,[104, 105] which ranked individuals according to the similarity of

their usual or habitual diet to the Mediterranean diet.[1, 97] This score is constructed through several subsequent steps. First, the components of a diet score are defined *a priori* based on the previous studies on the diet-disease association, dietary guidelines, and nutrition knowledge. These components are then *a priori* classified into the beneficial or harmful group regarding the cardiovascular system. Second, descriptive statistical analyses are used to define the cutoff point to dichotomize intakes of food items/groups or nutrients. Third, a value of either 0 or 1 is assigned to each intake category. Finally, the diet score is calculated as the sum of all the component values.

Three types of diet scores have been reported based on features of score components: 1) food items or groups as score components, such as recommended foods score (RFS)[106] and recommended foods and behavior score (RFBS);[107] 2) both food items and/or groups and nutrients as score components, such as Mediterranean dietary score (MDS), health diet indicator (HDI),[108] healthy eating index (HEI),[109] diet quality index (DQI)[110] and its variations including diet quality index-revised (DQI-R)[111] and diet quality index-International (DQI-I);[112] and 3) nutrients as score components, such as the composite dietary score.[113] For example, Schröder H et al. used folate, vitamin C, vitamin E, β-carotene, dietary fiber, cholesterol, saturated fatty acids, and sodium as components to construct the composite dietary score.[113] Some dietary indices derived from the intake of limited types of nutrients for a very specific purpose can be considered as the specific cases of the composite dietary score, but not a measure of the dietary pattern. For instance, Keys score was constructed using intakes of saturated, polyunsaturated fatty acids and cholesterol, and used to assess the dietary risk for CVD.[114]

Empirical dietary pattern Cluster analysis[102, 115] and factor analysis[98, 116, 117] are the most popular dimension-reduction statistical methods used to obtain the dietary pattern.[102] In recent years, other statistical methods have been used to derive the dietary pattern such as reduced rank regression.

Cluster analysis is an approach that classifies subjects into a limited number of subsets on the basis of similarities in quantitative intake of each food item/group or nutrient. The components within subsets share more similarities than those between subsets. Before performing cluster analysis, food items from the original dietary data usually are classified into food groups in order to reduce the numbers of variables. The cluster can be generated based on selected nutrients,[118, 119] culinary use,[115] and compositions of food and nutrients.[120] However, the selected nutrition variables for the cluster analysis should not be highly correlated, as the inclusion of multiple highly correlated measures may cause the factor they have in common to create its own cluster, even if it is not an important factor.[118] Prior to the cluster analysis, dietary data can be processed to obtain standardized food intakes in weight,[121] standardized number of servings,[120] percentage of calorie intake from each food group,[122] adjusted mean nutrient intakes,[123] or a combination of dietary and biochemical measures.[124] The standardized food intake in gram weight or in the number of servings can minimize the potential for greater effects from the variables with larger variances on the resulting clusters.[121] Usually less than ten runs of the cluster analysis are needed to determine the optimal number of clusters.[115, 120]

Factor analysis is another method to reduce the dimension, where a latent factor is used to explain variations among observed variables.[102] Principle component analysis

is the most widely used factor analysis to derive a statistical dietary pattern. In contrast to cluster analysis, principle component analysis generates dietary patterns (factors) based on the correlation rather than the similarity. Similar to cluster analysis, before conducting factor analysis, dietary data are processed, such as categorizing food items into food groups. As a consequence, dependent variables can be explained largely or entirely in terms of a much smaller number of variables called factors.[102]

Reduced rank regression (RRR) method is recently used as a statistical approach to generate the dietary pattern.[125, 126] It works simultaneously for predictors and responses.[127] RRR identifies linear functions of predicators that explain as much variation in the response variable as possible.[126] Hoffmann K and colleagues reported a dietary pattern derived from RRR could strongly explain variations in biomarkers related to the risk of coronary artery disease.[126]

Effects of variations in diet score constructions on associations between a diet score and biomarkers or diseases Fung TT et al.[128] evaluated the associations of five scores with biomarkers of inflammation and endothelial dysfunction using dietary data from the Nurses' Health Study in the US. These scores include Healthy Eating Index (HEI),[109] Alternate Healthy Eating Index (AHEI),[129] Diet Quality Index Revised (DQI-R),[111] Recommended Food Score (RFS),[106] and the alternate Mediterranean Diet Index (aMED).[128] All scores reflect the theoretically derived dietary pattern. The aMED was based on the Mediterranean diet score from the Trichopoulou's team[105] with some modifications. Fung TT et al. modified the original scale[105] by separating fruits and nuts into two groups, eliminating the dairy group, including whole-grain products only, including only red and processed meats for the meat group, and assigning alcohol intake

between 5 and 15 g/d for one point.[128] They found that higher AHEI and aMED scores were associated with lower concentrations of CRP, IL-6 and E-selectin, respectively, after adjusting for age, smoking status, physical activity, alcohol intake, total energy intake, and body mass index.[128] However, they did not provide data on the association between the original Trichopoulou's Mediterranean diet score[105] and biomarkers of inflammation and endothelial function.

In a national 5 year-follow up study in the US, Mitrou and collegues[2] found similar associations of adherence to the Mediterranean diet measured as either Trichopoulous's MED[105] or Fung's aMED[128] with death due to all causes, cancer or CVD.

III. The Mediterranean diet and cardiovascular disease

A large body of ecological, observational and experimental evidence supports the beneficial effect of the Mediterranean diet on the cardiovascular system. Consistent findings from epidemiological studies (1 ecological,[130] 1 case-control,[131] 1 cross-sectional,[132] and 8 cohort[2, 104, 105, 133-138]studies) show that the Mediterranean diet 1) decreases overall mortality[2, 104, 105, 133-138] and CHD death;[2, 105, 133] and 2) reduces the occurrence of first myocardial infarction[131] and acute coronary syndromes.[132] The protective magnitude of the Mediterranean diet is substantial. For each one-unit increase in the Mediterranean diet score (MDS) indicating greater adherence to the Mediterranean diet, mortality dropped between 7% and 31% in elders[104, 135-137] and in younger and older persons with coronary heart disease (CHD).[105] Decreased CHD death is a major contributor to the drop in the mortality; for each 2-point increment in MDS, the adjusted hazard ratio for death due to CHD was 0.75 (95% CI: 0.64 to 0.87) after controlling for

gender, age, waist-to-hip ratio, energy expenditure, years of education, smoking status, BMI, consumption of potatoes, consumption of eggs and total energy intake.[133] With the same MDS, the US national 5-year follow-up study showed that the adjusted hazard ratio comparing high to low adherence to the Mediterranean diet for cardiovascular disease (CVD) mortality were 0.76 (95% CI, 0.68-0.85) in men, and that a 20% decreased risk of CVD mortality was seen in women.[2]

Large-size randomized controlled trials in CVD patients support the cardioprotective effects of the Mediterranean diet in comparison with either the step I National Cholesterol Education Program (NCEP) prudent diet[3] or the prudent Western-type diet.[4] In the Lyon Heart Study, patients on the Mediterranean diet had 54% lower risk of death due to myocardial infarction and other CVD diseases than those on the prudent Western-type diet.[4] Of importance, the protective role of the Mediterranean diet against the recurrence after the first myocardial infarction lasted up to 4 years.[4]

IV. Mechanisms of the cardioprotective properties of the Mediterranean diet

The mechanisms of the cardioprotective properties of the Mediterranean diet are still unclear, although beneficial effects of the Mediterranean diet on the cardiovascular system may be through reducing low-grade inflammation[128, 139] and oxidative stress,[13, 14] both of which are the established pathophysiological pathway to atherosclerosis. Other possible mechanisms involve circulating lipids[3, 14] and endothelial function.[14, 140]

The Mediterranean diet and low-grade systemic inflammation

Large-scale cross-sectional studies suggest that the greater adherence to the Mediterranean diet is associated with the lower low-grade systemic inflammation in populations.[128, 139] The ATTICA study[139] showed that Greek people in the highest tertile of a Mediterranean diet score had the lower systemic-inflammation than those in the lowest tertile, measured as CRP levels, IL-6 levels, and white blood cell counts.[139] This association remained after adjusting for known CVD risk factors, including age, gender, smoking, physical activity, education status, presence of hypertension, diabetes, hypercholesterolemia, family history of coronary heart disease, and body mass index (BMI).[139]

The causal relationship between the Mediterranean diet and the low-grade systemic inflammation has been explored using randomized controlled trials (RCT).[9-11] Each of the trials studied a different European population – Italian,[9] Spanish[10] and German,[11] respectively, with inconsistent results. One of the RCTs, conducted among Italian patients with metabolic syndrome over 2 years, showed that the Mediterranean diet, compared with prudent diet, decreased the low-grade inflammation measured as IL-6 and CRP.[9] The Mediterranean diet emphasizing on olive oil, but not the Mediterranean diet emphasizing on nuts, in comparison to a low-fat diet, reduced CRP levels among the asymptomatic Spanish at high cardiovascular risk.[10] However, the Mediterranean diet did not attenuate CRP levels compared with the usual diet in German patients with established and treated coronary artery disease.[11]

The Mediterranean diet and oxidative stress

Research on the association of the Mediterranean diet with oxidative stress is very limited with inconsistent results. Three intermediate-term RCTs showed that the Mediterranean diet decreased oxidative stress and enhanced antioxidant defense, compared with a high fat diet in male volunteers after 3 month ingestion,[12] a low-fat isocalorie diet (2500 kcal for men, 2000 kcal for women) in kidney grafting patients over 6 month intervention,[13] or a low-fat diet in asymptomatic subjects at high risk for CHD over 3 month intervention,[141] respectively. On the contrary, the short-term RCTs failed to show the favorable effect of a Mediterranean diet on oxidative stress in comparison with a Swedish diet.[14, 15] One RCT was a 4-week intervention conducted in healthy subjects,[14] and the other was a 3-week intervention performed in patients with rheumatoid arthritis.[15] In these prior trials, oxidative stress has been measured with various markers, such as plasma antioxidants (vitamin C,[12] gamma-tocopherol,[15] beta-carotene,[15] lycopene,[15] and uric acid[15]), plasma lipid peroxidation products (TBARS[13] and MDA[15]), antioxidant enzymes (erythrocyte SOD[13]), plasma ox-LDL,[141] oxidative DNA damage,[12] and urinary F2-isoprostane.[14]

The Mediterranean diet, alpha-linolenic acid, cardiovascular disease, and inflammatory biomarkers of cellular response to interleukin-6

The rich content of alpha-linolenic acid (ALA) in the Mediterranean diet contributes to its cardioprotective feature at the nutrient level.[6] Several large-size observational studies[142-145] suggest a beneficial effect of dietary ALA on the cardiovascular system. To date, most randomized controlled trails (RCT) (1 primary[146] and 3 secondary prevention[3, 4, 147]), but not all (1 primary prevention[148]), indicated that a

high intake of dietary ALA reduced various adverse cardiac events.[3, 4, 147] A meta-analysis including 5 prospective studies and 3 RCTs shows that increasing intake of ALA by 1.2 g/d decreases the risk of fatal coronary heart disease by at least 20%.[149] Taken together, the weight of the evidence favors recommendations for modest dietary consumption of ALA (2 to 3 g per day) for the primary and secondary prevention of CHD.[6]

Dietary ALA is an essential n-3 fatty acid (18:3 n-3), because human beings can not synthesize ALA due to lack of the enzymes to introduce double bonds at carbon atoms beyond the ninth carbon from the carboxyl end.[150] Humans, therefore, must obtain enough ALA from diet to sustain the healthy status. The primary dietary sources for ALA are plant foods.

The metabolism of ALA *in vivo* (**Figure 3**) involves extensive interactions with other types of fatty acids including n-6, n-9 and *trans*-fatty acids. ALA is the precursor of all other n-3 long-chain polyunsaturated fatty acids (LCFA) including eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), and docosahexaenoic acid (DHA) in humans. ALA itself, its n-3 LCFA derivatives, and their metabolites such as series-3 prostaglandin (such as PGE_3, PGF_3, PGI_3, and TXA_3) and series-5 leukotrienes (such as LTA_5, LTB_5, LTC_5, LTD_5), are anti-inflammatory, anti-thrombotic, and anti-arrhythmic.[6]

A high ALA intake also inhibits the production of 5-lipoxygenase-mediated series-4 leukotrienes (such as LTA_4, LTB_4, LTC_4, LTD_4, and LTE_4) and cyclooxygenase 2-mediated series-2 prostaglandins, leukotrienes, and thromboxanes (such as PGD_2, PGE_2, PGF_2, and TXA_2), crucial inflammatory and thrombotic mediators. These series-4 and series-2 mediators are derived from eicosanoid metabolites of n-6 linoleic acid, mainly

arachidonic acid (20:4 n-6). Therefore, compared with n-6 fatty acids, ALA may reduce systemic inflammation and thrombosis.[6] Major antagonists for ALA to generate LCFA and their metabolites are n-6 linoleic acid (LA); minor antagonists are *trans*-fatty acids and n-9 monounsaturated fatty acids, because they compete with ALA for Δ^6 desaturase.

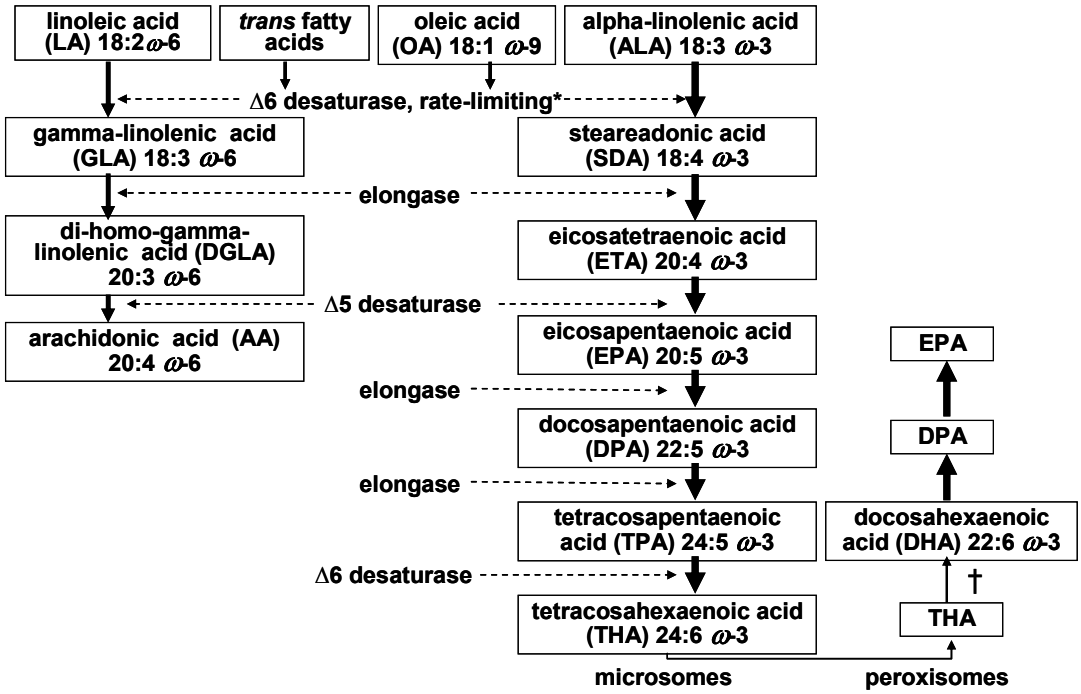

Figure 3. Conversion of alpha-linolenic acid into long chain ω-3 fatty acids competing with other fatty acids. * Affinities for Δ6 desaturase are ω-3> ω-6> trans->ω-9 fatty acids. †peroxisomal straight-chain acyl-CoA oxidase and D-bifunctional protein. Adapted from Wikipedia.[151]

N-3 LCFA are also feedback inhibitors in the ALA-LCFA metabolic pathway. Briefly, ALA may be the central switch toward anti-inflammatory milieu *in vivo*.

The mechanisms of cardioprotective properties of the Mediterranean diet through ALA are not completely clear. Limited RCT data[19, 152] suggest that systemic

inflammation related to cellular response to IL-6, including IL-6 and its soluble receptor (sIL-6R), may be the underlying mechanism. A higher dietary ALA intake providing 5% versus 0.5% energy intake lowered sIL-6R levels in the four-day RCT,[152] but had no affect on serum IL-6 concentrations in the 8-week RCT among healthy adults.[153] Another RCT showed that 15 ml of linseed oil (rich in ALA) per day versus 15 ml of safflower oil (rich in linoleic acid) per day in the Mediterranean diet decreased serum IL-6 concentrations in male dyslipidemic patients.[19] However, ALA attenuated serum IL-6 concentrations more profoundly when the background diet was rich in saturated fatty acids and poor in MUFA.[154]

Genetic factors, cardiovascular disease, inflammation and oxidative stress

Gene polymorphisms involved in inflammation[155-158] and oxidative stress[159] may contribute to CVD, where involve a variety of genes encoding cytokines, their receptors and other factors. For instance, variations in the IL-6 and its receptor gene may modulate the production of IL-6[160] and CRP[161] in response to inflammatory stimuli, and the cellular response to IL-6,[162] which may translate into elevated CVD risk.[163] Of two twin studies including monozygotic (MZ) and dizygotic (DZ) pairs,[164, 165] one indicated that genetic factors contributed to 17~26% of the variations in plasma levels of IL-6, CRP, and TNF after controlling for age, gender and BMI.[165] The other found that genetic factors accounted for about 44% of the variations in plasma concentration of fibrinogen, while the remaining variation was accounted for by individual-specific environmental factors.[164] In addition, variants in genes encoding enzymes involved in ROS generation

such as NAD(P)H oxidase[166, 167] and MPO[83, 168-170] as well as in ROS clearance such as glutathione peroxidase[171] may contribute to CVD.

Genetic factors, the Mediterranean diet, habitual dietary alpha-linolenic acid

To date, only one cohort study showed that genetic factors mediated the effect of the Mediterranean diet on CVD and/or CVD risk.[172] After controlling for potential confounders, adherence to the Mediterranean diet was associated with reduced homocysteine concentrations in persons with methylenetetrahydrofolate reductase gene 677C-->T mutation genotypes but not in persons without this gene mutation.[172] Heritable and familial determinants might affect diet preference and thus ALA intake in a number of ways, including dietary behaviors such as food preference,[173-180] food consumption frequency[180] and perception of hunger,[173] as shown in several twin studies.[173-175, 177, 178] However, genetic factors only account for 33-40% of dietary variability in middle-aged twins after the removal of influences of age and gender in regression analysis,[178] while the remaining 60% variation is attributable to environmental factors. Of the latter, individual-specific environmental factors explain greater than 50% variation.[178]

Other environmental factors

A wide range of environmental factors, including smoking,[181-185] obesity,[186-189] physical activity[61, 190-193] and psychological factors,[194, 195] may also contribute to low-grade systemic inflammation and oxidative stress and thus contribute to CVD risk.

References

1. Kris-Etherton P, Eckel RH, Howard BV, et al. AHA Science Advisory: Lyon Diet Heart Study. Benefits of a Mediterranean-style, National Cholesterol Education Program/American Heart Association Step I Dietary Pattern on Cardiovascular Disease. *Circulation.* Apr 3 2001;103(13):1823-1825.

2. Mitrou PN, Kipnis V, Thiebaut AC, et al. Mediterranean Dietary Pattern and Prediction of All-Cause Mortality in a US Population: Results From the NIH-AARP Diet and Health Study. *Arch Intern Med.* Dec 10 2007;167(22):2461-2468.

3. Singh RB, Dubnov G, Niaz MA, et al. Effect of an Indo-Mediterranean diet on progression of coronary artery disease in high risk patients (Indo-Mediterranean Diet Heart Study): a randomised single-blind trial. *Lancet.* Nov 9 2002;360(9344):1455-1461.

4. de Lorgeril M, Salen P, Martin JL, et al. Mediterranean diet, traditional risk factors, and the rate of cardiovascular complications after myocardial infarction: final report of the Lyon Diet Heart Study. *Circulation.* Feb 16 1999;99(6):779-785.

5. *Biochemical and Physiological Aspects of Human Nutrition* 1st ed. St. Louis. : W.B. Saunders Company; 2000.

6. Mozaffarian D. Does alpha-linolenic acid intake reduce the risk of coronary heart disease? A review of the evidence. *Altern Ther Health Med.* May-Jun 2005;11(3):24-30; quiz 31, 79.

7. *Diet and human immune function*. 1st ed. Totowa, N.J. : Humana Press; 2003.

8. Ghosh S, Novak EM, Innis SM. Cardiac proinflammatory pathways are altered with different dietary n-6 linoleic to n-3 {alpha}-linolenic acid ratios in normal, fat-fed pigs. *Am J Physiol Heart Circ Physiol.* Nov 2007;293(5):H2919-2927.

9. Esposito K, Marfella R, Ciotola M, et al. Effect of a mediterranean-style diet on endothelial dysfunction and markers of vascular inflammation in the metabolic syndrome: a randomized trial. *Jama.* Sep 22 2004;292(12):1440-1446.

10. Estruch R, Martinez-Gonzalez MA, Corella D, et al. Effects of a Mediterranean-style diet on cardiovascular risk factors: a randomized trial. *Ann Intern Med.* Jul 4 2006;145(1):1-11.

11. Michalsen A, Lehmann N, Pithan C, et al. Mediterranean diet has no effect on markers of inflammation and metabolic risk factors in patients with coronary artery disease. *Eur J Clin Nutr.* Apr 2006;60(4):478-485.

12. Leighton F, Cuevas A, Guasch V, et al. Plasma polyphenols and antioxidants, oxidative DNA damage and endothelial function in a diet and wine intervention study in humans. *Drugs Exp Clin Res.* 1999;25(2-3):133-141.

13. Stachowska E, Wesolowska T, Olszewska M, et al. Elements of Mediterranean diet improve oxidative status in blood of kidney graft recipients. *Br J Nutr.* Mar 2005;93(3):345-352.

14. Ambring A, Friberg P, Axelsen M, et al. Effects of a Mediterranean-inspired diet on blood lipids, vascular function and oxidative stress in healthy subjects. *Clin Sci (Lond).* May 2004;106(5):519-525.

15. Hagfors L, Leanderson P, Skoldstam L, et al. Antioxidant intake, plasma antioxidants and oxidative stress in a randomized, controlled, parallel,

Mediterranean dietary intervention study on patients with rheumatoid arthritis. *Nutr J.* Jul 30 2003;2:5.

16. Lopez-Garcia E, Schulze MB, Manson JE, et al. Consumption of (n-3) fatty acids is related to plasma biomarkers of inflammation and endothelial activation in women. *J Nutr.* Jul 2004;134(7):1806-1811.

17. Pischon T, Hankinson SE, Hotamisligil GS, et al. Habitual dietary intake of n-3 and n-6 fatty acids in relation to inflammatory markers among US men and women. *Circulation.* Jul 15 2003;108(2):155-160.

18. Bemelmans WJ, Lefrandt JD, Feskens EJ, et al. Increased alpha-linolenic acid intake lowers C-reactive protein, but has no effect on markers of atherosclerosis. *Eur J Clin Nutr.* Jul 2004;58(7):1083-1089.

19. Rallidis LS, Paschos G, Liakos GK, et al. Dietary alpha-linolenic acid decreases C-reactive protein, serum amyloid A and interleukin-6 in dyslipidaemic patients. *Atherosclerosis.* Apr 2003;167(2):237-242.

20. Wallace FA, Miles EA, Calder PC. Comparison of the effects of linseed oil and different doses of fish oil on mononuclear cell function in healthy human subjects. *Br J Nutr.* May 2003;89(5):679-689.

21. Zhao G, Etherton TD, Martin KR, et al. Dietary alpha-linolenic acid inhibits proinflammatory cytokine production by peripheral blood mononuclear cells in hypercholesterolemic subjects. *Am J Clin Nutr.* Feb 2007;85(2):385-391.

22. Rankinen T, Bouchard C. Genetics of Food Intake and Eating Behavior Phenotypes in Humans. *Annu Rev Nutr.* Apr 18 2006.

23. Larson NI, Neumark-Sztainer D, Hannan PJ, et al. Family meals during adolescence are associated with higher diet quality and healthful meal patterns during young adulthood. *J Am Diet Assoc.* Sep 2007;107(9):1502-1510.

24. Maynard M, Gunnell D, Ness AR, et al. What influences diet in early old age? Prospective and cross-sectional analyses of the Boyd Orr cohort. *Eur J Public Health.* Jun 2006;16(3):316-324.

25. NCHS. *Annual summary of births, marriages, divorces, and deaths: United States, 1990.* Vol 39, Monthly vital statistics report; no.13. Hyattsville, Maryland: US Department of Health and Human Services, Public Health Service, CDC; 1991.

26. AHA. *American Heart Association. Heart Disease and Stroke Statistics — 2006 Update.* Vol ©2006, . Dallas, Texas: American Heart Association.; 2006.

27. Speed C. The transposability of the Mediterranean-type diet in non-Mediterranean regions: application to the physician/allied health team. *Eur J Cancer Prev.* Dec 2004;13(6):529-534.

28. Ross R. Atherosclerosis--an inflammatory disease. *N Engl J Med.* Jan 14 1999;340(2):115-126.

29. Libby P. Inflammation in atherosclerosis. *Nature.* Dec 19-26 2002;420(6917):868-874.

30. Krieglstein CF, Granger DN. Adhesion molecules and their role in vascular disease. *Am J Hypertens.* Jun 2001;14(6 Pt 2):44S-54S.

31. *Molecular and Cellular Basis of Inflammation* Totowa, N.J. : Human Press; 1998.

32. Charo IF, Taubman MB. Chemokines in the pathogenesis of vascular disease. *Circ Res.* Oct 29 2004;95(9):858-866.

33. Weber C, Schober A, Zernecke A. Chemokines: key regulators of mononuclear cell recruitment in atherosclerotic vascular disease. *Arterioscler Thromb Vasc Biol.* Nov 2004;24(11):1997-2008.

34. Shoenfeld Y, Wu R, Dearing LD, et al. Are anti-oxidized low-density lipoprotein antibodies pathogenic or protective? *Circulation.* Oct 26 2004;110(17):2552-2558.

35. Ohsuzu F. The roles of cytokines, inflammation and immunity in vascular diseases. *J Atheroscler Thromb.* 2004;11(6):313-321.

36. Mehra VC, Ramgolam VS, Bender JR. Cytokines and cardiovascular disease. *J Leukoc Biol.* Oct 2005;78(4):805-818.

37. Watson C, Whittaker S, Smith N, et al. IL-6 acts on endothelial cells to preferentially increase their adherence for lymphocytes. *Clin Exp Immunol.* Jul 1996;105(1):112-119.

38. Raines EW, Ferri N. Thematic review series: The immune system and atherogenesis. Cytokines affecting endothelial and smooth muscle cells in vascular disease. *J Lipid Res.* Jun 2005;46(6):1081-1092.

39. Kerr R, Stirling D, Ludlam CA. Interleukin 6 and haemostasis. *Br J Haematol.* Oct 2001;115(1):3-12.

40. Yap SH, Moshage HJ, Hazenberg BP, et al. Tumor necrosis factor (TNF) inhibits interleukin (IL)-1 and/or IL-6 stimulated synthesis of C-reactive protein (CRP) and serum amyloid A (SAA) in primary cultures of human hepatocytes. *Biochim Biophys Acta.* Feb 19 1991;1091(3):405-408.

41. Ganapathi MK, Rzewnicki D, Samols D, et al. Effect of combinations of cytokines and hormones on synthesis of serum amyloid A and C-reactive protein in Hep 3B cells. *J Immunol.* Aug 15 1991;147(4):1261-1265.

42. Heinrich PC, Castell JV, Andus T. Interleukin-6 and the acute phase response. *Biochem J.* Feb 1 1990;265(3):621-636.

43. Du Clos TW, Mold C. C-reactive protein: an activator of innate immunity and a modulator of adaptive immunity. *Immunol Res.* 2004;30(3):261-277.

44. Chang MK, Binder CJ, Torzewski M, et al. C-reactive protein binds to both oxidized LDL and apoptotic cells through recognition of a common ligand: Phosphorylcholine of oxidized phospholipids. *Proc Natl Acad Sci U S A.* Oct 1 2002;99(20):13043-13048.

45. Fu T, Mukhopadhyay D, Davidson NO, et al. The peroxisome proliferator-activated receptor alpha (PPARalpha) agonist ciprofibrate inhibits apolipoprotein B mRNA editing in low density lipoprotein receptor-deficient mice: effects on plasma lipoproteins and the development of atherosclerotic lesions. *J Biol Chem.* Jul 2 2004;279(27):28662-28669.

46. van Tits L, de Graaf J, Toenhake H, et al. C-reactive protein and annexin A5 bind to distinct sites of negatively charged phospholipids present in oxidized low-density lipoprotein. *Arterioscler Thromb Vasc Biol.* Apr 2005;25(4):717-722.

47. Levenson J, Giral P, Razavian M, et al. Fibrinogen and silent atherosclerosis in subjects with cardiovascular risk factors. *Arterioscler Thromb Vasc Biol.* Sep 1995;15(9):1263-1268.

48. Ernst E, Resch KL. Fibrinogen as a cardiovascular risk factor: a meta-analysis and review of the literature. *Ann Intern Med.* Jun 15 1993;118(12):956-963.

49. Stoll G, Bendszus M. Inflammation and atherosclerosis: novel insights into plaque formation and destabilization. *Stroke.* Jul 2006;37(7):1923-1932.

50. Stocker R, Keaney JF, Jr. Role of oxidative modifications in atherosclerosis. *Physiol Rev.* Oct 2004;84(4):1381-1478.

51. Jones DP. Extracellular redox state: refining the definition of oxidative stress in aging. *Rejuvenation Res.* Summer 2006;9(2):169-181.

52. Girotti AW. Lipid hydroperoxide generation, turnover, and effector action in biological systems. *J Lipid Res.* Aug 1998;39(8):1529-1542.

53. Clapp BR, Hingorani AD, Kharbanda RK, et al. Inflammation-induced endothelial dysfunction involves reduced nitric oxide bioavailability and increased oxidant stress. *Cardiovasc Res.* Oct 1 2004;64(1):172-178.

54. Fichtlscherer S, Breuer S, Schachinger V, et al. C-reactive protein levels determine systemic nitric oxide bioavailability in patients with coronary artery disease. *Eur Heart J.* Aug 2004;25(16):1412-1418.

55. Dhaliwal BS, Steinbrecher UP. Cholesterol delivered to macrophages by oxidized low density lipoprotein is sequestered in lysosomes and fails to efflux normally. *J Lipid Res.* Oct 2000;41(10):1658-1665.

56. Maor I, Hayek T, Coleman R, et al. Plasma LDL oxidation leads to its aggregation in the atherosclerotic apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol.* Nov 1997;17(11):2995-3005.

57. Ryan KA, Smith MF, Jr., Sanders MK, et al. Reactive oxygen and nitrogen species differentially regulate Toll-like receptor 4-mediated activation of NF-kappa B and interleukin-8 expression. *Infect Immun.* Apr 2004;72(4):2123-2130.

58. Valen G, Yan ZQ, Hansson GK. Nuclear factor kappa-B and the heart. *J Am Coll Cardiol.* Aug 2001;38(2):307-314.

59. Rosenblat M, Coleman R, Aviram M. Increased macrophage glutathione content reduces cell-mediated oxidation of LDL and atherosclerosis in apolipoprotein E-deficient mice. *Atherosclerosis.* Jul 2002;163(1):17-28.

60. Jones D. Redox potential of GSH/GSSG couple: assay and biological significance. *Methods Enzymol.* 2002;348:93-112.

61. Abramson JL, Vaccarino V. Relationship between physical activity and inflammation among apparently healthy middle-aged and older US adults. *Arch Intern Med.* Jun 10 2002;162(11):1286-1292.

62. Lindmark E, Diderholm E, Wallentin L, et al. Relationship between interleukin 6 and mortality in patients with unstable coronary artery disease: effects of an early invasive or noninvasive strategy. *Jama.* Nov 7 2001;286(17):2107-2113.

63. Luc G, Bard JM, Juhan-Vague I, et al. C-reactive protein, interleukin-6, and fibrinogen as predictors of coronary heart disease: the PRIME Study. *Arterioscler Thromb Vasc Biol.* Jul 1 2003;23(7):1255-1261.

64. Ma J, Hennekens CH, Ridker PM, et al. A prospective study of fibrinogen and risk of myocardial infarction in the Physicians' Health Study. *J Am Coll Cardiol.* Apr 1999;33(5):1347-1352.

65. Ridker PM, Cushman M, Stampfer MJ, et al. Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. *N Engl J Med.* Apr 3 1997;336(14):973-979.

66. Ridker PM, Rifai N, Stampfer MJ, et al. Plasma concentration of interleukin-6 and the risk of future myocardial infarction among apparently healthy men. *Circulation.* Apr 18 2000;101(15):1767-1772.

67. Rost NS, Wolf PA, Kase CS, et al. Plasma concentration of C-reactive protein and risk of ischemic stroke and transient ischemic attack: the Framingham study. *Stroke.* Nov 2001;32(11):2575-2579.

68. St-Pierre AC, Cantin B, Bergeron J, et al. Inflammatory markers and long-term risk of ischemic heart disease in men A 13-year follow-up of the Quebec Cardiovascular Study. *Atherosclerosis.* Oct 2005;182(2):315-321.

69. Kushner I, MackiewiczI A. The acute phase response: an overview. In: Mackiewicz A, Kushner I, Baumann H, eds. *Acute Phase Proteins: Molecular Biology, Biochemistry and Clinical Applications*. Boca Raton: CRC Press 1993:3-19.

70. TNF-alpha(Tumor Necrosis Factor alpha). April 22. Available at: http://www.grt.kyushu-u.ac.jp/spad/account/ligand/tnf-a.html. Accessed April 22, 2005.

71. Mackiewicz A, Speroff T, Ganapathi MK, et al. Effects of cytokine combinations on acute phase protein production in two human hepatoma cell lines. *J Immunol.* May 1 1991;146(9):3032-3037.

72. Jones SA, Horiuchi S, Topley N, et al. The soluble interleukin 6 receptor: mechanisms of production and implications in disease. *Faseb J.* Jan 2001;15(1):43-58.

73. Rose-John S. Interleukin-6 biology is coordinated by membrane bound and soluble receptors. *Acta Biochim Pol.* 2003;50(3):603-611.

74. Husain T, Kim D. C-Reactive Protein and Erythrocyte Sedimentation Rate in Orthopaedics. *UPOJ.* Vol 15; 2002:13-16.

75. Bruunsgaard H, Pedersen BK. Age-related inflammatory cytokines and disease. *Immunol Allergy Clin North Am.* Feb 2003;23(1):15-39.

76. Nystrom T. C-reactive protein: a marker or a player? *Clin Sci (Lond).* Jul 2007;113(2):79-81.

77. Sesso HD, Wang L, Buring JE, et al. Comparison of interleukin-6 and C-reactive protein for the risk of developing hypertension in women. *Hypertension.* Feb 2007;49(2):304-310.

78. Fraley AE, Tsimikas S. Clinical applications of circulating oxidized low-density lipoprotein biomarkers in cardiovascular disease. *Curr Opin Lipidol.* Oct 2006;17(5):502-509.

79. Roberts LJ, 2nd, Morrow JD. Products of the isoprostane pathway: unique bioactive compounds and markers of lipid peroxidation. *Cell Mol Life Sci.* May 2002;59(5):808-820.

80. Nourooz-Zadeh J, Cooper MB, Ziegler D, et al. Urinary 8-epi-PGF2alpha and its endogenous beta-oxidation products (2,3-dinor and 2,3-dinor-5,6-dihydro) as

biomarkers of total body oxidative stress. *Biochem Biophys Res Commun.* May 13 2005;330(3):731-736.

81. Vassalle C, Petrozzi L, Botto N, et al. Oxidative stress and its association with coronary artery disease and different atherogenic risk factors. *J Intern Med.* Oct 2004;256(4):308-315.

82. Wu T, Rifai N, Willett WC, et al. Plasma fluorescent oxidation products: independent predictors of coronary heart disease in men. *Am J Epidemiol.* Sep 1 2007;166(5):544-551.

83. Zhang R, Brennan ML, Fu X, et al. Association between myeloperoxidase levels and risk of coronary artery disease. *Jama.* Nov 7 2001;286(17):2136-2142.

84. Gilbert HF. Molecular and cellular aspects of thiol-disulfide exchange. *Adv Enzymol Relat Areas Mol Biol.* 1990;63:69-172.

85. Jellema A, Plat J, Mensink RP. Weight reduction, but not a moderate intake of fish oil, lowers concentrations of inflammatory markers and PAI-1 antigen in obese men during the fasting and postprandial state. *Eur J Clin Invest.* Nov 2004;34(11):766-773.

86. Esterbauer H, Jurgens G. Mechanistic and genetic aspects of susceptibility of LDL to oxidation. *Curr Opin Lipidol.* 1993;1993(4):114-124. .

87. Prior RL. Plasma antioxidant measurements. *J Nutr.* Nov 2004;134(11):3184S-3185S.

88. Cao G, Prior RL. Comparison of different analytical methods for assessing total antioxidant capacity of human serum. *Clin Chem.* Jun 1998;44(6 Pt 1):1309-1315.

89. Harma MI, Harma M, Erel O. Are d-ROMs and FRAP tests suitable assays for detecting the oxidative status? *Eur J Obstet Gynecol Reprod Biol.* Aug 2006;127(2):271-272; author reply 272.

90. Abramson JL, Hooper WC, Jones DP, et al. Association between novel oxidative stress markers and C-reactive protein among adults without clinical coronary heart disease. *Atherosclerosis.* Jan 2005;178(1):115-121.

91. Al-Delaimy WK, Jansen EH, Peeters PH, et al. Reliability of biomarkers of iron status, blood lipids, oxidative stress, vitamin D, C-reactive protein and fructosamine in two Dutch cohorts. *Biomarkers.* Jul-Aug 2006;11(4):370-382.

92. Danesh J, Wheeler JG, Hirschfield GM, et al. C-reactive protein and other circulating markers of inflammation in the prediction of coronary heart disease. *N Engl J Med.* Apr 1 2004;350(14):1387-1397.

93. Van Hoydonck PG, Schouten EG, Temme EH. Reproducibility of blood markers of oxidative status and endothelial function in healthy individuals. *Clin Chem.* Jun 2003;49(6 Pt 1):963-965.

94. Browning LM, Krebs JD, Jebb SA. Discrimination ratio analysis of inflammatory markers: implications for the study of inflammation in chronic disease. *Metabolism.* Jul 2004;53(7):899-903.

95. Sakkinen PA, Macy EM, Callas PW, et al. Analytical and biologic variability in measures of hemostasis, fibrinolysis, and inflammation: assessment and implications for epidemiology. *Am J Epidemiol.* Feb 1 1999;149(3):261-267.

96. Macy EM, Hayes TE, Tracy RP. Variability in the measurement of C-reactive protein in healthy subjects: implications for reference intervals and epidemiological applications. *Clin Chem.* Jan 1997;43(1):52-58.

97. Keys Ae, Blackburn H MA, Buzina R, Mohacek I, Karvonen MJ, Punsar S, Aravanis C, Corcondilas A, Dontas AS, Lekos D, Fidanza F, Puddu V,Taylor HL, Monti M, Kimura N, Van Buchem, FSP, Djordjevic BS, Strasser T, Anderson JT, Den Hartog C, Pekkarine M, Roine P, Sdrin H. Coronary heart disease in seven countries. *Circulation.* 1970;41 (Suppl 1):1-211.

98. Newby PK, Muller D, Hallfrisch J, et al. Food patterns measured by factor analysis and anthropometric changes in adults. *Am J Clin Nutr.* Aug 2004;80(2):504-513.

99. van Dam RM, Grievink L, Ocke MC, et al. Patterns of food consumption and risk factors for cardiovascular disease in the general Dutch population. *Am J Clin Nutr.* May 2003;77(5):1156-1163.

100. Nicklas TA, Webber LS, Thompson B, et al. A multivariate model for assessing eating patterns and their relationship to cardiovascular risk factors: the Bogalusa Heart Study. *Am J Clin Nutr.* Jun 1989;49(6):1320-1327.

101. Hu FB. Dietary pattern analysis: a new direction in nutritional epidemiology. *Curr Opin Lipidol.* Feb 2002;13(1):3-9.

102. Newby PK, Tucker KL. Empirically derived eating patterns using factor or cluster analysis: a review. *Nutr Rev.* May 2004;62(5):177-203.

103. Supreme Scientific Health Council, Ministry of Health and Welfare of Greece. Dietary guidelines for adults in Greece. *Arch Hellenic Med.* 1999;16:516-524.

104. Trichopoulou A, Kouris-Blazos A, Wahlqvist ML, et al. Diet and overall survival in elderly people. *Bmj.* Dec 2 1995;311(7018):1457-1460.

105. Trichopoulou A, Bamia C, Trichopoulos D. Mediterranean diet and survival among patients with coronary heart disease in Greece. *Arch Intern Med.* Apr 25 2005;165(8):929-935.

106. Kant AK, Schatzkin A, Graubard BI, et al. A prospective study of diet quality and mortality in women. *Jama.* Apr 26 2000;283(16):2109-2115.

107. Kant AK, Graubard BI, Schatzkin A. Dietary patterns predict mortality in a national cohort: the National Health Interview Surveys, 1987 and 1992. *J Nutr.* Jul 2004;134(7):1793-1799.

108. Huijbregts P, Feskens E, Rasanen L, et al. Dietary pattern and 20 year mortality in elderly men in Finland, Italy, and The Netherlands: longitudinal cohort study. *Bmj.* Jul 5 1997;315(7099):13-17.

109. Weinstein SJ, Vogt TM, Gerrior SA. Healthy Eating Index scores are associated with blood nutrient concentrations in the third National Health And Nutrition Examination Survey. *J Am Diet Assoc.* Apr 2004;104(4):576-584.

110. Patterson RE, Haines PS, Popkin BM. Diet quality index: capturing a multidimensional behavior. *J Am Diet Assoc.* Jan 1994;94(1):57-64.

111. Haines PS, Siega-Riz AM, Popkin BM. The Diet Quality Index revised: a measurement instrument for populations. *J Am Diet Assoc.* Jun 1999;99(6):697-704.

112. Kim S, Haines PS, Siega-Riz AM, et al. The Diet Quality Index-International (DQI-I) provides an effective tool for cross-national comparison of diet quality as illustrated by China and the United States. *J Nutr.* Nov 2003;133(11):3476-3484.

113. Schroder H, Marrugat J, Covas M, et al. Population dietary habits and physical activity modification with age. *Eur J Clin Nutr.* Feb 2004;58(2):302-311.

114. Anderson JT, Jacobs DR, Jr., Foster N, et al. Scoring systems for evaluating dietary pattern effect on serum cholesterol. *Prev Med.* Sep 1979;8(5):525-537.

115. Newby PK, Hu FB, Rimm EB, et al. Reproducibility and validity of the Diet Quality Index Revised as assessed by use of a food-frequency questionnaire. *Am J Clin Nutr.* Nov 2003;78(5):941-949.

116. Schulze MB, Hoffmann K, Kroke A, et al. Risk of hypertension among women in the EPIC-Potsdam Study: comparison of relative risk estimates for exploratory and hypothesis-oriented dietary patterns. *Am J Epidemiol.* Aug 15 2003;158(4):365-373.

117. Dixon LB, Balder HF, Virtanen MJ, et al. Dietary patterns associated with colon and rectal cancer: results from the Dietary Patterns and Cancer (DIETSCAN) Project. *Am J Clin Nutr.* Oct 2004;80(4):1003-1011.

118. Diehr P, Beresford SA. The relation of dietary patterns to future survival, health, and cardiovascular events in older adults. *J Clin Epidemiol.* Dec 2003;56(12):1224-1235.

119. Quatromoni PA, Copenhafer DL, D'Agostino RB, et al. Dietary patterns predict the development of overweight in women: The Framingham Nutrition Studies. *J Am Diet Assoc.* Sep 2002;102(9):1239-1246.

120. Liese AD, Schulz M, Moore CG, et al. Dietary patterns, insulin sensitivity and adiposity in the multi-ethnic Insulin Resistance Atherosclerosis Study population. *Br J Nutr.* Dec 2004;92(6):973-984.

121. Haveman-Nies A, Tucker KL, de Groot LC, et al. Evaluation of dietary quality in relationship to nutritional and lifestyle factors in elderly people of the US Framingham Heart Study and the European SENECA study. *Eur J Clin Nutr.* Oct 2001;55(10):870-880.

122. Schwerin HS, Stanton JL, Smith JL, et al. Food, eating habits, and health: a further examination of the relationship between food eating patterns and nutritional health. *Am J Clin Nutr.* May 1982;35(5 Suppl):1319-1325.

123. Millen BE, Quatromoni PA, Copenhafer DL, et al. Validation of a dietary pattern approach for evaluating nutritional risk: the Framingham Nutrition Studies. *J Am Diet Assoc.* Feb 2001;101(2):187-194.

124. Schroll K, Carbajal A, Decarli B, et al. Food patterns of elderly Europeans. SENECA Investigators. *Eur J Clin Nutr.* Jul 1996;50 Suppl 2:S86-100.

125. Hoffmann K, Schulze MB, Schienkiewitz A, et al. Application of a new statistical method to derive dietary patterns in nutritional epidemiology. *Am J Epidemiol.* May 15 2004;159(10):935-944.

126. Hoffmann K, Zyriax BC, Boeing H, et al. A dietary pattern derived to explain biomarker variation is strongly associated with the risk of coronary artery disease. *Am J Clin Nutr.* Sep 2004;80(3):633-640.

127. SAS Institute Inc. *SAS/STAT user's guide, version 8*. Cary, NC: SAS Institute Inc; 1999.

128. Fung TT, McCullough ML, Newby PK, et al. Diet-quality scores and plasma concentrations of markers of inflammation and endothelial dysfunction. *Am J Clin Nutr.* Jul 2005;82(1):163-173.

129. McCullough ML, Feskanich D, Stampfer MJ, et al. Diet quality and major chronic disease risk in men and women: moving toward improved dietary guidance. *Am J Clin Nutr.* Dec 2002;76(6):1261-1271.

130. Fidanza F, Alberti A, Lanti M, et al. Mediterranean diet score: correlation with 25-year mortality from coronary heart disease in the Seven Countries Study. *Nutr Metab Cardiovasc Dis.* Dec 2004;14(6):397.

131. Martinez-Gonzalez MA, Fernandez-Jarne E, Serrano-Martinez M, et al. Mediterranean diet and reduction in the risk of a first acute myocardial infarction: an operational healthy dietary score. *Eur J Nutr.* Aug 2002;41(4):153-160.

132. Panagiotakos DB, Pitsavos C, Matalas AL, et al. Geographical influences on the association between adherence to the Mediterranean diet and the prevalence of acute coronary syndromes, in Greece: the CARDIO2000 study. *Int J Cardiol.* Apr 8 2005;100(1):135-142.

133. Trichopoulou A, Costacou T, Bamia C, et al. Adherence to a Mediterranean diet and survival in a Greek population. *N Engl J Med.* Jun 26 2003;348(26):2599-2608.

134. Trichopoulou A, Orfanos P, Norat T, et al. Modified Mediterranean diet and survival: EPIC-elderly prospective cohort study. *Bmj.* Apr 30 2005;330(7498):991.

135. Osler M, Schroll M. Diet and mortality in a cohort of elderly people in a north European community. *Int J Epidemiol.* Feb 1997;26(1):155-159.

136. Kouris-Blazos A, Gnardellis C, Wahlqvist ML, et al. Are the advantages of the Mediterranean diet transferable to other populations? A cohort study in Melbourne, Australia. *Br J Nutr.* Jul 1999;82(1):57-61.

137. Lasheras C, Fernandez S, Patterson AM. Mediterranean diet and age with respect to overall survival in institutionalized, nonsmoking elderly people. *Am J Clin Nutr.* Apr 2000;71(4):987-992.

138. Knoops KT, de Groot LC, Kromhout D, et al. Mediterranean diet, lifestyle factors, and 10-year mortality in elderly European men and women: the HALE project. *Jama.* Sep 22 2004;292(12):1433-1439.

139. Chrysohoou C, Panagiotakos DB, Pitsavos C, et al. Adherence to the Mediterranean diet attenuates inflammation and coagulation process in healthy adults: The ATTICA Study. *J Am Coll Cardiol.* Jul 7 2004;44(1):152-158.

140. Sondergaard E, Moller JE, Egstrup K. Effect of dietary intervention and lipid-lowering treatment on brachial vasoreactivity in patients with ischemic heart disease and hypercholesterolemia. *Am Heart J.* May 2003;145(5):E19.

141. Fito M, Guxens M, Corella D, et al. Effect of a traditional Mediterranean diet on lipoprotein oxidation: a randomized controlled trial. *Arch Intern Med.* Jun 11 2007;167(11):1195-1203.

142. Djousse L, Folsom AR, Province MA, et al. Dietary linolenic acid and carotid atherosclerosis: the National Heart, Lung, and Blood Institute Family Heart Study. *Am J Clin Nutr.* Apr 2003;77(4):819-825.

143. Albert CM, Oh K, Whang W, et al. Dietary alpha-linolenic acid intake and risk of sudden cardiac death and coronary heart disease. *Circulation.* Nov 22 2005;112(21):3232-3238.

144. Ascherio A, Rimm EB, Giovannucci EL, et al. Dietary fat and risk of coronary heart disease in men: cohort follow up study in the United States. *Bmj.* Jul 13 1996;313(7049):84-90.

145. Mozaffarian D, Ascherio A, Hu FB, et al. Interplay between different polyunsaturated fatty acids and risk of coronary heart disease in men. *Circulation.* Jan 18 2005;111(2):157-164.

146. Turpeinen O, Karvonen MJ, Pekkarinen M, et al. Dietary prevention of coronary heart disease: the Finnish Mental Hospital Study. *Int J Epidemiol.* Jun 1979;8(2):99-118.

147. Singh RB, Niaz MA, Sharma JP, et al. Randomized, double-blind, placebo-controlled trial of fish oil and mustard oil in patients with suspected acute myocardial infarction: the Indian experiment of infarct survival--4. *Cardiovasc Drugs Ther.* Jul 1997;11(3):485-491.

148. Natvig H, Borchgrevink CF, Dedichen J, et al. A controlled trial of the effect of linolenic acid on incidence of coronary heart disease. The Norwegian vegetable oil experiment of 1965-66. *Scand J Clin Lab Invest Suppl.* 1968;105:1-20.

149. Brouwer IA, Katan MB, Zock PL. Dietary alpha-linolenic acid is associated with reduced risk of fatal coronary heart disease, but increased prostate cancer risk: a meta-analysis. *J Nutr.* Apr 2004;134(4):919-922.

150. Spector A. Lipid metabolism: essential fatty acids. In: Stipanuk MH, ed. *Biochemical and physiological aspects of human nutrition*. Philadelphia: W.B. Saunders Company; 2000:365-401.

151. Essential fatty acid interactions.

152. Nelson TL, Hickey MS. Acute changes in dietary omega-3 fatty acid intake lowers soluble interleukin-6 receptor in healthy adult normal weight and overweight males. *Cytokine.* Jun 7 2004;26(5):195-201.

153. Nelson TL, Stevens JR, Hickey MS. Inflammatory markers are not altered by an eight week dietary alpha-linolenic acid intervention in healthy abdominally obese adult males and females. *Cytokine.* May 2007;38(2):101-106.

154. Paschos GK, Rallidis LS, Liakos GK, et al. Background diet influences the anti-inflammatory effect of alpha-linolenic acid in dyslipidaemic subjects. *Br J Nutr.* Oct 2004;92(4):649-655.

155. Pankow JS, Folsom AR, Cushman M, et al. Familial and genetic determinants of systemic markers of inflammation: the NHLBI family heart study. *Atherosclerosis.* Feb 15 2001;154(3):681-689.

156. Andreotti F, Porto I, Crea F, et al. Inflammatory gene polymorphisms and ischaemic heart disease: review of population association studies. *Heart.* Feb 2002;87(2):107-112.

157. Berg K. Molecular genetics and genetic epidemiology of cardiovascular diseases and diabetes. Introductory remarks: risk factor levels and variability. *Ann Med.* Oct 1992;24(5):343-347.

158. Nora J, Berg K, Nora A. *Cardiovascular diseases. Genetics, epidemiology and prevention.* . New York: Oxford University Press; 1991.

159. Nordlie MA, Wold LE, Kloner RA. Genetic contributors toward increased risk for ischemic heart disease. *J Mol Cell Cardiol.* Oct 2005;39(4):667-679.

160. Bennermo M, Held C, Stemme S, et al. Genetic predisposition of the interleukin-6 response to inflammation: implications for a variety of major diseases? *Clin Chem.* Nov 2004;50(11):2136-2140.

161. Vickers MA, Green FR, Terry C, et al. Genotype at a promoter polymorphism of the interleukin-6 gene is associated with baseline levels of plasma C-reactive protein. *Cardiovasc Res.* Mar 2002;53(4):1029-1034.

162. Galicia JC, Tai H, Komatsu Y, et al. Polymorphisms in the IL-6 receptor (IL-6R) gene: strong evidence that serum levels of soluble IL-6R are genetically influenced. *Genes Immun.* Sep 2004;5(6):513-516.

163. Rundek T, Elkind MS, Pittman J, et al. Carotid intima-media thickness is associated with allelic variants of stromelysin-1, interleukin-6, and hepatic lipase genes: the Northern Manhattan Prospective Cohort Study. *Stroke.* May 2002;33(5):1420-1423.

164. de Lange M, Snieder H, Ariens RA, et al. The genetics of haemostasis: a twin study. *Lancet.* Jan 13 2001;357(9250):101-105.

165. de Maat MP, Bladbjerg EM, Hjelmborg JB, et al. Genetic influence on inflammation variables in the elderly. *Arterioscler Thromb Vasc Biol.* Nov 2004;24(11):2168-2173.

166. Shimo-Nakanishi Y, Hasebe T, Suzuki A, et al. Functional effects of NAD(P)H oxidase p22(phox) C242T mutation in human leukocytes and association with thrombotic cerebral infarction. *Atherosclerosis.* Jul 2004;175(1):109-115.

167. Wyche KE, Wang SS, Griendling KK, et al. C242T CYBA polymorphism of the NADPH oxidase is associated with reduced respiratory burst in human neutrophils. *Hypertension.* Jun 2004;43(6):1246-1251.

168. Makela R, Karhunen PJ, Kunnas TA, et al. Myeloperoxidase gene variation as a determinant of atherosclerosis progression in the abdominal and thoracic aorta: an autopsy study. *Lab Invest.* Jul 2003;83(7):919-925.

169. Makela R, Laaksonen R, Janatuinen T, et al. Myeloperoxidase gene variation and coronary flow reserve in young healthy men. *J Biomed Sci.* Jan-Feb 2004;11(1):59-64.

170. Nikpoor B, Turecki G, Fournier C, et al. A functional myeloperoxidase polymorphic variant is associated with coronary artery disease in French-Canadians. *Am Heart J.* Aug 2001;142(2):336-339.

171. Kakko S, Paivansalo M, Koistinen P, et al. The signal sequence polymorphism of the MnSOD gene is associated with the degree of carotid atherosclerosis. *Atherosclerosis.* May 2003;168(1):147-152.

172. Dedoussis GV, Panagiotakos DB, Chrysohoou C, et al. Effect of interaction between adherence to a Mediterranean diet and the methylenetetrahydrofolate reductase 677C-->T mutation on homocysteine concentrations in healthy adults: the ATTICA Study. *Am J Clin Nutr.* Oct 2004;80(4):849-854.

173. de Castro JM. Heritability of hunger relationships with food intake in free-living humans. *Physiol Behav.* Aug 1999;67(2):249-258.

174. de Castro JM. Independence of heritable influences on the food intake of free-living humans. *Nutrition.* Jan 2002;18(1):11-16; discussion 91-12.

175. de Castro JM. Heritability of diurnal changes in food intake in free-living humans. *Nutrition.* Sep 2001;17(9):713-720.

176. de Castro JM. A twin study of genetic and environmental influences on the intake of fluids and beverages. *Physiol Behav.* Oct 1993;54(4):677-687.

177. Faith MS, Rha SS, Neale MC, et al. Evidence for genetic influences on human energy intake: results from a twin study using measured observations. *Behav Genet.* May 1999;29(3):145-154.

178. van den Bree MB, Eaves LJ, Dwyer JT. Genetic and environmental influences on eating patterns of twins aged >/=50 y. *Am J Clin Nutr.* Oct 1999;70(4):456-465.

179. Krondl M, Coleman P, Wade J, et al. A twin study examining the genetic influence on food selection. *Hum Nutr Appl Nutr.* Jun 1983;37 A(3):189-198.

180. Heitmann BL, Harris JR, Lissner L, et al. Genetic effects on weight change and food intake in Swedish adult twins. *Am J Clin Nutr.* Apr 1999;69(4):597-602.

181. Bakhru A, Erlinger TP. Smoking cessation and cardiovascular disease risk factors: results from the Third National Health and Nutrition Examination Survey. *PLoS Med.* Jun 2005;2(6):e160.

182. Wannamethee SG, Lowe GD, Shaper AG, et al. Associations between cigarette smoking, pipe/cigar smoking, and smoking cessation, and haemostatic and

inflammatory markers for cardiovascular disease. *Eur Heart J.* Sep 2005;26(17):1765-1773.

183. Zhang J, Liu Y, Shi J, et al. Side-stream cigarette smoke induces dose-response in systemic inflammatory cytokine production and oxidative stress. *Exp Biol Med (Maywood).* Oct 2002;227(9):823-829.

184. Ryder MI, Saghizadeh M, Ding Y, et al. Effects of tobacco smoke on the secretion of interleukin-1beta, tumor necrosis factor-alpha, and transforming growth factor-beta from peripheral blood mononuclear cells. *Oral Microbiol Immunol.* Dec 2002;17(6):331-336.

185. Wannamethee SG, Shaper AG, Whincup PH, et al. Smoking cessation and the risk of stroke in middle-aged men. *Jama.* Jul 12 1995;274(2):155-160.

186. Hashimoto K, Kasayama S, Yamamoto H, et al. Strong association of C-reactive protein with body mass index and 2-h post-challenge glucose in non-diabetic, non-smoker subjects without hypertension. *Diabet Med.* Jun 2004;21(6):581-585.

187. Schulz S, Schagdarsurengin U, Suss T, et al. Relation between the tumor necrosis factor-alpha (TNF-alpha) gene and protein expression, and clinical, biochemical, and genetic markers: age, body mass index and uric acid are independent predictors for an elevated TNF-alpha plasma level in a complex risk model. *Eur Cytokine Netw.* Apr-Jun 2004;15(2):105-111.

188. Sharman MJ, Volek JS. Weight loss leads to reductions in inflammatory biomarkers after a very-low-carbohydrate diet and a low-fat diet in overweight men. *Clin Sci (Lond).* Oct 2004;107(4):365-369.

189. Escobar-Morreale HF, Villuendas G, Botella-Carretero JI, et al. Obesity, and not insulin resistance, is the major determinant of serum inflammatory cardiovascular risk markers in pre-menopausal women. *Diabetologia.* May 2003;46(5):625-633.

190. Kasapis C, Thompson PD. The effects of physical activity on serum C-reactive protein and inflammatory markers: a systematic review. *J Am Coll Cardiol.* May 17 2005;45(10):1563-1569.

191. Covas MI, Elosua R, Fito M, et al. Relationship between physical activity and oxidative stress biomarkers in women. *Med Sci Sports Exerc.* May 2002;34(5):814-819.

192. Tauler P, Aguilo A, Cases N, et al. Acute phase immune response to exercise coexists with decreased neutrophil antioxidant enzyme defences. *Free Radic Res.* Oct 2002;36(10):1101-1107.

193. Tozzi-Ciancarelli MG, Penco M, Di Massimo C. Influence of acute exercise on human platelet responsiveness: possible involvement of exercise-induced oxidative stress. *Eur J Appl Physiol.* Jan 2002;86(3):266-272.

194. Miller GE, Stetler CA, Carney RM, et al. Clinical depression and inflammatory risk markers for coronary heart disease. *Am J Cardiol.* Dec 15 2002;90(12):1279-1283.

195. Elosua R, Molina L, Fito M, et al. Response of oxidative stress biomarkers to a 16-week aerobic physical activity program, and to acute physical activity, in healthy young men and women. *Atherosclerosis.* Apr 2003;167(2):327-334.

CHAPTER III

ADHERENCE TO THE MEDITERRANEAN DIET IS INVERSELY ASSOCIATED WITH CIRCULATING INTERLEUKIN-6 AMONG MIDDLE-AGED MALES: A TWIN STUDY

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Short title: Mediterranean diet, systemic inflammation, twins

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Abstract

Background The Mediterranean diet is protective against cardiovascular disease and a proposed mechanism is through reducing systemic inflammation. It is unknown to what extent the association between the Mediterranean diet and inflammation is due to genetic or other familial factors.

Methods and Results We administered the Willett food frequency questionnaire to 345 middle-aged male twins and assessed adherence to the Mediterranean diet using a published adherence score. Fasting plasma levels of interleukin-6 (IL-6), C-reactive protein (CRP), and known cardiovascular risk factors were measured. Mixed-effect regression analyses were used to examine the relationship between the diet score and inflammatory biomarkers after accounting for known cardiovascular risk factors. Adherence to the Mediterranean diet was associated with reduced levels of IL-6 ($P < 0.001$), but not CRP ($P = 0.10$), after adjusting for total energy intake, other nutritional factors, known cardiovascular risk factors, and use of supplements and medications. When the overall association of adherence to the diet with IL-6 levels was partitioned into between- and within-pair effects, the between-pair effect was not significant ($P = 0.9$) and the within-pair effect was highly significant ($P < 0.0001$). A one-unit within-pair absolute difference in the diet score was associated with a 9% (95% CI, 4.5 to 13.6) lower IL-6 level.

Conclusions Shared environmental and genetic factors are unlikely to play a major role in the association between adherence to the Mediterranean diet and systemic inflammation. These results support the hypothesis that reduced inflammation is an important mechanism linking Mediterranean diet to reduced cardiovascular risk.

Key Words: Mediterranean diet ■ inflammation ■ monozygotic ■ dizygotic ■ diet score

Introduction

Adherence to the Mediterranean diet appears to benefit the cardiovascular system,[1, 2] but the underlying mechanisms are unclear. An inverse relationship between adherence to the Mediterranean diet and biomarkers of systemic inflammation, such as interleukin-6 (IL-6) and C-reactive protein (CRP)[3, 4] has been reported, suggesting that inflammatory processes may be implicated. However, such relations were not consistently found in randomized controlled trials.[5-7] A possible reason for the inconsistent findings may be due to genetic differences in the response to diet, since each of the trials studied a different European population – Italian,[5] Spanish[6] and German,[7] respectively. In addition, since dietary habits are acquired while growing up, they are likely to be associated with other environmental conditions shared by members of the same family, such as unmeasured socioeconomic and lifestyle factors, which can also influence inflammation and thus act as confounders. However, no prior study has controlled for the influence of familial factors in the association between the Mediterranean diet and inflammation.

Twins are a powerful resource to dissect complex associations, because they allow to control for unmeasured and unknown confounding, such as genetic factors and socioeconomic, behavioral and lifestyle characteristics acquired by twins growing up in the same family. Using a sample of monozygotic (MZ) and dizygotic (DZ) middle-aged male twins raised in the same family, we examined the relationship between adherence to the Mediterranean diet and circulating biomarkers of inflammation. We sought to

determine if the association persisted after accounting for common genetic and environmental factors by comparing each twin with his co-twin.

Methods

Subjects

The Twins Heart Study (THS) is an investigation of psychological, behavioral and biological risk factors for subclinical cardiovascular disease using twins. It included 180 pairs of monozygotic (MZ) and dizygotic (DZ) male twins from the Vietnam Era Twin Registry[8] who were born between 1946 and 1956 and were free of symptomatic cardiovascular diseases based on survey data collected in 1990.[9] The Vietnam Era Twin Registry includes 7,369 middle-aged male-male twin pairs both of whom served in the United States military during the time of the Vietnam War. Zygosity was determined using similarity questionnaires supplemented with blood group typing data abstracted from military records.[10] For the THS, random samples of twins in two strata were selected from the Registry: one stratum included twins discordant for a lifetime history of major depression and in a second stratum neither twin had a history of depression. All twins were examined at the Emory University General Clinical Research Center between March 2002 and March 2006, where their medical history was updated. We excluded twins with missing dietary data, implausible energy intake ($\geq$ 6000 or <500 kcal/day),[11] or circulating IL-6 levels above 10 pg/mL, defined as a cutoff for low-grade systemic inflammation. The study protocol was approved by the Institutional Review Board at Emory University and informed consent was obtained from all subjects.

Diet Assessment

We used the Willett self-administered semiquantitative food frequency questionnaire[12] that collected dietary data over the past 12 months. The questionnaire classifies average food intake according to nine frequency categories ranging from "almost never or less than once per month" to "≥ 6 times/day", 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 following the nutrient database of the US Department of Agriculture. Daily food intake in grams was calculated from food intake frequency and portion sizes.

Mediterranean Diet Score (MDS)

The term Mediterranean diet refers to a dietary pattern typical of many regions in Greece and southern Italy in the early 1960s, including a high intake of fruits, vegetables, bread, other forms of cereals, potatoes, beans, nuts and seeds; low to moderate amounts of dairy products, fish, poultry and wine; low amounts of red meat; eggs consumed no more than four times weekly; and olive oil as an important fat source.[13] We measured adherence to the Mediterranean diet using a Mediterranean Diet Score (MDS) described by Trichopoulou et al. based on *a priori* assumptions about nine desirable or undesirable dietary components (Appendix Table A-I.)[1]: 1) seven desirable dietary components for health including cereals (excluding potatoes); vegetables; fruits and nuts; legumes; fish; dietary ratio of monounsaturated to saturated fatty acids (mainly due to using olive oil as the main cooking oil in the Mediterranean diet); and moderate alcohol consumption; and

2) two undesirable dietary components for health, including meat and dairy food products. The score was constructed using zygosity-specific, rather than gender-specific, median of food intake (adjusted to 2500 kcal),[14] in order to conduct analyses stratified by zygosity in our all-male sample. A value of 1 was assigned to a high intake (≥ median) of each desirable component; a value of 1 to a low intake (<median) of each undesirable foods; and a value of 0 to all other intakes.[14] For alcohol, a value of 1 was assigned to moderate consumption, that is, intake above the zygosity-specific median (2.38 for MZ and 1.72 gram/d for DZ) and at or below 33 g per day. The latter is the daily upper limit of alcohol intake considered to be "moderate" among American men,[15] and equals approximately two alcoholic drinks per day.[15, 16] The MDS was the sum of all values from the nine components, ranging from 0 to 9; the higher the score, the greater the adherence to the Mediterranean diet.

We also devised three slight variations of the MDS in order to evaluate the robustness of our findings. First, we followed an earlier method published by Trichopoulou et al. to calculate a score, MDS_1, ranging from 0 to 8, in which fish was included in the meat group[17] and potatoes and eggs were included among cereals and meats, respectively.[14] A second variant, MDS_2, was similar to the MDS_1 but fish was excluded from meat.[17] Fish intake as a covariate was forced into the models. In a third variation of the score, MDS_3, we excluded fish from the meat group and included fish as a desirable component.

Assessment of Known Cardiovascular Risk Factors

We assessed smoking (never smoked, current and past smoker), education and marital status using standardized questionnaires. Physical activity was evaluated with the Atherosclerosis Risk in Communities study Baecke questionnaire.[18] Waist and hip circumference were measured and used to calculate a waist-hip ratio. Systolic and diastolic blood pressures were measured using a mercury sphygmomanometer according to a standard protocol.[19] Hypertension was defined as systolic blood pressure $\geq$140 and/or diastolic blood pressure $\geq$ 90 mm Hg, or current use of antihypertensive medicines. Diabetes was defined as a fasting plasma glucose concentration $\geq$126 mg/dL,[20] or current treatment with insulin or oral hypoglycemic agents. Depressive symptoms were measured with the Beck Depression Inventory (BDI) which yielded a continuous score.[21] Current uses of aspirin and statins were also recorded.

Biochemical Analysis

Plasma samples were separated from nine-hour overnight fasting blood samples and stored at –80°C until analysis. Concentrations of glucose, triglycerides, and total, low- and high-density lipoprotein cholesterol were measured using standardized methods. Plasma IL-6 concentrations were determined using commercial enzyme-linked, high-sensitivity immunosorbent kits (R&D Systems, Minneapolis, MN). High-sensitivity CRP (hsCRP) concentrations were tested using the high-sensitive Beckman Coulter assay. The inter- and intra-assay variability for all assays was less than 10%, the samples were analyzed blindly, and twin pairs were assessed in the same analytical run.

Statistical Analysis

We ranked twins in each pair based on their MDS scores and calculated the within-pair absolute difference in the MDS as the difference between a twin with a higher MDS score and his twin brother with a lower MDS score. Inflammatory biomarkers were log-transformed due to skewed distributions. Intraclass correlation coefficients were calculated for the MDS and log-transformed inflammatory biomarkers in MZ and DZ twins. The association between the MDS and inflammatory markers was assessed by fitting linear regression models adapted for twin studies.[22] We first used the entire sample by treating twins as individuals and accounting for the twin pair clustering. The MDS was analyzed primarily as a continuous variable and secondarily as a four-level variable categorized as 0 to 3, 4, 5, and 6 to 9. Category midpoints were used for analyses.[23] Because the dependent variables were log-transformed, and the association was inverse, we expressed the results as percent difference of the non-transformed values by using the following formula: $[1-(\exp^{\beta})] \times 100$ (%); β is the regression coefficient and $\exp^{\beta}$ returns the exponential value of the parameter. The "base model" only adjusted for total energy intake and nutritional factors that were not part of the MDS, including potato and egg consumption.[1] To this model we subsequently added sociodemographic factors, including age, years of education, and current marital status; lifestyle factors, such as smoking, waist-to-hip ratio, and physical activity; comorbidity and cardiovascular risk factors, including previous coronary heart diseases, depressive symptoms (BDI score), fasting glucose, systolic blood pressure, low-density lipoprotein cholesterol, and high-density lipoprotein cholesterol; supplements and medications such as fish oil supplements, and use of aspirin and statins. Potential multicollinearity was investigated using condition

indices and variance decomposition proportions (VDP) by means of a SAS Macro, using criteria including both a condition index of 20 or more and at least two non-intercept variables with VDP values of 0.5 or higher.[24]

Next, we performed within-pair analyses to examine differences in inflammatory biomarkers between co-twins in each pair. The within-pair effects are inherently controlled for demographic, shared familial and environmental influences; in addition, environmental factors during the day of testing are controlled since co-twins were examined at the same time and under the same conditions. We fitted mixed effects models for twins[22] which allow for partitioning within- and between-pair differences in the dependent variable as a function of the independent variables. In these models the within-pair β coefficient describes the individual twin variation from the twin pair average; this formulation has the advantage of being independent of twin ordering; while the between-pair regression coefficient is just for the pair average. The within-pair coefficient is identical to the β coefficient from a model that fits the absolute difference between the co-twins.[22] Thus, the percent difference calculated from the within-pair coefficient can be interpreted as the difference in inflammatory biomarker concentrations per one-unit absolute difference in the MDS between co-twins in a pair. Since twin pairs were raised together, and MZ pairs share 100% of their genetic material, any association between the MDS and inflammatory markers within MZ pairs cannot be ascribed to genes or family environment. DZ pairs also share familial factors, but on average only share 50% of their genetic material and as such are less tightly matched. These models included separated variance components for twin type to accommodate the different residual correlation in MZ and DZ pairs. MZ and DZ twins, however, were also

examined separately. Obesity is tightly associated with systemic inflammation;[25] thus we further examined whether the diet-inflammation association was different among obese versus non-obese (body mass index ≥ 30 versus <30) twins. All analyses were conducted using mixed models to control for the pair clustering, using SAS software version 9.1 (SAS Institute). Significance levels were set at 0.05, two-sided. The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

Sample Characteristics

From the initial sample of 360 twins, we excluded 15 subjects (one with no dietary data, six with implausible energy intake, and eight with plasma IL-6 levels above 10 pg/mL). Therefore, our analyses were based on 345 twins (88 MZ and 77 DZ twin pairs, 5 MZ and 10 DZ unpaired twins). 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. The median biomarker concentration was 1.7 pg/mL (interquartile 1.1 to 2.7) for IL-6 and 1.3 mg/L (interquartile 0.5 to 2.7) for hsCRP. Twins with higher MDS were older, more educated, less likely to be smokers and more likely to use fish oil supplements (Table A.1). The mean within-pair absolute difference in the MDS was 1.6 (range, 5) in MZ and 1.8 (range, 7) in DZ twins. Intraclass correlation coefficients for the MDS, IL-6 and hsCRP were larger in MZ than DZ twin pairs (Table A.2), suggesting that genetic factors contribute to these traits.

Overall Associations

Increasing MDS was associated with decreasing levels of IL-6 and hsCRP in a dose-response fashion (Table A.3). When the MDS was treated as a continuous variable, for each 1-unit increase in the MDS, IL-6 and hsCRP levels decreased by 6.6% (P = 0.001) and 5.6% (P = 0.064), respectively, after adjusting for nutritional factors (Table A.3). After simultaneous adjustment for known cardiovascular risk factors the association between the MDS and hsCRP was no longer significant. However, even after full adjustment the relationship between the MDS and IL-6 remained strong (Model 4 in Table A.3): a one-unit increment in the MDS was associated with a 5.1% decrease in IL-6 concentration (P = 0.005). Similar results were observed with the MDS as an ordinal variable, confirming the presence of a dose-response association (Table A.3). In the fully adjusted model, twins in the highest MDS category had an IL-6 level 21.3% (95% CI 6.9~33.8%) lower than those in the lowest category, and there seem to be a threshold effect, such that subjects with a score at or above 6 had lower IL-6 levels than those with a score below 6.

Zygosity-Specific Within-Pair Results

For MZ pairs, a one-unit within-pair absolute difference in the MDS was associated with an 8.6% (95% CI 1% to 15%) and a 7.7% (95% CI 1% to 14%) lower IL-6 level before and after adjusting for the other risk factors, respectively (Table A.4)**.** The IL-6 results for DZ twins were similar. Neither MZ nor DZ twin pairs showed significant within-pair associations for hsCRP. The interaction between within-pair differences in the MDS and zygosity was not significant (Table A.4). The between-pair associations of

the MDS with inflammatory markers were not significant for either IL-6 or hsCRP (*P*=0.9 for IL-6 and *P*=0.5 for hsCRP in the fully adjusted model), suggesting that shared familial factors were not important.

Because inflammation is a strong correlate of obesity, we also examined whether the within-pair association between the MDS and inflammatory markers differed by obesity. The interaction between within-pair differences in the MDS and obesity was not significant for either IL-6 or hsCRP in the fully adjusted model for the entire sample and by zygosity status (all *P* values >0.25).

Finally, we repeated the analyses after excluding subjects with a previous history of coronary heart disease and by using published variations of the Mediterranean diet score (MDS_1, MDS_2 and MDS_3). The results were very similar and are not shown.

Discussion

We found an inverse association between adherence to the Mediterranean diet and inflammation as measured by IL-6, independent of a wide range of known cardiovascular risk factors. This finding persisted when comparing co-twins within pairs, either MZ or DZ, suggesting that shared familial factors do not confound the association between adherence to the Mediterranean diet and systemic inflammation as measured by IL-6. Results were robust and persisted after exclusion of persons with a previous history of coronary heart disease and when slight variations of the Mediterranean diet score were used.

Prior studies on the association between the Mediterranean diet and systemic IL-6 levels are limited. Inverse associations between adherence to the Mediterranean diet and

IL-6 were reported in observational studies among healthy subjects,[3, 4] and intermediate-[6] or long-term[5] randomized controlled trials among subjects at high risk for cardiovascular disease,[5, 6] but not in a short-term trial among healthy individuals,[26] perhaps due to lack of sufficient time to observe potential effects.

Although previous studies adjusted for socio-demographic and cardiovascular risk factors, no study has accounted for familial or genetic influences that may be shared between adherence to the Mediterranean diet and inflammatory response. Heritable and familial determinants affect both inflammatory biomarkers[27] and dietary behavior, including food preference,[28] food consumption frequency,[29] and perception of hunger.[30] A variety of other unmeasured environmental and behavioral factors that twins share, may confound the diet-inflammation association, and provide spurious associations. However, by comparing twins within pairs, we found that the MDS and IL-6 association was not diminished, which points to a conclusion that genetic influences or other familial factors do not play major roles.

We found an independent association between adherence to the Mediterranean diet and plasma levels of IL-6 but not CRP. In the base model, CRP was marginally associated with the Mediterranean diet score (P=0.06), but after controlling for known cardiovascular risk factors, this relationship was substantially reduced. Several studies have found that the association between CRP and cardiovascular disease is attenuated after adjustment for confounding factors.[31-35] One possible explanation is that IL-6 is a more sensitive indicator of atherosclerosis and cardiovascular risk than CRP.[36-39] IL-6 has numerous biological actions[40] that may promote atherosclerosis, including regulation of immune cells, recruitment of lymphocytes via stimulation of endothelial synthesis of

cellular adhesion molecules, pro-coagulant effects, and stimulation of the hepatic synthesis of CRP. Thus, IL-6 may have a direct atherogenic role,[36, 37, 39, 40] and CRP may mostly serve as a marker of IL-6 or of other atherogenic risk factors.[41]

While the within-pair effect was robust, the between-pair effect was weak and non-significant. This may be related to less confounding in the within-pair analyses. Since our twins were raised together, within-pair analyses naturally controlled for unmeasured confounding factors related to family environment or early development, which may act as negative confounders in this association. In general, a strong within-pair effect with a small or absent between-pair effect is consistent with a causal mechanism.[22]

There are some limitations to our study. The sample was restricted to middle-aged male Vietnam era veterans, and our results may not be generalizable to females. As in other common food frequency questionnaires used across the United States, combined food items, containing two or more components of the MDS, may misclassify individual MDS components. However, we carefully decomposed combined items into individual ingredients using appropriate recipes, therefore minimizing misclassification. Our results only reflect the cumulative effects of various foods in the Mediterranean diet score,[42] and our findings should not be extrapolated to a single food component. As in all observational studies, our results may be affected by unmeasured confounding. However, we controlled for many known lifestyle factors, and compared twins raised in the same family; thus it is unlikely that other behavioral factors confound substantially the association between the MDS and inflammation.

In conclusion, using a twin sample we were able to demonstrate that shared environmental and genetic factors do not confound the association between adherence to the Mediterranean diet and systemic inflammation. Our findings add weight to the biological plausibility of the cardioprotective effect of the Mediterranean diet, and substantiate the protective effect of the Mediterranean diet on cardiovascular risk. Furthermore, our data support the importance of behavioral interventions that encourage consumption of a healthier diet to prevent cardiovascular disease.

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Disclosures

None

References

1. Trichopoulou A, Costacou T, Bamia C, Trichopoulos D. Adherence to a Mediterranean diet and survival in a Greek population. *N Engl J Med.* 2003;348:2599-2608.

2. Serra-Majem L, Roman B, Estruch R. Scientific evidence of interventions using the Mediterranean diet: a systematic review. *Nutr Rev.* 2006;64:S27-47.

3. Chrysohoou C, Panagiotakos DB, Pitsavos C, Das UN, Stefanadis C. Adherence to the Mediterranean diet attenuates inflammation and coagulation process in healthy adults: The ATTICA Study. *J Am Coll Cardiol.* 2004;44:152-158.

4. Fung TT, McCullough ML, Newby PK, Manson JE, Meigs JB, Rifai N, Willett WC, Hu FB. Diet-quality scores and plasma concentrations of markers of inflammation and endothelial dysfunction. *Am J Clin Nutr.* 2005;82:163-173.

5. Esposito K, Marfella R, Ciotola M, Di Palo C, Giugliano F, Giugliano G, D'Armiento M, D'Andrea F, Giugliano D. Effect of a mediterranean-style diet on endothelial dysfunction and markers of vascular inflammation in the metabolic syndrome: a randomized trial. *Jama.* 2004;292:1440-1446.

6. Estruch R, Martinez-Gonzalez MA, Corella D, Salas-Salvado J, Ruiz-Gutierrez V, Covas MI, Fiol M, Gomez-Gracia E, Lopez-Sabater MC, Vinyoles E, Aros F, Conde M, Lahoz C, Lapetra J, Saez G, Ros E. Effects of a Mediterranean-style diet on cardiovascular risk factors: a randomized trial. *Ann Intern Med.* 2006;145:1-11.

7. Michalsen A, Lehmann N, Pithan C, Knoblauch NT, Moebus S, Kannenberg F, Binder L, Budde T, Dobos GJ. Mediterranean diet has no effect on markers of

inflammation and metabolic risk factors in patients with coronary artery disease. *Eur J Clin Nutr.* 2006;60:478-485.

8. Goldberg J, Curran B, Vitek ME, Henderson WG, Boyko EJ. The Vietnam Era Twin Registry. *Twin Res.* 2002;5:476-481.
9. Scherrer JF, Xian H, Bucholz KK, Eisen SA, Lyons MJ, Goldberg J, Tsuang M, True WR. A Twin study of depression symptoms, hypertension, and heart disease in middle-aged men. *Psychosom Med.* 2003;65:548-557.
10. Henderson WG, Eisen S, Goldberg J, True WR, Barnes JE, Vitek ME. The Vietnam Era Twin Registry: a resource for medical research. *Public Health Rep.* 1990;105:368-373.
11. van der AD, Peeters PH, Grobbee DE, Marx JJ, van der Schouw YT. Dietary haem iron and coronary heart disease in women. *Eur Heart J.* 2005;26:257-262.
12. Willett W. Chapter 5. Food frequency methods. In: *Nutritional epidemiology*. 2nd ed. New York, NY: Oxford University Press; 1998:74-91.
13. Kris-Etherton P, Eckel RH, Howard BV, St Jeor S, Bazzarre TL. AHA Science Advisory: Lyon Diet Heart Study. Benefits of a Mediterranean-style, National Cholesterol Education Program/American Heart Association Step I Dietary Pattern on Cardiovascular Disease. *Circulation.* 2001;103:1823-1825.
14. Trichopoulou A, Kouris-Blazos A, Wahlqvist ML, Gnardellis C, Lagiou P, Polychronopoulos E, Vassilakou T, Lipworth L, Trichopoulos D. Diet and overall survival in elderly people. *Bmj.* 1995;311:1457-1460.

15. The U.S. Department of Health and Human Services and the Department of Agriculture. Chapter 9. Alcoholic Beverages. In: Dietary Guidelines for Americans 2005. The USA; 2005.

16. Kerr WC, Greenfield TK. The average ethanol content of beer in the U.S. and individual states: estimates for use in aggregate consumption statistics. *J Stud Alcohol.* 2003;64:70-74.

17. Davidson S, Passmore R. *Human nutrition and dietetics.* ed. Edinburgh, NY: Churchill Livingstone; 1979.

18. Pols MA, Peeters PH, Bueno-De-Mesquita HB, Ocke MC, Wentink CA, Kemper HC, Collette HJ. Validity and repeatability of a modified Baecke questionnaire on physical activity. *Int J Epidemiol.* 1995;24:381-388.

19. Anonymous. The sixth report of the Joint National Committee on prevention, detection, evaluation, and treatment of high blood pressure. *Arch Intern Med.* 1997;157:2413-2446.

20. Barr RG, Nathan DM, Meigs JB, Singer DE. Tests of glycemia for the diagnosis of type 2 diabetes mellitus. *Ann Intern Med.* 2002;137:263-272.

21. Beck AT, Ward CH, Mendelson M, Mock J, Erbaugh J. An inventory for measuring depression. *Arch Gen Psychiatry.* 1961;4:561-571.

22. Carlin JB, Gurrin LC, Sterne JA, Morley R, Dwyer T. Regression models for twin studies: a critical review. *Int J Epidemiol.* 2005;34:1089-1099.

23. Rothman KJ, Greenland S. Chapter 17. Analysis of Polytomous Exposures and Outcomes. In: *Modern epidemiology*. 2nd ed. Philadelphia, PA: Lippincott William & Wilkins; 1998:301-328.

24. Luthi JC, Flanders WD, Pitts SR, Burnand B, McClellan WM. Outcomes and the quality of care for patients hospitalized with heart failure. *Int J Qual Health Care.* 2004;16:201-210.

25. Fontana L, Eagon JC, Trujillo ME, Scherer PE, Klein S. Visceral fat adipokine secretion is associated with systemic inflammation in obese humans. *Diabetes.* 2007.

26. Ambring A, Johansson M, Axelsen M, Gan L, Strandvik B, Friberg P. Mediterranean-inspired diet lowers the ratio of serum phospholipid n-6 to n-3 fatty acids, the number of leukocytes and platelets, and vascular endothelial growth factor in healthy subjects. *Am J Clin Nutr.* 2006;83:575-581.

27. de Craen AJ, Posthuma D, Remarque EJ, van den Biggelaar AH, Westendorp RG, Boomsma DI. Heritability estimates of innate immunity: an extended twin study. *Genes Immun.* 2005;6:167-170.

28. Krondl M, Coleman P, Wade J, Milner J. A twin study examining the genetic influence on food selection. *Hum Nutr Appl Nutr.* 1983;37 A:189-198.

29. Heitmann BL, Harris JR, Lissner L, Pedersen NL. Genetic effects on weight change and food intake in Swedish adult twins. *Am J Clin Nutr.* 1999;69:597-602.

30. de Castro JM. Heritability of hunger relationships with food intake in free-living humans. *Physiol Behav.* 1999;67:249-258.

31. Wilson PW, Nam BH, Pencina M, D'Agostino RB, Sr., Benjamin EJ, O'Donnell CJ. C-reactive protein and risk of cardiovascular disease in men and women from the Framingham Heart Study. *Arch Intern Med.* 2005;165:2473-2478.

32. Folsom AR, Pankow JS, Tracy RP, Arnett DK, Peacock JM, Hong Y, Djousse L, Eckfeldt JH. Association of C-reactive protein with markers of prevalent atherosclerotic disease. *Am J Cardiol.* 2001;88:112-117.

33. Makita S, Nakamura M, Hiramori K. The association of C-reactive protein levels with carotid intima-media complex thickness and plaque formation in the general population. *Stroke.* 2005;36:2138-2142.

34. Kivimaki M, Lawlor DA, Juonala M, Smith GD, Elovainio M, Keltikangas-Jarvinen L, Vahtera J, Viikari JS, Raitakari OT. Lifecourse socioeconomic position, C-reactive protein, and carotid intima-media thickness in young adults: the cardiovascular risk in Young Finns Study. *Arterioscler Thromb Vasc Biol.* 2005;25:2197-2202.

35. Wang TJ, Nam BH, Wilson PW, Wolf PA, Levy D, Polak JF, D'Agostino RB, O'Donnell CJ. Association of C-reactive protein with carotid atherosclerosis in men and women: the Framingham Heart Study. *Arterioscler Thromb Vasc Biol.* 2002;22:1662-1667.

36. Ridker PM, Rifai N, Stampfer MJ, Hennekens CH. Plasma concentration of interleukin-6 and the risk of future myocardial infarction among apparently healthy men. *Circulation.* 2000;101:1767-1772.

37. St-Pierre AC, Cantin B, Bergeron J, Pirro M, Dagenais GR, Despres JP, Lamarche B. Inflammatory markers and long-term risk of ischemic heart disease in men A 13-year follow-up of the Quebec Cardiovascular Study. *Atherosclerosis.* 2005;182:315-321.

38. Lee WY, Allison MA, Kim DJ, Song CH, Barrett-Connor E. Association of Interleukin-6 and C-Reactive Protein With Subclinical Carotid Atherosclerosis (the Rancho Bernardo Study). *Am J Cardiol.* 2007;99:99-102.

39. Cesari M, Penninx BW, Newman AB, Kritchevsky SB, Nicklas BJ, Sutton-Tyrrell K, Rubin SM, Ding J, Simonsick EM, Harris TB, Pahor M. Inflammatory markers and onset of cardiovascular events: results from the Health ABC study. *Circulation.* 2003;108:2317-2322.

40. Woods A, Brull DJ, Humphries SE, Montgomery HE. Genetics of inflammation and risk of coronary artery disease: the central role of interleukin-6. *Eur Heart J.* 2000;21:1574-1583.

41. Lowe GD, Pepys MB. C-reactive protein and cardiovascular disease: weighing the evidence. *Curr Atheroscler Rep.* 2006;8:421-428.

42. Hu FB. Dietary pattern analysis: a new direction in nutritional epidemiology. *Curr Opin Lipidol.* 2002;13:3-9.

TABLE A.1.

Age, Lifestyle, Clinical and Biochemical Characteristics of Subjects According to the Mediterranean Diet Score

	Mediterranean Diet Score				
Variable	**(0-3) (n=104)**	**(4) (n=70)**	**(5) (n=81)**	**(6-9) (n=90)**	***P* value***
Age, y	53.8±0.3	54.3±0.3	54.5±0.3	54.8±0.3	0.02
Education, y	13.8±0.2	14.1±0.2	14.7±0.2	14.5±0.2	0.003
Body mass index, kg/m^2	29.6±0.5	29.5±0.6	29.5±0.5	28.5±0.5	0.13
Waist to hip ratio	0.94±0.01	0.95±0.01	0.95±0.01	0.94±0.01	0.75
Physical activity, unit	7.15±0.16	7.34±0.19	7.69±0.18	7.49±0.17	0.07
Smoking status, n (%)					0.001
Former	29 (27.9)	24 (34.3)	33 (40.7)	44 (48.9)	
Current	41 (39.4)	34 (48.6)	13 (16.0)	9 (10.0)	
Never	34 (32.7)	12 (17.1)	35 (43.2)	37 (41.1)	
Married, n (%)	77 (74.0)	54 (77.1)	66 (81.5)	77 (85.6)	0.07
Employed, n (%)	78 (75.0)	58 (82.9)	64 (79.0)	74 (82.2)	0.28
Depressive symptoms, BDI score	6.04±0.62	4.44±0.74	4.15±0.70	3.98±0.67	0.02
Plasma glucose, mg/dL	98.2±1.8	102.7±2.1	100.0±2.0	103.3±1.9	0.052

TABLE A.1 (Con't.)

Age, Lifestyle, Clinical and Biochemical Characteristics of Subjects According to the Mediterranean Diet Score

	Mediterranean Diet Score				
Variable	**(0-3) (n=104)**	**(4) (n=70)**	**(5) (n=81)**	**(6-9) (n=90)**	***P* value***
Systolic blood pressure, mm Hg	130.3±1.6	129.5±1.8	128.5±1.7	129.3±1.6	0.61
Diastolic blood pressure, mm Hg	80.8±1.1	80.6±1.2	79.8±1.2	82.3±1.1	0.37
Blood Lipids, mg/dL					
Total triglycerides	176.6±10.0	192.9±11.6	179.2±11.6	182.5±10.5	0.75
Total Cholesterol	185.3±3.7	193.2±4.4	186.0±4.2	189.1±4.0	0.57
HDL-Cholesterol	38.9±0.9	38.4±1.0	39.7±1.0	37.7±1.0	0.41
LDL-Cholesterol	124.0±3.3	125.9±3.9	120.8±3.7	125.0±3.5	0.96
Previous coronary heart disease, n (%)	13 (12.5)	2 (2.9)	12 (14.8)	6 (6.7)	0.31
Diabetes mellitus, n (%)	8 (7.7)	8 (11.6)	11 (13.6)	10 (11.1)	0.35
Hypertension, n (%)	36 (34.6)	21 (30.0)	24 (29.7)	26 (28.9)	0.40
Use of fish oil supplements, n (%)	2 (1.9)	2 (2.9)	5 (6.2)	10 (11.1)	0.01
Use of statins, n (%)	26 (25.0)	11 (15.7)	27 (33.3)	22 (24.4)	0.79
Use of aspirin, n (%)	29 (27.9)	12 (17.1)	25 (30.9)	21 (23.3)	0.67

TABLE A.1 (Con't.)

Age, Lifestyle, Clinical and Biochemical Characteristics of Subjects According to the Mediterranean Diet Score

	Mediterranean Diet Score				
Variable	**(0-3) (n=104)**	**(4) (n=70)**	**(5) (n=81)**	**(6-9) (n=90)**	***P* value***
Plasma inflammatory biomarkers					
IL-6, pg/mL[†]	2.1 (1.8~2.3)	1.9 (1.6~2.2)	1.8 (1.6~2.1)	1.5 (1.3~1.7)	<0.0001
hsCRP, mg/L[†]	1.4 (1.1~1.7)	1.3 (1.0~1.7)	1.1 (0.9~1.4)	1.0 (0.8~1.3)	0.02

Results are expressed as means ± SEM when appropriate. Variables (%) are dichotomous except smoking status. BDI indicates Beck Depression Inventory; IL-6, interleukin-6; hsCRP, high-sensitivity CRP; HDL, high-density lipoprotein; and LDL, low-density lipoprotein.

[*] Test for trend across diet groups. All *P* values are corrected for pair clustering. Mixed models were used for continuous variables, generalized estimating equation (GEE) logistic models for dichotomous variables, and repeated proportional odds model with GEE for the three-level ordinal smoking variable.

[†] Geometric means (95% confidence intervals).

TABLE A.2.

Intraclass Correlation Coefficients*

	MZ	DZ
MDS, unit	0.26	0.0
IL-6, pg/mL	0.35	0.16
hsCRP, mg/L	0.70	0.26

Calculated using linear mixed model with SAS.

TABLE A.3.

Associations between the Mediterranean Diet Score (MDS) and Inflammatory Biomarker Concentrations in the Entire Sample.

Outcome	Difference (%) Per 1-Unit Increment in MDS (95% CI) (n=345)	*P* value	Geometric Means of IL-6 or hsCRP levels (95% CI) MDS=0-3 (n=104)	MDS=4 (n=70)	MDS=5 (n=81)	MDS=6-9 (n=90)	*P* value*
Model 1: Adjusted for zygosity and other nutritional factors not included in the MDS†.							
IL-6, pg/mL	-6.6 (-10.1, -2.9)	0.001	2.1 (1.8, 2.3)	1.9 (1.6, 2.2)	1.8 (1.6, 2.1)	1.5 (1.3, 1.7)	<0.0001
hsCRP, mg/L	-5.6 (-11.3, 0.3)	0.064	1.4 (1.1, 1.7)	1.3 (1.0, 1.7)	1.2 (0.9, 1.5)	1.0 (0.8, 1.3)	0.06
Model 2: Further adjusted for demographic and lifestyle factors‡.							
IL-6, pg/mL	-4.6 (-8.2, -0.9)	0.016	2.1 (1.9, 2.4)	2.1 (1.8, 2.4)	2.0 (1.8, 2.4)	1.7 (1.5, 1.9)	0.007
hsCRP, mg/L	-3.7 (-9.5, 2.4)	0.22	1.3 (1.1, 1.7)	1.3 (1.0, 1.7)	1.3 (1.0, 1.6)	1.1 (0.9, 1.4)	0.22

TABLE A.3 (Con't)

Associations between the Mediterranean Diet Score (MDS) and Inflammatory Biomarker Concentrations in the Entire Sample.

Outcome	Difference (%) Per 1-Unit Increment in MDS (95% CI) (n=345)	*P* value	Geometric Means of IL-6 or hsCRP levels (95% CI) MDS=0-3 (n=104)	MDS=4 (n=70)	MDS=5 (n=81)	MDS=6-9 (n=90)	*P* value*
Model 3: Further adjusted for comorbidity and cardiovascular risk factors§.							
IL-6, pg/mL	-5.1 (-8.6, -1.4)	0.007	2.1 (1.9, 2.4)	2.1 (1.8, 2.4)	2.1 (1.8, 2.4)	1.7 (1.5, 1.9)	0.005
hsCRP, mg/L	-4.0 (-9.7, 2.0)	0.18	1.4 (1.1, 1.7)	1.3 (1.0, 1.7)	1.3 (1.0, 1.7)	1.1 (0.9, 1.4)	0.17
Model 4: Further adjusted for use of supplements and medications‖.							
IL-6, pg/mL	-5.1 (-8.2, -0.9)	0.008	2.1 (1.7, 2.5)	2.0 (1.6, 2.5)	2.0 (1.7, 2.4)	1.6 (1.3, 1.9)	0.005
hsCRP, mg/L	-3.9 (-9.6, 2.2)	0.20	1.2 (0.9, 1.7)	1.2 (0.8, 1.7)	1.2 (0.9, 1.7)	1.0 (0.7, 1.3)	0.18

TABLE A.3 (Cont.)

The percent difference was calculated from the β coefficient of the MDS in mixed models treating twins as separate individuals but accounting for clustering within a pair. MDS indicates Mediterranean diet score; IL-6, interleukin-6; and hsCRP, high-sensitive C-reactive protein.

[*] Test for trend across diet groups.

[†] Total energy intake, egg and potato consumption.

[‡] Age, education, marital status, smoking, waist-to-hip ratio, and physical activity.

[§] Previous coronary heart disease, depressive symptom score, plasma glucose, systolic blood pressure, low- and high-density lipoprotein cholesterol.

[‖] Fish oil supplements, statins and aspirin.

TABLE A.4.

Within-Pair Differences in Inflammatory Biomarker Concentrations per 1-Unit Mediterranean Diet Score (MDS), Overall and by Zygosity

Outcome	MZ+DZ (n=345) Within-Pair Difference (%) (95% CI)	*P* Value	MZ (n=181) Within-Pair Difference (%) (95% CI)	*P* Value	DZ (n=164) Within-Pair Difference (%) (95% CI)	*P* Value	*P* Value‖
Model 1: Adjusted for zygosity and other nutritional factors not included in the MDS*.							
IL-6, pg/mL	-9.9 (-14.3, -5.2)	<0.0001	-8.6 (-15.4, -1.3)	0.022	-11.3 (-17.0, -4.9)	0.001	0.55
hsCRP, mg/L	-5.4 (-12.2 1.8)	0.14	-3.9 (-12.5, 6.2)	0.45	-6.8 (-17.4, 5.2)	0.26	0.69
Model 2: Further adjusted for demographic and lifestyle factors†.							
IL-6, pg/mL	-8.8 (-13.2 -4.1)	<0.0001	-8.6 (-15.4, -1.6)	0.018	-10.4 (-16.1, -3.6)	0.003	0.92
hsCRP, mg/L	-4.4 (-11.3 3.0)	0.21	-3.0 (-11.9, 7.4)	0.58	-6.8 (-17.1, 6.2)	0.28	0.66

TABLE A.4 (Con't).

Within-Pair Differences in Inflammatory Biomarker Concentrations per 1-Unit Mediterranean Diet Score (MDS), Overall and by Zygosity

	MZ+DZ (n=345)		MZ (n=181)		DZ (n=164)		
Outcome	Within-Pair Difference (%) (95% CI)	*P* Value	Within-Pair Difference (%) (95% CI)	*P* Value	Within-Pair Difference (%) (95% CI)	*P* Value	*P* Value‖
Model 3: Further adjusted for comorbidity and cardiovascular risk factors‡.							
IL-6, pg/mL	-9.2 (-13.6, -4.6)	<0.0001	-9.5 (-15.7, -2.6)	0.009	-11.3 (-16.8, -4.7)	0.001	0.85
hsCRP, mg/L	-4.4 (-11.2, 2.8)	0.22	-3.0 (-11.6, 6.6)	0.53	-6.8 (-17.5, 5.4)	0.26	0.57
Model 4: Further adjusted for use of supplements and medications§.							
IL-6, pg/mL	-9.2 (-13.6, -4.5)	<0.0001	-7.7 (-14.4, -0.8)	0.029	-11.3 (-17.1, -4.7)	0.001	0.80
hsCRP, mg/L	-4.1 (-10.8, 3.2)	0.26	-0.5 (-9.5, 9.5)	0.93	-7.7 (-18.0, 4.8)	0.22	0.46

TABLE A.4 (Cont.).

The within-pair difference (%) is calculated from the β coefficient and is expressed per 1-unit difference in the MDS between two twins in a pair. MDS indicates Mediterranean diet score; MZ, monozygotic twins; DZ, dizygotic twins; IL-6, interleukin-6; and hsCRP, high-sensitive C-reactive protein.

* Total energy intake, egg and potato consumption.

† Age, education, marital status, smoking, waist-to-hip ratio, and physical activity.

‡ Previous coronary heart disease, depressive symptom score, plasma glucose, systolic blood pressure, low- and high-density lipoprotein cholesterol.

§ Fish oil supplements, statins and aspirin.

‖ for interaction for with zygosity

APPENDIX

Appendix Table A-I.

Dietary Components Used to Construct the Mediterranean Diet Score

Dietary Component	Foods Included	Criteria for 1 Point
Desirable components		
Vegetables	All vegetables except potatoes	≥ median intake (g/d)
Legumes	Peas and beans	≥ median intake (g/d)
Fruits and nuts	All fruits and juices, all nuts and peanut butter	≥ median intake (g/d)
Cereals	All cereal foods included as follows: Cold cereals, white bread, dark bread, rice, pasta, as well as cereals in hotdog, pie, cake, cookie, and hamburger. Sugar and potatoes were excluded.	≥ median intake (g/d)
Fish	Fish	≥ median intake
Ratio of monounsaturated to saturated fatty acids	The whole diet	≥ median intake
Ethanol	Wine, beer and liquor	median~33 g/d
Undesirable components		
Dairy products	All dairy products	< median intake (g/d)

Appendix Table A-I (Con't)

Dietary Components Used to Construct the Mediterranean Diet Score

Dietary Component	Foods Included	Criteria for 1 Point
Meats	All meats excluding eggs	< median intake (g/d)

Medians for dietary components were zygosity-specific.

APPENDIX (Cont.)

Appendix Table A-II.

Medians of Dietary Components (adjusted to 2500 kcal) Used for the Mediterranean Diet Score Calculation or Otherwise Used in the Analysis.

Dietary Component	MZ	DZ
Dietary components for the score calculation		
Desirable components		
Vegetables (g/day)	138.4	125.7
Legumes (g/day)	34.6	33.1
Fruits and nuts (g/day)	298.3	259.5
Cereals (g/day)	131.2	127.2
Fish (g/day)	18.8	20.0
Ratio of monounsaturated to saturated fatty acids	1.106	1.091
Ethanol (g/day)	2.38	1.72
Undesirable components		
Dairy products (g/day)	308.1	319.5
Meats (g/day)	123.7	143.4

APPENDIX (Cont.)

Appendix Table A-II.

Medians of Dietary Components (adjusted to 2500 kcal) Used for the Mediterranean Diet Score Calculation or Otherwise Used in the Analysis.

Dietary Component	MZ	DZ
Food groups forced into models		
Eggs (g/day)	19.3	28.8
Potatoes (g/day)	139.0	156.9
Total energy intake (kcal/day)*	1426.7	1447.1

To convert values for energy intake to kJ, multiplied by 4.184.

MZ indicates monozygotic twins; DZ: dizygotic twins.

CHAPTER IV

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 is 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 paired twins and 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 oxidative stress. Mixed-effect regression analysis was used to partition the association into between- and within-twin pair differences. When examining within-pair effects, 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 adjusting for energy intake, other nutritional factors, cardiovascular 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 comparing the twin with the higher score to his co-twin with the lower score (P = 0.007). Results were similar in monozygotic and dizygotic twin pairs.

CONCLUSIONS: The association between 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 Mediterranean diet to reduced cardiovascular risk.

KEY WORDS: Mediterranean diet • identical twins • fraternal twins • reduced glutathione • oxidized glutathione

Introduction

Greater adherence to the Mediterranean diet is associated with lower risk for 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, since dietary habits in adult life are influenced by those 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 show that plasma GSH/GSSG decreases in response to tissue oxidative stress.[9] Oxidative stress is conceptualized as a disruption of redox signaling and control.[10] Therefore, the ratio of GSH to 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 may inhibit atherosclerosis.[12, 13] Additionally, a lower GSH/GSSG ratio 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 ratio was found in patients with acute myocardial infarction compared with controls,[14] and was related to the progression of atherosclerotic lesions after percutaneous coronary intervention.[15]

Using a sample of monozygotic (MZ) and dizygotic (DZ) 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 ratio. Twins are naturally matched for demographic, familial and other environmental influences while growing up. MZ pairs are also 100% matched for genetic factors, while DZ 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 environment/familial factors. If the association is observed within MZ 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 (MZ) and dizygotic (DZ) male twins from the Vietnam Era Twin Registry,[16] a registry of male-male twin pairs both of whom served in the United States military during the Vietnam era.[17] Twins selected for inclusion in the THS were born between 1946 and 1956, which represented >90% of the twins in the VET Registry. Using self-reported data from 1990, eligible twins were free of symptomatic cardiovascular diseases.[18] Zygosity information determined by DNA

analysis was available from all but 11 twin pairs. The zygosity of these 11 pairs was assessed using questionnaires supplemented with blood group typing data abstracted from military records,[19] which in our sample had an accuracy of 94%.

For the THS, random samples of twins in two strata were selected from the Registry: one stratum included twins discordant for a lifetime history of major depression; 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 one with no dietary data, four with implausible energy intake ($\geq$ 6000 or $<$500 kcal/day),[20] thirty-four with previous cardiovascular disease as assessed at our clinic visit, and two with missing GSH/GSSG data. A number of unpaired twins resulted from these exclusions; these were retained in the analyses. Inclusion of unpaired twins is common practice in twin modeling because it allows full use of all available data.[21] Therefore, our analyses were based on 297 twins, including 81 MZ and 57 DZ twin pairs, and 9 MZ and 12 DZ 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 semi-quantitative food frequency questionnaire[22] to collect dietary data over the previous 12 months. The questionnaire

classifies average food intake according to nine frequency categories ranging from "almost never or less than once per month" to "≥6 times/day", 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 using the nutrient database of the US Department of Agriculture.[22] Daily food intake in grams was calculated from food intake frequency and portion sizes.

Mediterranean Diet Score (MDS)

The Mediterranean diet is characterized by a high intake of fruits, 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 weekly; and use of olive oil as an important fat source.[23] We measured adherence to the Mediterranean diet using a Mediterranean Diet Score (MDS) described by Trichopoulou et al. based on *a priori* assumptions about nine desirable or undesirable dietary components for health[1] (**Appendix Table B-I**). The seven desirable components include cereals, vegetables, fruits 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 two undesirable components are meat and dairy food products. In order 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 2,500 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.[24] For alcohol, a value of 1 was assigned to moderate consumption, that is, an intake above the zygosity-specific median (1.91 gram/day for MZ or DZ) and at or below 33 g per

day. The latter is the upper limit of daily alcohol intake considered to be "moderate" among American men , and equals approximately two alcoholic drinks daily.[25, 26] The MDS was the sum of all values from the nine components, ranging from 0 to 9; the higher the score, the greater the adherence to the Mediterranean diet.

We also devised four slight variations of the MDS in order to evaluate the robustness of our findings. First, we followed an earlier method published by Trichopoulou et al. to calculate a score, MDS_1, ranging from 0 to 8, in which fish was included in the meat group[27] and was not considered as a desirable dietary component; potatoes were included with cereals; and eggs were included with meat.[24] In a second variant of the score, MDS_2, ranging from 0 to 8, fish was excluded from meat, and its intake was forced as a covariable into the models. In a third variant of the score, MDS_3, ranging from 0 to 8, fish and eggs were excluded from the meat group, and either ignored or included as separate covariates in the models. In a forth variation of the score, MDS_4, ranging from 0 to 9, fish was excluded from the meat group and included as a separate desirable component; potatoes were included with cereals; and eggs were included with meats.[24]

Assessment of Known Cardiovascular Risk Factors

We assessed smoking, education and marital status using standardized questionnaires. Physical activity was evaluated with the validated Baecke questionnaire.[28] Waist and hip circumferences were measured and used to calculate the waist-hip ratio. Systolic and diastolic blood pressures were measured using a mercury sphygmomanometer according to a standard protocol.[29] Hypertension was defined as systolic blood pressure $\geq$140 and/or diastolic blood pressure $\geq$90 mmHg, or current use of antihypertensive medicines. Diabetes was defined as a fasting plasma glucose concentration $\geq$126 mg/dL[30] or current treatment with insulin or oral

antihyperglycemic agents. Depressive symptoms were measured with the Beck Depression Inventory (BDI), which yielded a continuous score.[31] Current use of aspirin and statins was also recorded. Serum creatinine concentration was measured by a kinetic alkaline picrate method and used to calculate estimated glomerular filtration rate (eGFR) based on the formula: eGFR = 186 × (Serum creatinine concentrations)-1.154 × (age)-0.203 × (0.742 if female) × (1.210 if African-American).[32]

Biochemical Analysis

Blood samples for GSH and GSSG assays were assayed according to established procedures with twin pair samples assessed in the same analytical run.[11, 33] A nine-hour overnight fasting blood sample was collected into tubes containing 100 mM serine-borate (pH 8.5) containing (per ml) 0.5 mg sodium heparin, 1 mg bathophenanthroline disulfonate, and 2 mg iodoacetic acid to inhibit GSH auto-oxidation and degradation by γ-glutamyltranspeptidase. Following centrifugation, plasma was transferred to a microcentrifuge tube with 200 μl of a 10% (w/v) perchloric acid solution containing 0.2 M boric acid and 10 μM γ-Glu-Glu and stored at –80°C until analysis. GSH and GSSG concentrations were determined in a single run using a validated HPLC assay.[11, 33] The method for plasma GSH and GSSG assays included procedures to avoid hemolysis;[33] samples with evidence of hemolysis were discarded prior to analysis. The inter- and intra-assay variability for all assays was less than 10%. GSH and GSSG concentrations were used to calculate the GSH/GSSG ratio. We also used the Nernst equation to calculate the glutathione redox potential for the GSH/GSSG couple (E_h GSH/GSSG).[11] Because all values for E_h GSH/GSSG were negative, the absolute value, or aE_h GSH/GSSG, was used in the analysis. The higher the value of the GSH/GSSG ratio or the aE_h GSH/GSSG, the lower the oxidative

stress. Fasting plasma glucose, triacylglycerols, and total, low- and high-density lipoprotein cholesterol were measured 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 two levels:[21] between-subject and within-pair. Because dependent variables were log-transformed, the results were expressed as percent differences of the non-transformed values using the formula: $[(\exp^{\beta})-1] \times 100$ (%), where β is the regression coefficient and $\exp^{\beta}$ returns the exponential value of the parameter.

We first treated twins as individuals while accounting for twin pair clustering (**Table B.2**). The association at this level is the weighted average of within-pair and between-pair information.[21] 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 B.3**). The within-pair effects are inherently controlled for shared demographic, familial, and, in MZ pairs, genetic influences; additionally, environmental factors during the day of testing are controlled for since co-twins were examined at the same time. We fitted mixed models for twins,[21] which allow for partitioning within- and between-pair differences in the dependent variable as a function of the independent variables. In these models the within-pair β coefficient describes the individual twin variation from the twin pair average and it is

identical to the β coefficient from a model that fits the absolute difference between the co-twins.[21] Thus, the percent difference calculated from this parameter represents the percent 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 risk factors (depressive symptom scores, fasting glucose, systolic blood pressure, low-density lipoprotein cholesterol, the ratio of high-density lipoprotein 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 using SAS software version 9.1 (SAS Institute). Significance levels were set at 0.05, two-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 B.1**). 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, the ratio of GSH/GSSG, and the aE_h GSH/GSSG were greater among MZ than DZ twins, suggesting that genetic factors contribute to these traits (**Appendix Table B-II**).

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), the GSH/GSSG ratio was 8.0% higher (*P*=0.008), and the aE_h GSH/GSSG was 0.8% lower (*P*=0.03), respectively, after adjusting for nutritional factors not included in the Mediterranean diet score **(Model 1 in Table B.2)**. 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 B.2). In the fully adjusted model, twins in the highest MDS quartile had a GSH/GSSG ratio 31% higher (95% CI 2.1%, 69.7%) than those in the lowest quartile.

Within-Pair Results

The mean within-pair absolute difference in the MDS was 1.6 (range, 6) in MZ and 1.9 (range, 7) in DZ twins. The association between within-pair difference in the MDS and biomarkers were not different by zygosity (all *P*>0.4 for the interactions) (**Table B.3**), suggesting that genetic factors do not play a substantial role in these associations. In all the subsequent analyses we therefore pooled MZ and DZ pairs (Table B.3). In the combined sample of MZ and DZ pairs, within-pair associations of the MDS with GSSG concentrations and the GSH/GSSG ratio were statistically significant in all models, while the association with aE_h GSH/GSSG was nearly statistically significant (Table B.3). In the fully adjusted model (Model 2 in

Table B.3), 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 ratio (P=0.007).

Similar results were obtained using a three-level smoking variable (never smoked, current and past smoker), excluding four subjects with elevated concentrations of inflammatory biomarkers (three subjects with high sensitive C-reactive protein >30 mg/L and one subject with tumor necrosis factor-alpha >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 (MDS_1, MDS_2, MDS_3, and MDS_4), results remained similar.

Discussion

We found a robust inverse association between adherence to the Mediterranean diet and oxidative stress as measured by the GSH/GSSG ratio primarily due to lower GSSG concentrations, independent of a wide range of known cardiovascular risk factors. This finding persisted when comparing co-twins within pairs, either MZ or DZ, suggesting that shared familial and genetic factors do not confound the association between adherence to the Mediterranean diet and oxidative stress as measured by the GSH/GSSG ratio. Similar, but borderline significant, trends, were found for E_h GSH/GSSG. Results were robust to slight variations of 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[34-37] and one large trial,[38] however, have examined short- or intermediate-term effects of the Mediterranean diet on other circulating markers of oxidative stress, including urinary F2-isoprostanes,[34] plasma malondialdehyde,[35] oxidized low-density lipoproteins (LDL), and others.[36-38] In the largest of these trials, subjects assigned to the Mediterranean diet had lower oxidized LDL than those following the control diet.[38] 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 genetic background and other familial factors. Our twin study clearly overcomes this potential limitation, demonstrating an association in twin pairs matched for familial and (among MZ) 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 changing GSH concentrations.[39, 40] Our results also expand our recent finding, in this same population, of an inverse association between Mediterranean diet and inflammation,[41] since inflammation and oxidative stress are tightly inter-dependent.

Several possible mechanisms may explain an increased plasma GSH/GSSG ratio by the Mediterranean diet primarily through decreased GSSG concentrations. First, GSH is oxidized into GSSG by the enzyme glutathione peroxidase;[42] in this process, GSH quenches peroxides. GSSG reverts to GSH via gluthathione reductase with concomitant oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH).[43] Diverse nutrients and biofactors in foods characteristic of the

Mediterranean diet may provide higher NADPH[42] and upregulate gluthathione reductase activity,[44] leading to a decrease in GSSG and a resulting higher GSH/GSSG ratio. Second, a "sparing effect" on the GSH/GSSG redox cycle may also contribute to the increased GSH/GSSG ratio. By providing other antioxidants (such as vitamin C, vitamin E, carotenoids, polyphenols, zinc and selenium), and assuring adequate activity and efficiency of antioxidative 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.[45] Furthermore, the low content of pro-oxidants in the diet may also contribute to a higher GSH/GSSG ratio.[46]

Although there was a robust association with the GSH/GSSG ratio, the Mediterranean diet was weakly associated with the glutathione redox potential. Since glutathione redox potential is more sensitive to a concentration change of GSH than GSSG,[33] and adherence to the Mediterranean diet was not significantly associated with GSH concentrations, the observed results were not unexpected.

There are some limitations to our study. The sample was restricted to males, and therefore our results may not be generalizable to females. As our study was cross-sectional and observational, unmeasured confounding is possible. Our study addresses a pattern of diet that contains elements of, but is not necessarily equal to, the originally defined Mediterranean diet,[47] since the latter is rare in Western countries today. However, sufficient variations in diet across individuals allowed us to rank our subjects based on the similarity of their diet to the Mediterranean diet.[47] The Willett food frequency questionnaire may not accurately estimate absolute food and nutrient intake, but it is appropriate in our investigation on the diet-outcome relations after energy adjustment.[48] As in other common food frequency questionnaires used across the United States, combined food items, containing two or more components of the

MDS, may misclassify individual MDS components. However, we carefully decomposed combined items into individual ingredients using appropriate recipes, therefore minimizing misclassification.

On the other hand, a major strength of our study is 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 demonstrated a robust association between adherence to the Mediterranean diet and lower oxidative stress as indicated by the plasma GSH/GSSG ratio. 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 lowering oxidative stress.

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Author Contributions:

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Critical revision of the manuscript for important intellectual content: DJ, JG, TRZ, RMB, PWW, AKM, LS, LJ, VV

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References

1. Trichopoulou A, Costacou T, Bamia C, Trichopoulos D. Adherence to a Mediterranean diet and survival in a Greek population. N Engl J Med 2003;348:2599-608.
2. Mitrou PN, Kipnis V, Thiebaut AC, et al. Mediterranean Dietary Pattern and Prediction of All-Cause Mortality in a US Population: Results From the NIH-AARP Diet and Health Study. Arch Intern Med 2007;167:2461-8.
3. Visioli F, Galli C. The role of antioxidants in the Mediterranean diet. Lipids 2001;36 Suppl:S49-52.
4. Rankinen T, Bouchard C. Genetics of Food Intake and Eating Behavior Phenotypes in Humans. Annu Rev Nutr 2006.
5. Larson NI, Neumark-Sztainer D, Hannan PJ, Story M. Family meals during adolescence are associated with higher diet quality and healthful meal patterns during young adulthood. J Am Diet Assoc 2007;107:1502-10.
6. Maynard M, Gunnell D, Ness AR, Abraham L, Bates CJ, Blane D. What influences diet in early old age? Prospective and cross-sectional analyses of the Boyd Orr cohort. Eur J Public Health 2006;16:316-24.
7. Madamanchi NR, Vendrov A, Runge MS. Oxidative stress and vascular disease. Arterioscler Thromb Vasc Biol 2005;25:29-38.
8. Aw TY, Ookhtens M, Kaplowitz N. Inhibition of glutathione efflux from isolated rat hepatocytes by methionine. J Biol Chem 1984;259:9355-8.
9. Adams JD, Jr., Lauterburg BH, Mitchell JR. Plasma glutathione and glutathione disulfide in the rat: regulation and response to oxidative stress. J Pharmacol Exp Ther 1983;227:749-54.

10. Jones DP. Extracellular redox state: refining the definition of oxidative stress in aging. Rejuvenation Res 2006;9:169-81.

11. Jones D. Redox potential of GSH/GSSG couple: assay and biological significance. Methods Enzymol 2002;348:93-112.

12. Girotti AW. Lipid hydroperoxide generation, turnover, and effector action in biological systems. J Lipid Res 1998;39:1529-42.

13. Rosenblat M, Coleman R, Aviram M. Increased macrophage glutathione content reduces cell-mediated oxidation of LDL and atherosclerosis in apolipoprotein E-deficient mice. Atherosclerosis 2002;163:17-28.

14. Usal A, Acarturk E, Yuregir GT, et al. Decreased glutathione levels in acute myocardial infarction. Jpn Heart J 1996;37:177-82.

15. Tsuru R, Hojo Y, Gama M, Mizuno O, Katsuki T, Shimada K. Redox imbalance in patients with coronary artery disease showing progression of atherosclerotic lesions. J Cardiol 2006;48:183-91.

16. Goldberg J, Curran B, Vitek ME, Henderson WG, Boyko EJ. The Vietnam Era Twin Registry. Twin Res 2002;5:476-81.

17. Goldberg J, Curran B, Vitek ME, Henderson WG, Boyko EJ. The Vietnam Era Twin Registry. Twin Research 2002;5:476-81.

18. Scherrer JF, Xian H, Bucholz KK, et al. A Twin study of depression symptoms, hypertension, and heart disease in middle-aged men. Psychosomatic Medicine 2003;65:548-557.

19. Henderson WG, Eisen S, Goldberg J, True WR, Barnes JE, Vitek ME. The Vietnam Era Twin Registry: a resource for medical research. Public Health Rep 1990;105:368-73.

20. van der AD, Peeters PH, Grobbee DE, Marx JJ, van der Schouw YT. Dietary haem iron and coronary heart disease in women. Eur Heart J 2005;26:257-62.

21. Carlin JB, Gurrin LC, Sterne JA, Morley R, Dwyer T. Regression models for twin studies: a critical review. Int J Epidemiol 2005;34:1089-99.

22. Willett W. Nutritional Epidemiology. 2nd ed. New York, NY: Oxford University Press, 1998.

23. Kris-Etherton P, Eckel RH, Howard BV, St Jeor S, Bazzarre TL. AHA Science Advisory: Lyon Diet Heart Study. Benefits of a Mediterranean-style, National Cholesterol Education Program/American Heart Association Step I Dietary Pattern on Cardiovascular Disease. Circulation 2001;103:1823-5.

24. Trichopoulou A, Kouris-Blazos A, Wahlqvist ML, et al. Diet and overall survival in elderly people. Bmj 1995;311:1457-60.

25. US Department of Health and Human Services and the Department of Agriculture. Alcoholic Beverages. In: Dietary Guidelines for Americans 2005. 6th ed. Washington, DC: US Government Printing Office, 2005.

26. Kerr WC, Greenfield TK. The average ethanol content of beer in the U.S. and individual states: estimates for use in aggregate consumption statistics. J Stud Alcohol 2003;64:70-4.

27. Davidson S, Passmore R. Human nutrition and dietetics. Edinburgh, NY: Churchill Livingstone, 1979.

28. Pols MA, Peeters PH, Bueno-De-Mesquita HB, et al. Validity and repeatability of a modified Baecke questionnaire on physical activity. Int J Epidemiol 1995;24:381-8.

29. Anonymous. The sixth report of the Joint National Committee on prevention, detection, evaluation, and treatment of high blood pressure. Arch Intern Med 1997;157:2413-46.

30. Barr RG, Nathan DM, Meigs JB, Singer DE. Tests of glycemia for the diagnosis of type 2 diabetes mellitus. Ann Intern Med 2002;137:263-72.

31. Beck AT, Ward CH, Mendelson M, Mock J, Erbaugh J. An inventory for measuring depression. Arch Gen Psychiatry 1961;4:561-71.

32. Stevens L, Levey A. Frequently Asked Questions About GFR Estimates. National Kidney Foundation (the US), 2007.

33. Jones DP, Carlson JL, Samiec PS, et al. Glutathione measurement in human plasma. Evaluation of sample collection, storage and derivatization conditions for analysis of dansyl derivatives by HPLC. Clin Chim Acta 1998;275:175-84.

34. Ambring A, Friberg P, Axelsen M, et al. Effects of a Mediterranean-inspired diet on blood lipids, vascular function and oxidative stress in healthy subjects. Clin Sci (Lond) 2004;106:519-25.

35. Hagfors L, Leanderson P, Skoldstam L, Andersson J, Johansson G. Antioxidant intake, plasma antioxidants and oxidative stress in a randomized, controlled, parallel, Mediterranean dietary intervention study on patients with rheumatoid arthritis. Nutr J 2003;2:5.

36. Stachowska E, Wesolowska T, Olszewska M, et al. Elements of Mediterranean diet improve oxidative status in blood of kidney graft recipients. Br J Nutr 2005;93:345-52.

37. Leighton F, Cuevas A, Guasch V, et al. Plasma polyphenols and antioxidants, oxidative DNA damage and endothelial function in a diet and wine intervention study in humans. Drugs Exp Clin Res 1999;25:133-41.

38. Fito M, Guxens M, Corella D, et al. Effect of a traditional Mediterranean diet on lipoprotein oxidation: a randomized controlled trial. Arch Intern Med 2007;167:1195-203.

39. Alvarez P, Alvarado C, Mathieu F, Jimenez L, De la Fuente M. Diet supplementation for 5 weeks with polyphenol-rich cereals improves several functions and the redox state of mouse leucocytes. Eur J Nutr 2006;45:428-38.

40. Davis L, Stonehouse W, Loots du T, et al. The effects of high walnut and cashew nut diets on the antioxidant status of subjects with metabolic syndrome. Eur J Nutr 2007;46:155-64.

41. Dai J, Miller AH, Bremner JD, et al. Adherence to the mediterranean diet is inversely associated with circulating interleukin-6 among middle-aged men: a twin study. Circulation 2008;117:169-75.

42. Le CT, Hollaar L, Van der Valk EJ, et al. Protection of myocytes against free radical-induced damage by accelerated turnover of the glutathione redox cycle. Eur Heart J 1995;16:553-62.

43. Fico A, Paglialunga F, Cigliano L, et al. Glucose-6-phosphate dehydrogenase plays a crucial role in protection from redox-stress-induced apoptosis. Cell Death Differ 2004;11:823-31.

44. Fang YZ, Yang S, Wu G. Free radicals, antioxidants, and nutrition. Nutrition 2002;18:872-9.

45. Rebrin I, Zicker S, Wedekind KJ, Paetau-Robinson I, Packer L, Sohal RS. Effect of antioxidant-enriched diets on glutathione redox status in tissue homogenates and mitochondria of the senescence-accelerated mouse. Free Radic Biol Med 2005;39:549-57.

46. Roy P, Sajan MP, Kulkarni AP. Lipoxygenase-mediated glutathione oxidation and superoxide generation. J Biochem Toxicol 1995;10:111-20.

47. Blackburn H, Menotti A, Buzina R, et al. (Keys, A. ed.). Coronary heart disease in seven countries. Circulation 1970;41 (Suppl 1):1-211.

48. Subar AF, Thompson FE, Kipnis V, et al. Comparative validation of the Block, Willett, and National Cancer Institute food frequency questionnaires : the Eating at America's Table Study. Am J Epidemiol 2001;154:1089-99.

TABLE B.1.

Characteristics according to the Mediterranean diet score in the entire sample

Variable	Mediterranean Diet Score				Trend *P* value[1]
	(0-3) (n = 88)	(4) (n = 63)	(5) (n = 66)	(6-9) (n = 80)	
Age (y)	54.0 (51.5, 56.0)	55.0 (53.0, 57.0)	55.5 (53.0, 57.0)	55.0 (54.0 57.0)	0.63
Education (y)	13.0 (12.0, 14.5)	14.0 (13.0, 16.0)	15.0 (13.0, 16.0)	14.5 (13.0, 16.0)	<0.001
Married [*n* (%)]	71 (76.3)	50 (73.5)	56 (78.9)	73 (83.9)	0.37
Current smoker [*n* (%)]	26 (28.0)	14 (20.6)	7 (9.9)	10 (11.5)	0.002
Ethanol intake (g/d)	0 (0, 10.9)	1.1 (0, 13.6)	2.5 (0, 9.9)	4.0 (0.5, 10.9)	0.013
Body mass index (kg/m^2)	29.6 ± 5.4	29.2 ± 5.0	29.3 ± 4.1	28.4 ± 3.5	0.16
Waist to hip ratio[3]	0.94 ± 0.06	0.95 ± 0.07	0.93 ± 0.06	0.94 ± 0.06	0.83

TABLE B.1.

Characteristics according to the Mediterranean diet score in the entire sample (Con't)

Variable	Mediterranean Diet Score (0-3) (n = 88)	(4) (n = 63)	(5) (n = 66)	(6-9) (n = 80)	Trend *P* value[1]
Physical activity (unit)[2]	7.20 (5.93, 8.32)	7.41 (6.59, 8.38)	7.82 (6.93, 8.69)	7.63 (7.07, 8.34)	0.02
Clinical and biochemical features					
Depressive symptoms (BDI score)[2]	4.0 (1.0, 8.5)	2.0 (0.0, 6.0)	1.0 (0.0, 4.0)	3.0 (0.0, 5.0)	0.03
Plasma glucose concentration (mg/dL)[2,4]	97 (90, 104)	99 (91, 110)	97 (93, 103)	99 (93, 105)	0.24
Systolic blood pressure (mm Hg)[3]	131 ± 15	128 ± 17	127 ± 16	132 ± 14	0.89
Diastolic blood pressure (mm Hg)[3]	81 ± 11	79 ± 10	80 ± 11	83 ± 10	0.10
Total triacylglycerols concentration (mg/dL)[2,5]	172 (124, 231)	145 (104, 200)	158 (108, 215)	162 (127, 223)	0.85
Total Cholesterol concentration (mg/dL)[6]	188 ± 33	191 ± 41	191 ± 35	190 ± 43	0.57

TABLE B.1. (Con't)

Characteristics according to the Mediterranean diet score in the entire sample

	Mediterranean Diet Score				Trend
	(0-3)	(4)	(5)	(6-9)	*P*
Variable	**(n = 88)**	**(n = 63)**	**(n = 66)**	**(n = 80)**	**value[1]**
HDL-Cholesterol concentration (mg/dL)[2,6]	37 (31, 45)	39 (31, 48)	39 (35, 46)	36 (32, 42)	0.93
LDL-Cholesterol concentration (mg/dL)[6]	125 ± 32	124 ± 34	124 ± 30	127 ± 37	0.60
Take fish oil supplement [*n* (%)]	2 (2.2)	2 (2.9)	5 (7.0)	7 (8.0)	0.07
Take statins [*n* (%)]	21 (22.6)	12 (17.7)	15 (21.1)	18 (20.7)	0.76
Take aspirin [*n* (%)]	19 (20.4)	14 (20.6)	14 (19.7)	19 (21.8)	0.89
Take antihypertensives [n (%)]	16 (18.2)	14 (22.2)	15 (22.7)	18 (22.5)	0.52
Take antihyperglycemics [n (%)]	6 (6.5)	8 (11.9)	7 (9.9)	10 (11.5)	0.31
Plasma oxidative stress biomarkers[2]					
GSH (μM)	1.41 (0.86, 2.02)	1.16 (0.89, 1.89)	1.17 (0.86, 1.96)	1.45 (1.00, 2.45)	0.31

TABLE B.1. (Con't)
Characteristics according to the Mediterranean diet score in the entire sample

	Mediterranean Diet Score				Trend
	(0-3)	(4)	(5)	(6-9)	*P*
Variable	**(n = 88)**	**(n = 63)**	**(n = 66)**	**(n = 80)**	**value[1]**
GSSG (μM)	0.07 (0.02, 0.10)	0.05 (0.03, 0.07)	0.04 (0.03, 0.08)	0.04 (0.02, 0.09)	0.31
GSH/GSSG	24.2 (14.5, 47.0)	28.4 (15.8, 48.8)	28.5 (18.5, 48.3)	32.3 (19.3, 64.1)	0.03
aE_h GSH/GSSG (mV)	130 (122, 140)	133 (124, 142)	132 (122, 141)	135 (125, 146)	0.02

Abbreviations: BDI, Beck Depression Inventory; HDL, high-density lipoprotein; LDL, low-density lipoprotein; GSH, glutathione; GSSG, glutathione disulfide; GSH/GSSG, the ratio of GSH to GSSG; aE_h GSH/GSSG, absolute value of the redox potential for the GSH/GSSG couple.

[1] Test for trend across diet score groups. All *P* values are corrected for clustering within a twin pair according to the twin type using linear mixed models for continuous variables, and generalized estimating equation logistic models for dichotomous variables. Means and medians presented are raw values.

[2]Median (25^{th}, 75^{th} percentile).

[3]$x \pm$ SD

TABLE B.1. (Con't)

Characteristics according to the Mediterranean diet score in the entire sample

[4] To convert values for plasma glucose to mmol/L, multiply by 0.0555.

[5] To convert values for plasma triacylglycerols to mmol/L, multiply by 0.0113.

[6] To convert values for plasma cholesterol to mmol/L, multiply by 0.0259.

TABLE B.2.
Associations between the Mediterranean diet score and plasma concentrations of oxidative stress biomarkers in the entire sample

Outcome	Geometic mean difference (%) (95% CI) per 1-unit increment in MDS[1] (n = 297)	*P* value	Geometric Means (95% CI) of Biomarkers				Trend *P* value[4]
			Diet Score = 0-3 (n = 88)	Diet Score = 4 (n = 63)	Diet Score = 5 (n = 66)	Diet Score = 6-9 (n = 80)	
Model 1: Adjusted for zygosity and nutritional factors not included in the Mediterranean diet score[2].							
GSH (μM)	0.9 (-3.9, 5.9)	0.71	1.58 (1.15, 2.19)	1.50 (1.14, 2.18)	1.40 (1.02, 1.93)	1.72 (1.24, 2.35)	0.54
GSSG (μM)	-6.4 (-12.8, 0.5)	0.07	0.07 (0.05, 0.12)	0.06 (0.04, 0.1)	0.06 (0.03, 0.09)	0.06 (0.04, 0.09)	0.14
GSH/GSSG	8.0 (2.1, 14.3)	0.008	21.3 (14.6, 31.0)	25.8 (17.6, 37.9)	24.5 (16.8, 35.6)	29.1 (20.1, 42.4)	0.01
aE_h GSH/GSSG (mV)	0.8 (0.1, 1.6)	0.03	129 (122, 135)	132 (125, 138)	129 (122, 135)	133 (127, 140)	0.03

TABLE B.2. (Con't)
Associations between the Mediterranean diet score and plasma concentrations of oxidative stress biomarkers in the entire sample

Outcome	Geometic mean difference (%) (95% CI) per 1-unit increment in MDS[1] (n = 297)	*P* value	Geometric Means (95% CI) of Biomarkers				Trend *P* value[4]
			Diet Score = 0-3 (n = 88)	Diet Score = 4 (n = 63)	Diet Score = 5 (n = 66)	Diet Score = 6-9 (n = 80)	
Model 2: Adjusted for zygosity; nutritional factors not included in the Mediterranean diet score; demographic, lifestyle, and coronary risk factors; and use of medications[3].							
GSH (μM)	-1.2 (-6.0, 3.8)	0.63	1.43 (1.01, 2.01)	1.34 (0.95, 1.90)	1.17 (0.83, 1.65)	1.39 (0.99, 1.97)	0.89
GSSG (μM)	-7.3 (-13.9, -0.3)	0.04	0.07 (0.04, 0.11)	0.05 (0.03, 0.09)	0.05 (0.03, 0.08)	0.05 (0.03, 0.08)	0.09
GSH/GSSG	6.8 (0.8, 13.2)	0.03	21.1 (14.1, 31.4)	25.0 (16.6, 37.6)	25.0 (16.7, 37.2)	27.7 (18.5, 41.5)	0.03
aE_h GSH/GSSG (mV)	0.5 (-0.2, 1.3)	0.17	126 (120, 133)	129 (122, 136)	126 (120, 133)	130 (123, 137)	0.15

TABLE B.2 (Con't)

Associations between the Mediterranean diet score and plasma concentrations of oxidative stress biomarkers in the entire sample

Abbreviations: CI, confidence interval; MDS, the Mediterranean diet score; GSH, glutathione; GSSG, glutathione disulfide; GSH/GSSG, the ratio of GSH to GSSG; aE_h GSH/GSSG, absolute value of redox potential for the GSH/GSSG couple.

[1]Values are % differences in geometric means (95% CI) calculated from the β coefficient of the MDS in linear mixed models treating twins as separate individuals but accounting for different clustering within a twin pair according to zygosity. A negative value of the % difference indicates an inverse association between the MDS and the biomarker, while a positive value reflects a positive association.

[2]Total energy intake (continuous), egg and potato consumption (continuous), oxidative stress-related nutritional factors [supplements containing fish oil (yes/no), vitamins and minerals (ordinal), magnitude of habitual removal of visible fat on meat (ordinal), frequency of consumption of punches and fruit drinks (ordinal), frequency of consumption of fried food and types of cooking oils (ordinal)].

[3]Further controlling for age (continuous), education (continuous), marital status (yes/no), current smoking (yes/no), waist-to-hip ratio (continuous), physical activity (continuous), depressive symptom score (continuous), plasma glucose (continuous), systolic

TABLE B.2 (Con't)

Associations between the Mediterranean diet score and plasma concentrations of oxidative stress biomarkers in the entire sample

blood pressure (continuous), low density lipoprotein cholesterol (continuous), ratio of high density lipoprotein cholesterol to triacylglycerols (continuous), and use of statins (yes/no) and aspirin (yes/no).

[4]Test for trend across diet groups

TABLE B.3

Within-pair percent difference in geometric means of plasma oxidative stress biomarker concentrations per 1-unit within-pair difference in Mediterranean diet score, overall and by zygosity[1]

Outcome	MZ + DZ (n = 297)[2] Within-pair Difference (%) (95% CI)[1]	*P* value	MZ (n = 171)[3] Within-pair Difference (%) (95% CI)	*P* value	DZ (n = 126)[4] Within-pair Difference (%) (95% CI)	*P* value	Interaction with Zygosity *P* value
Model 1: Adjusted for zygosity and nutritional factors not included in the Mediterranean diet score[3].							
GSH (*μM*)	-0.6 (-6.5, 5.7)	0.85	2.0 (-6.6, 10.7)	0.69	-3.0 (-12.1, 8.0)	0.61	0.92
GSSG (*μM*)	-9.2 (-16.9, -0.7)	0.03	-7.7 (-18.2, 3.3)	0.15	-16.5 (-29.0, -1.8)	0.03	0.78
GSH/GSSG	9.5 (2.2, 17.4)	0.01	10.5 (0.1, 21.3)	0.048	16.2 (3.7, 31.0)	0.01	0.77
aE_h GSH/GSSG (*mV*)	0.8 (-0.1, 1.7)	0.07	1.0 (-0.1, 2.6)	0.07	1.0 (-0.4, 2.3)	0.15	0.94

TABLE B.3 (Con't)

Within-pair percent difference in geometric means of plasma oxidative stress biomarker concentrations per 1-unit within-pair difference in Mediterranean diet score, overall and by zygosity[1]

Outcome	MZ + DZ (n = 297)[2] Within-pair Difference (%) (95% CI)[1]	*P* value	MZ (n = 171)[3] Within-pair Difference (%) (95% CI)	*P* value	DZ (n = 126)[4] Within-pair Difference (%) (95% CI)	*P* value	Interaction with Zygosity *P* value
Model 2: Adjusted for zygosity; nutritional factors not included in the Mediterranean diet score; demographic and lifestyle factors[4].							
GSH (*μM)*	-1.3 (-7.3, 5.1)	0.68	1.0 (-7.6, 9.5)	0.89	-3.0 (-13.5, 8.7)	0.58	0.99
GSSG (*μM)*	-10.6 (-18.4, -2.1)	0.02	-9.5 (-19.6, 1.3)	0.08	-18.9 (-32.6, -3.3)	0.02	0.62
GSH/GSSG	10.7 (3.2, 18.8)	0.005	10.5 (1.3, 21.4)	0.02	19.7 (5.5, 36.2)	0.007	0.49
aE_h GSH/GSSG (*mV)*	0.9 (-0.1, 1.8)	0.07	1.0 (-0.1, 2.4)	0.08	1.0 (-0.4, 2.6)	0.15	0.78

TABLE B.3 (Con't)

Within-pair percent difference in geometric means of plasma oxidative stress biomarker concentrations per 1-unit within-pair difference in Mediterranean diet score, overall and by zygosity[1]

Outcome	MZ + DZ (n = 297)[2] Within-pair Difference (%) (95% CI)[1]	*P* value	MZ (n = 171)[3] Within-pair Difference (%) (95% CI)	*P* value	DZ (n = 126)[4] Within-pair Difference (%) (95% CI)	*P* value	Interaction with Zygosity *P* value
Model 3: Adjusted for zygosity; nutritional factors not included in the Mediterranean diet score; demographic, lifestyle, and coronary risk factors[5].							
GSH (*μM)*	-1.4 (-7.3, 4.9)	0.65	1.0 (-7.5, 9.2)	0.90	-2.0 (-12.8, 10.0)	0.71	0.93
GSSG (*μM)*	-10.4 (-18.1, -2.1)	0.02	-10.4 (-19.7, 0.8)	0.07	-17.3 (-30.8, -1.3)	0.04	0.71
GSH/GSSG	10.2 (2.8, 18.1)	0.007	11.6 (1.7, 21.5)	0.02	17.4 (3.0, 32.7)	0.02	0.73
aE_h GSH/GSSG (*mV)*	0.8 (-0.1, 1.7)	0.08	1.0 (-0.1, 2.4)	0.06	1.0 (-0.6, 2.5)	0.21	0.98

TABLE B.3 (Con't)

Within-pair percent difference in geometric means of plasma oxidative stress biomarker concentrations per 1-unit within-pair difference in Mediterranean diet score, overall and by zygosity[1]

Outcome	MZ + DZ (n = 297)[2] Within-pair Difference (%) (95% CI)[1]	*P* value	MZ (n = 171)[3] Within-pair Difference (%) (95% CI)	*P* value	DZ (n = 126)[4] Within-pair Difference (%) (95% CI)	*P* value	Interaction with Zygosity *P* value
Model 4: Adjusted for zygosity; nutritional factors not included in the Mediterranean diet score; demographic, lifestyle, and coronary risk factors; and use of medications[6].							
GSH (*μM)*	-1.3 (-7.1, 5.0)	0.68	1.0 (-7.5, 9.4)	0.89	-4.9 (-15.0, 6.8)	0.39	0.76
GSSG (*μM)*	-10.1 (-17.7, -1.8)	0.02	-9.5 (-19.5, 1.2)	0.08	-18.9 (-32.4, -3.6)	0.02	0.62
GSH/GSSG	10.1 (2.7, 18.0)	0.007	11.6 (1.5, 21.7)	0.02	17.4 (2.8, 33.0)	0.02	0.78
aE_h GSH/GSSG (*mV)*	0.8 (-0.1, 1.7)	0.09	1.0 (-0.1, 2.4)	0.06	1.0 (-1, 2.1)	0.46	0.77

TABLE B.3 (Con't)

Abbreviations: MZ, monozygotic twins; DZ, dizygotic twins; MDS, the Mediterranean diet score; CI, confidence interval; GSH, glutathione; GSSG, glutathione disulfide; GSH/GSSG, the ratio of GSH to GSSG; aE_h GSH/GSSG, absolute value of redox potential for the GSH/GSSG couple.

[1]Values are within-pair % differences in geometric means (95% CI) calculated from the β coefficient given a 1-unit within-pair difference in the MDS. A negative within-pair % difference indicates that the twin with the higher score has lower biomarker concentrations than his co-twin, while a positive value indicates that the twin with the higher score has higher biomarker concentrations. Linear mixed models included the MDS and covariates as fixed effects and the twin pair as a random effect, accounted for clustering within a pair and allowed for different correlation in MZ and DZ pairs in the pooled samples.

[2]Including 138 twin pairs and 21 unpaired twins

[3]Including 81 MZ twin pairs and 9 MZ unpaired twins

[4]Including 57 DZ twin pairs and 12 DZ unpaired twins

[5]Total energy intake (continuous), egg and potato consumption (continuous), oxidative stress-related nutritional factors [supplements containing fish oil (yes/no), vitamins and minerals (ordinal), magnitude of habitual removal of visible fat on meat (ordinal),

TABLE B.3 (Con't)

frequency of consumption of punches and fruit drinks (ordinal), frequency of consumption of fried food and types of cooking oils (ordinal)].

[6]Age (continuous), education (continuous), marital status (yes/no), current smoking (yes/no), waist-to-hip ratio (continuous), and physical activity (continuous).

[7]Depressive symptom score (continuous), plasma glucose (continuous), systolic blood pressure (continuous), low density lipoprotein cholesterol (continuous), ratio of high density lipoprotein cholesterol to triacylglycerols (continuous).

[8]Use of statins (yes/no) and aspirin (yes/no)

Appendix TABLE B-I

Dietary components (adjusted to 2,500 kcal[1]) used for the Mediterranean diet score calculation or otherwise used in the analysis

Dietary Component	Foods Included	Criteria for 1 Point	Median[2]	
			MZ	DZ
Dietary components for the score calculation				
Desirable components				
Vegetables (g/day)	All vegetables except potatoes	≥ median intake (g/d)	135.1	129.8
Legumes (g/day)	Peas and beans	≥ median intake (g/d)	34.6	32.5
Fruits and nuts (g/day)	All fruits and juices, all nuts and peanut butter	≥ median intake (g/d)	298.3	247.8
Cereals (g/day)	All cereal foods included as follows: Cold cereals, white bread, dark bread, rice,	≥ median intake (g/d)	134.6	121.5

Appendix TABLE B-I (Con't)

Dietary components (adjusted to 2,500 kcal[1]) used for the Mediterranean diet score calculation or otherwise used in the analysis

Dietary Component	Foods Included	Criteria for 1 Point	Median[2]	
			MZ	DZ
Dietary components for the score calculation				
Cereals (g/day)	pasta, as well as cereals in hotdog, pie, cake, cookie, and hamburger. Sugar and potatoes were excluded.	≥ median intake (g/d)	134.6	121.5
Fish (g/day)	Fish	≥ median intake	18.6	20.2
Ratio of monounsaturated to saturated fatty acids	The whole diet	≥ median intake	1.098	1.102
Ethanol (g/day)	Wine, beer and liquor	median~33 g/d	1.91	1.91

Appendix TABLE B-I (Con't)

Dietary components (adjusted to 2,500 kcal[1]) used for the Mediterranean diet score calculation or otherwise used in the analysis

			Median[2]	
Dietary Component	**Foods Included**	**Criteria for 1 Point**	**MZ**	**DZ**
Undesirable components				
Dairy products (g/day)	All dairy products	< median intake (g/d)	318.4	281.5
Meat (g/day)	All meats excluding eggs and fish	< median intake (g/d)	229.1	230.6
Food groups forced into models				
Eggs (g/day)	eggs	*N/A*	20.4	28.8
Potatoes (g/day)	potatoes	*N/A*	147.2	153.5
Total energy intake (kcal/day)[3]	*N/A*	*N/A*	1442	1394

Abbreviations: MZ, monozygotic twins; DZ, dizygotic twins.

[1] using the formula: food intake × 2,500/energy intake (kcal).

Appendix TABLE B-I (Con't)

Dietary components (adjusted to 2,500 kcal[1]) used for the Mediterranean diet score calculation or otherwise used in the analysis

[2]Medians for dietary components were calculated according to zygosity (rather than sex) in order to conduct analyses stratified by zygosity in our all-male sample, since MZ twins share 100% genes while DZ twins share on average 50% genes.

[3]Unadjusted total energy intake; to convert values for total energy intake to kJ, multiply by 4.184.

Appendix TABLE B-II

Correlations for MDS, IL-6, and sIL-6R between MZ and DZ co-twins.

Variable	MZ	DZ
MDS	0.36	0.00
GSH (μM)	0.36	0.37
GSSG (μM)	0.45	0.23
GSH/GSSG	0.50	0.30
aE_h GSH/GSSG (mV)	0.49	0.44

Abbreviations: MZ, monozygotic twins; DZ, dizygotic twins; MDS, the Mediterranean diet score; GSH, glutathione; GSSG, glutathione disulfide; GSH/GSSG, the ratio of GSH to GSSG; aE_h GSH/GSSG, absolute value of the redox potential for the GSH/GSSG couple.

The intraclass correlation coefficient is calculated using unconditional means model, and ranges from 0 to 1. The greater the intraclass correlation coefficient, the higher phenotypic similarity between co-twins. Genetic influence on the phenotype is suggested if the MZ twin correlation is greater than the DZ correlation.

CHAPTER V

A HIGH HABITUAL DIETARY ALPHA-LINOLENIC ACID INTAKE IS ASSOCIATED WITH DECREASED SOLUBLE INTERLEUKIN-6 RECEPTOR LEVELS AMONG MALE TWINS

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Potential Financial Conflicts of Interest

None disclosed

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Abstract

Objectives: To examine whether higher habitual dietary intake of alpha-linolenic acid (ALA), an essential ω-3 fatty acid, is associated with lower circulating inflammatory biomarkers related to cellular response to IL-6 controlling for common familial factors in adult males.

Background: ALA and its derivatives have anti-inflammatory properties. It is unknown whether the association between habitual dietary ALA intake and inflammation is independent of genetic or other familial factors.

Methods: We administered the Willett food frequency questionnaire to 312 male middle-aged twins free of cardiovascular disease. Fasting plasma interleukin-6 (IL-6) and its soluble receptor (sIL-6R), and cardiovascular risk factors were measured. Mixed-effect regression analyses were used to examine the association between habitual dietary ALA intake and inflammatory biomarkers related to cellular response to IL-6 after accounting for known cardiovascular risk factors.

Results: A one gram increment in habitual dietary ALA intake was associated with 11.4% lower levels of sIL-6R (P=0.009), but not IL-6 (P=0.61) after adjusting for known cardiovascular risk factors including total energy intake, nutritional factors, cardiovascular risk factors, and medications. A within-pair analysis between co-twins, comparing the twin with higher ALA intake to his brother with lower intake, showed that a 1-gram within-pair absolute difference in dietary ALA intake was associated with 12.2% (95% CI, 3.8 to 19.7%) lower sIL-6R levels (P=0.005).

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Conclusions: An inverse association between habitual dietary ALA intake and sIL-6R is independent of shared family environmental influences and genetic factors. Lowering sIL-6R may be an important mechanism underlying the cardioprotective effects of habitual dietary ALA.

Condensed Abstract

A High Habitual Dietary Alpha-Linolenic Acid Intake Is Associated with Decreased Soluble Interleukin-6 Receptor Levels among Male Twins

In a study of male middle-aged twins without cardiovascular disease, we examined whether habitual dietary intake of alpha-linolenic acid (ALA), an essential omega-3 fatty acid, was associated with lower cellular response to interleukin-6 (IL-6) and whether the association was independent of familial or genetic factors. A twin with a 1-gram higher habitual dietary ALA intake than his co-twin had 12% lower soluble IL-6 receptor (sIL-6R) levels. Lowering sIL-6R may be an important mechanism underlying the cardio-protective effects of habitual dietary ALA. Our results suggest that increasing ALA intake may help preventing atherosclerotic vascular disease.

Abbreviations: ALA, alpha-linolenic acid; IL-6, interleukin-6; sIL-6R, soluble interleukin-6 receptor; MZ , monozygotic; DZ , dizygotic; mIL-6R, membrane-bound-IL-6 receptors; THS, the Twins Heart Study; BDI, Beck Depression Inventory; ω-3 ≥ 20C-PUFA, ω-3 long-chain polyunsaturated fatty acids.

Introduction

Dietary alpha-linolenic acid (ALA) has been suggested to be protective against cardiovascular diseases.[1] ALA, a plant 18-carbon ω-3 long-chain polyunsaturated fatty acid (18:3ω-3), is essential for humans because it cannot be synthesized *in vivo* due to lack of Δ12 and Δ15 desaturases. Humans can synthesize ω-3 long-chain polyunsaturated fatty acid derivatives with 20 or more carbons (ω-3≥ 20C-PUFA) from ALA, including prominently eicosapentaenoic (20:5ω-3) and docosapentaenoic acid (22:5ω-3), but also limited amounts of docosahexaenoic acid (22:6ω-3). Although marine foods and fish oils represent good sources of ω-3≥ 20C-PUFA fatty acids, particularly, docosahexaenoic acid (22:6ω-3), they may have disadvantages as a routine dietary component due to the potential for oxidation[2] or for contamination with heavy metals such as mercury.[3] Therefore, plant-originated ALA, which is derived primarily from linseed (flaxseed), canola, and soybean oils, and secondarily from nuts, green leafy vegetables, and some fruits, may be important dietary constituents for cardiovascular prevention.

The cardioprotective mechanisms of ALA are not completely understood. ALA and its derivatives inhibit inflammation in a variety of experimental settings via suppressing the arachidonic acid cascade, which produces thrombotic and inflammatory prostaglandins and leukotrienes.[4] Although dietary deficiency of essential fatty acids, either linoleic (18:3ω-6) or linolenic acid, is rare in the US general population, greater intake of linoleic acid relative to ALA results in an imbalance of ω-6 to ω-3 fatty acids, which may create a pro-inflammatory milieu,[5] and predispose to atherosclerosis.

Interleukin-6 (IL-6) has been suggested to play a role in arachidonic acid cascade-related inflammation.[6] The soluble IL-6 receptor enhances and broadens cellular response to IL-6,[7] and thus may regulate the IL-6 inflammatory action related to arachidonic acid cascade. However, previous studies of the association of dietary ALA with inflammatory biomarkers related to cellular response to IL-6, including IL-6 and sIL-6R, have yielded inconsistent results.[8-13] One possible explanation for these inconsistencies is that genetic factors may confound the association between habitual dietary ALA and the inflammatory response. For example, genetic influences are observed for dietary preferences[14] and are related to circulating levels of sIL-6R[15] and IL-6.[16] In addition, since dietary habits in adult life are influenced by those acquired during childhood and youth,[17-19] these are likely to be associated with other environmental conditions or lifestyle factors shared by members of the same family; the latter, therefore, can confound the association between diet and disease. In this context, the study of adult twins raised in the same family is especially useful, because it can help to disentangle specific environmental associations from shared familial and genetic factors.

The aim of this study, therefore, was to examine the association between habitual dietary ALA intake and circulating inflammatory biomarkers related to cellular response to IL-6. We used a sample of monozygotic (MZ) and dizygotic (DZ) middle-aged male twins raised in the same family to account for familial and genetic confounding.

Methods

Subjects

The Twins Heart Study (THS) is an investigation of psychological, behavioral and biological risk factors for subclinical cardiovascular disease using twins. The THS includes 180 pairs of monozygotic (MZ) and dizygotic (DZ) male twins from the Vietnam Era Twin Registry, a registry of 7,369 middle-aged male-male twin pairs both of whom served in the United States military during the Vietnam era (1964-1975).[20] Twins selected for inclusion in the THS were born between 1946 and 1956 which represented >90% of the twins in the VET Registry. In addition, eligible twins were selected if they were without a history of symptomatic cardiovascular disease, including a previous diagnosis of heart attack/myocardial infarction, coronary artery disease, angina, congestive heart failure or stroke, or previous coronary angioplasty or coronary bypass surgery according to self-reported data from a 1990 survey.[21] For the THS, random samples of twins in two strata were selected from the Registry: one stratum included twins discordant for a lifetime history of major depression and the other included twins with no history of depression.

Once selected, twin pairs were examined together at the Emory University General Clinical Research Center between March 2002 and March 2006. The assessment included a comprehensive history and physical exam during which we obtained updated information about previous diagnoses symptomatic cardiovascular disease. Zygosity information, determined by DNA analysis, was available from all but 11 twin pairs. The zygosity of these 11 pairs was assessed using questionnaires supplemented with blood group typing data abstracted from military records.[22] A circulating IL-6 level above 10 pg/ml was defined as a cutoff for low-grade systemic inflammation,[23] and we excluded 8 subjects with IL-6 above this cutoff. Furthermore, we excluded 1 subject with no dietary

data, 6 with implausible energy intake (≥ 6000 or <500 kcal/day), and 33 with a previous history of cardiovascular disease. Therefore, our analyses were based on 312 twins (79 MZ and 63 DZ twin pairs, 10 MZ and 18 DZ unpaired twins). The study protocol was approved by the Institutional Review Board of Emory University and informed consent was obtained from all subjects.

Assessment of diet

We used the Willett self-administered semiquantitative food frequency questionnaire[24] that collected habitual dietary intakes including supplements over the previous 12 months. The questionnaire classifies average food intake according to nine frequency categories ranging from "almost never or less than once per month" to "≥ 6 times/day". Standardized portion sizes are used 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 using the nutrient database of the US Department of Agriculture. [24] The nutrient data used in the analysis included total energy intake, intakes of saturated, monounsaturated, trans-fatty acids, ALA, linoleic acid, gamma-linolenic acid, and ω-3≥ 20C-PUFA (either from diet or from fish oil supplements) (C20:5, C22:5, and C22:6). Daily food intake in grams was calculated from food intake frequency and portion sizes.

Assessment of known cardiovascular risk factors

We assessed smoking, education, and marital status using standardized questionnaires. Physical activity was evaluated with the validated Baecke questionnaire.[25]

Waist and hip circumference were measured by interviewers and used to calculate the waist-hip ratio. Systolic and diastolic blood pressures were measured using a mercury sphygmomanometer according to a standard protocol. Hypertension was defined as systolic blood pressure ≥140 mm Hg and/or diastolic blood pressure ≥90 mm Hg, or current use of antihypertensive medications. Diabetes was defined as a fasting plasma glucose concentration ≥126 mg/dL,[26] or current treatment with insulin or oral hypoglycemic agents. Depressive symptoms were measured with the Beck Depression Inventory (BDI) which yielded a continuous score.[27] Current use of aspirin and statins was also recorded.

BIOCHEMICAL ANALYSIS

Plasma samples were separated from nine-hour overnight fasting blood samples and stored frozen at −80°C until analysis. Laboratory personnel were blinded to the subject identification and status, and twin pairs were assessed in the same analytical run. Fasting plasma concentrations of glucose, triglycerides, and total, low- and high-density lipoprotein cholesterol were measured using standardized methods. Fasting plasma levels of IL-6 and total sIL-6R (free sIL-6R plus sIL-6R bound to IL-6) were measured using commercial human enzyme-linked immunosorbent assay (ELISA) kits (Quantikine, R & D systems, Inc., Minneapolis, USA). The inter- and intra-assay variability for all assays was less than 10%. The 10 pg/mL of plasma IL-6 concentration was defined as the cutoff for low-grade systemic inflammation, based on previous data.[23]

Statistical analysis

Inflammatory biomarkers were log-transformed to improve normality. To estimate whether genetic factors influence dietary ALA intake and inflammatory biomarkers, intraclass correlation coefficients were calculated. We defined "within-pair absolute differences" as differences between a twin with a higher habitual dietary ALA intake and his twin brother with a lower intake.

The association between habitual dietary ALA intake and inflammatory biomarkers was assessed by fitting linear regression models adapted for twin studies at two levels:[28] between-subjects, i.e., treating twins as separate individuals (without controlling for shared genetic and environmental factors) and within-pair (controlling for these shared factors). Because dependent variables were log-transformed, the results were expressed as percent difference of the non-transformed values using the formula: $[(\exp^{\beta})-1] \times 100$ (%), where β is the regression coefficient and $\exp^{\beta}$ returns the exponential value of the parameter.[29]

At the between-subject level, we treated twins as individuals while accounting for twin pair clustering (**Table C.2**).[28] Habitual dietary ALA intake was analyzed primarily as a continuous variable and secondarily as a categorical variable according to quintiles with levels of 0.16 to 0.38, 0.39 to 0.52, 0.53 to 0.68, 0.69 to 0.89 and 0.90 to 2.31 gram/day.

Next, we performed within-pair analyses to examine differences in inflammatory biomarkers between co-twins in each pair (**Table C.3**). The within-pair effects are inherently controlled for shared demographic, familial, and, in MZ pairs, genetic influences; additionally, environmental factors during the day of testing are controlled for since co-twins were examined at the same time. In these models the within-pair β

coefficient describes the individual twin variation from the twin pair average; this formulation is independent of twin ordering and results are identical to a model that fits the absolute difference between the co-twins.[28] The percent difference calculated from this parameter represents the percent increment/decrement in biomarker concentrations per one-gram absolute difference in dietary ALA intake between twin brothers, comparing a twin with one-gram higher ALA intake to his twin brother with a lower intake. Regression analyses included separated variance components for twin type to accommodate the different residual correlation in MZ and DZ pairs. MZ and DZ twins were also examined separately.

The "base model" included total energy intake and intakes of fatty acids inherently related to ALA metabolism *in vivo*, including linoleic acid, gamma-linolenic acid, trans-, monounsaturated, and saturated fatty acids, and ω-3 ≥ 20C-PUFA (C20:5, C22:5, and C22:6). To this model we subsequently added sociodemographic factors, including age, years of education, and current marital status; lifestyle factors, such as current smoking, waist-to-hip ratio, and physical activity; cardiovascular risk factors, including depressive symptoms (BDI score), fasting glucose, systolic blood pressure, cholesterol in low- and high-density lipoproteins, and medications including current use of aspirin and statins. Potential multicollinearity was investigated using condition indices (CI) and variance decomposition proportions. To rule out model overfitting, we fitted parsimonious models after backward elimination. However, our associations of interest from the parsimonious models and the full models were similar. All analyses were conducted using SAS software version 9.1 (SAS Institute). Significance level was set at 0.05, two-sided.

Results

Sample Characteristics

Subjects with a higher habitual dietary intake of ALA were more likely to use fish oil supplements, and to have a higher total energy intake, and higher energy-adjusted intakes of gamma-linolenic acid, linoleic acid, ω-3 ≥ 20C-PUFA, and monounsaturated fatty acids (**Table C.1**). The sample was 94% Non-Hispanic White, 3% African-American, and 3% other racial/ethnic groups; this distribution reflected the racial distribution of the Vietnam Era Twin Registry from which it was sampled. Intraclass correlation coefficients, measuring between co-twin correlations, for habitual dietary ALA intake, plasma IL-6 or sIL-6R concentrations were larger in MZ than in DZ. These correlations were more modest for habitual dietary ALA intake or plasma IL-6 than for sIL-6R (**Figure C**).

Overall Associations

Greater habitual dietary ALA intake was associated with lower levels of sIL-6R, but not IL-6, in a dose-response fashion (**Table C.2**). When habitual dietary ALA intake was treated as a continuous variable, a one-gram increase in dietary ALA intake was significantly associated with 10.6% lower sIL-6R levels after controlling for nutritional factors (P=0.017, Model 1). Even after full adjustment, the inverse association between habitual dietary ALA intake and sIL-6R remained strong: a one-gram higher habitual dietary ALA intake was significantly associated with 11.4% lower sIL-6R concentrations (P=0.009, Model 4). Similar results were observed when dietary ALA intake was treated as an ordinal variable (P=0.03), confirming that there was a dose-response association. In

the fully adjusted model, twins in the highest quintile of dietary ALA intake had plasma sIL-6R levels that were 10.4% (95% CI 0.8 ~ 19.7%) lower than those in the lowest quintile.

Within-Pair Results

The within-pair inverse association between dietary ALA intake and plasma sIL-6R was statistically significant in the combined sample of MZ and DZ pairs while there was no association between dietary ALA intake and plasma IL-6 levels (**Table C.3**). A one-gram within-pair absolute difference in habitual dietary ALA intake was significantly associated with 11.2% (P=0.01, Model 1) and 12.2% (P=0.005, Model 4) lower sIL-6R levels before and after adjusting for other risk factors. Among MZ pairs, a one-gram within-pair absolute difference in habitual dietary ALA intake was significantly associated with 13.1% (P=0.008, Model 1) and 12.2% (P=0.008, Model 4) lower sIL-6R levels before and after adjusting for other risk factors. Results for the DZ pairs were overall similar, although the association between dietary ALA and plasma sIL-6R was marginally significant in DZ pairs (P=0.08) due to larger confidence intervals. Dietary ALA intake, however, was more strongly and marginally statistically associated with plasma IL-6 levels within the DZ pairs (P=0.049, Model 4) than within the MZ pairs.

Similar results were obtained after further controlling for medications for hypertension and diabetes mellitus.

Discussion

We found a strong inverse dose-response association of habitual dietary ALA intake with plasma sIL-6R concentrations, independent of a wide range of known

cardiovascular risk factors in this adult male cohort. The association persisted when comparing twins within a pair and when the analysis was restricted to MZ twin pairs. These findings indicate that the association between habitual dietary ALA and sIL-6R is independent of genetic and other familial factors.

The soluble interleukin-6 receptor (sIL-6R) in blood enhances and broadens IL-6 actions on target cells; thus, sIL-6R and IL-6 work in concert to exert pro-inflammatory effects at cellular level.[7] Through the formation of the IL-6/sIL-6R complex, sIL-6R prolongs the IL-6 half-life, amplifies responses of cells with membrane-bound-IL-6 receptors (mIL-6R) to IL-6, enables cells without mIL-6R to respond to IL-6,[30] and augments IL-6 actions on many aspects of atherosclerosis.[31] Blood concentrations of both IL-6 and sIL-6R have been related to increased susceptibility to cardiovascular diseases.[23,32]

Previous studies have rarely investigated the association between habitual dietary ALA intake and plasma sIL-6R levels. One four-day randomized trial among healthy adults found that a diet in which ALA provided 5% of total energy intake lowered plasma sIL-6R levels compared to a control diet in which ALA provided 0.5% of total energy intake.[33] Our study shows for the first time that this finding applies also to habitual ALA dietary intake at much lower levels of ALA intake, which in our study ranged from 0.16 to 2.3 g per day (from 0.14% to 0.93% of total energy intake).

Although we found a robust association between dietary ALA and sIL-6R, we did not find a statistically significant association between habitual dietary ALA intake and IL-6 levels in blood. The association of dietary ALA intake with plasma IL-6 levels has been examined in previous studies, but yield inconsistent results from either

observational reports[8,9] or randomized trials.[10-13] Most of these studies, however, were small and short-term. The randomized trial with the largest sample size and the longest intervention duration (2 years) reported that an intake of 5.9 g/day of ALA (2.3% of total energy intake) did not significantly affect IL-6 levels compared with 1 g/day of ALA (0.4% of total energy intake).[10] These findings are consistent with our study results.

It is unclear why habitual dietary ALA is associated with plasma sIL-6R, but not IL-6. As endothelial and smooth muscle cells lack of mIL-6R and rely on sIL-6R to respond to IL-6,[30] sIL-6R may be a sensitive indicator of the inflammatory process at the cellular level in tissues most affected by atherosclerosis. These include endothelial and smooth muscle cells in the vascular wall. An alternative explanation is that the association of ALA with IL-6 may be genetically modulated while that with sIL-6R is not; if this is the case, it would be more difficult to show such an association within related individuals such as twins, particularly if they are genetically identical like the MZ twins. The within-pair association for sIL-6R (but not IL-6) was twice as large in DZ compared with MZ twins, suggesting that shared genetic factors are important in modulating the association between habitual ALA intake and sIL-6R.

The possible underlying biochemical mechanism through which habitual dietary ALA lowers sIL-6R is unclear. *In vivo*, sIL-6R is produced by two mechanisms: differential mRNA splicing and shedding (proteolytic cleavage).[34] The sIL-6R from differential mRNA splicing contributes to basal levels of sIL-6R, while shed sIL-6R is regulatory. sIL-6R shedding is triggered by elevated intracellular calcium levels,[35] which are regulated by arachidonic acid levels.[36] Dietary ALA may reduce sIL-6R shedding by decreasing arachidonic acid levels, which lowers intracellular calcium levels. ALA

decreases arachidonic acid levels by competing with linoleic acid for common enzymes and, thus, reduces the derivation of arachidonic acid from linoleic acid.[1]

Our study has some limitations. The Willett food frequency questionnaire may not be optimal for estimating absolute intakes; however, it is appropriate in our investigation, where we assessed diet-disease relations after energy adjustment.[37] Subjects with higher dietary intakes of ALA were more likely to use fish oil supplements and to have higher intakes of linoleic acid, trans-, ω-3 long-chain, and monounsaturated fatty acids. However, we controlled for these and other factors, making it unlikely that these behavioral factors confound the association. The sample was restricted to middle-aged males, and our results may not be generalizable to females. Nevertheless, our study was strengthened by using a twin design to control for unmeasured and unknown confounding variables, such as genetic factors and socioeconomic, behavioral, and lifestyle characteristics acquired by twins raised within the same family.

In conclusion, our data indicate that a higher habitual dietary ALA intake is strongly associated with reduced inflammation as measured by plasma sIL-6R. Shared familial factors, including genetic variables and early environment factors, do not confound this dietary ALA intake-plasma sIL-6R association. Our findings support the hypothesis that sIL-6R is a key locus in inflammation related to cellular response to IL-6, which potentially links dietary ALA to its cardioprotective effects. Our results are relevant from a clinical and public health standpoint, since 1.0 g increase in daily dietary ALA intake is easily achievable. For example, one tablespoon (15 mL) of canola oil, non-hydrogenatd soybean oil, or one teaspoon (5 mL) of linseed (flaxseed) oil will provide

1.0 g of ALA. Our findings support the potential importance of increasing dietary ALA in the habitual diet for preventing cardiovascular disease.

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References

1. Mozaffarian D. Does alpha-linolenic acid intake reduce the risk of coronary heart disease? A review of the evidence. Altern Ther Health Med 2005;11:24-30; quiz 31, 79.
2. Hulbert AJ, Faulks SC, Buffenstein R. Oxidation-resistant membrane phospholipids can explain longevity differences among the longest-living rodents and similarly-sized mice. J Gerontol A Biol Sci Med Sci 2006;61:1009-18.
3. Domingo JL, Bocio A. Levels of PCDD/PCDFs and PCBs in edible marine species and human intake: a literature review. Environ Int 2007;33:397-405.
4. Calder P. Polyunsaturated fatty acids and inflammation. Biochemical Society Transactions 2005;33:423-7.
5. Ghosh S, Novak EM, Innis SM. Cardiac proinflammatory pathways are altered with different dietary n-6 linoleic to n-3 {alpha}-linolenic acid ratios in normal, fat-fed pigs. Am J Physiol Heart Circ Physiol 2007;293:H2919-27.
6. Bagga D, Wang L, Farias-Eisner R, Glaspy JA, Reddy ST. Differential effects of prostaglandin derived from omega-6 and omega-3 polyunsaturated fatty acids on COX-2 expression and IL-6 secretion. Proc Natl Acad Sci U S A 2003;100:1751-6.
7. Lopez-Garcia E, Schulze MB, Manson JE, et al. Consumption of (n-3) fatty acids is related to plasma biomarkers of inflammation and endothelial activation in women. J Nutr 2004;134:1806-11.

8. Pischon T, Hankinson SE, Hotamisligil GS, Rifai N, Willett WC, Rimm EB. Habitual dietary intake of n-3 and n-6 fatty acids in relation to inflammatory markers among US men and women. Circulation 2003;108:155-60.

9. Bemelmans WJ, Lefrandt JD, Feskens EJ, et al. Increased alpha-linolenic acid intake lowers C-reactive protein, but has no effect on markers of atherosclerosis. Eur J Clin Nutr 2004;58:1083-9.

10. Rallidis LS, Paschos G, Liakos GK, Velissaridou AH, Anastasiadis G, Zampelas A. Dietary alpha-linolenic acid decreases C-reactive protein, serum amyloid A and interleukin-6 in dyslipidaemic patients. Atherosclerosis 2003;167:237-42.

11. Wallace FA, Miles EA, Calder PC. Comparison of the effects of linseed oil and different doses of fish oil on mononuclear cell function in healthy human subjects. Br J Nutr 2003;89:679-89.

12. Zhao G, Etherton TD, Martin KR, Gillies PJ, West SG, Kris-Etherton PM. Dietary alpha-linolenic acid inhibits proinflammatory cytokine production by peripheral blood mononuclear cells in hypercholesterolemic subjects. Am J Clin Nutr 2007;85:385-91.

13. van den Bree MB, Eaves LJ, Dwyer JT. Genetic and environmental influences on eating patterns of twins aged >/=50 y. Am J Clin Nutr 1999;70:456-65.

14. Galicia JC, Tai H, Komatsu Y, Shimada Y, Akazawa K, Yoshie H. Polymorphisms in the IL-6 receptor (IL-6R) gene: strong evidence that serum levels of soluble IL-6R are genetically influenced. Genes Immun 2004;5:513-6.

15. de Maat MP, Bladbjerg EM, Hjelmborg JB, Bathum L, Jespersen J, Christensen K. Genetic influence on inflammation variables in the elderly. Arterioscler Thromb Vasc Biol 2004;24:2168-73.

16. Larson NI, Neumark-Sztainer D, Hannan PJ, Story M. Family meals during adolescence are associated with higher diet quality and healthful meal patterns during young adulthood. J Am Diet Assoc 2007;107:1502-10.

17. Maynard M, Gunnell D, Ness AR, Abraham L, Bates CJ, Blane D. What influences diet in early old age? Prospective and cross-sectional analyses of the Boyd Orr cohort. Eur J Public Health 2006;16:316-24.

18. Rankinen T, Bouchard C. Genetics of food intake and eating behavior phenotypes in humans. Annu Rev Nutr 2006;26:413-34.

19. Goldberg J, Curran B, Vitek ME, Henderson WG, Boyko EJ. The Vietnam Era Twin Registry. Twin Research 2002;5:476-81.

20. Scherrer JF, Xian H, Bucholz KK, et al. A twin study of depression symptoms, hypertension, and heart disease in middle-aged men. Psychosom Med 2003;65:548-57.

21. Eisen S, Neuman R, Goldberg J, Rice J, True W. Determining zygosity in the Vietnam Era Twin Registry: an approach using questionnaires. Clin Genet 1989;35:423-32.

22. Ridker PM, Rifai N, Stampfer MJ, Hennekens CH. Plasma concentration of interleukin-6 and the risk of future myocardial infarction among apparently healthy men. Circulation 2000;101:1767-72.

23. Willett W. Nutritional Epidemiology. 2nd ed. New York, NY: Oxford University Press, 1998.

24. Pols MA, Peeters PH, Bueno-De-Mesquita HB, et al. Validity and repeatability of a modified Baecke questionnaire on physical activity. Int J Epidemiol 1995;24:381-8.

25. Barr RG, Nathan DM, Meigs JB, Singer DE. Tests of glycemia for the diagnosis of type 2 diabetes mellitus. Ann Intern Med 2002;137:263-72.

26. Beck AT, Ward CH, Mendelson M, Mock J, Erbaugh J. An inventory for measuring depression. Arch Gen Psychiatry 1961;4:561-71.

27. Carlin JB, Gurrin LC, Sterne JA, Morley R, Dwyer T. Regression models for twin studies: a critical review. Int J Epidemiol 2005;34:1089-99.

28. Dai J, Miller AH, Bremner JD, et al. Adherence to the mediterranean diet is inversely associated with circulating interleukin-6 among middle-aged men: a twin study. Circulation 2008;117:169-75.

29. Kallen KJ. The role of transsignalling via the agonistic soluble IL-6 receptor in human diseases. Biochim Biophys Acta 2002;1592:323-43.

30. Rose-John S. Interleukin-6 biology is coordinated by membrane bound and soluble receptors. Acta Biochim Pol 2003;50:603-11.

31. Libby P. Inflammation in atherosclerosis. Nature 2002;420:868-74.

32. St-Pierre AC, Cantin B, Bergeron J, et al. Inflammatory markers and long-term risk of ischemic heart disease in men A 13-year follow-up of the Quebec Cardiovascular Study. Atherosclerosis 2005;182:315-21.

33. Nelson TL, Hickey MS. Acute changes in dietary omega-3 fatty acid intake lowers soluble interleukin-6 receptor in healthy adult normal weight and overweight males. Cytokine 2004;26:195-201.

34. Jones SA, Horiuchi S, Topley N, Yamamoto N, Fuller GM. The soluble interleukin 6 receptor: mechanisms of production and implications in disease. Faseb J 2001;15:43-58.

35. Jones SA, Horiuchi S, Novick D, Yamamoto N, Fuller GM. Shedding of the soluble IL-6 receptor is triggered by Ca2+ mobilization, while basal release is predominantly the product of differential mRNA splicing in THP-1 cells. Eur J Immunol 1998;28:3514-22.

36. Huang JM, Xian H, Bacaner M. Long-chain fatty acids activate calcium channels in ventricular myocytes. Proc Natl Acad Sci U S A 1992;89:6452-6.

37. Subar AF, Thompson FE, Kipnis V, et al. Comparative validation of the Block, Willett, and National Cancer Institute food frequency questionnaires : the Eating at America's Table Study. Am J Epidemiol 2001;154:1089-99.

Table C.1

Nutritional, Sociodemographic, Lifestyle, Clinical and Biochemical Characteristics of Subjects According to Dietary Intake of Alpha-linolenic Acid

	Quintiles of ALA (g/d)					Trend
Variable	**0.16-0.38 (n = 62)**	**0.39-0.52 (n = 62)**	**0.53-0.68 (n = 65)**	**0.69-0.89 (n = 60)**	**0.90-2.31 (n = 63)**	***P* value***
Nutritional factors (mean ± SEM)						
Total energy intake, kcal/d	982 ± 61	1,238 ± 61	1,457 ± 60	1,716 ± 62	2,321 ± 61	<0.0001
Fatty acid intakes adjusted for total energy intake using models, g/d (mean ± SEM)						
Linoleic acid†	4.8 ± 0.3	6.2 ± 0.3	6.5 ± 0.2	7.9 ± 0.3	11.0 ± 0.3	<0.0001
Gamma-linolenic acid	0.01 ± 0.0	0.01 ± 0.0	0.01 ± 0.0	0.01 ± 0.0	0.01 ± 0.0	0.84
ω-3 ≥20C-PUFA						
including supplements	0.14 ± 0.03	0.13 ± 0.03	0.18 ± 0.03	0.21 ± 0.03	0.25 ± 0.03	0.002
excluding supplements	0.12 ± 0.02	0.13 ± 0.02	0.17 ± 0.02	0.20 ± 0.02	0.20 ± 0.03	0.002
Trans-fatty acids	1.8 ± 0.1	1.9 ± 0.1	1.8 ± 0.1	1.7 ± 0.1	2.0 ± 0.1	0.12

Table C.1. (Con't)

Nutritional, Sociodemographic, Lifestyle, Clinical and Biochemical Characteristics of Subjects According to Dietary Intake of Alpha-linolenic Acid

	Quintiles of ALA (g/d)					Trend
Variable	**0.16-0.38 (n = 62)**	**0.39-0.52 (n = 62)**	**0.53-0.68 (n = 65)**	**0.69-0.89 (n = 60)**	**0.90-2.31 (n = 63)**	***P* value***
Saturated fatty acids	21.8 ± 0.9	22.1 ± 0.9	22.1 ± 0.8	21.2 ± 0.9	22.9 ± 1.0	0.11
Monounsaturated fatty acids‡	19.8 ± 0.9	21.3 ± 0.8	21.8 ± 0.8	21.9 ± 0.8	27.2 ± 1.0	<0.0001
Sociodemographic factors						
Age, yrs (mean ± SEM)	54.5 ± 0.4	54.7 ± 0.4	54.4 ± 0.4	54.4 ± 0.4	53.8 ± 0.4	0.88
Education, yrs (mean ± SEM)	14.2 ± 0.3	14.1 ± 0.3	14.3 ± 0.3	14.3 ± 0.3	14.1 ± 0.3	0.97
Married, n (%)	43 (69.4)	47 (75.8)	57 (87.7)	51 (85.0)	48 (76.2)	0.31
Lifestyle factors						
Waist to hip ratio, (mean ± SEM)	0.93 ± 0.01	0.95 ± 0.01	0.94 ± 0.01	0.94 ± 0.01	0.95 ± 0.01	0.32
Body mass index, kg/m^2, (mean ± SEM)	29.2 ± 0.5	28.5 ± 0.5	29.3 ± 0.5	28.6 ±0.5	30.0 ± 0.5	0.10

Table C.1. (Con't)

Nutritional, Sociodemographic, Lifestyle, Clinical and Biochemical Characteristics of Subjects According to Dietary Intake of Alpha-linolenic Acid

	Quintiles of ALA (g/d)					Trend
Variable	**0.16-0.38 (n = 62)**	**0.39-0.52 (n = 62)**	**0.53-0.68 (n = 65)**	**0.69-0.89 (n = 60)**	**0.90-2.31 (n = 63)**	***P* value***
Physical activity, unit (mean ± SEM)	7.1 ± 0.2	7.1 ± 0.2	7.8 ± 0.2	8.0 ± 0.2	7.3 ± 0.2	0.21
Current smokers, n (%)	11 (17.7)	13 (21.0)	14 (21.5)	8 (13.3)	8 (12.7)	0.28
Clinical and biochemical features (mean ± SEM)						
Depression symptom score, BDI	4.6 ± 0.8	4.6 ± 0.8	4.5 ± 0.8	4.9 ± 0.8	4.9 ± 0.8	0.79
Plasma glucose, mg/dL	97.7 ± 2.0	99.7 ± 2.0	102.0 ± 2.0	101.0 ± 2.0	99.9 ± 2.0	0.42
Systolic blood pressure, mm Hg	129.7 ± 1.9	127.6 ± 2.0	128.1 ± 1.9	130.4 ± 2.0	130.7 ± 2.0	0.36
Diastolic blood pressure, mm Hg	81.2 ± 1.3	79.8 ± 1.3	80.3 ± 1.3	81.3 ± 1.4	82.1 ± 1.3	0.33

Table C.1.

Nutritional, Sociodemographic, Lifestyle, Clinical and Biochemical Characteristics of Subjects According to Dietary Intake of Alpha-linolenic Acid (Con't)

	Quintiles of ALA (g/d)					Trend
Variable	**0.16-0.38 (n = 62)**	**0.39-0.52 (n = 62)**	**0.53-0.68 (n = 65)**	**0.69-0.89 (n = 60)**	**0.90-2.31 (n = 63)**	***P* value***
Plasma lipids, *mg/dL*						
Total triglycerides	186.5 ± 12.0	175.0 ± 11.9	184.6 ± 11.9	172.3 ± 11.7	200.4 ± 12.2	0.16
Total-cholesterol	190.7 ± 4.7	190.6 ± 4.7	188.2 ± 4.6	197.4 ± 4.7	188.9 ± 4.7	0.97
High-density lipoprotein cholesterol	40.5 ± 1.1	38.6 ± 1.1	38.1 ± 1.1	40.3 ± 1.1	38.2 ± 1.1	0.41
Low-density lipoprotein cholesterol	123.9 ± 4.1	125.2 ± 4.1	122.5 ±4.0	133.8 ± 4.2	124.9 ± 4.1	0.63
Use of statins, n (%)	16 (25.8)	12 (19.4)	16 (24.6)	11 (18.3)	9 (14.3)	0.17
Use of aspirin, n (%)	10 (16.1)	12 (19.4)	17 (26.2)	13 (21.7)	12 (19.0)	0.74
Use of antihypertensives, n (%)	9 (14.5)	11 (17.7)	14 (21.5)	15 (25.0)	15 (23.8)	0.11
Use of antihyperglycemics, n (%)	2 (3.2)	7 (11.3)	6 (9.2)	6 (10.0)	3 (4.8)	0.85

Table C.1.

Nutritional, Sociodemographic, Lifestyle, Clinical and Biochemical Characteristics of Subjects According to Dietary Intake of Alpha-linolenic Acid (Con't)

Abbreviation: ALA = alpha-linolenic acid; ω-3 ≥20C-PUFA = ω-3 20 carbon or more long-chain polyunsaturated fatty acids (20:5ω-3, 22:5ω-3 and 22:6ω-3, including fish oil supplements); BDI = Beck Depression Inventory.

Plus–minus values are the least squared mean ± SEM estimated from mixed models accounting for clustering within a pair while otherwise indicated.

SI conversion factors: to convert glucose values to micromoles, multiply by 0.055; to convert triglycerides values to micromoles, multiply by 0.011; and to convert cholesterol to micromoles, multiply by 0.026.

* Test for trend across diet groups: all *P* values are corrected for clustering within a twin pair using mixed models for continuous variables, and generalized estimating equation logistic models for dichotomous variables.

† Exclude *trans*- and *trans*- *cis*- linoleic acid

ǂ Exclude *trans*-monounsaturated fatty acid

Table C.2.

Associations between Dietary ALA and Plasma Concentrations of Interleukin-6 and its Soluble Receptor sIL-6R

Outcome	Difference (%)* (95% CI) 1-gram increment in dietary ALA	*P* value	Adjusted Geometric Means (95% CI) of Biomarkers (g/d) ALA (0.16 - 0.38) (n = 62)	ALA (0.39 - 0.52) (n = 62)	ALA (0.53 - 0.68) (n = 65)	ALA (0.69 - 0.89) (n = 60)	ALA (0.90 - 2.31) (n = 63)	Trend *P* value†
Model 1: Adjusted for zygosity and nutritional factors‡								
IL-6, pg/mL	-12.6 (-43.1, 34.2)	0.54	1.8 (1.5, 2.2)	1.8 (1.5, 2.1)	1.8 (1.5, 2.1)	1.7 (1.4, 2.0)	1.8 (1.4, 2.2)	0.84
sIL-6R, ng/mL	-10.6 (-18.5, -2.0)	0.017	28.8 (27.0, 30.8)	27.7 (26.1, 29.4)	28.2 (26.8, 30.0)	27.4 (25.9, 29.1)	26.1 (24.1, 28.1)	0.04
Model 2: Adjusted for all above plus sociodemographic and lifestyle factors§								
IL-6, pg/mL	-5.3 (-37.7, 44.3)	0.80	2.0 (1.7, 2.5)	2.0 (1.7, 2.4)	2.1 (1.8, 2.5)	2.0 (1.7, 2.4)	1.9 (1.5, 2.4)	0.64
sIL-6R, ng/mL	-11.5 (-19.2, -3.1)	0.009	28.5 (26.6, 30.7)	27.4 (25.6, 29.2)	28.2 (26.5, 30.0)	27.4 (25.6, 29.1)	26.1 (24.0, 28.1)	0.03

Table C.2

Associations between Dietary ALA and Plasma Concentrations of Interleukin-6 and its Soluble Receptor sIL-6R (Con't)

Outcome	Difference (%)* (95% CI) 1-gram increment in dietary ALA	*P* value	Adjusted Geometric Means (95% CI) of Biomarkers (g/d) ALA (0.16 - 0.38) (n = 62)	ALA (0.39 - 0.52) (n = 62)	ALA (0.53 - 0.68) (n = 65)	ALA (0.69 - 0.89) (n = 60)	ALA (0.90 - 2.31) (n = 63)	Trend *P* value†
Model 3: Adjusted for zygosity and nutritional factors‖								
IL-6, pg/mL	-10.3 (-40.9, 35.8)	0.60	2.2 (1.8, 2.6)	2.1 (1.7, 2.5)	2.1 (1.8, 2.5)	2.1 (1.7, 2.5)	1.9 (1.5, 2.4)	0.38
sIL-6R, ng/mL	-11.7 (-19.2, -3.4)	0.007	28.5 (26.6, 30.8)	27.4 (25.6, 29.2)	28.2 (26.5, 30.1)	27.4 (25.6, 29.1)	26.1 (24.0, 28.0)	0.02
Model 4: Adjusted for all above plus medications¶								
IL-6, pg/mL	-10.1 (-40.8, 36.4)	0.61	2.1 (1.7, 2.6)	2.0 (1.7, 2.4)	2.1 (1.8, 2.5)	2.0 (1.7, 2.5)	1.9 (1.5, 2.4)	0.38
sIL-6R, ng/mL	-11.4 (-19.1, -3.0)	0.009	28.8 (26.5, 31.1)	27.4 (25.4, 29.4)	28.2 (26.3, 30.1)	27.1 (25.4, 29.1)	25.5 (23.7, 27.7)	0.03

Table C.2.

Associations between Dietary ALA and Plasma Concentrations of Interleukin-6 and its Soluble Receptor sIL-6R (Con't)

Abbreviations: IL-6 = interleukin 6; sIL-6R = soluble Interleukin-6 receptor; ALA = alpha-linolenic acid.

* Values are % geometric mean differences (95% CI) calculated from the β coefficient of ALA intake in mixed models treating twins as separate individuals but accounting for different clustering within a twin pair according to zygosity. A negative value of the % difference indicates an inverse association between ALA intake and the biomarker, while a positive value reflects a positive association.

† Test for trend across diet groups accounting for clustering within a pair and different correlations across twin types. An ordinal variable was generated with a mean of the ALA intake as the rank value.

‡ Total energy intake (continuous); linoleic (excluding trans- and trans-cis-linoleic acid) (continuous); gamma-linolenic (continuous); ω-3 20 carbon or more long-chain polyunsaturated fatty acids (including 20:5, 22:5, 22:6 from fish oil supplements) (continuous); and trans-, saturated, and monounsaturated fatty acids (excluding trans-monounsaturated fatty acids) (continuous).

§ Age (continuous), education (continuous), waist-to-hip ratio (continuous), physical activity (continuous), current smoking (yes/no), and marital status (yes/no).

Table C.2.

Associations between Dietary ALA and Plasma Concentrations of Interleukin-6 and its Soluble Receptor sIL-6R (Con't)

‖Depressive symptom score (continuous), plasma glucose (continuous), systolic blood pressure (continuous), low- and high-density lipoprotein cholesterol (continuous).

¶Use of statin (yes/no) and aspirin (yes/no).

Table C.3.

Within-Pair Percent Differences in Plasma Concentrations of Interleukin-6 and its Soluble Receptor sIL-6R per 1-gram Within-pair Absolute Difference in Habitual Dietary ALA Intake

Outcome	Within-pair Difference (%) (95% CI)* MZ + DZ (n = 312)	*P* value	Within-pair Difference (%) (95% CI)* MZ (n = 182)	*P* value	Within-pair Difference (%) (95% CI)* DZ (n = 130)	*P* value	*P* Value for Interaction With Zygosity
Model 1: Adjusted for zygosity and nutritional factors†							
IL-6, pg/mL	-10.7 (-42.3, 38.2)	0.61	6.2 (-41.5, 91.4)	0.85	-25.9 (-63.1, 49)	0.39	0.27
sIL-6R, ng/mL	-11.2 (-18.9, -2.7)	0.011	-13.1 (-21.2, -3.6)	0.008	-15.6 (-34.2, 7)	0.15	0.27
Model 2: Adjusted for all above plus sociodemographic and lifestyle factors‡							
IL-6, pg/mL	-5.0 (-38.1, 46.0)	0.82	23.4 (-31.1, 122.3)	0.47	-37.5 (-68.8, 25.6)	0.18	0.12
sIL-6R, ng/mL	-12.2 (-19.8, -3.9)	0.005	-13.9 (-21.7, -4.9)	0.004	-19.7 (-37.3, 3.8)	0.09	0.26
Model 3: Adjusted for all above plus known clinical cardiovascular risk factors§							
IL-6, pg/mL	-9.2 (-40.6, 38.9)	0.66	20.9 (-32.0, 116.8)	0.51	-48.3 (-74.2, 3.8)	0.063	0.054
sIL-6R, ng/mL	-12.4 (-19.8, -4.1)	0.004	-13.1 (-21.0, -4.2)	0.005	-18.9 (-37.1, 5.4)	0.12	0.26

Table C.3. (Con't)

Within-Pair Percent Differences in Plasma Concentrations of Interleukin-6 and its Soluble Receptor sIL-6R per 1-gram Within-pair Absolute Difference in Habitual Dietary ALA Intake

Outcome	Within-pair Difference (%) (95% CI)* MZ + DZ (n = 312)	*P* value	Within-pair Difference (%) (95% CI)* MZ (n = 182)	*P* value	Within-pair Difference (%) (95% CI)* DZ (n = 130)	*P* value	*P* Value for Interaction With Zygosity
Model 4: Adjusted for all above plus medications‖							
IL-6, pg/mL	-8.8 (-40.4, 39.6)	0.67	20.9 (-31.6, 114.0)	0.51	-49.8 (-74.6, 1.3)	0.049	0.055
sIL-6R, ng/mL	-12.2 (-19.7, -3.8)	0.005	-12.2 (-20.7, -3.6)	0.008	-19.7 (-37.1, 2.6)	0.08	0.31

Abbreviations: IL-6 = interleukin 6; sIL-6R = soluble Interleukin-6 receptor; ALA = alpha-linolenic acid.

* Values are within-pair % geometric mean differences (95% CI) calculated from the β coefficient and expressed for per 1-gram difference in ALA intake comparing the twin with a higher ALA intake to his brother with a lower intake. A negative value indicates that the twin with a higher intake has lower levels while a positive value reflects that he has higher levels, compared with his twin brother with a lower intake. Mixed models included ALA intake and covariates as fixed effects and the twin pair as a random effect, accounting for clustering within a pair and allowing for different correlations within MZ and DZ pairs in the pooled samples.

Table C.3.

Within-Pair Percent Differences in Plasma Concentrations of Interleukin-6 and its Soluble Receptor sIL-6R per 1-gram Within-pair Absolute Difference in Habitual Dietary ALA Intake (Con't).

† Total energy intake (continuous); linoleic (excluding trans- and trans-cis-linoleic acid) (continuous); gamma-linolenic (continuous); ω-3 20 carbon or more long-chain polyunsaturated fatty acids (including 20:5, 22:5, 22:6 from fish oil supplements) (continuous); and trans-, saturated, and monounsaturated fatty acids (excluding trans-monounsaturated fatty acids) (continuous).

‡ Age (continuous), education (continuous), waist-to-hip ratio (continuous), physical activity (continuous), current smoking (yes/no), and marital status (yes/no).

§ Depressive symptom score (continuous), plasma glucose (continuous), systolic blood pressure (continuous), low- and high-density lipoprotein cholesterol (continuous).

‖ Use of statin (yes/no) and aspirin (yes/no).

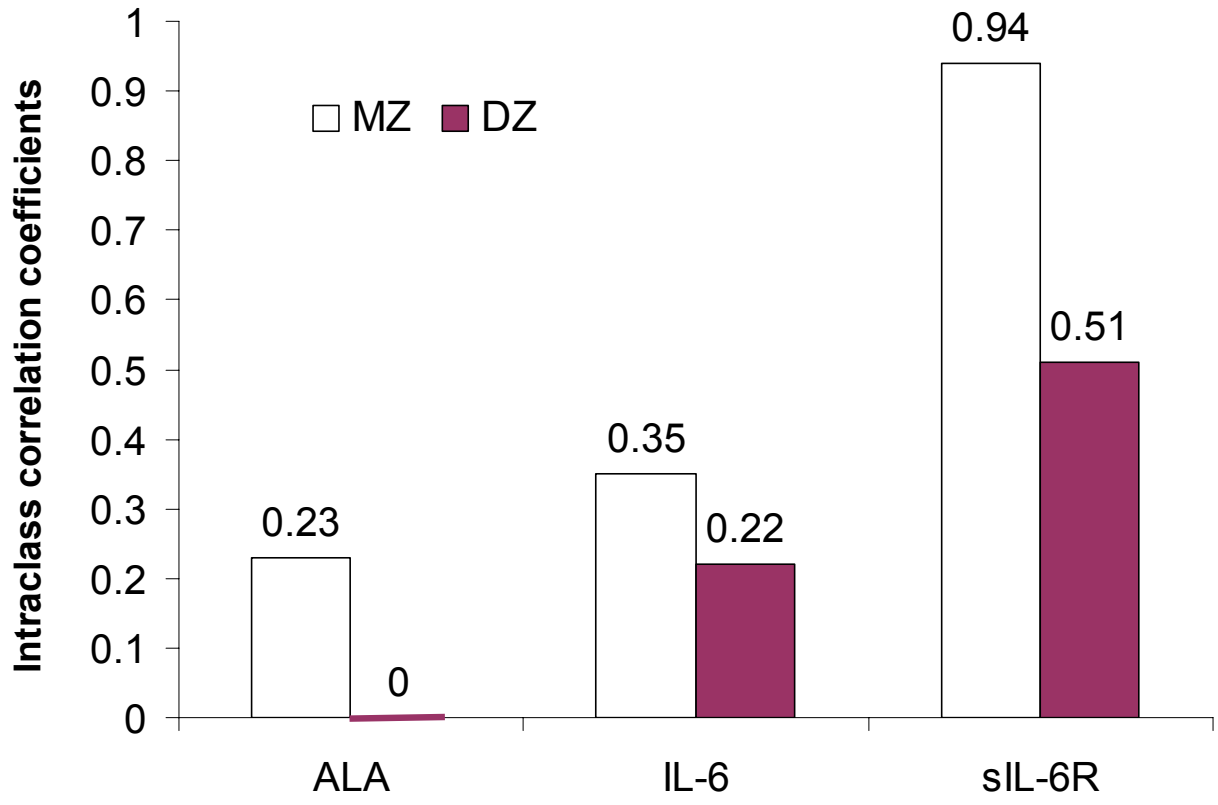

Figure C. Correlations for ALA, IL-6, and sIL-6R between MZ and DZ co-twins.

Abbreviations: MZ = monozygotic twins; DZ = dizygotic twins; ALA = alpha-linolenic acid; IL-6 = interleukin-6; sIL-6R = soluble Interleukin-6 receptor.

The intraclass correlation coefficient is calculated using unconditional means model, and ranges from 0 to 1. The greater the intraclass correlation coefficient, the higher the phenotypic similarity between co-twins. Genetic influence on the phenotype is suggested if the MZ twin correlation is greater than the DZ correlation.