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[QA1] Intervention Trials with the Mediterranean Diet in Cardiovascular Prevention: Understanding Potential Mechanisms through Metabolomic Profiling^{1–3}

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Abstract

Large observational epidemiologic studies and randomized trials support the benefits of a Mediterranean dietary pattern on cardiovascular disease (CVD). Mechanisms postulated to mediate these benefits include the reduction of low-grade inflammation, increased adiponectin concentrations, decreased blood coagulation, enhanced endothelial function, lower oxidative stress, lower concentrations of oxidized LDL, and improved apolipoprotein profiles. However, the metabolic pathways through which the Mediterranean diet influences CVD risk remain largely unknown. Investigating specific mechanisms in the context of a large intervention trial with the use of high-throughput metabolomic profiling will provide more solid public health messages and may help to identify key molecular targets for more effective prevention and management of CVD. Although metabolomics is not without its limitations, the techniques allow for an assessment of thousands of metabolites, providing wide-ranging profiling of small molecules related to biological status. Specific candidate plasma metabolites that may be associated with CVD include branched-chain and aromatic amino acids; the glutamine-to-glutamate ratio; some short- to medium-chain acylcarnitines; gut flora metabolites (choline, betaine, and trimethylamine *N*-oxide); urea cycle metabolites (citrulline and ornithine); and specific lipid subclasses. In addition to targeted metabolites, the role of a large number of untargeted metabolites should also be assessed. Large intervention trials with the use of food patterns for the prevention of CVD provide an unparalleled opportunity to examine the effects of these interventions on plasma concentrations of specific metabolites and determine whether such changes mediate the benefits of the dietary interventions on CVD risk. *J Nutr* 2016;146(Suppl):1S–7S.

Keywords: feeding trials, olive oil, nuts, cardiovascular disease, coronary heart disease

Strong Evidence Supports the Beneficial Vascular Effect of the Mediterranean Diet

The leading global, modifiable cause of morbidity and mortality is suboptimal quality of the dietary pattern (1). In contrast to the

isolated single-nutrient approach, the most relevant characteristics of healthful diets are the overall patterns of foods consumed. In the context of high-quality overall dietary patterns, current

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⁹ Abbreviations used: CHD, coronary heart disease; COX-1, cyclooxygenase 1; COX-2, cyclooxygenase 2; CRP, C-reactive protein; CVD, cardiovascular disease; EVOO, extra-virgin olive oil; LRP1, LDL receptor-related protein 1; MCP-1, monocyte chemoattractant protein 1; MedDiet: Mediterranean diet; PREDIMED, PREvención con Dieta MEDiterránea; T2D, type 2 diabetes.

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knowledge points to traditional Mediterranean-style diets' being causally related to substantial health benefits. The traditional Mediterranean diet (MedDiet)⁹ can be defined as the dietary pattern prevailing in the olive tree–growing areas of the Mediterranean basin before the mid-1960s (2). It is characterized by high consumption of plant-based foods, such as vegetables, fruits, nuts, legumes, and unprocessed cereals; low consumption of meat and meat products; and low consumption of dairy products (with the [AQ5] exception of yogurt and the long-preservable cheeses). Alcohol consumption is included, in moderation and in the form of wine and, as a rule, during meals. Total intake of lipids can be high (around or in excess of 40% of total energy intake), but the ratio of the beneficial monounsaturated to the nonbeneficial saturated lipids is high, because of the high monounsaturated content of liberally used olive oil, which is a hallmark of the MedDiet and is its main culinary fat. In the past, fish consumption has been a function of the distance from the sea, but intake has been, overall, at a moderate level (2).

A large body of observational epidemiologic evidence from large and well-characterized prospective cohort studies supports the fact that high adherence to a dietary pattern score appraising [AQ6] a traditional MedDiet is associated with decreased risk of fatal and nonfatal clinical events from cardiovascular disease (CVD), including both coronary heart disease (CHD) and stroke (3–7). These large cohort studies have been conducted not only in the Mediterranean area, but also in other geographic regions, such as the United States, Europe, Japan, and Australia.

According to the series of systematic reviews on the relation between dietary patterns and health outcomes conducted by the USDA (7), scores that were strongly associated with decreased risk of CVD, CHD, or stroke were the original Mediterranean Diet Score proposed by Trichopoulou et al. (8) or the Alternate Mediterranean Diet Score (9–10). Similarly, other high-quality dietary patterns, such as the Healthy Eating Index–2005, the Alternative Healthy Eating Index, the updated Alternative Healthy Eating Index–2010, the Recommended Food Score, and the Dietary Approaches to Stop Hypertension score were also associated with a reduced risk of CVD. The decreased risk of CHD ranged from 29% to 61% with increased adherence to a Mediterranean dietary pattern, from 24% to 31% for increased adherence to a dietary healthy guidelines–related pattern, and from 14% to 27% for adherence to the Dietary Approaches to Stop Hypertension (7). A recent systematic review of observational cohort studies reported that, after removing studies that only included fatal cardiovascular endpoints, each 2-point increment in the Mediterranean Diet Score (0–9 score) was associated with a 13% relative reduction in the incidence of CVD (RR: 0.87; 95% CI: 0.85–0.90), without any evidence of heterogeneity (3).

Moreover, beyond observational studies, 2 large randomized trials, the Lyon trial in secondary prevention (11), and the PREvención con Dieta MEDiterránea (PREDIMED) trial in primary prevention (12, 13), have shown strong protective effects from the Mediterranean dietary pattern against CVD. Therefore, the association between closer adherence to the MedDiet and a substantial reduction in incident CVD outcomes shows a remarkable consistency of findings between both observational and experimental studies.

Mechanistic Studies: Main Findings

In addition to their observed effects on clinical cardiovascular events, the MedDiet interventions in the PREDIMED diet appear to exert favorable effects on the physiologic processes

underlying CVD, supporting their observed beneficial effects on incident CVD and changes in the levels of classic CVD risk factors (14–18). In the development and progression of atherosclerosis, from its initial to its final lesions, low-grade, chronic inflammation is a key contributor to the pathologic process (19). Increased expression of C-reactive protein (CRP), proinflammatory cytokines such as IL-6, or adhesion molecules such as intracellular adhesion molecule 1 are important biomarkers frequently used to assess this chronic inflammatory process, which is inherent to the development and progression of atherosclerosis. In fact, elevated concentrations of CRP, a marker of chronic inflammation, predict future occurrence of CVD clinical events (20). A meta-analysis of randomized clinical trials testing the MedDiet reported that high-sensitivity CRP concentrations and other inflammatory biomarkers were significantly more decreased with MedDiets than with control diets, with the following weighted mean differences—CRP: -0.98 mg/L, 95% CI: $-1.5, -0.49$, $P < 0.0001$ (14 trials); IL-6: -0.42 pg/mL, 95% CI: $-0.73, -0.11$, $P = 0.008$ (6 trials); and intracellular adhesion molecule 1: -23.7 μ g/L, 95% CI: $-41.2, -6.22$, $P = 0.008$ (2 trials) (21). Several substudies from the PREDIMED trial have reported a strong anti-inflammatory effect from both intervention groups [i.e., the MedDiet supplemented with either extra-virgin olive oil (EVOO) or tree nuts] compared with the control group allocated to receive advice on a low-fat diet (22–28). Therefore, the anti-inflammatory properties of some nutrients included in the Mediterranean dietary pattern are likely to be key in atherosclerosis-related pathways explaining the observed reductions in clinical outcomes of CVD. Interestingly, the strongest [AQ7] reduction in clinical outcomes with the MedDiet in the PREDIMED trial was observed for peripheral artery disease (29), which is closely related to the development and progression of atherosclerosis, with less involvement of other factors, such as coagulation-related mechanisms.

Oxidative stress, and, particularly, the oxidation of LDL, is considered to be another strong contributor to the genesis of atherosclerosis from its initial steps, and to the development of atherosclerotic clinical events (30). Oxidized LDL is a biomarker for CVD development in the general population. The observed inverse association between adherence to the MedDiet and atherosclerotic clinical disease has been attributed, at least in part, to the richness of antioxidants in this food pattern. In the PREDIMED trial, after 3 mo of intervention, both MedDiets apparently exerted a beneficial effect on in vivo LDL oxidation, with significant reductions in oxidized LDL compared with the control diet. Observed changes were -10.6 U/L (95% CI: $-14.2, -6.1$) for the MedDiet + EVOO group, and -7.3 U/L (95% CI: $-11.2, -3.3$) for the MedDiet + nuts group, without changes in the control group [-2.9 U/L (95% CI: $-7.3, 1.5$)] (31). Other substudies of the PREDIMED trial have also shown longer-term antioxidant effects from the intervention with the MedDiet (32–34). Moreover, the assessment of transcriptomic profiles also suggested that the effects of the MedDiet on gene expression may partially explain the beneficial effects of the MedDiet, especially with respect to genes involved in inflammation [cyclooxygenase 1 (COX-1), cyclooxygenase 2 (COX-2), and monocyte chemoattractant protein 1 (MCP-1)] and genes involved in foam cell formation [LDL receptor–related protein 1 (LRP1), LDL receptor, and CD36] (35, 36). In addition, the [AQ8] rich polyphenol content of the MedDiet was found to be associated with increased production of plasma NO (37).

The 2 characteristic components of the MedDiet that were emphasized in the PREDIMED trial (EVOO and nuts) have been

shown to have independent beneficial biological properties. EVOO possesses a healthy FA profile and contains myriad bioactive phenolic compounds (38). These compounds have been shown to deliver an anti-inflammatory effect (39), decrease the total-to-HDL cholesterol ratio (40), lower markers of oxidative stress (31, 40), exert an antiplatelet effect (41), and stimulate mitochondrial biogenesis for the promotion of mitochondrial function (42). Nuts also contain a beneficial FA profile, with high amounts of MUFAs and PUFAs, minerals, vitamins, essential amino acids, and fiber. Nut consumption has been shown to lower LDL, non-HDL, and total cholesterol and apoB-100 concentrations (43), and to possess anti-inflammatory properties (44). Nuts also may exert an antioxidant effect, have benefits on cardiac rhythm, and improve platelet and endothelial function (45).

However, the specific metabolic pathways involved in the protection apparently exerted by the MedDiet in the PREDIMED trial and other studies are not yet fully understood. Investigating specific mechanisms will provide more solid public health messages and allow for the identification of key molecular targets for more effective prevention and management of CVD. Thus, in both the PREDIMED and other large intervention trials aimed at the primary prevention of CVD, technologies capable of identifying the effects of intervention diets on the thousands of circulating small molecule metabolites—i.e., metabolomics—will contribute to a better understanding of the effects of these interventions.

Systems Epidemiology and Metabolomics

Recent technologic developments in molecular biology currently allow the use of high-throughput techniques for the determination of genomic, transcriptomic, proteomic, and metabolomic traits. This new and powerful armamentarium, coupled with traditional and recent advances in methodologies used by epidemiologists, can provide an unprecedented opportunity to unlock the full potential of epidemiologic research to ascertain causal relations. This integrated approach has been referred to as “systems epidemiology” (46).

In this context, metabolomic techniques allow a feasible, high-throughput assessment of a large number of metabolites resulting from genomic, transcriptomic, and proteomic variability. Metabolomics is the comparative analysis of metabolite flux and how it relates to biological phenotypes. As an intermediate phenotype, metabolite signatures capture a unique aspect of cellular dynamics that is not typically interrogated, providing a distinct perspective on cellular homeostasis (47). Metabolomics provides a metabolic profile related to biological status reflecting genetic and environmental interactions.

Although metabolomics is emerging as a powerful approach to identifying metabolic biomarkers of diet and nutrition, as well as health, there are some limitations of metabolomics that deserve to be emphasized. Because of the high amount of information acquired with these procedures, the interpretation of metabolomics data can be challenging not only because of the large number of metabolic compounds identified, but also because of a high level of cellular compartmentalization, as well as different cell types, tissues, and organs (48). Metabolomics reflects a snapshot of metabolites at one point in time, and not necessarily longer-term exposure. That said, many metabolite concentrations, particularly in storage tissues such as adipose, may be the result of the cumulative effects of longer-term exposures. In addition, there can be considerable variations

from one laboratory to another, because of both the type of instrumentation used and also the particular panel of metabolites targeted. Because of the relative novelty of these techniques, issues related to between-laboratory reproducibility and to consistency of the results obtained in different studies using metabolomics approaches remain to be resolved. Many metabolomic analysis issues are reminiscent of those of the early days of genomic data analysis, including concepts such as the “winner’s curse.” Lessons learned from over a decade of genetic epidemiology point to caution in interpreting metabolome-wide results. Tools such as adjusting for multiple comparisons and including replication samples help to ensure a sensible and cautious approach. Multiple comparison issues, for example, can be addressed with the use of the false discovery rate procedure by Benjamini and Yekutieli (49), taking into account the frequently high intercorrelations between the metabolites. The false discovery rate approach controls for the proportion of incorrectly rejected null hypotheses among all those considered significant.

Despite these limitations, the integration of metabolomics with population-based studies and large randomized trials may be instrumental to our understanding of the molecular pathophysiologic processes of CVD, defining metabolic changes from diet and their associations with disease risk, and discovering novel biomarkers and new targets for intervention.

There is increasing evidence that the human metabolome responds to acute, short-term changes in diet (50–52). In a small recent trial assessing lipoproteins and low-molecular-weight metabolites, a diet rich in fatty fish increased polyunsaturation in plasma FAs, especially in n-3 (ω -3) (PUFAs, whereas n-6 and n-7 FAs decreased; in addition, changes in HDL particles were also observed, with a subclass distribution toward larger particles (52). Other investigations have shown that polyunsaturated fat- and fiber-rich foods are associated with less saturation and longer carbon chain length of phosphatidylcholines in human serum (53). Dietary saturated fat may also influence the proportion of SFAs in serum phospholipids and the degree of saturation of the constituent acyl group of plasma lysophosphatidylcholines; overweight/obese men have been reported to show higher concentrations of stearic acid and lower concentrations of oleic acid in serum phospholipids and higher concentrations of lysophosphatidylcholine C14:0 and lysophosphatidylcholine C18:0 but lower concentrations of lysophosphatidylcholine C18:1 than do lean subjects (54). Coffee intake has been reported to be positively associated with specific classes of sphingomyelins and negatively associated with long- and medium-chain acylcarnitines in plasma (55, 56). Fruit and vegetable intake has been reported to show a strong association with a glycerophospholipid (phosphatidylcholine diacyl C38:6, $P = 1.39 \times 10^{-9}$) and a sphingolipid (sphingomyelin C26:1, $P = 6.95 \times 10^{-13}$). Protein intake was positively associated with plasma valine, phenylalanine, and tyrosine, and inversely associated with glutamine in cross-sectional studies (56). In addition, some cross-sectional studies have included metabolomic components in nutritional epidemiologic research (56–58).

Although these data are promising, they are limited by their cross-sectional design, small sample sizes, and assessment of only a small number of metabolites. To our knowledge, no large trial has examined the long-term effects of randomized dietary interventions on a wide range of metabolite changes.

In this context, the PREDIMED trial provides an unparalleled opportunity to examine the effects of Mediterranean dietary interventions on changes in plasma concentrations of metabolites, and to determine whether such changes mediate the

benefits of these dietary interventions on CVD risk. Compared with a control diet, the traditional MedDiets, supplemented with either EVOO or tree nuts, were shown to reduce CVD risk by ~30% (4, 13) in the PREDIMED trial. It is unclear whether changes in traditional risk factors such as blood lipids (14, 15, 31) and blood pressure (17, 18) fully account for this CVD benefit. Although the 2 intervention diets (i.e., EVOO and nuts) were equally protective against CVD, the metabolic pathways underlying these benefits may differ because of variations in nutrient profiles. Although it may be challenging to ascribe the benefits of the PREDIMED interventions to a single nutrient or food, given the trial's focus on overall dietary patterns, the pattern approach is more applicable to public health messaging.

Candidate Metabolites that May Predict CVD Risk

The application of cutting-edge LC-MS metabolomics methods allows profiling of both targeted and untargeted metabolites. In the context of the PREDIMED trial, we have begun analyses that are first focusing on candidate metabolites and metabolic pathways that have been previously associated with CVD risk. In particular, BCAAs, including leucine, isoleucine, and valine, as well as aromatic (phenylalanine and tyrosine) amino acid concentrations have been reported to be positively associated with obesity and prediabetes, and are considered to be an early marker of insulin resistance (59–69). Circulating concentrations of BCAAs tend to be higher in obese individuals and are associated with worse metabolic health and future insulin resistance or type 2 diabetes (T2D) (70). A hypothesized mechanism linking increased concentrations of BCAAs to T2D involves the leucine-mediated activation of the mammalian target of rapamycin complex 1, which results in the uncoupling of insulin signaling at an early stage (70). Wang et al. (71) observed relations between BCAAs and incident T2D, independent of conventional risk factors, with a >5-fold higher risk for individuals in the top quartile of BCAA concentrations. Higher fasting blood concentrations of these amino acids may indicate a reduced catabolism in adipose tissue and liver, resulting in limited tissue concentrations of amino acid derivatives, which are key to normal metabolism such as oxidation of long-chain FAs. It is likely that the accumulation of mitotoxic metabolites (and not BCAAs per se) might induce β -cell mitochondrial dysfunction, stress signaling, and apoptosis, leading to T2D. Circulating amino acids may also directly promote insulin resistance via disruption of insulin signaling in skeletal muscle.

Recently, several studies have examined BCAAs or aromatic amino acids and CVD outcomes and found that BCAAs and aromatic amino acids were positively associated with atherosclerosis. Furthermore, inclusion of BCAAs and aromatic amino acids in prediction models added to the discriminative capability of traditional clinical risk factors (72–75). Further prospective investigations are warranted to confirm these findings and to assess the potential dietary modifications that may be able to counteract the detrimental cardiovascular effects of elevated concentrations of these amino acids.

Another group of potential amino acid targets are glutamine and glutamate. Cross-sectionally, cardiometabolic risk factors such as insulin resistance, obesity, and blood pressure are positively associated with plasma glutamate, but inversely associated with plasma glutamine and the glutamine-to-glutamate ratio (68, 69). An excess of glutamine relative to glutamate in blood circulation has also been associated with a

reduced risk of incident T2D (68). Glutamine supplementation in humans and animals improves glucose homeostasis and blood pressure (76–77). Enhanced release of glucagon-like peptide 1, externalization of glucose transporters, transcription of insulin-dependent enzymes, pancreatic β -cell insulin secretion, and insulin sensitivity of adipose tissue are proposed mechanisms underlying the beneficial effects of glutamine. It would be interesting to assess whether the ratio of glutamine to glutamate is also prospectively associated with the risk of CVD and not only of T2D.

In addition to amino acids, elevated concentrations of short- and medium-chain acylcarnitines have been linked to obesity, insulin resistance, and T2D (62, 78, 79). Acylcarnitine esters facilitate the transport of long-chain FAs across the mitochondrial membrane for β -oxidation (80). Their accumulation may reflect dysregulated FA oxidation, which in turn contributes to metabolic disorders (80). A prospective study of 314 participants reported a positive association between acylcarnitines and the risk of CHD, in which medium-chain acylcarnitines were weakly positively associated with atherosclerosis in both the discovery and replication datasets (74). Dicarboxyl-acylcarnitines were predictive of CVD events in individuals with known atherosclerosis.

Gut microbiota may be associated with metabolic disturbances by increasing energy bioavailability from the diet, modifying host gene expression, and increasing metabolic endotoxemia and inflammation (81–86). For example, altering the composition of the gut flora may reduce the ability to ferment carbohydrates and metabolize bile acids. Bile acids emulsify ingested lipids and facilitate their transport, but a novel function of bile acids suggests that they have a role as metabolic integrators of whole-body energy homeostasis. Wang et al. (85) observed that concentrations of choline, betaine, and trimethylamine *N*-oxide were significantly higher in CVD cases than in controls (total 75 pairs), and were positively correlated with an angiographically assessed atherosclerotic burden. The assessment of these metabolites in the context of a nutritional intervention may greatly contribute to our understanding of the complex relations between nutritional status, genetic variation, metabolomic profiling, and the microbial genome, which may help to explain the successful results of clinical trials of nutritional interventions with whole dietary patterns in contrast with the often negative results of single-nutrient trials (87).

Plasma urea cycle metabolites, such as citrulline and ornithine, have been implicated in impaired glucose tolerance (88, 89). Shah et al. (74) found that urea cycle metabolites (arginine, citrulline, and histidine) were positively associated with CHD events.

Dietary *n*-6 and *n*-3 PUFAs have been consistently associated with lower CVD risk, supporting the role of FA saturation and elongation in the etiology of CHD (90, 91). In the context of the MedDiet, in which the main source of MUFAs is olive oil (2), the ratio of MUFA to SFA intake is associated with a reduced CVD risk (92). It is possible that these FA properties may extend to other lipids that serve as important components of cell membrane phospholipids, energy stores, and signaling molecules. Metabolomics especially has been useful in advancing our knowledge of the role of specific lipid subclasses. In a nested case-control analysis, cholesterol esters, sphingomyelins, lysophosphatidylcholines, phosphatidylcholines, lysophosphatidylethanolamines, and TGs with a lower carbon number and lower double bond content were associated with a higher risk of diabetes, whereas lipids of higher carbon number and higher double bond content were associated with a lower risk (93).

Similar patterns of association have also been observed for other cardiometabolic risk factors, such as obesity and insulin resistance, in cross-sectional metabolomic studies.

Conclusions

Taken together, these observations raise the possibility that alterations in plasma metabolite concentrations may be implicated in the onset and progression of CVD, and could serve as proximal precursors to CVD. However, most studies to date have been small, have not followed a prospective design, and have measured only a limited number of metabolites. A gap in research potentially more critical to our understanding of intervention effects on CVD risk is that, to our knowledge, none of the studies to date have used repeated measures of metabolomic profiles over a follow-up period or before and after the implementation of a dietary or other intervention.

The PREDIMED study was a rigorously conducted primary prevention trial, with excellent compliance, very high follow-up rates, and low drop-out rates (12, 13) The availability of plasma samples not only at baseline, but also at 1 y of follow-up, will allow for the assessment of the long-term effect of the randomized dietary interventions on metabolite profiles. Furthermore, this assessment will permit the evaluation of which metabolic markers are most likely to mediate the effect of the dietary interventions on CVD risk. In collaboration with the Harvard T. H. Chan School of Public Health and the Broad Institute, and funded by an NIH grant, we are addressing important public health issues related to the effectiveness of population-wide approaches to apply dietary interventions in primary cardiovascular prevention. The application of cutting-edge metabolomics technology will allow us to delineate the causal pathways between diet and CVD events, taking advantage of the methodologic strengths of the design of the PREDIMED trial. The approach includes a case-cohort design, including the incident cases of CVD occurring during the PREDIMED trial, and a randomly selected subcohort of 10% of the trial participants. With this approach, the PREDIMED trial may contribute to advancements in this field, which can inform not only future effective interventions, but also key metabolite and pathway targets to prevent the development of CVD.

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- [AQ10] MAM-G and FBH conceived of and designed the study; MAM-G wrote the first draft; MAM-G and MR-C conducted the literature search; and MAM-G, MR-C, AH, LL, AT, and FBH [AQ11] conducted a critical review of the manuscript. All authors read and approved the final manuscript.

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