Plasma Arginine/Asymmetric Dimethylarginine Ratio and Incidence of Cardiovascular Events: A Case-Cohort Study

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ABBREVIATIONS:

ADMA, asymmetric dimethylarginine

ANOVA, analysis of variance

CHD, coronary heart disease

CI, confidence interval

CVD, cardiovascular disease

EVOO, extra virgin olive oil

HR, hazard ratio

MD, Mediterranean diet

NADH, nicotinamide adenine dinucleotide

NADPH, nicotinamide adenine dinucleotide phosphate

NMMA, N-monomethyl arginine

NO, nitric oxide

PREDIMED, PREvención con DIeta MEDiterránea

SD, standard deviation

SDMA, symmetric dimethylarginine

ABSTRACT

CONTEXT: Arginine, its methylated metabolites and other metabolites related to the urea cycle have been independently associated with cardiovascular risk, but the potential causal meaning of these associations (positive for some metabolites and negative for others) remains elusive due to a lack of studies measuring metabolite changes over time.

OBJECTIVE: To examine the association between baseline and 1-year concentrations of urea cycle metabolites and cardiovascular disease in a case-cohort setting.

DESIGN: A case-cohort study was nested within the PREvención con DIeta MEDiterránea (PREDIMED) trial. We used liquid chromatography-tandem mass spectrometry to assess metabolite levels at baseline and after 1-year follow-up. The primary CVD outcome was a composite of myocardial infarction, stroke and cardiovascular death. We used weighted Cox regression models (Barlow weights), to estimate multivariable-adjusted hazard ratios (HR) and their 95% confidence intervals (CI).

SETTING: Multi-center randomized trial in Spain.

PARTICIPANTS: 984 participants accruing 231 events over 4.7 years median follow-up.

MAIN OUTCOME MEASURE: Incident cardiovascular disease.

RESULTS: Among Baseline arginine/asymmetric dimethylarginine ratio (HR per SD=0.80, 95% CI=0.67 - 0.96), and global arginine availability (arginine/(ornithine+ citrulline)) (HR per SD=0.83 (0.69 - 1.00), were significantly associated with lower risk of CVD. We observed no significant association for 1-year changes in these ratios nor any effect modification by the Mediterranean diet intervention.

CONCLUSIONS: A higher baseline arginine/asymmetric dimethylarginine ratio was associated with lower CVD incidence in a high cardiovascular risk population. The intervention with the Mediterranean diet did not change 1-year levels of these metabolites.

INTRODUCTION

Cardiovascular disease (CVD) continues to be a major cause of death in most countries[1]. Development of CVD is associated with changes in many metabolites[2], but which ones predict ischemic events have not been completely characterized. Nitric oxide (NO) is a well-studied molecule known to confer a protective effect on the vasculature system by dilating blood vessels, inhibiting leukocyte adhesion, reducing platelet aggregation, and preventing plaque formation[3]. NO is synthesized via nitric oxide synthase (NOS), which converts 2 O_2 + Arginine + NADPH into citrulline + NADP + 2 NO[4]. This pathway draws arginine from the urea cycle, the details of which have been reviewed elsewhere[5]. Briefly, after arginine is converted to NO and citrulline, arginosuccinate synthetase converts citrulline into argininosuccinate, which is then converted into arginine by arginosuccinate lysase (**Figure 1**). The global bioavailability of arginine has been reported to be associated with lower risk of coronary artery disease and major cardiovascular events[6]. Alternatively, arginine may undergo proteolysis to become asymmetric dimethylarginine (ADMA), which acts as an endogenous competitive inhibitor of NOS and causes local vasoconstriction when infused[7]. ADMA has been proposed as an independent biomarker of endothelial dysfunction and a potential biomarker for higher risk of future CVD events[8]. Consequentially, the arginine/ADMA ratio provides information on arginine bioavailability for production of NO[9] and has been identified as a marker for the severity of chronic heart failure[10], diastolic blood pressure[11], and recently as a predictor for atherosclerosis[12].

A Mediterranean dietary pattern rich in unsaturated vegetable fats and whole grains and low in refined sugars and saturated fats has been shown to improve markers of vascular function[13]. In the PREvención con DIeta MEDiterránea (PREDIMED) trial, being allocated to a Mediterranean diet (MD) supplemented with extra-virgin olive oil (EVOO) or with nuts

significantly decreased the risk of CVD compared to a control group[14]. Furthermore, from the same trial, those consuming the MD experienced reduced 24-hour ambulatory blood pressure[15]. In another smaller randomized trial, a significant increase in the arginine/ADMA ratio was observed in participants assigned to a MD compared to those in a low-fat control diet after 3 months of follow-up[16]. Thus, it can be hypothesized that the beneficial effects of the MD may partially be mediated by changes in the arginine/ADMA ratio.

In the present study, we evaluated 1) the effect of the PREDIMED intervention with a Mediterranean diet on 1-year changes in these metabolites in a random sample of the entire PREDIMED cohort, and 2) the association of both baseline levels and changes in these metabolites on incident clinical events of CVD following a case-cohort design.

METHODS

Study population and design

The PREDIMED trial is a parallel-group, multicenter, randomized trial of dietary interventions of MD supplemented with either nuts or extra-virgin olive oil (EVOO) for the primary prevention of CVD, compared to a low-fat control group (www.predimed.es). The protocol, design, and primary results are detailed elsewhere[15,17]. Briefly, 7,447 eligible men and women (55–80 years old) at high risk of CVD were randomly assigned to a MD supplemented with 1 L/week of extra-virgin olive oil (MD + EVOO), a MD supplemented with 30 g/day mixed nuts (15 g walnuts, 7.5 g hazelnuts, and 7.5 g almonds) (MD + nuts), or a control diet consisting of advice to reduce the intake of all types of fat (control group) from October 2003 to December 2010, a median of 4.7 years for all participants. Medical conditions and lifestyle risk factors were collected using questionnaires at the first screening visit and yearly during follow-up. Weight,

height, waist circumference, and blood pressure were directly measured following a standardized protocol[17] by PREDIMED trained personnel. The primary endpoint was a composite of CVD events, defined as non-fatal stroke, non-fatal myocardial infarction, or death from cardiovascular causes. Stroke events included all non-stroke and cases of stroke-related mortality. Non-stroke events included all non-fatal myocardial infarction and all death from vascular causes not related to stroke. Information on primary endpoints was collected by study physicians, who were blinded to the intervention, and from other sources of information, such as the National Death Index. All participants provided written informed consent.

For the present study, to maintain the integrity of trial randomization, we used a case-cohort design consisting of a random sample of approximately 10% of all PREDIMED participants and all of the incident CVD cases occurring during follow-up through December 1, 2010, who had available plasma samples at baseline. A random selection of 790 participants was included at baseline (to constitute the subcohort), of which 37 individuals experienced major cardiovascular events. Incident CVD cases included 231 individuals who experienced an event during follow-up (total sample size: 790+231-37=984). Among the 231 cases, 118 experienced a stroke (113 ischemic and 5 hemorrhagic). In addition, 926 participants out of the 984 (i.e., 749 controls and 177 cases) also had a 1-year follow-up sample and were included in the 1-year change analyses. Data from the PREDIMED trial and previous clinical trials suggest that changes in plasma biomarkers at 1 year might be sufficient to predict later CVD[15,18-21].

Quantification of Metabolites

Fasting plasma EDTA tubes were collected for all participants, and aliquots were coded and kept refrigerated until they were stored at −80 °C. Pairs of samples (baseline and first-year

visits from each participant) were randomly ordered and shipped to the Broad Institute of Harvard and MIT (Cambridge, MA, USA) for metabolomics analyses. Liquid chromatography tandem mass spectrometry (LC-MS/MS) on a system comprised of a Shimadzu Nexera ×2 U-HPLC (Shimadzu Corp.) coupled to a Q Exactive hybrid quadrupole orbitrap mass spectrometer (Thermo Fisher Scientific) was used to quantitatively profile metabolites such arginine, ornithine, citrulline, asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA), and N-methylarginine (NMMA) in fasting plasma collected at baseline and year 1 of the intervention. Metabolite extracts were precipitated from plasma samples (10 μL) with 9 volumes of 74.9:24.9:0.2 (volume:volume:volume) of acetonitrile:methanol:formic acid–containing stable isotope-labeled internal standards [valine-d8 (Sigma-Aldrich) and phenylalanine-d8 (Cambridge Isotope Laboratories)]. The samples were then centrifuged for 10 min at 9000 × *g* in 4°C, and the supernatant was injected onto a 150 × 2-mm, 3-μm Atlantis HILIC column (Waters). The column was eluted at a flow rate of 250 μL/min with 5% mobile phase A (10 mmol ammonium formate/L and 0.1% formic acid in H_2O) for 0.5 min followed by a linear gradient to 40% mobile phase B (acetonitrile with 0.1% formic acid) for 10 minutes. Quantification of metabolites were carried out with the use of electrospray ionization in positive-ion mode with use of a full-scan analysis over 70–800 *m*/*z* at 70,000 resolution and a 3-Hz data-acquisition rate. Reference standards were used to confirm metabolite identities. Raw data were processed with the use of TraceFinder 3.1 (Thermo Fisher Scientific) and Progenesis CoMet v2.0 (Nonlinear Dynamics)

Statistical Analysis

Rank-based inverse normal transformations were used to transform the non-normal distributions of arginine and ADMA[22]. We used the arginine/ADMA ratio (by dividing the raw values and then taking inverse normal transformations), as previously described in the literature[9,10,12,23], as well as a global arginine availability score (by dividing the raw values of arginine by the sum of ornithine and citrulline) as described by Tang et al.[24] We also assessed associations of CVD with alternate ratios such as arginine/citrulline and other ratios built using symmetric dimethylarginine (SDMA) and NG-monomethylarginine (NMMA) including ADMA/SDMA, and ADMA/NMMA.

Baseline data by case status are presented as means (± standard deviations) for continuous variables and N and percentages for categorical variables. Baseline characteristics were compared between cases and non-cases using chi-squared tests for categorical variables and one-way analysis of variance (ANOVA) for continuous variables.

We used weighted Cox regression models with controls up-weighted by their sampling fraction, and used robust variance to account for correlation between observations[25]. We calculated multivariable-adjusted hazard ratios (HRs) and their 95% confidence intervals (CIs) for the composite CVD endpoint, and also separately for stroke. Follow-up time was calculated from the date of enrollment to the date of diagnosis of CVD for cases, and to the date of the last visit (provided that this visit took place before December 1, 2010) or death for non-cases. In model 1, we adjusted for age, sex, family history of CHD, smoking status, and body mass index (BMI), and stratified by the intervention group. In model 2, we additionally adjusted for baseline hypertension, dyslipidemia, and diabetes. All individual metabolites, the arginine/ADMA ratio and the arginine availability score were also analyzed according to quartiles. Quartile cutpoints were generated on the basis of the distributions of these metabolites among controls in the

subcohort. We conducted tests of linear trend by examining an ordinal score on the basis of the median value in each quartile in the multivariable models.

We conducted joint analyses and interaction tests for the arginine / ADMA ratio and the intervention groups (MD + EVOO and MD + nuts vs control group). We additionally conducted analyses stratified by sex, age (<65 vs. ≥65 yrs old), intervention group, obesity (BMI <30 vs. ≥ 30 kg/m^2), smoking status (current/former vs. never), family history of CHD, baseline type 2 diabetes, baseline hypertension, and baseline dyslipidemia status. We used a likelihood ratio test for models without interaction terms vs. models with interaction terms to assess the significance of interaction between stratifying variables and the arginine/ADMA ratio.

To assess changes in metabolites from baseline to 1 year according to intervention group, we calculated adjusted means stratified by age (<65, ≥65 years old), sex (male, female), and body mass index (<30.0 kg/m^2, ≥30.0 kg/m^2) in the randomly selected subcohort. If no significant changes were observed among intervention groups, we performed our primary analyses in the entire study population (i.e. combining all participants in the three intervention arms). We examined the associations between 1-year changes in the arginine/ADMA ratio and CVD risk by calculating the difference between baseline and 1-year ratio concentrations, then normalized this difference with an inverse normal transformation.

All statistical analyses were performed with SAS (v9.4, SAS Institute, Cary, NC) and R (v2.13.0, R Foundation, Vienna, Austria).

RESULTS

The flow diagram of the present study is depicted in **Supplemental Figure 1**. Among the 7,447 participants recruited in the PREDIMED trial, 288 experienced a CVD before December

1st, 2010. Out of the 288 cases, 231 had available plasma samples at baseline and 177 had available plasma samples *both at baseline and* after 1-year of follow-up. The present study population consisted of 790 participants randomly selected to the subcohort at baseline and the 231 cases from the entire trial (37 of whom were in the subcohort), for a total of 984 participants at baseline with a median follow-up time of 4.7 years.

Descriptive statistics of the study population at baseline by subcohort and case status are presented in **Table 1.** Cases tended to be older, male, not dyslipidemic, diabetic, and more likely to smoke compared to the randomly chosen subcohort.

Figure 2 depicts a heat map of correlation coefficients of the metabolites included in the present study. Metabolites of the urea cycle (arginine, citrulline, and ornithine) were correlated with each other, with r=0.30 to 0.50 for each pair of metabolites ($p<0.0001$ for all correlations) Arginine metabolites were poorly correlated with citrulline, but moderately correlated with ornithine and arginine. ADMA, SDMA, and NMMA were strongly correlated ($p<0.0001$ for all correlations) with each other, with r=0.60 to 0.80 for each pair of metabolites.

Association of individual metabolites and CVD risk

Table 2 presents the associations of both baseline and 1-year changes in urea cycle metabolites (arginine, ornithine, and citrulline) with incident composite CVD. A 1-SD increase in baseline arginine was associated with a decreased risk of CVD in model 1 (HR=0.84 (95% CI: 0.71–0.99)), but the association was attenuated after adjusting for baseline hypertension, dyslipidemia, and diabetes (HR=0.86 (0.72–1.02)). Compared to the bottom quartile, higher quartiles of baseline arginine appeared to be associated with lower CVD risk, but the test for linear trend was not significant in the fully adjusted model (p for trend=0.17). Neither baseline

citrulline nor baseline ornithine levels were significantly associated with CVD risk in either continuous or quartile analyses.

When examining 1-year changes in the levels of these metabolites, increasing quartiles of citrulline were significantly associated with lower risk of CVD in the fully adjusted model (p for trend=0.03). One-year changes of arginine as a continuous variable exhibited a similar trend for the association with CVD (HR=0.87 (0.73–1.04)).

Table 3 presents the associations of baseline and 1-year changes in arginine metabolites (ADMA, SDMA, NMMA) with CVD. None of them was significantly associated with incident CVD.

Supplemental Figures 2 and 3 depict adjusted mean metabolite changes according to intervention group after 1 year of follow-up. No significant differences were noted for changes in levels of any metabolite between intervention groups (p=0.32 for arginine, p=0.09 for ornithine, p=0.18 for citrulline, p=0.77 for ADMA, p=0.86 for SDMA, p=0.60 for NMMA). Therefore, the intervention had no significant effect on changes in these metabolites or their ratios.

Association of the arginine/ADMA ratio with cardiovascular disease

Associations of the baseline arginine/ADMA ratio and arginine availability score with CVD risk are presented in **Table 4**. A 1-SD increase in baseline arginine/ADMA ratio was associated with 20% lower risk of CVD (HR=0.80 (0.67–0.96)) in the fully adjusted model. Quartile analysis revealed independent inverse associations of quartiles 2 (HR=0.55 (0.35–0.86)) and 3 (HR=0.56 (0.36–0.88)) versus quartile 1, with risk of CVD, but the test of linear trend was not significant. Similarly, the global arginine availability score was associated with lower risk of CVD. However, this association was close to the limit of statistical significance (p=0.048). A 1-

SD increase in the score was associated with a HR=0.83 (0.69–0.999). None of the 1-year changes in these traits was associated with incident CVD risk. These results did not appreciably differ when examining stroke or non-stroke events as separate endpoints (data not shown). Subgroup analyses (**Supplemental Table 1**) indicated no statistically significant interaction by variables of interest in the relationship between arginine/ADMA ratio and CVD risk.

Exploratory analyses involving alternate ratios (ADMA/SDMA ratio, ADMA/NMMA ratio, and arginine/citrulline ratio) are presented in **Supplemental Table 2**. Baseline ADMA/NMMA ratio was positively associated with incident CVD as a continuous variable (HR=1.19 (1.01 – 1.39) per 1 SD). None of the 1-year changes in alternative ratios were significantly associated with CVD. We proceeded with further analysis of the arginine/ADMA ratio in light of its stronger observed inverse association and the better established biological plausibility for the association of this ratio with atherosclerosis[7-12].

Effects of dietary interventions on arginine/ADMA ratio and arginine in incident CVD

Supplemental Figure 4 depicts the hazard ratios of arginine/ADMA ratio stratified by intervention group. The MD+EVOO and the MD+nuts groups were combined to increase statistical power. Using participants in the lowest baseline arginine/ADMA ratio quartile who were randomized to a MD intervention as the reference group, those in quartiles 2-4 had a non-significant lower risk of CVD. Participants in the lowest quartile randomized to the control diet had a significantly higher risk than the reference group, presumably due to the differences due to the intervention, while those in quartiles 2-4 had a comparatively lower risk, signifying no strong effect modification by the dietary intervention. **Supplemental Figure 5** uses 1-year changes in

arginine/ADMA ratio instead of baseline values. We observed no significant associations or effect modification when stratifying by dietary intervention.

DISCUSSION

In the present case-cohort study in the framework of the PREDIMED trial, we observed an inverse association between both the baseline arginine/ADMA ratio, as well as the baseline global arginine bioavailability ratio proposed by Tang et al.[24] with incident CVD. However, we found no significant associations of changes in these ratios with incident CVD after 1 year of follow-up. Although changes in plasma metabolites may not become apparent in some participants within 1 year, several short-term randomized trials of arginine supplementation demonstrate significant differences between treatment arms within 6 months[19-21]. To our knowledge, no prior studies have assessed changes in urea cycle or methylarginine metabolite concentrations in relation with the incidence of primary clinical events of CVD.

Our results are in line with previous studies investigating arginine and ADMA and their association with CVD. A study from the Framingham Offspring cohort reported a significantly lower risk of CVD associated with the arginine/ADMA ratio adjusted for other established CVD risk factors and biomarkers[26]. A recent meta-analysis of 22 prospective cohort studies with a mean follow-up time of 7.1 years reported a robust association between ADMA concentrations and higher risk of subsequent CVD events[27], with individuals in the highest ADMA tertile experiencing a ~40% increased CVD risk compared to those in the lowest tertile. High ADMA in concert with low NO has been reported to propagate a variety of harmful detrimental processes biologically related to atherosclerosis, including free radical generation, smooth cell proliferation, systemic inflammation, and endothelial dysfunction[8].

Regarding primary urea cycle metabolites, we found a significant inverse relationship for 1-year changes in citrulline and vascular events but not for ornithine nor arginine (although we note that arginine was borderline significant). Arginine has been suggested as a molecule of therapeutic interest for its role as the precursor to NO. L-arginine (and only L-arginine) is the required substrate for all isoforms of the enzyme nitric oxide synthase (NOS) to produce NO. NO is acknowledged as a powerful short-life vasodilator with an important defensive role against ischemic disease through endothelial smooth muscle relaxation[28]. In rodent models, chronic arginine treatment alleviates metabolic and cardiovascular complications in obese rats[29], and protects against oxidative stress and improves myocardial energetics in hypoxic rat hearts[30]. Experimental studies aimed to increase the bioavailability of arginine have reported promising results. Inhibition of arginase, a competitive inhibitor of arginine, improves vascular integrity, and protects against ischemia-induced injury[31]. In recent randomized supplementation trials, patients receiving arginine experienced improvements in anthropometric and biochemical indices associated with later CVD[32], and alleviations in postprandial endothelial dysfunction[33]. Few studies have been published regarding the role of citrulline in the pathophysiology of heart disease, but it is known that citrulline is a product of NO synthesis and can be reconverted back to arginine in the kidneys to increase arginine bioavailability[34,35]. Joint therapy of both arginine and citrulline has also been recommended, as arginine supplementation alone appears to increase arginase expression and reduce bioavailability of arginine, whereas citrulline supplementation does not[34]. In addition to the arginine pathway, it has been reported that citrulline has anti-inflammatory properties and may exert beneficial pleiotropic effects on various impaired functions due to aging[36]. Thus, future investigations of citrulline as a therapeutic agent for arginine deficient diseases are warranted.

We did not observe significant effect modification by a dietary intervention on the association between the arginine/ADMA ratio and incident CVD. Previous studies have hypothesized that the cardioprotective benefit of the MD, particularly when supplemented with nuts, is in part mediated by arginine intake[37-39]. This hypothesis is biologically plausible and it is in agreement with our results for non-stroke events. Dietary sources of arginine consist mainly of meat, poultry, fish, dairy, and nuts[40], and higher intake of some of these foods, especially nuts and fish, have been found to be associated with subsequently reduced CVD risk[41,42]. Studies of dietary arginine intake have been inconclusive. In a study of elderly men, Oomen et al. reported a similar nonsignificant inverse association with coronary heart disease mortality[43]. More recently, Bahadoran et al. concluded that plant-derived arginine conferred a benefit for CHD events and blood pressure, whereas animal-derived arginine was a risk factor for these outcomes[44]. Given the emerging evidence of arginine as a cardioprotective molecule, it will be important for future epidemiologic and clinical studies to assess the potential causal link between arginine and CVD.

We note several strengths of the present study. Our cohort assessed the incidence of major CVD clinical events as the primary endpoint, as well as stroke and non-stroke cases as secondary endpoints. The prospective assessment of urea cycle and methylarginine metabolites, as well as the adjustment for hypothesized confounders measured among individuals under close follow-up within a randomized trial, adds to the potential causal interpretation of our findings. Limitations of the present study also warrant discussion. First, residual confounding by other unknown or unmeasured variables cannot be ruled out. Second, individuals who donated blood at baseline but not at 1 year of follow-up may have been systematically different from those who donated at both time points. Third, we were assuming a determined induction period of 1-year,

and perhaps a 1-year follow-up is not a sufficiently long period to observe an effect of the dietary pattern on the metabolites tested. Fourth, the PREDIMED trial consisted of high-risk participants living in the Mediterranean region, and our findings may not be generalizable to populations with different demographic distributions or exposure ranges.

CONCLUSION

Results from a case-cohort study nested within the PREDIMED trial indicated that baseline arginine / ADMA ratio, as well as global arginine availability, were associated with lower risk of future CVD events. One-year changes in citrulline were inversely associated with subsequent CVD incidence, but neither arginine / ADMA ratio nor global arginine availability were significantly associated with CVD outcomes.

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Figure 1. Diagram of the urea cycle, nitric oxide synthesis, and degradation of arginine

Figure 1 Sub-Caption: NO, Nitric oxide; NOS, Nitric oxide synthetase; NMMA, N-monomethylarginine; ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine

Figure 2. Correlation coefficient heatmap of metabolites under study. Abbreviations: ADMA, asymmetric dimethylarginine; NMMA, N-methylarginine; SDMA, symmetric dimethylarginine.

Figure 2 Sub-Caption: All correlation coefficients had p-values <0.0001.