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Circulating citric acid cycle metabolites and risk of cardiovascular disease in the PREDIMED study

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Abstract *Background and aim:* Plasma citric acid cycle (CAC) metabolites might be likely related to cardiovascular disease (CVD). However, studies assessing the longitudinal associations between circulating CAC-related metabolites and CVD risk are lacking. The aim of this study was to evaluate the association of baseline and 1-year levels of plasma CAC-related metabolites with CVD incidence (a composite of myocardial infarction, stroke or cardiovascular death), and their interaction with Mediterranean diet interventions.
Methods and results: Case-cohort study from the PREDIMED trial involving participants aged 55–80 years at high cardiovascular risk, allocated to MedDiets or control diet. A subcohort of 791 participants was selected at baseline, and a total of 231 cases were identified after a median follow-up of 4.8 years. Nine plasma CAC-related metabolites (pyruvate, lactate, citrate, aconitate, isocitrate, 2-hydroxyglutarate, fumarate, malate and succinate) were measured using liquid chromatography-tandem mass spectrometry. Weighted Cox multiple regression was used to

Abbreviations: CVD, Cardiovascular disease; TCA, Tricarboxylic acid cycle; CAC, Citric Acid Cycle; MedDiet, Mediterranean Diet; HR, Hazard Ratio; CI, Confidence Interval; SD, Standard deviation; FDR, False discovery rate; HFM, 2-hydroxyglutarate (H), fumarate (F) and malate (M); LC-MS, Liquid chromatography-tandem mass spectrometry; MI, Myocardial infarction; PREDIMED, PREvención con DIeta MEDiterránea.

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calculate hazard ratios (HRs). Baseline fasting plasma levels of 3 metabolites were associated with higher CVD risk, with HRs (for each standard deviation, 1-SD) of 1.46 (95%CI:1.20−1.78) for 2-hydroxyglutarate, 1.33 (95%CI:1.12−1.58) for fumarate and 1.47 (95%CI:1.21−1.78) for malate (p of linear trend <0.001 for all). A higher risk of CVD was also found for a 1-SD increment of a combined score of these 3 metabolites (HR = 1.60; 95%CI: 1.32−1.94, p trend <0.001). This result was replicated using plasma measurements after one-year. No interactions were detected with the nutritional intervention.

Conclusion: Plasma 2-hydroxyglutarate, fumarate and malate levels were prospectively associated with increased cardiovascular risk.

Clinical trial number: ISRCTN35739639

1. Introduction

Cardiovascular diseases (CVDs) are the main cause of death worldwide, being most of them myocardial infarction (MI) or stroke (http://www.who.int) [1]. An increased number of biomarkers derived from genomics, epigenetics, imaging and clinical biochemistry (inflammation-, thrombosis- and lipid-related) are being proposed as predictors of diverse cardiovascular outcomes. In this multiomic context, the study of the circulating metabolome (complete set of plasma small molecules) has been proposed as one of the most promising strategies for the early identification of biomarkers of cardiovascular diseases [2].

The mitochondrial citric acid cycle (CAC), also named tricarboxylic acid cycle (TCA) or Krebs cycle, is critical in ATP production, and it is a central metabolic hub connecting a web of multiple catabolic, anabolic and anaplerotic reactions. CAC-related metabolites also participate in a diverse set of physiological processes such as signaling molecules, epigenetic effectors and modulators of immune and hypoxic responses [3]. In myocardial ischemia and heart damage, lack of oxygen profoundly alters mitochondrial CAC flux in the heart muscle, reducing ATP production leading to intracellular accumulation of CAC intermediates [3−7]. Interestingly, CAC intermediates and related metabolites are also measurable in plasma, where they represent sensitive biomarkers of nutritional challenges such as glucose loads [8] or are associated with metabolic diseases [9,10]. Moreover, circulating CAC-related metabolites have been associated with the development of a variety of cardiovascular adverse events in both humans and animal models [2,5,6,11−26]. It is possible that plasma changes of CAC-related metabolites represent causes of increased CVD risk connected to pathophysiological actions, or alternatively a consequence of an cardiac damage. In any case, it is relevant to test whether such circulating metabolites may behave as biomarkers in the prediction of future CVD events. We prospectively evaluated the association between baseline and 1-year changes in plasma CAC-related metabolites with CVD incidence in the PREDIMED trial metabolomics project (www.predimed.es) [27], in which several multi-metabolite scores have been previously developed [28−32]. We also tested the potential interaction between CAC-related metabolites and Mediterranean diet (MedDiet) interventions on the development of CVD.

2. Methods

2.1. Study design, participants and clinical assessment

The present research is a case-cohort study nested within the PREDIMED study (ISRCTN35739639). In brief, the PREDIMED study was a cardiovascular primary intervention trial conducted from 2003 to 2010 involving a large sample of participants (7447 at baseline) aged 55−80 years (57% women) with high CVD risk, but without previous diagnosis of CVD [27,33]. At the beginning of the trial, participants were allocated into three intervention arms and followed for a median of 4.8 years to monitor CVD incidence: two Mediterranean diets (enriched with either nuts or extra-virgin olive oil) and a control diet advising participants to adhere to a low-fat. The primary end point of the PREDIMED trial was a composite CVD event of nonfatal myocardial infarction, nonfatal stroke, and death from cardiovascular causes. A case-cohort study was designed within the PREDIMED study to assess the association between plasma metabolites and the risk of CVD, as the primary outcome of the PREDIMED study [31]. In the present case-cohort study, 288 CVD cases occurred during follow-up were selected together with a random subcohort of 794 PREDIMED participants at baseline (10.7% of the PREDIMED study participants). Among CVD cases, n = 57 did not have available plasma samples at baseline, leaving a sample of n = 231 CVD cases. The final sample size of the subcohort was n = 791 after discarding 3 samples with no available valid measurements of CAC-related metabolites. The subcohort included 37 overlapping CVD cases (also included in the group of cases as part of the case-cohort selection) and n = 754 non-cases. Then, the total number of participants were 231 + 754 = 985. Among the n = 231 CVD cases, 117 had a non-fatal stroke and 78 non-fatal myocardial infarction (MI). After one-year of follow-up, n = 907 participants (n = 776 in the subcohort) also had

available measurements of CAC-related metabolites in plasma (Figs. S1 and S2). Additional details on the design, sample size, inclusion/exclusion criteria and analysis of the case-cohort study involving metabolomics within PREDIMED study are available in previous articles [29,31].

Study physicians collected information on CVD outcomes based on continuous contact with participants, comprehensive yearly review of medical records of all participants (blinded to intervention), and periodic consultation of the National Death Index [27]. This anonymized information was submitted to the Clinical Endpoint Committee, which adjudicated the events blinded to the intervention group. The present study was approved by the research ethics committees at all study locations, and participants provided written informed consent.

2.2. Plasma metabolomic profiling and biochemical determinations

Both at baseline and after 1-year, blood samples were collected in study participants using EDTA-containing tubes after >8 h of fasting. Blood samples were processed within 2 h after extraction and used to separate plasma aliquots that were stored at −80 °C. Matched samples (baseline and one-year after) were randomly allocated before being shipped on dry ice to the Broad Institute (Boston, MA, USA) for metabolomic analysis. Pooled plasma samples and reference samples were inserted every 20 samples. Liquid Chromatography-Mass Spectrometry (LC-MS) was used for quantitative profiling. The system is composed by a Shimadzu Nexera X2 U-HPLC (Shimadzu Corp.; Marlborough, MA) coupled to a Q Exactive hybrid quadrupole orbitrap mass spectrometer (Thermo Fisher Scientific; Waltham, MA). Raw data were processed using MultiQuant software (AB SCIEX) to integrate chromatographic peaks [10,34].

We used plasma levels of a panel of 9 CAC-related metabolites: 6 CAC-intermediates (citrate, aconitate, isocitrate, fumarate, malate and succinate), 1 CAC-related metabolite (D/L enantiomers of 2-hydroxyglutarate, connected with α-ketoglutarate) and 2 metabolites of the glycolytic pathway closely linked to the CAC (pyruvate and lactate) (Fig. S3; see http://www.hmdb.ca/). The intra-assay coefficient of variation (CV) of individual plasma metabolite determinations remained low (<3%) for most metabolites, ranging from 0.9% (malate) to 2.9% (lactate), with the exception of pyruvate (CV = 11.5%) [10].

2.3. Statistical analysis

Individual metabolites were normalized and scaled to 1 standard deviation (SD) using rank-based inverse normal transformations. Weighted proportional hazards Cox regression models using Barlow weights [10] were used to estimate hazard ratios (HRs) and their 95% confidence intervals (CIs). Follow-up time was calculated from the date of enrollment to the date of CVD diagnosis for cases, and to the date of the last visit or the end of the follow-up period (December 1, 2010) for non-cases. All regression models were stratified by recruitment center and adjusted for age, sex, intervention group, smoking status (never/current/former), body mass index (kg/m^2), leisure-time physical activity [metabolic equivalent tasks (METs) min/day], dyslipidemia, hypertension, diabetes, and family history of premature coronary heart disease (yes/no). We carried out all analyses using robust variance estimators to account for intra-cluster correlation. The same multivariate models were used to assess the association between either 1-year plasma metabolite levels or changes in individual metabolites with CVD risk (using as outcome only cases of CVD occurring after 1-year follow-up).

Multimetabolite scores were created by adding plasma levels of metabolites using weights derived from their respective coefficients obtained in multivariable Cox regression models. To generate weights, we used the leave-one-out cross-validation approach to avoid overfitting [10]. For this purpose, Cox regression models were applied to all-but-one observations, and the estimated regression coefficient was used as the weight applied to the left-out observation in order to calculate the multimetabolite score for this particular observation.

To assess effect modification (interaction), Cox regression analyses were stratified by intervention group (either using three interventions as categories, or in 2 categories, i.e. both MedDiet groups merged together vs. the control group). The likelihood ratio test was used to assess the significance of the 1-degree of freedom interaction product-term (effect modification in multiplicative scale). When appropriate, p-values were corrected for multiple comparisons using the Simes procedure.

The Framingham Heart Study (FHS) multivariable risk function was also applied to PREDIMED participants at baseline to calculate 10-year risk for CVD [35]. Equations were originally developed for individuals 30−74 years old and without CVD at the baseline. Predictions from HFS equations were converted into a CVD risk score through logarithmic transformation and scaled to mean = 0 and SD = 1. Multivariate logistic models with CVD as outcome, together with Receiver Operating Characteristic (ROC) curve analyses, were used to assess the performance of predictions containing only the FHS risk score versus the FHS risk score plus the multi-metabolite score.

All statistical analyses were carried out using Stata 17 (Stata Corp; www.stata.com), at a two-tailed α of 0.05.

3. Results

Table 1, Fig. S1 and, Fig. S2 show sample size, distribution and characteristics of participants, including total CVD cases (n = 231), and subcohort (n = 791), the latter including overlapping cases (n = 37) and non-cases (n = 754). Table 2 shows HR (95%CI) for the association between plasma metabolites and CVD, both by 1-SD increase and by groups defined by quartiles. Baseline fasting plasma levels of 2-hydroxyglutarate, with HR = 1.46 (95%CI: 1.20−1.78) for each 1 standard deviation (1-SD) increase, fumarate, 1.33 (95%CI: 1.12−1.58), and malate, 1.47 (95%CI: 1.21−1.78), were the only metabolites achieving statistical significance for

Table 1 Baseline participant characteristics in the random subcohort and cardiovascular disease (CVD) cases in the case-cohort PREDIMED study.

	Subcohort[a]	CVD Cases	*P*-value
n	791	231	
Age (years)	67.2 (5.9)	69.5 (6.5)	<0.001
Sex (% women),	57.0	39.4	<0.001
Intervention group, %			
MedDiet + EVOO	37.2	35.5	
MedDiet + nuts	33.1	28.1	
Control	29.7	36.4	
Hypertension, %	83.6	82.7	
Diabetes %	47.1	64.6	<0.001
Dyslipidemia, %	73.6	58.4	<0.001
Smoking, %			
Never	62.2	45.0	<0.001
Former	25.4	35.1	
Current	12.4	19.9	
Waist circumference, cm	99.9 (10.0)	101.8 (10.8)	
Body mass index, kg/m^2	29.8 (3.6)	29.6 (3.7)	
Physical activity, METs/d	258 (258)	238 (237)	
Education, %			
Elementary or lower	76.2	80.5	
Secondary or higher	23.8	19.5	
Total energy intake, kcal/d	2336 (615)	2369 (686)	
Mediterranean diet score[b]	8.8 (1.9)	8.4 (1.8)	<0.05
Family history of CHD	25.0	19.1	<0.001

EVOO, Extra-virgin olive oil; MET, metabolic equivalent. Values are mean (SD) or percentage.
To avoid problems derived from the existence of 37 cases being included both in the subcohort and cases, we compared the observed means (or proportions) in the cases vs the expected values (expected = mean or proportion observed in the subcohort).
[a] The randomly selected subcohort included 37 CVD cases.
[b] This score is based on the 14-item PREDIMED screener of adherence to the Mediterranean Diet.

increased CVD risk ($p < 0.001$ for linear trend for all). Analysis of groups defined by quartiles revealed significantly increased risk in the upper quartile for these 3 metabolites as compared to the first-quartile.

A global CAC multimetabolite score yielded a significant increased risk for CVD (HR = 1.29; 95%CI: 1.08−1.53 for each SD; p = 0.004). Metabolites 2-hydroxyglutarate (or H), fumarate (or F) and malate (or M) were collectively termed herein as HFM. The multimetabolite score built with such three metabolites (HFM score) exhibited a higher estimate (HR = 1.60; 95% CI: 1.32−1.94 for each SD) and HR_{Q4vsQ1} = 2.63 (95% CI: 1.11−4.58) for the comparison of extreme quartiles ($p < 0.001$ for linear trend). Additional statistical adjustments were carried out controlling the models for previously published multi-metabolite risk scores for CVD, such as arginine/asymmetric dimethylarginine ratio [28], acylcarnitines [29], ceramides [30], branched-chain amino acids [31], as well as two scores based on complex circulating lipids [32]. Adding such six scores as covariates to multivariate models yielded HR for 1-SD increment of HFM score in relation to CVD of 1.52 (95% CI: 1.23−1.89). Statistical adjustment by medication use include: antihypertensive agents (ACE-inhibitors, diuretics, calcium channel blockers, and other hypertensive agents), statins, hypoglycemic agents and anti-platelet agents. Adding such four medication use variables to multivariate models yielded HR for 1-SD increment of HFM score in relation to CVD of 1.58 (95% CI: 1.29−1.93).

Table 3 shows HRs estimated for HFM metabolites stratified by MedDiet intervention in relation to CVD risk. No statistically significant interaction between metabolite levels and the dietary intervention (categorized either as the three diets, or merging the two MedDiet groups) was found. Likewise, no significant interaction between baseline HFM score metabolites with type 2 diabetes or family history of CVD was detected.

Multivariate logistic models showed, as expected, that the FHS score significantly predicted a higher CVD risk, with an odds ratio (OR) of 1.82 (95%CI: 1.30−2.55) for each 1-SD increase (p = 0.007 for linear trend) and OR_{Q4vsQ1} = 5.02 (95%CI: 1.98−12.75) for CVD (Table S1). The Area Under the ROC curve (AUC-ROC) for the multivariate logistic model containing only the FHS risk score was 0.80 (95%CI: 0.77−0.84). In contrast, the AUC-ROC for the multivariate logistic model containing FHS predictions plus the multimetabolite score of 3 metabolites (HMF) was 0.82 (95%CI: 0.79−0.85). Testing equality of AUCs revealed that both curves were significantly different (p-value = 0.022) (Fig. 1).

Table 4 shows HR by 1-SD increase of each metabolite for non-fatal stroke and non-fatal MI. Only baseline plasma 2-hydroxyglutarate, fumarate and malate achieved nominal statistical significance for their association with both stroke and non-fatal MI. For stroke, only HFM metabolites still remained significant after correcting for multiple testing (p-values of 0.006, 0.006 and 0.007 for H, F and M respectively). A nominal significant association of low magnitude was also found for plasma pyruvate (HR = 1.35; 95%CI = 1.01−1.82), although such significance disappeared after correcting for multiple testing. HRs for 1-SD increment of HFM metabolite score in relation to non-fatal stroke was 1.77 (95%CI: 1.36−2.32). For the non-fatal MI outcome, no metabolites remained significant after correction for multiple comparisons, although nominal significant associations were again found for plasma 2-hydroxyglutarate, fumarate and malate (Table 4). The HR for 1-SD HFM score in relation to nonfatal MI was 1.54 (95%CI: 1.10−2.16). No significant interactions between HFM metabolite scores and diet interventions were detected in relation to nonfatal stroke or nonfatal MI (data not shown).

Using data from 1-year after baseline, and after discarding CVD events (n = 16) occurring during the first year, we found a nominal significant association between plasma levels of 2-hydroxyglutarate, malate, fumarate and succinate with CVD in the same direction that those estimated with baseline levels. However, such statistical significance was lost and remained only marginal (p-values from 0.05 to 0.08) after correction for multiple testing (Table S2). Multimetabolite HFM scores calculated from data obtained 1-year after baseline remained significantly associated with a higher CVD incidence, with HRs

Table 2 Hazard ratios for incident cardiovascular disease (CVD) by baseline plasma levels of citric acid cycle (CAC)-related metabolites in the PREDIMED trial (2003–2010).

					Hazard Ratio[b] (95% CI)			
	N[a]	CVD cases	Hazard Ratio[b] per 1 SD increment[c] (95% CI)	*P*-value[d]	Q1	Q2	Q3	Q4
Pyruvate	727	155	1.08 (0.85–1.36)	0.264	Ref.	0.97 (0.54–1.76)	2.33 (1.44–3.75)	1.23 (0.67–2.29)
Lactate	985	231	1.13 (0.94–1.36)	0.543	Ref.	1.05 (0.60–1.82)	1.27 (0.73–2.20)	1.33 (0.81–2.18)
Citrate	985	231	1.14 (0.95–1.36)	0.264	Ref.	0.92 (0.56–1.50)	0.78 (0.47–1.29)	1.34 (0.84–2.12)
Aconitate	985	231	1.11 (0.94–1.31)	0.264	Ref.	1.11 (0.67–1.85)	1.42 (0.88–2.30)	1.17 (0.73–1.88)
Isocitrate	985	231	1.06 (0.88–1.28)	0.543	Ref.	1.14 (0.68–1.92)	0.86 (0.52–1.43)	1.21 (0.74–1.98)
2-Hydroxyglutarate (H)	985	231	1.46 (1.20–1.78)	0.001	Ref.	1.02 (0.57–1.80)	1.75 (1.00–3.06)	1.93 (1.10–3.38)
Fumarate (F)	985	231	1.33 (1.12–1.58)	0.004	Ref.	0.70 (0.39–1.27)	1.40 (0.83–2.36)	2.09 (1.29–3.38)
Malate (M)	985	231	1.47 (1.21–1.78)	0.001	Ref.	1.33 (0.77–2.29)	1.94 (1.13–3.33)	2.20 (1.29–3.75)
Succinate	985	231	1.16 (0.98–1.38)	0.211	Ref.	1.51 (0.89–2.59)	1.47 (0.85–2.53)	1.66 (0.98–2.83)
CAC metabolite score[e]	985	231	1.29 (1.08–1.53)	0.004	Ref.	1.41 (0.80–2.46)	2.06 (1.22–3.48)	1.84 (1.08–3.14)
HFM metabolite score	985	231	1.60 (1.32–1.94)	<0.001	Ref.	1.29 (0.70–2.38)	2.17 (1.21–3.88)	2.63 (1.51–4.58)

SD: Standard Deviation. CI: Confidence interval.

[a] N refers to the total number of independent participants in the analysis resulting from summing subjects of the subcohort (n = 791) plus cases (n = 231 for most metabolites, except pyruvate), and subtracting n = 37 overlapping cases resulting from case-cohort selection procedure.

[b] Weighted proportional hazards Cox regression models using Barlow weights, adjusted for age (years), sex (male, female), intervention group (MedDiet + EVOO, MedDiet + nuts), body mass index (kg/m^2), smoking (never, current, former), leisure-time physical activity (metabolic equivalent tasks in minutes/day), dyslipidemia, hypertension, diabetes, and family history of premature CHD, as well as with robust standard errors to account for intracluster.

[c] A rank-based inverse normal transformation was applied to raw values of plasma metabolite levels.

[d] False discovery rate (FDR)-corrected p-value.

[e] Weighted metabolite scores were generated using plasma levels of CAC intermediates plus 2-hydroxyglutarate, and Cox regression coefficients as weights, and applying the leave-one-out cross-validation procedure.

Table 3 Incident cardiovascular disease (CVD) by baseline plasma levels of selected Citric Acid Cycle (CAC)-related metabolites stratified by mediterranean diet interventions in the PREDIMED trial, 2003–2010.

	Hazard Ratio per 1-SD increment (95%CI)[a,b] Control diet	*P*-value	Hazard Ratio per 1-SD increment (95%CI)[a,b] MedDiet + EVOO	*P*-value	Hazard Ratio per 1-SD increment (95%CI)[a,b] MedDiet + nuts	*P*-value	*P*-value interaction	*P*-value interaction[c]
2-Hydroxyglutarate (H)	1.60 (1.14–2.25)	0.006	1.37 (0.95–1.99)	0.092	1.49 (1.08–2.07)	0.016	0.297	0.693
Fumarate (F)	1.51 (1.14–1.99)	0.004	1.35 (1.04–1.76)	0.023	1.51 (1.08–2.10)	0.016	0.913	0.972
Malate (M)	1.58 (1.16–2.16)	0.004	1.36 (1.00–1.84)	0.051	1.68 (1.21–2.34)	0.002	0.688	0.795
HFM metabolite score[d]	1.72 (1.25–2.36)	0.001	1.58 (1.16–2.17)	0.004	1.78 (1.29–2.46)	0.001	0.836	0.624

Abbreviations: SD: Standard Deviation. CI: Confidence interval.

[a] Adjusted for age (years), sex (male, female), intervention group (MedDiet + EVOO, MedDiet + nuts), body mass index (kg/m2), smoking (never, current, former), leisure-time physical activity (metabolic equivalent tasks in minutes/day), dyslipidemia, hypertension, diabetes, and family history of premature CHD, as well as with robust standard errors to account for intracluster correlations.

[b] A rank-based inverse normal transformation was applied to raw values of metabolites.

[c] MedDiet + EVOO/MedDiet + nuts together.

[d] A weighted metabolite score was generated using Cox regression coefficients and applying the leave-one-out cross-validation procedure.

(expressed as per 1-SD increase) of 1.46 (95%CI: 1.15–1.84; p < 0.001 for linear trend) (Table S2). Additional adjustment by published multimetabolite scores [28–32] or medication use did not modify substantially HRs for HFM score in relation to CVD risk using metabolites after 1-year of follow-up (Table S2). Table S3 shows medication use (both basal and after 1-year) of participants of the subcohort by quartiles of the HFM score.

Changes in individual plasma metabolite levels from baseline to 1-year were not significantly associated with CVD incidence, after adjustment for relevant covariates in Cox regression models, and without detecting any interaction with the dietary interventions (data not shown). Linear regression analysis showed that plasma levels of metabolites at baseline were the only significant determinant of their own plasma levels after 1-year, after multivariable-adjustment. Fig. 2 shows adjusted mean values (and 95% CIs) for 2-hydroxyglutarate, fumarate and malate at baseline and after 1 year of follow-up, by intervention group. A decrease in the levels of these metabolites was only observed in the MedDiet + EVOO group, while an increase was observed in the other two arms of the trial. No significant interactions were observed between baseline levels and dietary interventions on plasma metabolite levels at 1-year (Fig. 2 and S4).

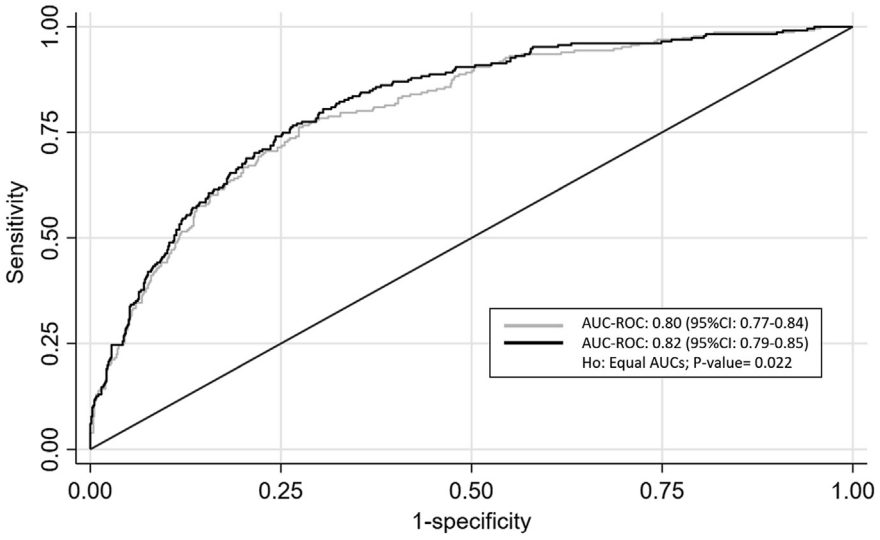

Figure 1 Receiver Operating Characteristic curve analysis comparing multivariate logistic predictions for CVD in PREDIMED participants using a model including covariates, FHS risk score and HFM metabolite score (black curve) versus a model containing only covariates and FSH risk score (grey curve). Multivariate logistic models were adjusted for age (years), sex (male, female), intervention group (MedDiet + EVOO, MedDiet + nuts), body mass index (kg/m2), smoking (never, current, former), leisure-time physical activity (metabolic equivalent tasks in minutes/day), dyslipidemia, hypertension, diabetes, family history of CHD and study center.

Table 4 Hazard ratios for incident stroke and non-fatal myocardial infarction (MI) by baseline plasma levels of Citric Acid Cycle (CAC)-related metabolites in the PREDIMED trial (2003–2010).

	N[a]	Nonfatal Stroke cases	Hazard Ratio [b] per 1 SD increment[c] (95% CI)	N[a]	Non-fatal MI cases	Hazard Ratio [b] per 1 SD increment[c] (95% CI)
Pyruvate	648	117	1.35 (1.01–1.82)	627	78	0.73 (0.48–1.12)
Lactate	871	117	1.29 (0.97–1.70)	832	78	0.98 (0.73–1.32)
Citrate	871	117	1.13 (0.85–1.50)	832	78	1.35 (0.99–1.84)
Aconitate	871	117	1.20 (0.93–1.55)	832	78	1.13 (0.87–1.46)
Isocitrate	871	117	1.33 (1.01–1.76)	832	78	0.87 (0.63–1.20)
2-Hydroxyglutarate (H)	871	117	1.60 (1.20–2.14)	832	78	1.40 (1.04–1.89)
Fumarate (F)	871	117	1.48 (1.17–2.14)	832	78	1.44 (1.07–1.95)
Malate (M)	871	117	1.57 (1.18–2.08)	832	78	1.58 (1.12–2.23)
Succinate	871	117	1.24 (0.98–1.56)	832	78	1.08 (0.81–1.45)
CAC metabolite score[d]	871	117	1.38 (1.06–1.81)	832	78	1.07 (0.80–1.43)
HFM metabolite score	871	117	1.77 (1.36–2.32)	832	78	1.54 (1.10–2.16)

[a] N refers to the total number of independent participants in the analysis resulting from summing subjects of the subcohort (n = 791) + cases (n = 117 for non-fatal stroke, or n = 78 for non-fatal MI, for most metabolites, except pyruvate), and subtracting n = 37 overlapping cases resulting from case-cohort selection procedure.

[b] Adjusted for age (years), sex (male, female), intervention group (MedDiet + EVOO, MedDiet + nuts), body mass index (kg/m^2), smoking (never, current, former), leisure-time physical activity (metabolic equivalent tasks in minutes/day), dyslipidemia, hypertension, diabetes, and family history of CHD, as well as with robust standard errors to account for intracluster.

[c] A rank-based inverse normal transformation was applied to raw values of plasma metabolite levels.

[d] Weighted metabolite scores were generated using plasma levels of CAC intermediates plus 2-hydroxyglutarate, and Cox regression coefficients obtained after applying the leave-one-out cross-validation procedure Abbreviations: SD: Standard Deviation. CI: Confidence interval.

4. Discussion

Using a comprehensive panel of citric acid cycle (CAC) intermediates and related metabolites, we found that baseline fasting plasma levels of 2-hydroxyglutarate, fumarate and malate (collectively termed as HFM) were associated with higher CVD risk in the PREDIMED trial. In both humans and animal models, CAC intermediates and CAC-related metabolites such as lactate, citrate, succinate, malate or fumarate had been previously associated with a variety of cardiovascular risk factors and cardiovascular adverse events, including myocardial infarction, infarct size, stroke, cardiac hypertrophy, heart failure, ischemia-reperfusion injury, atrial fibrillation, and cardiovascular-related mortality [2,5,6,11–26]. Despite the capital importance of the CAC cycle in ischemia and CVD, no previous studies have been conducted to assess longitudinal associations between a comprehensive panel of circulating CAC-related metabolites and CVD incidence in the context of a MedDiet intervention. We found that the inclusion of a

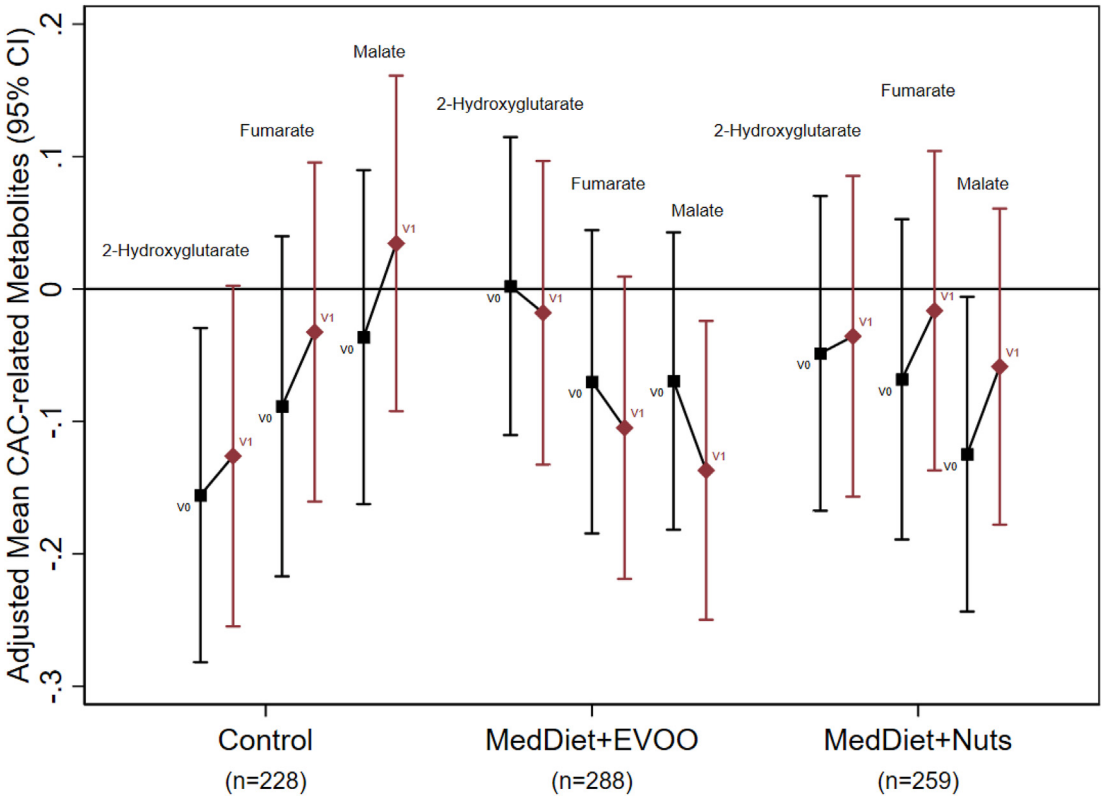

Figure 2 Changes in plasma 2-hydroxyglutarate, fumarate and malate after 1 year of intervention in the subcohort, by MedDiet intervention groups. V0 and V1: baseline and 1-year plasma levels of 2-hydroxyglutarate, fumarate and malate.

multimetabolite score combining 2-hydroxyglutarate, fumarate and malate (HFM score) modestly but significantly improved the prediction of CVD as compared to the multivariate equations derived from the Framingham study [35]. Using either baseline data or after 1-year of follow-up, we have found that the magnitude of the association between the HFM score and CVD risk is not affected by medication use or the incorporation of other previously-published multimetabolite risk scores [28–32].

Hypoxia profoundly impacts mitochondrial CAC flux, critically affecting intermediary metabolism, electron transport-chain flux, and leading to intracellular accumulation of CAC-related metabolites [3,4]. Succinate is typically the CAC intermediate that accumulates after ischemia in diverse tissues such as heart, brain, kidney and liver [36]. In pigs with transient coronary artery occlusion, it has been shown that levels of succinate, fumarate, malate, citrate and lactate are notably increased in the great cardiac vein (not in femoral vein) following ischemia, in a magnitude proportional to infarct size [11]. In an untargeted metabolomic study, it was found that fumarate, malate and succinate were elevated in cardiac-arrest patients post-resuscitation versus controls in plasma [12]. In that research, a comparative study of humans and mice revealed notable similarities in the accumulation of such circulating CAC metabolites in both species, supporting biological plausibility for a relevant role of circulating CAC-related metabolites as biomarkers for cardiovascular outcomes [12]. In the mouse, a complete analysis of energy metabolites in plasma and tissues revealed systemic metabolic alterations in heart failure, including changes in CAC-related metabolites [6]. In our study, we also found that plasma fumarate and malate are associated with both non-fatal MI, non-fatal stroke, indicating that such metabolites precede the development of CVD.

We also found a consistent association between plasma levels of 2-hydroxyglutarate and CVD risk in PREDIMED participants, including significant associations with non-fatal stroke and nominal associations for a higher risk of non-fatal MI. 2-hydroxyglutarate is not a CAC intermediate, but considered a oncometabolite generated from the reduction of α-ketoglutarate in hypoxia [37,38]. The D-2-hydroxyglutarate enantiomer is derived from tumors harboring mutant isocitrate dehydrogenase, while L-2-hydroxyglutarate enantiomer can be also produced from α-ketoglutarate by the promiscuous activity of enzymes such as lactate dehydrogenase A and malate dehydrogenase [37]. Apart from its role in tumorigenesis, L-2-hydroxyglutarate accumulates in hypoxia and acidic pH, extending its role as a hypoxia-inducible factor signaling, possibly involved in cardiovascular dysfunction through unknown mechanisms [37,39]. Also, hypoxia-induced elevations of L-2-hydroxyglutarate in response to mitochondrial dysfunction may inhibit electron transport and glycolysis [40]. Recently, it has been reported that plasma 2-hydroxyglutarate, as well as other CAC intermediates (malate, aconitate and isocitrate) are significantly associated with prospective events of atrial fibrillation and heart

failure [19], also supporting the hypothesis that increased plasma levels of 2-hydroxyglutarate and CAC-related metabolites may precede CVD events.

We have not found significant metabolite-by-diet interactions in relation to CVD risk in our study. Only a non-significant mild reduction of the hazard ratio for such metabolites was observed in the group of MedDiet supplemented with extra-virgin olive oil, in comparison with the control group or the group of MedDiet supplemented with nuts. Taken as a whole, the results of our study show that significant associations between HFM metabolites and increased CVD risk are not modified by MedDiet interventions.

Our study has several strengths and limitations. The blinded assessment by the Endpoint Adjudication Committee of CVD events avoided potential information biases in outcomes. In addition, CAC-related metabolites were measured both at baseline and 1-year after the start of the study with little number of missing values and adequate precision, with the exception of pyruvate. We internally replicated the positive association of baseline plasma metabolites with CVD, stroke and non-fatal MI using 1-year measurements of plasma metabolites. The appropriateness of the case-cohort design and the relative reduced number of drop-outs during the study are also strengths of this study. The use of a random sample of 10% of the original PREDIMED as a subcohort provides the opportunity to assess metabolite-by-diet interactions, retaining the advantage of the random allocation, and allowing the extension of the conclusions to the whole cohort [41,42]. Among the weaknesses of our study, the separation of CVD events in stroke and non-fatal MI, involved statistical analyses with sharp reductions in sample size that limited the conclusions in relation to such outcomes. Although the PREDIMED trial provides the possibility to adjust for a great variety of known confounders, residual confounding may still persist. Finally, external validity of our study is limited, since a direct application of our results is restricted to elderly subjects at high cardiovascular risk who were assigned to different dietary interventions.

In conclusion, we found that baseline fasting plasma levels of 2-hydroxyglutarate, fumarate and malate were significantly associated with higher CVD risk in the PREDIMED trial. We also found that a 3-metabolite score was significantly associated with non-fatal stroke and non-fatal MI. In all scenarios, hazard ratios provided by multi-metabolite HFM scores were of equivalent or greater magnitude compared to HRs for each individual metabolite. Moreover, we internally replicated such associations by estimating similar HRs (in terms of magnitude and direction of association) using metabolite measurements obtained after 1-year. We also found that the 3-metabolite score provides significantly better prediction compared to Framingham Heart Study risk scores.

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Declaration of competing interest

Authors declare no conflict of interest in this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.numecd.2023.01.002.

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