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Urinary polyphenol signature of the Mediterranean diet is associated with lower cardiovascular disease risk: the PREDIMED trial

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Abstract

Background The Mediterranean diet (MedDiet) is strongly associated with lower cardiovascular disease (CVD) risk and is particularly rich in polyphenols, bioactive compounds with potential cardioprotective effects. However, the specific phenolic compounds underlying these benefits remain unclear. The objective of this study was to develop a urinary multi-metabolite signature of phenolic compounds reflecting MedDiet adherence and to evaluate its prospective association with CVD risk.

Methods In a case–cohort nested study within the PREDIMED trial, we measured 62 phenolic metabolites in spot urine by liquid chromatography–high-resolution mass spectrometry at baseline and after 1 year in 1180 individuals: 653 incident CVD cases (stroke, myocardial infarction, CVD death, or heart failure) and a random subcohort of 603 participants (76 overlapping cases). We applied elastic net regression to derive a urinary multi-metabolite signature prospectively associated with MedDiet adherence, measured by the validated 14-item Mediterranean Diet Adherence Screener (MEDAS). Multivariable Cox models were used to estimate hazard ratios (HRs) of CVD by levels of the multi-metabolite signature.

Results The urinary multi-metabolite signature, comprising eight phenolic compounds selected by elastic net regression, was inversely associated with CVD risk in a dose–response pattern (HR per SD = 0.80 (0.68–0.94); HR Q4 vs Q1 = 0.48 (0.30–0.78); *p*-trend = 0.002). The metabolites included in the signature were derived from foods typical

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of the MedDiet, particularly virgin olive oil, wine, nuts, fruits, and vegetables. After 1 year, MedDiet interventions significantly increased urolithin A metabolites (derived from walnuts) compared to the control group.

Conclusions We identified a urinary multi-metabolite signature of MedDiet adherence that is prospectively associated with lower CVD incidence. These findings support that polyphenols derived from the MedDiet showed inverse associations with cardiovascular outcomes.

Trial registration The study was registered with the International Standard Randomized Controlled Trial Number (ISRCTN) 35739639.

Keywords Hydroxytyrosol, Urolithins, Naringenin, Metabolomics, Cardiovascular health, Mediterranean diet

Background

Cardiovascular disease (CVD) is the leading cause of death and disability worldwide [1]. The Mediterranean diet (MedDiet) has emerged as one of the healthiest dietary patterns for CVD prevention. Seminal, large-scale intervention trials with a traditional MedDiet such as the PREDIMED [2], along with many well-conducted observational studies, have provided strong evidence that the MedDiet is effective in preventing CVD, as well as in improving its prognosis [3]. Several individual components of the traditional MedDiet are beneficial for cardiovascular health, including higher consumption of fruits and vegetables, extra-virgin olive oil (EVOO), nuts, and moderate wine consumption [4–7]. Interestingly, many of these foods are also high in (poly)phenol content [8].

(Poly)phenols are plant-derived compounds linked to lower risks of mortality, CVD, cancer, and cognitive decline [9, 10]. Dietary unabsorbed (poly)phenols are metabolized by gut microbiota into phenolic metabolites and further modified in the liver through conjugation [11]. Traditional self-reported dietary assessment methods may present limitations to capture interindividual variability in dietary (poly)phenol's metabolism [12]. Importantly, to our knowledge, no study has applied metabolomics to urinary (poly)phenol metabolites in large populations to investigate their relevance for cardiovascular health.

In the present study, we aimed to identify a multi-metabolite signature of phenolic compounds measured in urine reflecting adherence to the traditional MedDiet, and to prospectively assess the association of this multi-metabolite signature with subsequent CVD events in the PREDIMED trial. In secondary analyses, we examined the association between the individual metabolites of the multi-metabolite signature with CVD risk, as well as with individual items of the MedDiet.

Methods

Study design

A prospective case-cohort analysis was conducted using baseline and 1-year data from the PREDIMED (PREvención con DIeta MEDiterránea) study, a well-known randomized, controlled intervention trial conducted in Spain [2, 13]. Eligible participants included men (aged 55–80 years) and women (aged 60–80 years) with type 2 diabetes or exhibiting at least three of the following risk factors: current smoking, hypertension, dyslipidemia, overweight/obesity, and/or a family history of premature CVD. It involved 7447 participants at high CVD risk, and its aim was to assess the effects of a MedDiet enriched with EVOO or nuts on the incidence of CVD events. After the active intervention period, an extended follow-up until December 2017 was planned, consisting of the review of medical records for the selected end-points.

We included all the identified incident cases of CVD with available urine samples ($n=653$), and a random subsample of 10% of the PREDIMED trial participants (referred to as the sub-cohort) representative of the full PREDIMED cohort (Additional file 1: Table S1). This case-cohort design included a total of 1180 participants, 653 incident cases of CVD and a random sub-cohort of 603 participants that included 527 non-cases and 76 overlapping incident CVD cases. Cases were the participants who developed a clinical cardiovascular event (cardiovascular death, myocardial infarction, stroke, or heart failure) during the active trial (2003–2010) plus an extended follow-up period until December 2017. During a median follow-up of 9.0 years, 189 cases of heart failure, 96 cases of non-fatal myocardial infarction, 144 cases of non-fatal stroke, and 224 cardiovascular deaths were adjudicated by the independent Clinical Event Ascertainment Committee.

Ascertainment of CVD cases

During the active trial, at every recruitment center, medical experts, blinded to the intervention, performed yearly assessments of participants' medical files to detect potential cardiovascular events. To identify new cases, four independent sources of information were used: regular communication with participants, collaboration with their primary care physicians, yearly reviews of medical records, and consultation of the Spanish National Death Index. Following this, de-identified data were sent to a central Event Ascertainment Committee, also blinded

to the intervention, which conducted the ultimate event adjudication. During the extended follow-up, CVD case detection was based on the review of medical records and the corresponding confirmation by the central Event Ascertainment Committee. Cases were defined based on the first diagnosed CVD event in each participant.

Covariate assessment

Food intake was assessed through a validated, semi-quantitative FFQ with 137 items under the guidance of trained dietitians and delivered in face-to-face interviews [14]. Nutrient intake was determined using Spanish food composition tables by skilled dietitians with expertise in nutritional epidemiology. Compliance with the MedDiet was evaluated using a 14-item questionnaire (Mediterranean Diet Adherence Screener, MEDAS), with each dietary component scored as either 0 or 1 [13].

Anthropometric measurements, such as weight and height, were performed by trained personnel using established techniques, allowing for the calculation of body mass index (BMI) in kg/m^2. Physical activity levels were assessed using a validated Spanish version of the Minnesota physical activity questionnaire, quantifying metabolic equivalent tasks per minute per day (METs min/day) [15].

Urinary phenolic metabolites

Biological samples were collected after an overnight fast, coded, and stored at − 80 °C until analysis. Phenolic compounds were determined following dilution and acidification of the spot urine samples (Additional File 1: Methods S1) [16]. A list of the standards and their commercial sources used to identify and quantify the phenolic compounds can be found in Additional file 1: Table S2. The compounds whose standard was not available were identified by comparison with reference spectra [16].

The analysis was performed in a liquid chromatograph Agilent 1290 Infinity II coupled to a high-resolution mass spectrometer Agilent 6560 Ion Mobility QTOF LC/MS (Santa Clara, CA, USA), available at the Separation Techniques Unit of the Scientific and Technological Centers (CCiTUB), Universitat de Barcelona. Agilent Mass Hunter Software (Version 10.0) was used to identify and quantify the phenolic compounds (Additional file 1: Table S3 [17]). Further details can be found in Additional file 1: Methods S1.

Statistical analyses

After data preprocessing, 58 urinary phenolic compounds were included in the linear elastic net regression model to identify a signature of adherence to the MedDiet. A total of 8 compounds were selected for the final signature prospectively associated with adherence to the MedDiet. Further details on the data preprocessing and development of the signature can be found in Additional File 1: Methods S2 [18, 19].

Baseline characteristics of participants were described as means and standard deviations (SDs) for quantitative variables and as percentages for categorical variables. We categorized all participants into quartiles of the urinary multi-metabolite signature of the MedDiet.

Cox regression models with Barlow weights [20] (to account for oversampling cases in the case-cohort design) were used to calculate hazard ratios (HR) and their 95% confidence interval (CI) for the association between the urinary multi-metabolite signature of the MedDiet with the risk of the composite CVD outcome. The CVD composite included heart failure, myocardial infarction, stroke, and CVD death. Model 1 was minimally adjusted for age and sex, and stratified by recruitment center; Model 2 was further adjusted for smoking habit (no/former/current), physical activity (continuous), waist-to-height ratio (quartiles), hypertension (yes/no), hypercholesterolemia (yes/no), diabetes (yes/no), medication for dyslipidemia (yes/no) and hypertension (yes/no), family history of CVD (yes/no), and total energy intake (continuous). Models were stratified by recruitment center, randomized intervention group, and educational level (5 categories), using the Stata command *strata*. Linear trends were assessed by modeling quartile medians as continuous variables. The same adjustment models were used to assess the association between MedDiet adherence measured by the MEDAS with the risk of CVD events. We evaluated the association between 1-year changes in the urinary multi-metabolite signature and CVD risk, adjusting for baseline values and assessing interaction with intervention groups. The interaction between the 1-year changes in the urinary multi-metabolite signature of the MedDiet and intervention groups was assessed using the likelihood ratio test. Associations between the standardized individual metabolites and CVD were assessed using the same multivariable models described above, with *p*-values corrected for multiple testing using the Simes procedure [19].

Multivariable linear regressions were used to assess associations between MEDAS (independent variable) and the baseline urinary multi-metabolite signature of the MedDiet (dependent variable). Correlations between the 8 individual standardized metabolites that made up the urinary multi-metabolite signature and MEDAS (0–14), as well as with the main food groups, were assessed using the Pearson correlation coefficient. Detailed descriptions of all study methods are provided in Additional File 1: Methods S2.

P-values < 0.050 were considered statistically significant. All statistical analyses were performed with Stata

16.0 (Stata-Corp LP, Tx. USA) and R 4.5.0 version, using glmnet 4.1.8, survival 3.8.3, and tidyverse 2.0.0 packages.

Results

Characteristics of study participants

We analyzed 1180 participants, including 653 subjects who suffered incident CVD events during the follow-up. Baseline characteristics of the population are described in Table 1. The mean age of participants was 68y and the proportion of women was higher in the lowest quartile of the urinary multi-metabolite signature of the MedDiet. Participants in the highest quartile of urinary multi-metabolite signature had lower BMI and lower waist-to-height ratio. Additional file 1: Fig. S1 depicts the flowchart of the study.

Identification and description of the phenolic compounds

Our analysis identified and quantified a total of 62 compounds. Among them, phenolic acids were the predominant class, accounting for 30 (48%) of the selected metabolites. They were followed by 7 flavonoids (11%), 6 lignans, hydroxycoumarins, and tyrosols (each constituting 10%). Additionally, there were 2 stilbenes (3%) and 1 secoiridoid (2%), along with 4 metabolites from other categories.

The elastic net regressions selected a combination of 8 urinary compounds associated with adherence to MedDiet (Table 2): catechol, vanillin glucuronide, urolithin A metabolites, *m*-coumaric acid, naringenin glucuronide, hydroxytyrosol, protocatechuic acid,

Table 1 General characteristics of the study population at baseline according to quartiles of the urinary multi-metabolite signature prospectively associated with MedDiet adherence ($n = 1180$)

Urinary multi-metabolite signature*	Q1 ($n = 295$) − 1.14 (− 2.98 to − 0.74)	Q2 ($n = 295$) − 0.33 (− 0.73 to + 0.02)	Q3 ($n = 295$) + 0.32 (+ 0.02 to + 0.68)	Q4 ($n = 295$) + 1.16 (0.69 to + 2.98)	*p*-value
Age, years	69.1 ± 6.4	68.3 ± 6.3	67.7 ± 6.1	68.3 ± 5.8	0.032
Women, *n* (%)	175 (59.3)	153 (51.9)	133 (45.1)	169 (57.3)	0.002
BMI, kg/m^2	30.4 ± 4.0	30.3 ± 3.9	29.9 ± 3.5	29.5 ± 3.6	0.017
Waist-to-height ratio	0.64 ± 0.07	0.64 ± 0.07	0.63 ± 0.06	0.63 ± 0.07	< 0.001
Diabetes Mellitus, *n* (%)	171 (58.0)	162 (54.9)	150 (50.8)	154 (52.2)	0.317
Dyslipidemia, *n* (%)	195 (66.1)	196 (66.4)	207 (70.2)	212 (71.9)	0.349
Hypertension, *n* (%)	243 (82.4)	252 (85.4)	245 (83.1)	242 (82.0)	0.687
Educational level, *n* (%)					0.047
- Low (none or primary)	245 (83.1)	219 (74.2)	223 (75.6)	233 (79.0)	
- High and medium (secondary/university)	50 (16.9)	76 (25.8)	72 (24.4)	62 (21.0)	
Smoking habit, *n* (%)					0.036
- Current smokers	37 (12.5)	36 (12.2)	50 (17.0)	38 (12.9)	
- Former smokers	84 (28.5)	83 (28.1)	98 (33.2)	69 (48.3)	
Total energy intake, kcal/day	2239 ± 609	2283 ± 663	2357 ± 589	2299 ± 591	0.134
MEDAS score	8.2 ± 2.2	8.4 ± 2.0	8.7 ± 1.8	9.0 ± 1.9	< 0.001
Physical activity, MET·min/day	217 ± 242	239 ± 234	250 ± 251	259 ± 224	0.151
Family history of early-onset CHD, *n* (%)	59 (20.0)	69 (23.4)	71 (24.1)	61 (20.7)	0.562
Lipid-lowering medication, *n* (%)	131 (44.4)	141 (47.8)	136 (46.1)	133 (45.1)	0.855
Anti-hypertensive agents, *n* (%)	216 (73.4)	233 (79.1)	220 (74.6)	214 (72.7)	0.268
Intervention group, *n* (%)					0.151
- MedDiet + EVOO	103 (34.9)	90 (30.5)	107 (36.3)	111 (37.6)	
- MedDiet + nuts	85 (28.8)	92 (31.2)	95 (32.2)	101 (34.2)	
- Control diet	107 (36.3)	113 (38.3)	93 (31.5)	83 (28.1)	

BMI body mass index, *METS* metabolic task equivalents, *CVD* cardiovascular disease, *CHD* coronary heart disease, *MEDAS* Mediterranean Diet Adherence Screener, *MedDiet* Mediterranean diet, *EVOO* extra-virgin olive oil

Values are percentages for categorical variables and means ± SD for continuous variables

One-ANOVA factor was used for continuous variables, and chi-square test was used for categorical variables. $p < 0.05$ was considered significant

* The urinary multi-metabolite signature is presented with the interquartile range (Q1–Q3)

Table 2 Urinary phenolics included in the urinary multi-metabolite signature of the Mediterranean diet

Metabolite	Phenolic class—subclass	Coefficient
Catechol	Other	0.079
Vanillin glucuronide	Other—hydroxybenzaldehydes	0.067
Urolithin A metabolites	Other—hydroxycoumarins	0.055
m-Coumaric acid	Phenolic acid—hydroxycinnamic acid	0.055
Naringenin glucuronide	Flavonoid—flavanones	0.052
Hydroxytyrosol	Other-tyrosol	0.043
Protocatechuic acid	Phenolic acid—hydroxybenzoic acid	0.031
Hydroxytyrosol sulfate 2	Other—tyrosol	0.030

MEDAS Mediterranean diet adherence screener (0 to 14 points)

Coefficients for the 8 metabolites selected for the urinary multi-metabolite signature prospectively associated with MedDiet adherence (MEDAS) using elastic net linear regression

and hydroxytyrosol sulfate 2. Their dietary and metabolic sources are described in Fig. 1. Additional file 1: Figure S2 shows their Pearson's pairwise correlation coefficients for the metabolites selected in the urinary multi-metabolite signature, and Additional file 1: Fig. S3 includes the Pearson's correlation coefficients of all the metabolites.

Urinary phenolic metabolites, metabolomics signature, and MedDiet adherence

Additional file 1: Table S4 presents 1-year changes in each phenolic compound in the MedDiet groups as compared to the control group. In multivariable analyses, only urolithin A metabolites maintained significant 1-year increases in the MedDiet groups (vs. control) after correcting for multiple testing. One-year increases in the urinary multi-metabolite signature of the MedDiet were higher in the two groups randomized to the MedDiet intervention as compared to the control group, but this association was not statistically significant.

In Additional file 1: Table S5, a positive and linear association was observed between MedDiet adherence and the baseline urinary multi-metabolite signature ($\beta = 0.15$ (0.06; 0.24) per 1-SD increment, p-trend = 0.006). This significant and linear relationship was also found when evaluating 1-year changes in both the signature and adherence to the MedDiet, after adjusting for potential confounders and baseline measurements. Consistently, most selected metabolites showed a direct correlation

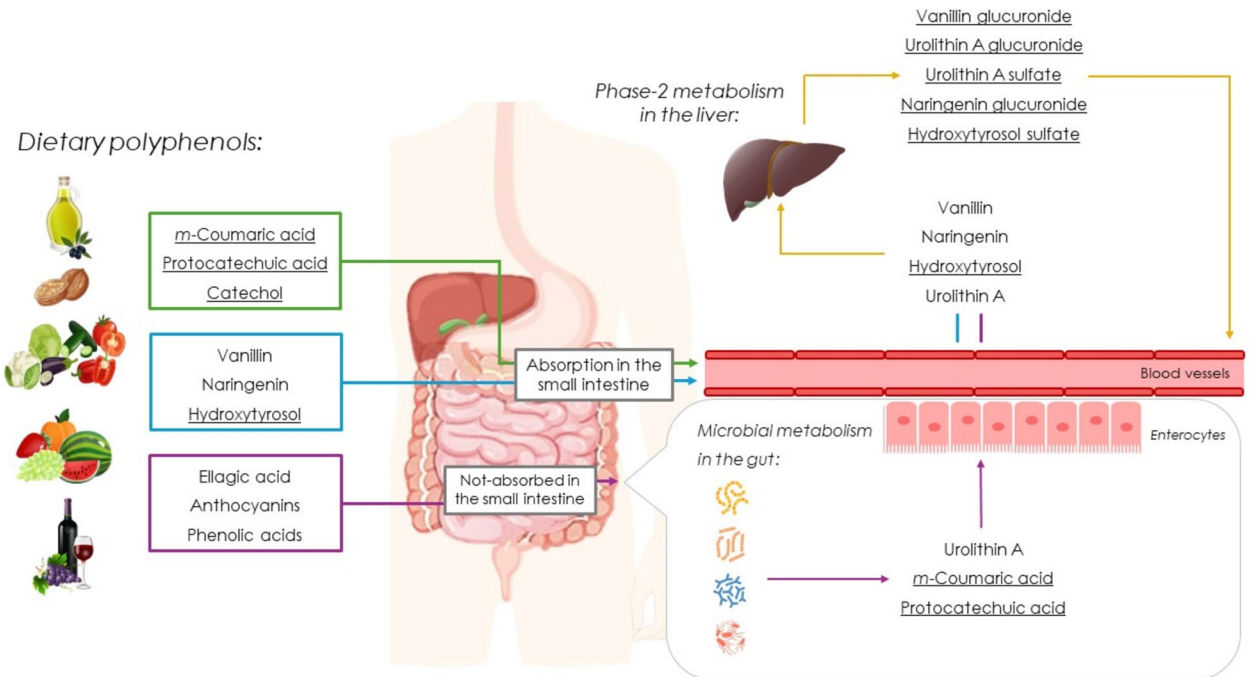

Fig. 1 Overview of the occurrence of metabolites in the urinary multi-metabolite signature (underlined). Dietary polyphenols are found in plant foods like olives and olive oil (vanillin, hydroxytyrosol), walnuts (ellagic acid), fruits (anthocyanins, naringenin, vanillin, phenolic acids, ellagic acid), vegetables (phenolic acids), coffee (phenolic acids, catechol), and wine (hydroxytyrosol). Vanillin, hydroxytyrosol, catechol, and phenolic acids (e.g., protocatechuic, m-coumaric acid) are absorbed in the small intestine and enter the bloodstream. Ellagic acid, anthocyanins, and some phenolic acids reach the gut, where microbiota convert them into urolithin A and phenolic acids before absorption. These compounds undergo liver phase-2 metabolism, conjugated with glucuronide or sulfate by glucuronosyltransferases and sulfotransferases enzymes

with the MEDAS score and its individual components, as shown in Additional file 1: Fig. S4. When examining continuous food intake data, the urinary multi-metabolite signature correlated most strongly with fruit consumption ($r=0.15$), followed by nuts ($r=0.11$), vegetables ($r=0.10$), and wine ($r=0.09$) (Additional file 1: Fig. S5A). The highest correlation was observed between nut consumption and the excretion of urolithin A metabolites ($r=0.20$). Similar patterns were found in the 1-year data, where the signature showed the strongest associations with nuts ($r=0.16$) and fruits ($r=0.14$) (Additional file 1: Fig. S5B). In contrast, correlations between 1-year changes in reported food consumption and both urinary metabolites and the overall signature were weaker, likely reflecting the limited variation in changes in dietary intake during the first year of intervention (Additional file 1: Fig. S5C).

Urinary metabolomics signature and CVD events

Table 3 shows that baseline values of the urinary multi-metabolite signature of the MedDiet were strongly and inversely associated with the risk of subsequent CVD events. The signature showed a highly significant inverse association with CVD (HR = 0.80 (0.68; 0.94) per SD). The multivariable adjusted associations for the upper versus the lowest quartile rendered a 52% relative risk reduction (95% CI: 22% to 70%; p for trend = 0.002). Similar, although weaker, associations were found between adherence to the MedDiet measured by the MEDAS and CVD events (Additional file 1: Table S6).

Individually, two single compounds included in the signature showed significant inverse associations with CVD (Fig. 2). These metabolites were hydroxytyrosol sulfate 2 (HR = 0.75 (0.63; 0.90)) and vanillin glucuronide (HR = 0.84 (0.71; 0.99)). However, none of them retained significant associations after correcting for multiple testing (Additional file 1: Table S7).

Additional file 1: Table S8 shows the HRs for each individual type of CVD event. We observed an inverse association between the urinary multi-metabolite signature (per 1-SD increment) and the risk of heart failure (HR = 0.72; 95% CI: 0.56–0.94, p for trend = 0.005) and myocardial infarction (HR = 0.60; 95% CI: 0.42–0.86, p for trend = 0.003). The urinary multi-metabolite signature of the MedDiet was also associated with a lower risk of the primary combined endpoint of the original trial including myocardial infarction, stroke, and CVD death (HR = 0.84; 95% CI: 0.70–1.00 per SD).

CVD risk by 1-year changes in the urinary multi-metabolite signature of the MedDiet is shown in Additional file 1: Table S9. We found a non-linear pattern, showing an increased risk of CVD for the first quartile of change, with lower risk at the second quartile, that subsequently plateaued. Therefore, in the categorical analysis, quartiles 2–4 were merged and used as the reference group in order to assess the impact of suboptimal changes (lowest quartile) in excretion of phenolic compounds. Participants with 1-year reductions in the excretion of phenolic compounds (Q1) were at higher risk of CVD as compared to those with higher excretion levels (Q2–Q4) (multivariable-adjusted HR = 1.61 (1.01; 1.96)). When stratifying by intervention group, this relationship was observed only among participants in the control group. However, the p for interaction was not significant (p for interaction = 0.151).

Table 3 Risk of CVD according to the baseline urinary multi-metabolite signature of the traditional Mediterranean diet (continuous and categorized in quartiles)

	Continuous per 1-SD (*n* = 1180)		**Urinary multi-metabolite signature, quartiles**				
			Q1 (*n* = 295)	**Q2 (*n* = 295)**	**Q3 (*n* = 295)**	**Q4 (*n* = 295)**	***p* linear trend**
Cases	*653*		*183*	*172*	*165*	*133*	
Person-years	*6195*		*1318*	*1457*	*1557*	*1864*	
	HR (95% CI)	*p*-value		HR (95% CI)	HR (95% CI)	HR (95% CI)	
Model 1	0.85 (0.74; 0.98)	0.027	Ref	0.92 (0.63; 1.35)	0.78 (0.53; 1.13)	0.56 (0.38; 0.84)	0.003
Model 2	0.80 (0.68; 0.94)	0.007	Ref	1.05 (0.67; 1.62)	0.85 (0.55; 1.29)	0.48 (0.30; 0.78)	0.002

CVD cardiovascular disease, *Q* quartile, *HR* hazard ratio

The subcohort included 76 overlapping cases

Model 1 was adjusted for age and sex and stratified by recruitment center

Model 2 was further adjusted for smoking habit, physical activity, total energy intake, waist-to-height ratio (quartiles), hypercholesterolemia, hypertension, diabetes, medication for hypertension and dyslipidemia, family history of CHD, and stratified by educational level and intervention group (MedDiet vs control)

P-trend was assessed by modeling medians of each quartile as a continuous variable

The metabolites included in the urinary multi-metabolite signature and their coefficients were: catechol (0.079), vanillin glucuronide (0.067), the mean of 4 urolithin A metabolites (0.055), *m*-coumaric acid (0.055), naringenin glucuronide (0.052), hydroxytyrosol (0.043), protocatechuic acid (0.031), and hydroxytyrosol sulfate 2 (0.030)

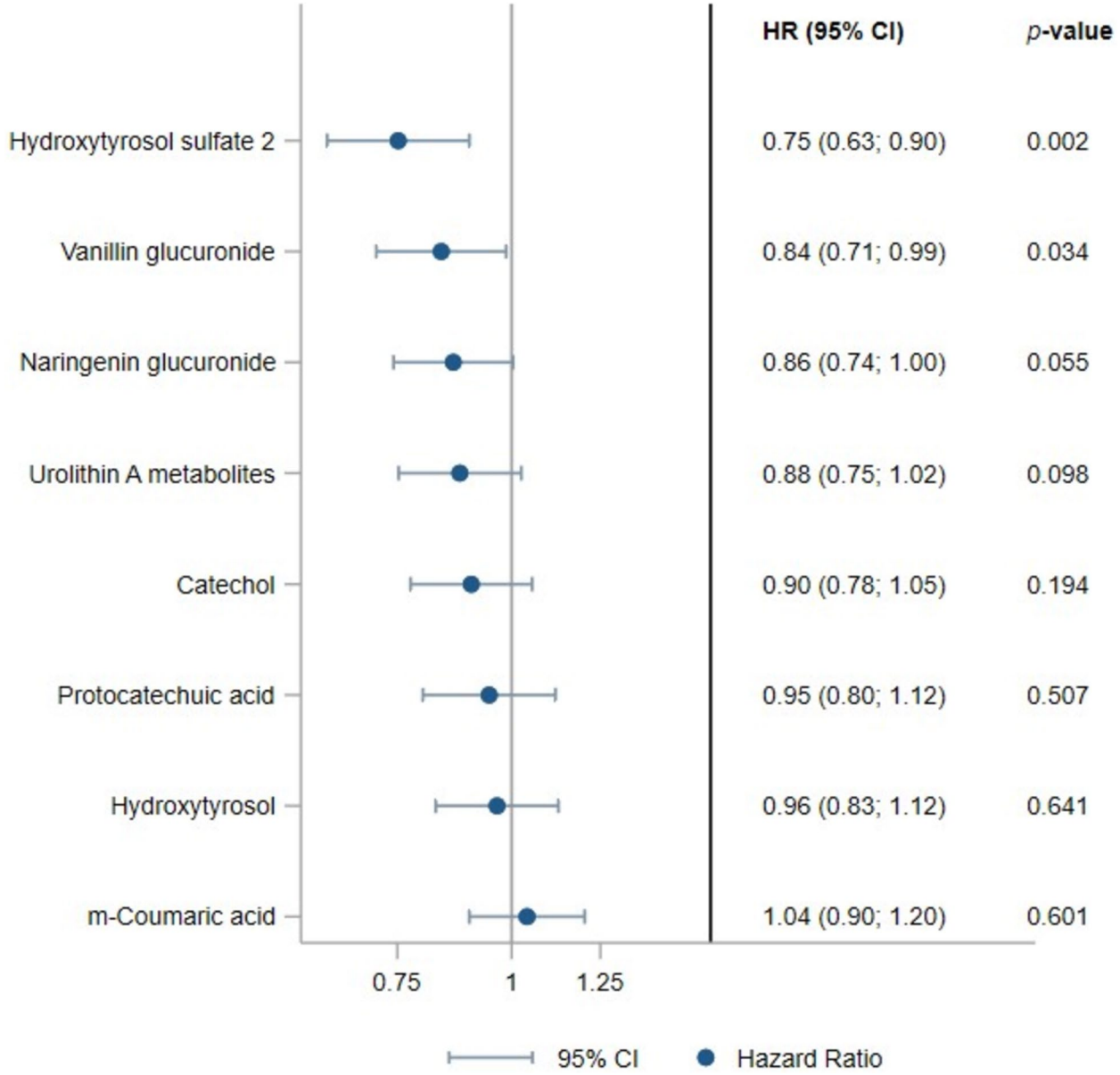

Fig. 2 Association of the 8 metabolites in the urinary multi-metabolite signature at baseline with CVD ($n = 1180$). Urolithin A metabolites were combined into a single score due to the strong positive pairwise correlations observed among them. The subcohort included 76 overlapping cases. Adjusted for age, sex, smoking habit, physical activity, total energy intake, waist-to-height ratio (quartiles), hypercholesterolemia, hypertension, diabetes, medication for hypertension and dyslipidemia, family history of CHD, and stratified by recruitment center, educational level, and intervention group (MedDiet vs Control)

Discussion

In this prospective case-cohort study nested in the PREDIMED trial, we identified a multi-metabolite signature, composed of 8 compounds, that reflects adherence to the MedDiet and is prospectively associated with lower risk of CVD events. By linking objective biomarkers of (poly)phenol-rich food intake to hard clinical cardiovascular outcomes, our findings provide novel evidence that a specific combination of phenolic compounds derived from the MedDiet is associated with a lower risk of CVD.

Previous research has reported the benefits of individual (poly)phenols on cardiovascular health through reducing inflammation by inhibiting proinflammatory enzymes and transcription factors [21], while boosting the activity of antioxidant enzymes and signaling pathways [22]. However, due to their chemical diversity and

complex metabolism, it has been challenging to identify which compounds contribute most to cardiovascular protection in humans.

Previous studies have developed metabolomic signatures related to adherence to the MedDiet or to some of its characteristic foods, but they have generally neglected minor components, such as (poly)phenols [23, 24]. In our novel targeted analysis, the urinary multi-metabolite signature associated with greater adherence to the MedDiet was prospectively linked to a lower risk of CVD. This association was stronger when using the multi-metabolite signature than that observed for self-reported MedDiet adherence, underscoring the potential of urinary polyphenols as reliable and objective biomarkers of MedDiet components likely beneficial for cardiovascular health. However, the interventions conducted in PREDIMED did not significantly increase the urinary multi-metabolite signature, suggesting that recent dietary changes over 1 year might not be sufficient to induce meaningful changes in the combined metabolic signature. Furthermore, we used spot urine samples, which can increase within-person variability in metabolite levels and make urinary concentrations less reflective of long-term intake.

Several components of the MedDiet pattern are relevant sources of (poly)phenols. Since most (poly)phenols are not produced endogenously, they provide good opportunities to examine their association with the consumption of certain foods and to identify biomarkers of intake. Walnuts, a rich source of ellagic acid, are metabolized in the gut microbiota into urolithins [25]. Consistent with this, nut consumption (both as dichotomous, > 3 servings/day, and on a continuous scale) was positively correlated with urolithin A metabolites. Wine, well-known for its high content of (poly)phenols [26], was correlated with some urinary (poly)phenols, particularly hydroxytyrosol sulfate 2, and catechol. Fruit consumption above > 3 servings/day was correlated with higher levels of phenolic compounds, including naringenin glucuronide—a flavonoid commonly found in citrus fruits [26]—and vanillin glucuronide. When fruit consumption was modeled as a continuous variable, vanillin glucuronide showed the strongest correlation. As expected, this metabolite was also linked to the consumption of vegetables and of olive oil [26, 27], although these associations were weaker in the continuous models. Additionally, *m*-coumaric acid showed a correlation with the preferential use of olive oil as the main culinary fat source, which is consistent with previous findings [26].

The most strongly associated metabolite with lower risk of CVD was hydroxytyrosol sulfate 2, a phase-II metabolite of the tyrosol class. Hydroxytyrosol is typically derived from the hydrolysis of the oleuropein found in EVOO, although it is also present in red wine [28]. Of note, our findings suggest that wine was also an important source of hydroxytyrosol, which aligns with previous studies conducted in Europe [29]. Consistent evidence supports the cardiovascular benefits of hydroxytyrosol [30], particularly its protective effect against low-density lipoprotein (LDL) oxidation, as recognized by the European Commission with a health claim [31].

The microbial metabolite urolithin A has been less studied than other urinary metabolites. Dietary ellagitannins and ellagic acid can be found in various foods such as pomegranates and berries but are particularly abundant in walnuts [26], and we found a correlation between nuts consumption and these metabolites. Although the ability to produce urolithin A depends on the composition of an individual's gut microbiota, it is estimated that most adults can generate it, mainly from walnuts [32]. Indeed, most participants in the present study were able to produce it. Urolithin A and their metabolites were included in the urinary multi-metabolite signature and showed inverse associations with CVD events. In a prior cross-sectional study in children, urolithin A was linked to lower blood pressure [33]. Most studies, however, have been conducted in animal models or in vitro, suggesting cardioprotective mechanisms of the aglycone urolithin A such as the induction of mitophagy and the inhibition of Akt kinase phosphorylation, an overactivated pathway in type 2 diabetes [34].

Naringenin glucuronide was the only flavonoid in the urinary multi-metabolite signature that was prospectively associated with lower CVD risk. This phase II metabolite of naringenin is found in citrus fruits like oranges, lemons, and grapefruits [26]. Preclinical studies have demonstrated that naringenin possesses lipid-lowering and insulin-sensitizing properties [35], along with notable anti-inflammatory effects [36]. Although its poor bioavailability [37] has limited consistent results in humans, small clinical trials with specific populations reported improvements in CVD risk markers and reduced arterial stiffness following naringenin supplementation [38, 39]. However, our study is the first to link urinary naringenin with lower CVD events in a large population.

The best documented source of vanillin is EVOO [26, 40], although it has also been identified in other foods (fruits, vegetables, and cereals) and used by the food industry as a flavoring agent, derived naturally from vanilla beans [41]. Consistent with this, we observed a positive correlation between vanillin glucuronide and olive oil. Importantly, vanillin glucuronide was inversely associated with CVD risk in our study. Its precursor, vanillin, has shown promising antioxidant and anti-inflammatory properties in vitro and in animal studies, which could help reduce the oxidative stress and inflammation

linked to CVD [42]. Vanillin glucuronidation is recognized as a key metabolic pathway in vivo [43]; however, its potential health effects are unknown. Moreover, the wide array of products containing vanillin prevents us from attributing its presence solely to natural food sources.

Other metabolites in the signature—catechol, protocatechuic acid sulfate, and *m*-coumaric acid—were not individually associated with lower CVD risk. Catechol likely reflects the breakdown of multiple complex polyphenols [44, 45], while protocatechuic acid and *m*-coumaric acid may originate from both direct dietary intake and microbial metabolism [46, 47]. Their presence in the signature suggests that they act as integrative markers of overall MedDiet adherence rather than as specific bioactive contributors to cardiovascular protection.

Overall, adherence to the MedDiet is associated with bioactive phenolic compounds, which individually support cardiovascular health and may act synergistically when consumed together within the dietary pattern. Importantly, most of the phenolic compounds associated with lower CVD risk are derived from gut microbial metabolism. These findings underscore the importance of measuring phenolic compounds in biological samples, as the transformation of dietary (poly)phenols generates a range of metabolites with potential bioactive and cardioprotective effects.

The strengths of this study include its large sample size and case-cohort study design nested within a well-known randomized intervention trial of primary prevention of CVD through MedDiet interventions. The prospective design with repeated 1-year measurements of phenolics, while not confirming causality, helps to reduce the concerns about reverse causation. We measured urinary phenolic compounds, avoiding the subjectivity associated with self-reported dietary assessment tools. These biomarkers reflect not only dietary intakes of (poly)phenols, but also absorption and metabolism of these compounds. The study has well-defined CVD outcomes and includes detailed covariate data to control for potential confounding factors.

The study also has limitations. The metabolites identified were derived from a pool of 150 annotated phenolic compounds, and we cannot exclude that further relevant phenolic metabolites could be absent in our analyses. Stool samples from the participants were not collected; thus, we could not examine the gut microbiome involved in the production of the metabolites detected. In the PREDIMED, only spot urine samples were available, and samples were only collected 1 day alone (both pre and post intervention), rather than in repeated samples across multiple days. Using single time-point spot urine samples may have introduced some degree of imprecision in the measurement of urinary phenolic compounds, thereby reducing statistical power and potentially contributing to the attenuated associations between 1-year changes in the multi-metabolite signature and CVD. Nevertheless, prior studies have reported high correlations between (poly)phenol excretion measured in spot urine samples and in 24-h urine collection [48]. Also, similar correlations have been reported between fruits and vegetables intake with (poly)phenols determined in spot and 24-h urine samples [49]. In addition, given that most cohorts collect spot urine samples rather than 24-h urine collections [50], our approach enhances the comparability of our findings with the broader literature. Finally, the study was conducted in an older Mediterranean population at high CVD risk; therefore, the results should be replicated in other populations.

Conclusions

In conclusion, we developed a urinary multi-metabolite signature prospectively associated with higher adherence to the MedDiet, which was inversely associated with prospective CVD risk. These findings suggest that the combination of specific (poly)phenols derived from the MedDiet pattern is associated with a lower risk of CVD.

Abbreviations

CVD	Cardiovascular disease
LOD	Limit of detection
LOQ	Limit of quantification
EVOO	Extra virgin olive oil
FFQ	Food-frequency questionnaire
LDL	Low-density lipoprotein
MedDiet	Mediterranean diet
MEDAS	Mediterranean diet adherence screener

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12916-025-04587-w.

Additional file 1: Expanded Methods—Urinary phenolic compounds measurement and statistical analyses. Supplementary tables and figures. Table S1—Baseline characteristics of the full PREDIMED cohort (n = 7,447) and the representative subcohort included in the present study (n = 603, including 76 overlapping cases). Table S2—Commercial sources of the standards used in the analysis. Table S3—Identification of the 62 phenolic compounds according to their standards, exact mass, and MS/MS fragments. Table S4—One-year changes of phenolic compounds (in units of standardized values as SD) in the MedDiet groups vs Control diet (reference) groups. Table S5—Multivariable adjusted linear regression assessing the association between baseline MEDAS (independent variable) and the urinary multi-metabolite signature (outcome, in units of standardized values as SD). Table S6—Risk of CVD according to the baseline adherence to the Mediterranean diet (continuous and categorized in quartiles). Table S7—Associations between individual urinary phenolic metabolites (per 1-SD) and risk of CVD. Table S8—Risk of each type of clinical cardiovascular event included in the composite end-point, according to the baseline urinary multi-metabolite signature of the MedDiet (continuous and categorized in quartiles). Table S9—Risk of CVD according to the one-year changes in the urinary multi-metabolite signature predictive of adherence to the Mediterranean diet. Figure S1—Flowchart of study

participants. Figure S2—Heat map (Pearson's correlation coefficients) for the eight metabolites selected in the urinary multi-metabolite signature reflecting adherence to the MedDiet. Figure S3—Heat map (Pearson's correlation coefficients) for the 58 metabolites included in the analysis. Figure S4—Pearson's pairwise correlations of the 8 selected metabolites with each of the MEDAS components and the total MEDAS score at baseline. Figure S5—Heat map (Pearson's correlation coefficients) for the eight metabolites selected in the urinary multi-metabolite signature reflecting adherence to the MedDiet and the main food groups at baseline (A), one-year of follow-up (B), and one-year changes (C).

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Authors' contributions
MAM-G, FBH, and RML-R designed research; ID-L, PG, CP, and OJ performed the experimental analyses; ID-L, GF-D, and MAM-G carried out the statistical analyses; ID-L, MAM-G, FHB, and RML-R interpreted the data. ID-L drafted the manuscript. CR, JS-S, LT-S, MF1, DC, MF, MF2 JL, EG-G, XP, MR-C, OC, LL, QS, LS-M, ER, MAM-G, RE, FBH, and RML-R contributed to the critical review of the paper. All authors read and approved the final manuscript.

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Data availability
Investigators of PREDIMED will provide access to the PREDIMED dataset (including data dictionaries), making possible the replication of the main analyses used for the present article. Due to the restrictions imposed by the Informed Consent and the Institutional Review Board, bona fide investigators interested in analyzing the PREDIMED dataset used for the present article may submit a brief proposal and statistical analysis plan to the PREDIMED Steering Committee. Upon approval from the PREDIMED Steering Committee and Institutional Review Boards, the data will be made available to them using an onsite secure access data enclave.

Declarations

Ethics approval and consent to participate
The Research Ethics Committees of all recruitment centers approved the overall PREDIMED trial design according to the ethical guidelines of the Declaration of Helsinki (Clinical trial registry number: Controlled-Trials.com number, ISRCTN35739639). All participants provided informed consent and signed a written consent form.

Consent for publication
Not applicable.

Competing interests
J.S-S reports serving on the board of and receiving grant support through his institution from the International Nut and Dried Fruit Council, serving on the board of the Instituto Danone Spain and the International Danone institute. E.R. reports grants, personal fees, non-financial support and other from the California Walnut Commission while the study was carried out; grants, personal fees, non-financial support and other from Alexion; and personal fees and other from Sociedad Española de Arteriosclerosis and Fundación de Dieta Mediterránea, both from Spain, outside the submitted work. R.M.L.-R. reports personal fees from Cerveceros de España, UNIDECO SA, personal fees, and other from Adventia, Wine in Moderation, Ecoveritas S.A., outside the submitted work. R.E. reports grants from the Fundación Dieta Mediterránea (Spain), and Cerveza y Salud (Spain), and personal fees for given lectures from Brewers of Europe (Belgium), the Fundación Cerveza y Salud (Spain), Pernaud-Ricard (Mexico), Instituto Cervantes (Alburquerque, USA), Instituto Cervantes (Milan, Italy), Instituto Cervantes (Tokyo, Japan), Lilly Laboratories (Spain), and the Wine and Culinary International Forum (Spain), as well as non-financial support for the organization of a National Congress on Nutrition and feeding trials with products from Grand Fountain and Uriach Laboratories (Spain). The remaining authors have nothing to disclose.

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