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Association of microbiota polyphenols with cardiovascular health in the context of a Mediterranean diet

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ABSTRACT

Background and aims: The Mediterranean diet (MedDiet) is rich in polyphenols, phytochemicals that are beneficial for cardiovascular health. Phenolic compounds have poor bioavailability but they are extensively metabolized by the gut microbiota. Therefore, we aimed to assess the association of microbial phenolic metabolites (MPM) with adherence to the MedDiet, and their relationship with ideal cardiovascular health (ICVH) and cardiovascular risk factors.

Methods and results: This cross-sectional substudy within the PREDIMED trial included 200 participants from the Barcelona-Clinic recruitment center. Five MPM were identified and quantified using a novel method based on liquid chromatography coupled to mass spectrometry: protocatechuic acid (PCA), enterodiol glucuronide (EDG), enterolactone glucuronide (ELG), vanillic acid glucuronide (VAG) and urolithin B glucuronide (UBG). Multivariable-adjusted regressions were used to evaluate the associations between MPM and MedDiet adherence, ICVH score, biochemical parameters, and blood pressure. Additionally, an MPM score was calculated as the weighted sum of MedDiet adherence and ICVH and found to be directly associated. Among individual polyphenols, UBG was inversely associated with LDL-cholesterol.

Conclusions: A score of urinary MPM was associated with higher adherence to the MedDiet and ICVH, and individual MPM were related to better cardiovascular health. These findings suggest that the MedDiet may affect gut microbiota, whose metabolites are linked with cardiovascular health.

1. Introduction

Cardiovascular diseases (CVD), which include various heart and circulatory system disorders, are the leading cause of premature death in Europe (Francula-Zaninovic & Nola, 2018). Individual characteristics and habits, such as smoking, hypertension, hypercholesterolemia, diabetes mellitus, obesity, and an unhealthy diet, have been identified as CVD risk factors (Joseph et al., 2017; Piepoli & Villani, 2017). In 2010, the American Heart Association (AHA) proposed an ideal cardiovascular health (ICVH) score with the purpose of improving overall health and reducing deaths from CVD (Lloyd-Jones et al., 2010). The ICVH score is based on 7 parameters of ideal health behaviors (nonsmoking, body

Abbreviations: CVD, Cardiovascular diseases; DBP, diastolic blood pressure; EDG, enterodiol glucuronide; ELG, enterolactone glucuronide; HDL-cholesterol, high-density lipoprotein-cholesterol; ICVH, ideal cardiovascular health; LTQ-Orbitrap-MS, linear ion trap quadrupole-Orbitrap-mass spectrometry; LDL-cholesterol, low-density lipoprotein-cholesterol; MedDiet, Mediterranean diet; MPM, microbial phenolic metabolites; PCA, protocatechuic acid; TG, triglycerides; SBP, systolic blood pressure; UBG, urolithin B glucuronide; VAG, vanillic acid glucuronide.

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mass index (BMI) < 25 kg/m2, appropriate physical activity, and healthy diet) and ideal health factors (total cholesterol < 200 mg/dL, blood pressure < 120/80 mmHg, and fasting blood glucose < 100 mg/dL). The benefits of meeting the 7 health metrics have been demonstrated in numerous epidemiological studies in different countries and populations, adherence being inversely associated with CVD (Ahmad et al., 2019; Aneni et al., 2017).

Diet and cardiovascular health are closely related, as certain nutrients and phytochemicals play a major role in the prevention or development of CVD (GBD, 2013). As the effects of individual nutrients depend on interactions with other components of food, it can be more useful to assess their impact within the context of a diet or dietary pattern rather than separately (Rinaldi de Alvarenga et al., 2019).

The Mediterranean diet (MedDiet) is proven to reduce the risk of developing CVD (Estruch et al., 2018). Based on the consumption of extra virgin olive oil, nuts, fruits, vegetables, whole grains, legumes, fish, and moderate quantities of wine (Bach-Faig et al., 2011), this dietary pattern is rich in healthy fats and other antioxidant bioactive molecules such as polyphenols (Tresserra-Rimbau et al., 2013). Although the intake of polyphenols has been demonstrated to improve cardiovascular health (Li et al., 2020; Tresserra-Rimbau, Rimm, Medina-Remón, Martínez-González, López-Sabater, et al., 2014), their bioavailability in the small intestine is low and 90–95 % of them reach the large intestine/colon, where they undergo enzymatic transformations catalyzed by the gut microbiota (Cardona et al., 2013; Marhuenda-Muñoz et al., 2019). After absorption, these microbial phenolic metabolites (MPM) may exert biological effects but to date, studies assessing the positive benefits of different classes of MPM on cardiovascular health are scarce.

The present study applied an innovative method to determine and quantify MPM in a subpopulation of the PREDIMED trial using a linear ion trap quadrupole-Orbitrap-mass spectrometer (LTQ-Orbitrap-MS), which provides accurate structural information to identify and quantify novel compounds. We aimed to assess the relationship between MPM and MedDiet adherence, as well as to evaluate their association with the ICVH score and cardiovascular risk factors in an elderly Mediterranean population.

2. Methods

2.1. Study design

A cross-sectional analysis was carried out using baseline data from the PREDIMED (PREvención con DIeta MEDiterránea) trial, a large, parallel-group, multicenter, randomized, controlled, five-year clinical trial that examined the effect of the traditional MedDiet on the primary prevention of CVD (https://www.predimed.es). The details of the study design can be found elsewhere (M. Á. Martínez-González et al., 2012). A total of 7,447 participants were recruited in Spain between October 2003 and December 2010, the men aged 55–80 years and women, 60–80 years. Eligible participants were free of CVD at baseline and presented type-2 diabetes or at least three of the following cardiovascular risk factors: current smoking, hypertension, dyslipidaemia, overweight/obesity or family history of premature CVD.

In the present study, 200 randomly selected participants from the PREDIMED-Hospital Clinic recruitment center (Barcelona) were included. Extreme total energy intake (>3500 or < 500 kcal/day in women or > 4000 or < 800 kcal/day in men) was an exclusion criterion for this subanalysis (Fernández-Ballart et al., 2010).

The Institutional Review Board of the Hospital Clinic (Barcelona, Spain), accredited by the US Department of Health and Human Services (DHHS) update for Federal-wide Assurance for the Protection of Human Subjects for International (Non-US) Institutions #00000738, approved the study protocol on July 16, 2002. All participants provided informed consent and signed a written consent form.

2.2. Covariate assessment

Dietary intake was assessed using a validated, semi-quantitative 137-item food frequency questionnaire with the assistance of trained dietitians (Fernández-Ballart et al., 2010). To assess MedDiet adherence, a 14-item questionnaire with a value of 0 or 1 for each dietary component was used. Each item is related to a specific feature of the MedDiet, and the higher the overall score, the greater the adherence (Martínez-González, Buil-Cosiales, et al., 2019).

Trained personnel recorded the anthropometric and clinical measurements of the participants, including height, weight, waist circumference, and blood pressure. BMI was calculated by dividing the body weight in kilograms by the squared height in cm. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured in triplicate with a validated semi-automatic oscillometer (Omron HEM-705CP, Lake Forest, IL, USA). The Minnesota Leisure-Time Physical Activity Questionnaire was used to assess physical activity (metabolic equivalent tasks per minute per day, METS min/day) of the participants (Elosua et al., 2000).

During the first screening visit, data on medical conditions, family history of disease, and risk factors were collected through a questionnaire. Biological samples (plasma and urine) were taken at baseline after a 12 h overnight fast and stored at −80 °C until analysis. Blood glucose, total cholesterol, triglycerides (TG), and high-density lipoprotein-cholesterol (HDL-cholesterol) were determined by standard enzymatic methods, and low-density lipoprotein-cholesterol (LDL-cholesterol) was calculated by the Friedewald equation (Estruch et al., 2006).

2.3. Microbial phenolic metabolite analysis

2.3.1. Standards and reagents

Protocatechuic acid (PCA), enterodiol, urolithin-A, and urolithin-B were purchased from Sigma-Aldrich (St. Louis, MO, USA). The internal standard (+)*cis,trans*-abscisic acid d_6 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Vanillic acid, enterolactone and creatinine were obtained from Fluka (St. Louis, MO, USA). Standards were stored in powder form and protected from light.

The reagents were purchased from the following commercial suppliers: methanol of LC-MS grade and acetonitrile of HPLC grade from Sigma-Aldrich and formic acid (≥98 %) from Panreac Química S.A. (Barcelona, Spain). Ultrapure water (Milli-Q) was generated by a Millipore system (Bedford, MA, USA).

2.3.2. Sample preparation

Urinary MPM were determined following a method previously validated by our research group with minor modifications (Laveriano-santos et al., 2022). Briefly, urine samples (50 μL) diluted 1:20 (*v:v*) with Milli-Q Water and 100 μL of the internal standard abscisic acid d_6 were acidified with 2 μL of formic acid. After centrifugation at 14,000 g for 4 min at 4 °C, the acidified urines underwent solid-phase extraction in Water Oasis HLB 96-well plates 30 μm (30 mg) (Water Oasis, Milford, MA, USA) to extract the phenolic metabolites and eliminate unwanted compounds. After activating the plate with 1.5 M formic acid, the samples were loaded and cleaned with 1.5 M formic acid and 0.5 % methanol. The MPM were eluted with methanol acidified with 0.1 % formic acid, evaporated to dryness with nitrogen gas and reconstituted with 100 μL of 0.05 % formic acid. Finally, the samples were vortexed for 20 min and filtered through 0.22 μm polytetrafluoroethylene 96-well plate filters (Millipore, Massachusetts, USA).

Urine concentrations of MPM were corrected by urine creatinine, which was measured according to the adapted Jaffé alkaline picrate method for 96-well plates described by Medina-Remón et al. (2009). Urine concentrations of MPM were expressed as μmol per mg creatinine.

2.3.3. LTQ Orbitrap ESI analysis

The analysis was performed on an LTQ Orbitrap Velos mass

Table 1
General characteristics of the participants ($n = 200$).

Characteristics	
Women, %	54.5
Age, years	66.1 ± 5.3
Weight, kg	76.6 ± 11.7
BMI, kg/m^2	29.5 ± 3.4
Diabetes Mellitus, %	51.0
Dyslipidaemia, %	79.0
Hypertension, %	76.0
Current smoker, %	15.0
Education level, %	
Low	66.5
Medium & High	33.5
Physical activity, METS-min/day	291.0 ± 266.9
Total energy intake, kcal/day	2394.1 ± 500.8

METS, Metabolic Equivalents.
Continuous variables are shown as means ± SDs, and categorical variables are shown as percentages.

Table 2
Means and SD of urine concentrations of MPM (micromole per mg of creatinine) of the participants ($n = 200$).

Metabolites (Mean ± SD)	
Protocatechuic acid	0.009 ± 0.020
Vanillic acid glucuronide	0.222 ± 0.393
Enterodiol glucuronide	0.003 ± 0.004
Enterolactone glucuronide	0.020 ± 0.026
Urolithin B glucuronide	0.158 ± 0.605

MPM, microbial phenolic metabolites.

Table 3
Multivariable adjusted regression between Mediterranean diet adherence diet and MPM.

		β (95 % CI)	*p*-value
Protocatechuic acid	*Model 1*	0.68 (0.14; 1.22)	0.013*
	Model 2	0.62 (0.09; 1.15)	0.022
Vanillic acid glucuronide	*Model 1*	0.41 (-0.15; 0.97)	0.150
	Model 2	0.34 (-0.23; 0.91)	0.244
Enterodiol glucuronide	*Model 1*	0.36 (-0.27; 0.98)	0.260
	Model 2	0.30 (-0.31; 0.93)	0.347
Enterolactone glucuronide	*Model 1*	0.64 (-0.10; 1.18)	0.019*
	Model 2	0.55 (0.01; 1.10)	0.046
Urolithin B glucuronide	*Model 1*	0.35 (-0.17; 0.88)	0.181
	Model 2	0.42 (-0.12; 0.96)	0.125
MPM Score	*Model 1*	0.70 (0.14; 1.26)	0.021
	Model 2	0.62 (0.06; 1.18)	0.041

MPM, microbial phenolic metabolites.
β, difference between groups; CI, confidence interval.
*This difference remained statistically significant after adjusting for multiple comparisons.
Model 1: sex and age.
Model 2: sex, age, smoking habit, educational level, BMI, physical activity, and total energy intake.

spectrometer (Thermo Scientific, Hemel Hempstead, UK) equipped with an ESI source working in negative mode, as described elsewhere (Laveriano-santos et al., 2022). Chromatographic separation was performed on a Kinetex F5 100 Å (50 × 4.6 mm × 2.6 μm) from Phenomenex (Torrance, CA, USA). Mobile phases A and B were, respectively, 0.05 % formic acid in water and 0.05 % formic acid in acetonitrile. The following linear gradient was used: held at 98 %A for 1.7 min, decreased to 92 %A for 3 min, decreased to 80 %A for 1.3 min, decreased to 70 %A for 1.3 min, decreased to 50 % for 0.1 min, decreased to 0 % for 1.3 min, then returned to the initial conditions for 1.7 min and re-equilibrated for 3 min. The flow rate was set at 0.750 mL/min and the injection volume was 5 μL.

Table 4
Frequency of participants with higher scores for overall ICVH and individual metrics.

Metrics	*n* (%)
Overall ICVH score	
0	1 (0.5)
1	16 (8.0)
2	67 (33.5)
3	63 (31.5)
4	43 (21.5)
5	10 (5.0)
Individual metrics	
Smoking status	164 (82.0)
Body mass index	12 (6.0)
Physical activity	167 (83.5)
Health diet score	83 (41.5)
Total cholesterol	64 (32.0)
Blood pressure	8 (4.0)
Glucose	63 (31.5)

ICVH, ideal cardiovascular health.

Table 5
Multivariable adjusted regression between overall ICVH and individual metrics and MPM score.

	β (95 % CI)	*p*-value
Overall ICVH score	1.29 (0.52; 2.07)	0.001
Individual Metrics		
Smoking status	0.07 (-0.18; 0.35)	0.523
Body mass index	0.02 (-0.18; 0.23)	0.839
Physical activity	0.20 (-0.11; 0.51)	0.201
Diet	0.65 (0.28; 1.01)	0.001
Blood pressure	0.09 (-0.07; (0.25)	0.275
Total cholesterol	−0.13 (-0.49; (0.22)	0.457
Glucose	0.38 (0.01; 0.75)	0.042

MPM, microbial phenolic metabolites.
β, difference between groups; CI, confidence interval.
Adjusted for sex and age.

2.3.4. Identification and quantification of MPM

Trace Finder software version 4.1 (Thermo Fisher Scientific, San Jose, CA) was used to identify and quantify urinary MPM. The glucuronidated and sulfated metabolites were quantified using their respective aglycone equivalents due to the unavailability of standards.

Metabolic differences and high inter-variability are major concerns when assessing metabolites derived from gut microbiota. Due to the wide variability of metabolites identified across the 200 participants, we focused our study on those with less than 20 % of missing values. Thus, the 5 MPM included in the statistical analysis were: PCA, enterodiol glucuronide (EDG), enterolactone glucuronide (ELG), urolithin B glucuronide (UBG), and vanillic acid glucuronide (VAG). When values for these 5 metabolites were missing, half the minimum detectable value was used.

2.4. Ideal cardiovascular health

The ICVH score was calculated based on the 7 health metrics proposed by the AHA (Lloyd-Jones et al., 2010). Thus, a value of 1 was assigned when the participants met the following criteria: never smoked or quit > 12 months ago; ≥ 9 points of adherence to the MedDiet; ≥ 150 min/week of moderate or ≥ 75 min/week of vigorous physical activity equivalent to ≥ 500 METS-min/week (Organization, 2020); BMI < 25 kg/m^2; total cholesterol < 200 mg/dL; blood pressure < 120/80 mmHg and fasting glucose < 100 mg/dL. The MedDiet was selected as a healthy dietary pattern considering the extensive and robust evidence for its beneficial effect on cardiovascular health (Martínez-González, Gea, et al., 2019). The 9 cut-point score was used to define good adherence to the MedDiet in accordance with previous studies (Hu et al., 2013).

Table 6
Multivariable adjusted regression between biochemical parameters and MPM.

	LDL, mg/dL		HDL, mg/dL		TG, mg/dL		Glucose, mg/dL	
	β (95 % CI)	*p*-value	β (95 % CI)	*p*-value	β (95 % CI)	*p*-value	β (95 % CI)	*p*-value
Protocatechuic acid								
Model 1	0.09 (−3.98; 4.17)	0.964	−0.01 (−0.04; 0.03)	0.688	−0.02 (−0.08; 0.04)	0.600	−0.01 (−0.05; 0.03)	0.595
Model 2	0.93 (−2.99; 4.84)	0.641	−0.01 (−0.04; 0.02)	0.374	0.01 (−0.05; 0.07)	0.859	−0.01 (−0.02; 0.04)	0.554
Model 3	1.80 (−2.34; 5.94)	0.393	−0.01 (−0.04; 0.03)	0.688	−0.02 (−0.08; 0.04)	0.576	<0.01 (−0.03; 0.03)	0.866
Vanillic acid								
Model 1	−1.73 (−5.71; 2.26)	0.394	−0.01 (−0.04; 0.03)	0.707	0.05 (0.01; 0.11)	0.102	0.01 (−0.03; 0.05)	0.594
Model 2	−1.54 (−5.37; 2.29)	0.429	−0.01 (−0.04; 0.02)	0.574	0.06 (<0.01; 0.12)	0.039	0.01 (−0.01; 0.04)	0.338
Model 3	−1.46 (−5.55; 2.62)	0.481	−0.01 (−0.04; 0.03)	0.604	0.06 (<−0.01; 0.12)	0.054	−0.01 (−0.02; 0.04)	0.581
Enterodiol glucuronide								
Model 1	−1.01 (−5.08; 3.05)	0.624	0.01 (−0.02; 0.04)	0.604	<0.01 (−0.06; 0.06)	0.921	−0.03 (−0.07; <−0.01)	0.071
Model 2	−0.26 (−4.09; 3.56)	0.893	<0.01 (−0.03; 0.03)	0.790	0.01 (−0.05; 0.06)	0.857	−0.03 (−0.05; <0.01)	0.071
Model 3	−0.58 (−4.77; 3.61)	0.786	0.01 (−0.02; 0.04)	0.604	<0.01 (−0.06; 0.06)	0.991	−0.03 (−0.06; <0.01)	0.066
Enterolactone glucuronide								
Model 1	2.71 (−1.24; 6.66)	0.178	0.02 (−0.01; 0.05)	0.238	−0.03 (−0.09; 0.03)	0.311	−0.04 (−0.08; <−0.01)	0.041
Model 2	2.05 (−1.75; 5.86)	0.289	0.02 (−0.01; 0.05)	0.201	−0.01 (−0.07; 0.05)	0.766	<−0.01 (−0.03; 0.03)	0.958
Model 3	2.18 (−2.01; 6.37)	0.307	0.02 (−0.01; 0.05)	0.238	−0.02 (−0.08; 0.04)	0.542	<−0.01 (−0.04; 0.03)	0.773
Urolithin B glucuronide								
Model 1	−3.46 (−7.40; 0.49)	0.086	<−0.01 (−0.03; 0.03)	0.962	0.03 (−0.03; 0.09)	0.302	0.01 (−0.03; 0.05)	0.644
Model 2	−4.83 (−8.63–1.02)	0.013	<0.01 (−0.03; 0.03)	0.905	0.01 (−0.05; 0.07)	0.825	<0.01 (−0.03; 0.03)	0.940
Model 3	−5.58 (−9.66;−1.50)	0.008*	<−0.01 (−0.03; 0.03)	0.962	0.03 (−0.03; 0.10)	0.268	<0.01 (−0.03; 0.03)	0.963

MPM, microbial phenolic metabolites; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TG, triglycerides.
*This difference remained statistically significant after adjusting for multiple comparisons.
Model 1: sex and age.
Model 2: sex, age, smoking habit, educational level, BMI, physical activity, diabetes, hypercholesterolemia, hypertension and medication (cholesterol-lowering, antidiabetic and antihypertensive agents).
Model 3: sex, age, smoking habit, educational level, BMI, physical activity, diabetes, hypercholesterolemia, hypertension, medication (cholesterol-lowering, antidiabetic and antihypertensive agents), total energy intake and Mediterranean diet adherence.

Table 7
Multivariable adjusted regression between blood pressure and phenolic metabolites.

	DBP, mmHg		SBP, mmHg	
	β (95 % CI)	*p*-value	β (95 % CI)	*p*-value
Protocatechuic acid glucuronide				
Model 1	0.75 (−0.68; 2.18)	0.297	0.01 (−0.01; 0.03)	0.274
Model 2	0.96 (−0.49; 2.40)	0.198	0.01 (−0.01; 0.03)	0.321
Model 3	1.01 (−0.52; 2.54)	0.193	0.01 (−0.01; 0.03)	0.233
Vanillic acid glucuronide				
Model 1	1.00 (−0.39 2.39)	0.157	0.02 (<−0.01; 0.03)	0.117
Model 2	1.26 (−0.15; 2.68)	0.079	0.02 (<−0.01; 0.03)	0.081
Model 3	1.48 (−0.02; 2.98)	0.054	0.02 (<−0.01; 0.04)	0.061
Enterodiol glucuronide				
Model 1	−0.52 (−1.93; 0.90)	0.472	<0.01 (−0.02; 0.02)	0.865
Model 2	−0.42 (−1.83; 0.99)	0.553	<0.01 (−0.02; 0.02)	0.682
Model 3	−0.61 (−2.16; 0.93	0.436	<0.01 (−0.02; 0.02)	0.900
Enterolactone glucuronide				
Model 1	−0.89 (−2.27; 0.49)	0.204	−0.01 (−0.03; 0.01)	0.221
Model 2	−0.43 (−1.84; 0.98)	0.547	<−0.01 (−0.02; 0.01)	0.682
Model 3	−0.60 (−2.15; 0.95)	0.443	<−0.01 (−0.02; 0.02)	0.711
Urolithin B glucuronide				
Model 1	1.25 (−0.11; 2.62)	0.072	0.01 (−0.01; 0.02)	0.399
Model 2	1.16 (0.26; 2.57)	0.109	0.01 (−0.01; 0.02)	0.417
Model 3	1.53 (−0.01; 3.06)	0.048	0.01 (−0.01; 0.03)	0.375

MPM, microbial phenolic metabolites; DBP, diastolic blood pressure; SBP, systolic blood pressure.
*This difference remained statistically significant after adjusting for multiple comparisons.
Model 1: sex and age.
Model 2: sex, age, smoking habit, educational level, BMI, physical activity, diabetes, hypercholesterolemia, hypertension, and medication (cholesterol-lowering, antidiabetic and antihypertensive agents).
Model 3: sex, age, smoking habit, educational level, BMI, physical activity, diabetes, hypercholesterolemia, hypertension, medication (cholesterol-lowering, antidiabetic and antihypertensive agents), total energy intake and Mediterranean diet adherence.

Table A1
Daily intake of foods for the total population and according to tertiles of MPM.

	All ($n = 200$)	T1 ($n = 67$)	T2 ($n = 67$)	T3 ($n = 66$)	*p*-value
Olive oil (g)	40.0 ± 13.5	40.0 ± 13.2	41.7 ± 13.3	39.3 ± 14.1	0.446
Nuts (g)	9.1 ± 11.4	10.9 ± 13.6	9.5 ± 11.3	6.9 ± 8.7	0.122
Fruits (g)	479.2 ± 232.9	451.8 ± 237.4	452.3 ± 227.0	534.3 ± 227.9	0.063
Vegetables (g)	406.6 ± 175.9	426.3 ± 219.5	397.5 ± 156.7	395.7 ± 142.4	0.531
Legumes (g)	17.8 ± 7.4	18.1 ± 7.2	18.1 ± 6.7	17.2 ± 8.4	0.732
Fish (g)	117.5 ± 44.1	119.5 ± 40.0	124.1 ± 49.1	108.8 ± 42.0	0.122
Meat or meat products (g)	148.8 ± 53.7	149.3 ± 56.7	146.7 ± 47.0	150.3 ± 57.6	0.924
Pastries (g)	21.0 ± 25.9	23.7 ± 28.5	17.84 ± 25.4	21.6 ± 23.6	0.417
Dairy products (g)	338.4 ± 207.4	364.3 ± 179.5	306.9 ± 208.0	344.1 ± 231.0	0.268
Alcohol (g)	11.7 ± 17.2	8.6 ± 12.9[a]	15.9 ± 22.3[b]	10.5 ± 14.2[a,b]	0.038
Fiber (g)	27.4 ± 7.3	26.9 ± 7.6	27.2 ± 7.6	28.2 ± 6.7	0.612
Cholesterol (g)	407.0 ± 113.1	416.0 ± 117.9	412.3 ± 110.7	392.7 ± 110.9	0.445
Sodium (mg)	2523.9 ± 779.6	2529.1 ± 856.4	2621.7 ± 847.7	2419.3 ± 604.4	0.327
Folic acid (μg)	458.7 ± 103.6	457.5 ± 108.2	456.5 ± 105.0	462.2 ± 98.7	0.946
Total polyphenols (mg)	999.8 ± 316.5	971.9 ± 300.5	991.4 ± 332.6	1036.2 ± 316.7	0.491

MPM, microbial phenolic metabolites.
p-values were calculated using the test one-way ANOVA.
Different lower-case letters indicate a significant difference among groups and were calculated analysed using Bonferroni post-hoc test.

Table A2
Multivariable adjusted regression between overall ICVH and individual metrics and MPM.

	β (95 % CI)	*p*-value
Protocatechuic acid		
Overall ICVH	0.10 (-0.06; 0.25)	0.214
Individual Metrics		
Smoking status	<0.01 (-0.06; 0.07)	0.875
Body mass index	0.01 (-0.02; 0.05)	0.379
Physical activity	−0.01 (-0.07; 0.05)	0.764
Diet	0.09 (-0.03; 0.16)	0.007
Blood pressure	<-0.01 (-0.03; 0.02)	0.803
Total cholesterol	0.01 (-0.06; 0.07)	0.863
Blood glucose	−0.01 (-0.08; 0.07)	0.842
Vanillic acid glucuronide		
Overall ICVH	−0.16 (-0.29; −0.02)	0.026
Individual Metrics		
Smoking status	−0.04 (-0.09; 0.01)	0.166
Body mass index	0.01 (-0.02; 0.04)	0.448
Physical activity	−0.04 (-0.10; 0.02)	0.227
Diet	−0.01 (-0.08; 0.06)	0.806
Blood pressure	−0.01 (-0.03; 0.01)	0.406
Total cholesterol	−0.01 (-0.08; 0.05)	0.717
Blood glucose	−0.07 (-0.13; <-0.01)	0.052
Enterodiol glucuronide		
Overall ICVH	0.03 (-0.11; 0.17)	0.689
Individual Metrics		
Smoking status	0.01 (-0.03; 0.06)	0.596
Body mass index	−0.01 (-0.04; 0.03)	0.713
Physical activity	−0.05 (-0.10; 0.01)	0.096
Diet	0.10 (0.03; 0.16)	0.003
Blood pressure	0.01 (-0.01; 0.04)	0.343
Total cholesterol	−0.02 (-0.09; 0.04)	0.504
Blood glucose	−0.02 (-0.09; 0.05)	0.623
Enterolactone glucuronide		
Overall ICVH	0.09 (-0.06; 0.24)	0.227
Individual Metrics		
Smoking status	−0.02 (-0.07; 0.02)	0.321
Body mass index	0.02 (-0.01; 0.05)	0.156
Physical activity	<0.01 (-0.06; 0.06)	0.913
Diet	0.11 (0.05; 0.18)	0.001
Blood pressure	0.02 (-0.01; 0.05)	0.148
Total cholesterol	−0.07 (-0.13; −0.01)	0.027
Blood glucose	0.03 (-0.04; 0.09)	0.452
Urolithin B glucuronide		
Overall ICVH	−0.05 (-0.8; 0.09)	0.483
Individual Metrics		
Smoking status	0.03 (-0.03; 0.08)	0.349
Body mass index	0.01 (-0.02; 0.04)	0.630
Physical activity	−0.04 (-0.09; 0.01)	0.120
Diet	−0.04 (-0.11; 0.03)	0.224
Blood pressure	<0.01 (-0.02; 0.02)	0.827
Total cholesterol	0.01 (-0.05; 0.07)	0.790
Blood glucose	−0.01 (-0.08; 0.06)	0.746

ICVH, ideal cardiovascular health; MPM, microbial phenolic metabolites.
β, difference between groups; CI, confidence interval.
Adjusted for sex and age.

Overall scores ranged from 0 to 7, with a higher score indicating a better ICVH profile.

2.5. Statistical analyses

Baseline characteristics of the participants are presented as means ± standard deviation (SD) for continuous variables and percentages for categorical values. Individual baseline values of urine metabolites were normalized and scaled in multiples of 1-SD with Blom inverse normal transformation (Blom, 1960). Multivariable adjusted linear regression was used to assess the association of urinary MPM with MedDiet adherence, ICVH scores (both overall and individual metrics), and cardiovascular risk factors. To evaluate the MPM relationship with MedDiet adherence, measured with a 14-item dietary screener, two models of increasing complexity were used. Multivariable model 1 was adjusted for age and sex, and model 2 was additionally adjusted for smoking habit, educational level, BMI, physical activity, and energy intake. Additionally, we calculated an MPM score as the weighted sum of the concentrations of the 5 quantified metabolites (PCA, VAG, EDG, ELG, and UBG). The weight for each metabolite was the regression coefficient for a 1-SD increment in urine from the multivariable adjusted regression model. The associations of MPM with ICVH scores (overall and individual metrics) were adjusted for age and sex, and an MPM score was also calculated as described. To analyse the association of MPM with biochemical parameters (LDL-cholesterol, HDL-cholesterol, TG, and glucose) and blood pressure, the normality of the outcome variables was assessed with the Shapiro-Wilk test, and those that did not follow normal distribution were transformed into logarithms (HDL-cholesterol, TG, glucose and SBP). Three models of increasing complexity were employed: model 1 was adjusted for sex and age; model 2 was further adjusted for smoking habit, educational level, BMI (except anthropometric analyses), physical activity, diabetes, hypercholesterolemia, hypertension, and medication (cholesterol-lowering, antidiabetics, and antihypertensive agents); model 3 was further adjusted for total energy intake and MedDiet adherence. We used the procedure described by Simes to correct for multiple testing of the multivariable-adjusted associations that included the 5 MPM (Simes, 1986). All statistical analyses were performed using Stata 16.0 (Stata-Corp LP, Tx. USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. General characteristics

Table 1 shows the main characteristics of all the participants. Among the 200 participants, 109 were women and 91 men, and the overall mean age was 66.1 ± 5.3 years. As expected from the inclusion criteria of the PREDIMED trial, participants were at high cardiovascular risk; 51.0 % had T2D, 79.0 % had dyslipidaemia and 76.0 % had hypertension. A total of 15.0 % of the volunteers were current smokers and most had received a low level of education. Table A.1 describes the average food consumption of the participants according to tertiles of MPM. Food nutrient and polyphenol intake was similar across the three groups, except for alcohol, which was higher in the second tertile.

The concentrations of the 5 MPM, corrected for urinary creatinine, are presented in Table 2. The predominant metabolite was VAG (0.222 ± 0.393 μmol/mg of creatinine), followed by UBG (0.158 ± 0.605 μmol/mg of creatinine), and the least abundant was EDG (0.003 ± 0.004 μmol/mg of creatinine).

3.2. MPM and MedDiet adherence

Table 3 shows the association of MedDiet adherence with individual MPM and the MPM score. In the fully adjusted model, participants with higher MPM scores also reported greater adherence to the MedDiet (β = 0.62 (0.06; 1.18), *p*-value = 0.041). Regarding individual metabolites, PCA and ELG were positively associated with the MedDiet adherence score (β = 0.62 (0.09; 1.15) per 1-SD increase, *p*-value = 0.022 and β = 0.55 (0.01; 1.10) per 1-SD increase, *p*-value = 0.046, respectively). These associations were maintained after adjusting for multiple testing in Model 1, but the significance was lost after applying Model 2.

3.3. MPM score and ICVH

The ICVH metrics and the frequency of higher ICVH scores in participants are shown in Table 4. As could be expected from the nature of the PREDIMED trial, most participants obtained low ratings for overall ICVH. The majority achieved a score of 2 (33.5 %) or 3 (31.5 %), and none responded positively for>5 health items. Most of the participants did not obtain an ideal score in the individual metrics, except for smoking status and physical activity.

The association of the MPM score with the ICVH score, both overall

and individual metrics, is presented in Table 5. A positive association was found between the MPM score and the overall ICVH score (β = 1.29 (0.52; 2.07), *p*-value = 0.001). Regarding individual metrics, ratings for diet and blood glucose were significantly higher in participants with higher MPM scores (β = 0.65 (0.28; 1.01), *p*-value = 0.001; β = 0.38 (0.01; 0.75), *p*-value = 0.042, respectively). However, no differences were observed for the other items. Results for individual MPM are presented in Table A.2.

3.4. MPM and biochemical variables and blood pressure

The associations between individual MPM with LDL-cholesterol, HDL-cholesterol, TG, and glucose are shown in Table 6. Participants with higher urinary concentrations of UBG had lower levels of LDL-cholesterol in the fully adjusted model (β = -5.58 mg/dL (-9.66; −1.50) per 1-SD increase, *p*-value = 0.008), and this association was maintained after correcting for multiple testing. In addition, EDG was associated with lower levels of blood glucose in Model 1 (β = -0.04 mg/dL (-0.08; <-0.01) per 1-SD increase, *p*-value = 0.041), but this result did not remain significant after using more complex adjustment models. No significant association was found for any metabolite with HDL-cholesterol and TG, although we observed a strong tendency towards a positive association between VAG and TG.

As shown in Table 7, SBP was not significantly associated with any urinary MPM. Unexpectedly, UBG was directly associated with DBP (β = 1.53 mmHg (-0.01; −3.06) per 1-SD increase, *p*-value = 0.048), although the relationship did not remain significant after correcting for multiple testing.

4. Discussion

In this cross-sectional substudy of the PREDIMED trial, we observed that higher urinary MPM scores were associated with greater MedDiet adherence and a better ICVH score. We also found a strong inverse association between urinary concentrations of UBG and LDL-cholesterol. These findings suggest that the MedDiet is associated with phenolic metabolites that have a positive impact on cardiovascular health. To our knowledge, this is the first study to evaluate the link between diet, urinary MPM, and cardiovascular health using a high-resolution analytical technique (LTQ-Orbitrap-MS).

In the current study, the level of adherence to the MedDiet was found to be associated with the MPM score. The MedDiet is reported to modulate the microbiome ecosystem and the microbial metabolites produced (de Filippis et al., 2016; Ghosh et al., 2020). Studies on gut microbiota have reported that greater adherence to the MedDiet is linked to higher amounts of *Bacteroidetes* (Garcia-Mantrana et al., 2018; Gutiérrez-Díaz et al., 2016), including members of the genus *Prevotella* (de Filippis et al., 2016). Several gut microbiota species are involved in polyphenol metabolism, some of which belong to the *Bacteroidetes* phylum (Achterholt et al., 2000; Clavel et al., 2006; Selma et al., 2014; Venturi et al., 1989). Altogether, these results suggest that the MedDiet promotes a favourable microbial environment for the production of MPM potentially beneficial for human health.

In our subsample of the PREDIMED trial, no participant achieved the highest ICVH rating, and therefore none met all 7 health metrics. These results are consistent with a previous study within the PREDIMED trial, in which only 0.3 % of the participants achieved scores of 6 and 7 (Díez-Espino et al., 2020). The results are not surprising due to the nature of the PREDIMED trial, which is focused on an elderly Mediterranean population at high risk of CVD. However, other studies conducted in Mediterranean countries and the United States found that approximately 20 % of the adult population met at least 5 metrics (Fernandez-Lazaro et al., 2022; Younus et al., 2016). It is well-known that polyphenols have multiple benefits on cardiovascular health, mostly due to their anti-inflammatory properties [24]. Accordingly, a high polyphenol intake is reported to reduce mortality and provide cardioprotective benefits (Salazar et al., 2022; Tresserra-Rimbau, Rimm, Medina-Remón, Martínez-González, de la Torre, et al., 2014). Furthermore, urinary polyphenols are associated with lower DBP and SBP, and higher HDL-cholesterol in individuals following a Mediterranean diet (Medina-Remón et al., 2015). However, there is a lack of research on the effect of phenolic compounds detected in biological samples on the overall ICVH. The present findings suggest that ICVH is associated with multiple MPM. Regarding individual health metrics, diet and blood glucose were the most positively linked to the MPM score, which is in accordance with the association found between the MPM score and MedDiet adherence (Table 3). These results are in accordance with a recent report that polyphenols and their metabolites are associated with a lower risk of developing T2DM and reduced insulin resistance (Chiva-Blanch et al., 2013; Marhuenda-muñoz et al., 2022). Notably, individual MPM did not seem to benefit overall ICVH, suggesting that the phenolic metabolites may have a positive impact on cardiovascular health in combination rather than individually. Interestingly, total cholesterol was not associated with the MPM score, even though a negative association was observed between UBG and LDL-cholesterol in subsequent analyses. This calls into question if total cholesterol is the most appropriate item to include in the ICVH assessment, considering that LDL-cholesterol has a negative impact on health but HDL-cholesterol does not (Ference et al., 2018).

Urolithins are microbial products synthesized from ellagitannins and ellagic acid, whose main food sources are walnuts and pomegranates. Urolithin production is particularly subject to interindividual variability, as there are 3 urolithin metabotypes in the population (Tomás-Barberán et al., 2014). In our elderly PREDIMED cohort, we mainly detected metabolites of urolithin B, the glucuronidated conjugate of urolithin B being predominant. This is consistent with Cortés-Martín et al., who reported that metabotype B, the producer of urolithin B, increases with age, whereas urolithin A decreases (Cortés-Martín et al., 2018). Interestingly, in a previous clinical trial administering a phenolic supplement, LDL-cholesterol decreased only in participants with phenotype B (González-Sarrías et al., 2010). An *in vitro* study demonstrated that urolithin B may decrease the lipid plaque deposition by modulating the expression of genes involved in reverse cholesterol pathways (Zhao et al., 2019). This mechanism could explain the inverse association observed between UBG and LDL-cholesterol.

Dietary lignans, the precursors of enterodiol and enterolactone, are mainly obtained from cereal fibre, and their effect on adiposity and biochemical parameters has been assessed by numerous studies. Two intervention trials reported that dietary lignan supplementation improved glycaemic control (Pan et al., 2007; Zhang et al., 2008). In our study, we found that glucose tended to be negatively associated with EDG but not with ELG. It should be noted that dietary intake of lignans correlates poorly with enterolignans measured in biological samples, as their bioavailability may be affected by gut microbiota composition, intestinal transit time or antibiotic use (Peterson et al., 2010).

Previous publications have reported that polyphenol intake can reduce blood pressure in a population at high cardiovascular risk (Medina-Remón et al., 2013, 2015). In contrast, our data do not support this beneficial effect, as significant associations were not observed for most MPM, and the direct association between UBG and DBP was weak. It is plausible that polyphenol effects on blood pressure are caused by those absorbed in the small intestine and not metabolized by gut microbiota.

Our study has three main limitations. As all participants were elderly Mediterranean individuals at high cardiovascular risk, the results may not be applicable to other populations. Also, the sample size is relatively small, although comparable to those in similar studies performing targeted metabolomics (Marhuenda-muñoz et al., 2022). Since fecal samples were not collected in the PREDIMED trial, we could not assess the microbiota composition of our participants. Finally, the cross-sectional nature of the study does not allow causality to be determined.

On the other hand, the strengths of the study include the analysis of

biological samples, which provides reliable information on the metabolism of participants, unavailable through intake questionnaires. Importantly, the analytical equipment used (LTQ-Orbitrap) allows a precise and accurate elucidation and quantification of novel compounds.

In conclusion, we found that urinary MPM were directly associated with MedDiet adherence and a healthier cardiovascular status. These results suggest that the MedDiet is associated with phenolic metabolites that possess beneficial properties for cardiovascular health. In addition, they show that MPM effects need to be considered in combination rather than individually when assessing their health benefits. This may be helpful in establishing dietary recommendations that favour a healthier colonic microbial environment. However, more studies are needed to confirm these potential MPM-derived benefits.

CRediT authorship contribution statement

Inés Domínguez-López: Conceptualization, Methodology, Investigation, Writing – original draft. **Camila Arancibia-Riveros:** Investigation. **María Marhuenda-Muñoz:** Methodology, Writing – review & editing. **Anna Tresserra-Rimbau:** Conceptualization, Writing – review & editing. **Estefanía Toledo:** Writing – review & editing. **Montserrat Fitó:** Writing – review & editing. **Emilio Ros:** Writing – review & editing. **Ramon Estruch:** Writing – review & editing. **Rosa M. Lamuela-Raventós:** Conceptualization, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki and was approved by the Institutional Review Board of the 11 participating centres. The study was registered with the International Standard Randomized Controlled Trial Number (ISRCTN) 35739639.

Appendix A

See Tables A1 and A2.

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