Metabolomic pattern analysis after Mediterranean diet intervention in a non-diabetic population: a 1- and 3-year follow-up in the PREDIMED study

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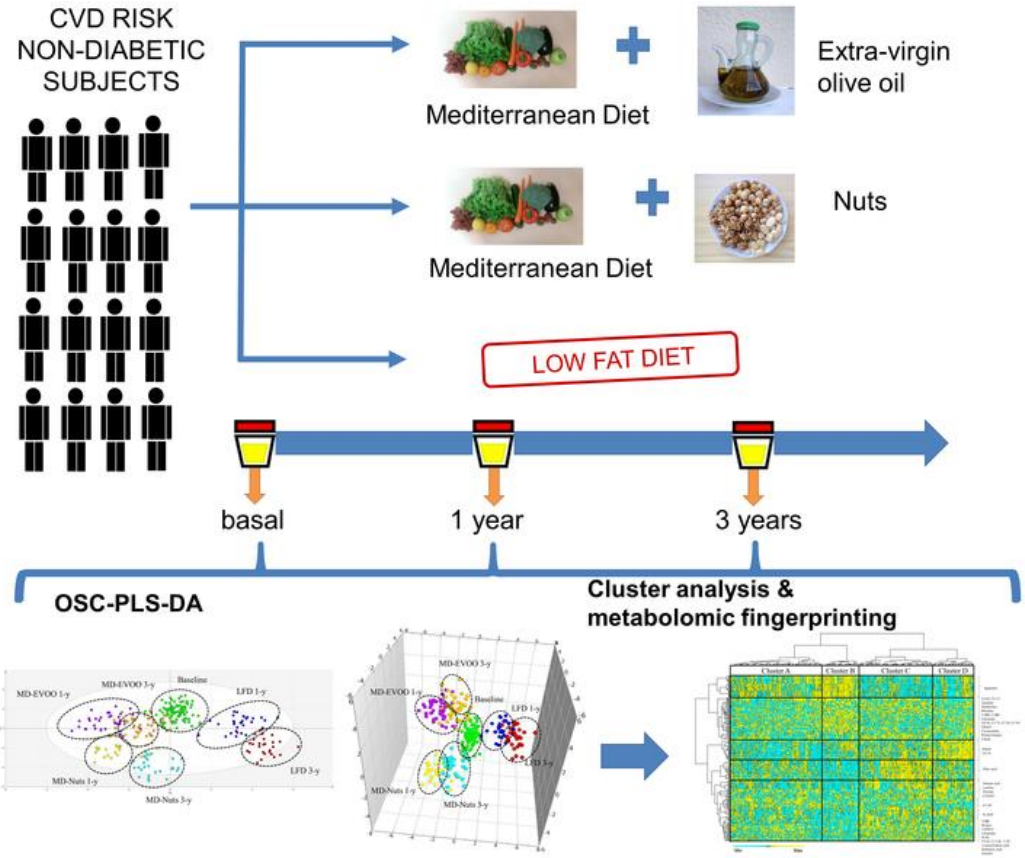
ABSTRACT

The Mediterranean diet (MD) is considered a dietary pattern with beneficial effects on human health. The aim of this study was to assess the effect of an MD on urinary metabolome by comparing subjects at 1 and 3 years of follow-up, after an MD supplemented with either extra-virgin olive oil (MD+EVOO) or nuts (MD+Nuts), to those on advice to follow a control low-fat diet (LFD). Ninety-eight non-diabetic volunteers were evaluated, using metabolomic approaches, corresponding to MD+EVOO (n=41), MD+Nuts (n=27) or LFD (n=30) groups. The ^{1}H-NMR urinary profiles were examined at baseline, after 1 and 3 years of follow-up. Multivariate data analysis (OSC-PLS-DA and HCA) methods were used to identify the potential biomarker discriminating groups, exhibiting a urinary metabolome separation between MD groups against baseline and LFD. Results revealed that the most prominent hallmarks concerning MD groups were related to the metabolism of carbohydrates (3-hydroxybutyrate, citrate, cis-aconitate), creatine, creatinine, amino acids (proline, N-acetylglutamine, glycine, branched-chain amino acids and derived metabolites), lipids(oleic and suberic acids), and microbial co-metabolites (phenylacetylglutamine, *p*-cresol). Otherwise, hippurate, trimethylamine-N-oxide, histidine and derivates (methylhistidines, carnosine, anserine) and xanthosine were predominant after LFD. The application of NMR-based metabolomics enabled the classification of individuals regarding their dietary pattern and highlights the potential of this approach for evaluating changes in the urinary metabolome at different time points of follow-up in response to specific dietary interventions.

Keywords: nutrimetabolomics, Mediterranean diet, nuclear magnetic resonance, low-fat diet, olive oil, nuts.



INTRODUCTION

Nowadays, the importance of a healthy diet is widely highlighted, along with the lifestyle needed to prevent several metabolic disorders and nutrition-linked illnesses such as obesity, diabetes and cardiovascular disease. Traditionally, studies centred on diet have focused on a single nutrient or dietary component in the evaluation of dietary intake (1, 2). However, the assessment of dietary patterns to elucidate the beneficial effects in complex disease processes is increasing. For instance, the Mediterranean diet (MD) is a dietary pattern that has recently shown beneficial effects on human health (3). This particular diet is based on the consumption of vegetables, fish, legumes, nuts and cereals. Other representative features are the consumption of olive oil as the main source of fat, moderate wine intake, relatively low amounts of meat (mainly poultry, instead of beef and pork) and low to moderate consumption of dairy products (4). Likewise, recently studies reported that MD supplemented with extra-virgin olive oil (EVOO) or nuts can reduce the incidence and prevalence of major cardiovascular events (3, 5-8). Moreover, frequency of nut consumption was recently shown to be inversely associated with the prevalence of obesity, metabolic syndrome and diabetes (9), and lower incidence of mortality (10) in subjects at high cardiovascular risk from the PREDIMED study. Similarly, extra-virgin olive oil consumption was recently reported

to be inversely related to cardiovascular disease mortality (11), and reduced the risk of atrial fibrillation (12).

Metabolomics is an interesting tool for assessing the nutritional status of an individual, as well as for studying the biological consequences or metabolic mechanisms following a nutritional intervention (13-15). For instance, population-based studies have shown marked differences in metabolic profiles within and between populations that reflect dietary differences (16, 17). A number of metabolic profiling studies have been undertaken to assess the metabolic phenotype of humans due to their dietary pattern (18, 19). Moreover, a metabolomic approach has been proposed to evaluate the intricate relationship between nutrition and health (20).

In the present study, we aimed to assess the impact on urine metabolites of a long-term intervention with a complex diet in a cohort study population. Specifically, we evaluated the effect on the urine metabolome of a 1- and 3-year dietary intervention with an MD plus either EVOO or nuts as supplements in a sub-cohort from PREDIMED non-diabetic subjects using high-throughput screening ^{1}H-NMR spectroscopy. To our knowledge, this is the first time that a long-term MD interventional study has been evaluated on the metabolic profile of individuals using a metabolomic approach.

MATERIAL AND METHODS

Study design and participants

The PREDIMED study is a parallel-group, single-blind, multicentre, randomized, controlled, 5-year feeding trial assessing the effects of a Mediterranean diet (MD) supplemented either with EVOO (MD+EVOO) or mixed nuts (MD+Nuts) on the primary prevention of cardiovascular disease (CVD) compared to a low-fat diet (LFD). Details of the study protocol were published previously (21-23). The present sub-study is an analysis using 98 clinically evaluated non-diabetic subjects at high CVD risk, recruited from the Barcelona (IDIBAPS) and Valencia centres of the PREDIMED study (3) (ISRCTN 35739639). These participants had at least three cardiovascular risk factors (current smoking, hypertension, hypercholesterolemia, body mass index (BMI) $\geq$25 kg/m^2, or a family history of premature cardiovascular disease). Exclusion criteria were type 2 diabetes mellitus, cardiovascular disease, any severe chronic illness, drug or alcohol addiction, history of allergy, or intolerance to olive oil or nuts. Urine samples were obtained at baseline and after 1 and 3 years of the intervention period, along with other clinical and dietary data, collected according to the PREDIMED study protocols.

Dietary intervention

Participants allocated to the low-fat diet were advised to reduce all types of fat and were given written recommendations according to the American Heart Association guidelines. MD group participants received instructions directed at upscaling the 14-item score measuring adherence to MD, including the use of EVOO for cooking and dressing; increased consumption of vegetables, nuts and fish products; consumption of white meat instead of red or processed meat; and following a moderate pattern of red wine consumption (Supporting Information, Table S1). Neither energy restriction nor physical activity promotion were suggested for any intervention group. MD group subjects were given free allotments of EVOO (1 L/week) or mixed nuts (30 g/day, as 15

g of walnuts, 7.5 g of hazelnuts and 7.5 g of almonds). All participants had free access to their dietitian throughout the study. The fatty acid and minor component composition of the EVOO and nuts employed in the study was published elsewhere (21). The local research ethics committee approved the study protocol, and all participants provided written informed consent.

Metabolomic analysis of urine samples

^{1}-NMR Sample Preparation Data Acquisition and Processing

A procedure based on previously published methodology was applied for the metabolomic analysis. Briefly, the initial volume of urine samples from the study participants was 300 µl diluted with 200 µl of H_2O/D_2O (8:2) and mixed with a buffer solution (24). The spectral data processed were intelligent, bucketed in domains of 0.005 ppm and integrated using ACD/NMR Processor 12.0 software (Advanced Chemistry Development, Inc.). The spectral region between 4.75 and 5.00 ppm was excluded from the data set to avoid spectral interference from residual water. The ^{1}H-NMR experiment was processed with 128 scans with a spectral width of 14 ppm, an acquisition time of 3.2 s and a relaxation delay of 3 s. To exclude data points showing little variance across experimental conditions, data were interquartile range (IQR) filtered, row-wise normalized by sum to reduce systematic bias during sample collection and cube root transformed by a corresponding tool provided by *MetaboAnalyst* (25).

Exploratory data analysis and orthogonal signal correction partial least squares discriminant analysis (OSC-PLS-DA)

Data were imported to SIMCA-P+13.0 software (Umetrics, Umeå, Sweden) and Pareto-scaled prior to being analysed. An exploratory data analysis, principal component analysis (PCA), which provides a summary overview of all observations in the data, was performed. After the initial overview of the data, the aim was to build a model that could be useful for classifying new samples, and at the same time could allow identification of biomarkers from the distinct groups (26). Then, the data set was preprocessed using orthogonal signal correction (OSC) filtration before PLS-DA analysis to reduce the variability not associated with the diet effect (27). The ability to classify each individual in the correct group was assessed by the proportion of the variance of the response variable that is explained by the model (R^2Y) and the predictive ability parameter (Q^2), which was calculated by a seven-round internal cross-validation of the data using a default option of the SIMCA-P+ 13.0 software. The values of $Q^2 < 0$ suggest a model with no predictive ability, and $0 < Q^2 < 1$ suggests some predictive character, with the reliability increasing as Q^2 approaches 1 (28). Validation of the models and the evaluation of the degree of overfitting were then crucial to ensure that models were robust and not overfitted, as well as to exclude models that are just due to chance. For this purpose, a response permutation test (n = 200) was performed, and the correlation coefficient between the original Y and the permuted Y plotted against the cumulative R^2 and Q^2 was calculated. Generally, the R^2- and Q^2-intercept limits for a valid model should be less than 0.4 and 0.05, respectively (29). Despite this validation, the predictive ability of the OSC-PLS-DA models was also evaluated. In this context, the analysis with a training set (75% of the samples), removing 25% of the population (as validation set), was repeated four times, and the ability to classify each

individual in the correct group was also assessed each time (30). The percentage of correctly classified individuals for the validation set was further evaluated. Potential markers of interest were extracted from variable importance in projection (VIP) scores that were constructed from the OSC-PLS-DA analysis, and markers were chosen based on their contribution to the variation and correlation within the data set. VIP value is defined as the influence that each variable has in the PLS-DA model. Thus, the higher the VIP value, the more relevant is the variable in the model. Variables with a VIP value >1.5 were considered important in discriminating between groups (31) and were selected as the most relevant to explain the differences in metabolic profile. While a VIP >1 threshold is generally accepted (32-34), the cut-off applied in this study is more restrictive, reducing the possibility of obtaining false positive results.

Two-way hierarchical clustering analysis (two-way HCA)

The data set composed of the important signals (VIP>1.5) obtained from the OSC-PLS-DA model was submitted to two-way HCA using PermutMatrix version 1.9.3 (35). PermutMatrix is a freely available program (http://www.lirmm.fr/~caraux/PermutMatrix/EN/index.html). Two-way clustering means that the NMR signals (rows) and urine samples/individuals (columns) are clustered simultaneously to obtain groups of samples (individuals) and NMR signals that behave as similarly as possible (36). Groups of individuals with similar patterns are adjacent in this tree (37). Two-way HCA was carried out using Euclidean distance, and aggregation of the observations was performed using Ward's method. A heat map of the peak intensities of the metabolites and samples of the individuals was obtained to visualize and characterize urinary metabolome constructed based on the potential candidates of importance.

Statistical analysis of covariance (ANCOVA)

Before submitting the data to ANCOVA analysis, the normality of continuous variables derived from the VIP scores was assessed by the Kolmogorov-Smirnov test. Then, statistical analysis was used to evaluate differences among the three intervention groups and the time periods during the study. Changes among interventions were analysed by ANCOVA (covariance test) with a subsequent Bonferroni post hoc test. Potential confounding was controlled by baseline values. After 1 and 3 years, changes for each treatment were assessed using a *t*-test. Pearson correlation was also applied for significant metabolites and FFQ data in order to find significant correlations between metabolites and food records ($r>0.3$, $p<0.05$). The SPSS 20 statistical package (SPSS, Chicago, IL, USA) was used for the analysis. Differences were considered significant at $p<0.05$.

Metabolite identification and interpretation

Significant metabolites were tentatively identified using the Chenomx NMR Suite 7.6 profiler software (Chenomx Inc. Edmonton, Canada), by comparing NMR spectral data to those available in databases such as the Human Metabolome Database (HMDB), the Biological Magnetic Resonance Data Bank (BMRB) and the Madison Metabolomics Consortium Database (MMCD), along with the existing NMR-based metabolomics literature. Secondly, features were correlated using Pearson's correlation coefficient ($r \geq 0.7$ as cut-off, $p<0.001$) to identify clusters of features that were likely related. Finally,

the biological interpretation was carried out using information from the HMDB and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases, and the published research reports.

RESULTS

The current study was assessed with 98 non-diabetic men and women, aged 53 to 79 years. A flow chart of the participants allocated in the present study (Figure S1) and descriptive data of the initially eligible individuals **(**Table S2) are presented in the Supplementary Material.

Metabolomic differences between baseline, 1 and 3 years of follow-up

Orthogonal signal correction-PLS-DA (OSC-PLS-DA) analysis

The initial overview of the ^{1}H-NMR urine data by PCA analysis showed substantial separation of signals from the data corresponding to acetaminophen and derived metabolites (Supporting Information Figure S2). To allow differentiation of spectra without the overriding influence of the strong acetaminophen peaks, these signals from the NMR data were removed before performing further metabolomic analysis (38). Then, the OSC-PLS-DA analysis resulted in a model with $R^2Y=0.75$ and $Q^2= 0.58$. The permutation test displayed an R^2 intercept of 0.28 and a Q^2 intercept of -0.28. To further assess the modelling quality process, a training subset of the individuals was taken (75% of participants) and a validation subset (25%) was used to test the model. This analysis was repeated four times, with each sample appearing once in the validation set. The mean value of R^2Y from this analysis was 0.75 and the mean Q^2(cum) was 0.54. The mean percentage of correctly classified individuals performed in the validation set was 96.94%. The validation set fitted reasonably, confirming the validation of the model (Supporting Information, Table S3).VIP values, which indicate the contribution of metabolites to the classification of samples, were calculated for each variable in the OSC-PLS-DA model. Discriminant individuals of MD vs. LFD and baseline groups were observed on the score plot graphic (Supporting Information, Figure S3).

Identification of features from the multivariate analysis

The selected features (VIP>1.5) for the partial least squares discriminant analysis were tentatively identified using the procedure explained above. The information concerning the tentative identification, chemical shifts and multiplicity of each metabolite is provided in Table 1.

(VIP>1.5) of NMR signal intensities in urine. For the direction of change in ANCOVA analysis, (↑) indicates a relatively higher metabolite urinary excretion; (↓) indicates a relatively lower excretion. A dash indicates no statistical significance in ANCOVA analysis (p <0.05). * Statistical significance from the baseline (*t-test*, p<0.05).

Metabolite ID	δ (multiplicity)	1-year ANCOVA (*p*)			3-year ANCOVA (*p*)		
		E vs. L	N vs. L	E vs. N	E vs. L	N vs. L	E vs. N
MD associated metabolites							
3-HB	1.20 (d)	↑*	↑*	-	↑*	↑*	-
Leucine	0.93 (d)	↑*	↑*	-	↑*	↑*	-
Isobutyric acid[1,2]	1.06 (d)	↑*	↑*	-	↑*	↑*	-
2-oxoisovaleric[3,7]	1.14 (d)	-	-	-	↑*	-	-
4-DTEA[4]	1.22 (d)	↑*	↑*	-	↑*	↑*	-
N-Ac[5,9]	2.06 (s)	↑*	-	-	↑*	-	-
Glycine	3.57 (s)	-	-	-	↑	↑	-
***p*-cresol**[2]	2.34 (s) 7.22 (d) 7.27 (d)	↑*	-	-	-	↑*	↓*
Suberic acid	1.29 (m) 1.60 (t)	↑*	↑*	-	↑*	↑*	↓*
Oleic acid	1.33 (m) 1.63 (m) 1.80 (m) 2.00 (m)	↑*	↑*	-	↑*	↑*	-
Proline	1.95 (m) 2.06 (m) 3.38dt 4.12dd	↑*	↑*	-	↑*	↑*	-
U3.46	3.46 (s)	↑*	↑	-	↑*	↑*	-
U3.33	3.33 (m)	-	-	-	-	-	-
U3.50	3.50 (s)	-	-	-	↑*	↑*	-
U3.81	3.81(s)	-	↑	-	↑*	↑*	-
Metabolite ID	**δ**(multiplicity)	**1-year ANCOVA (*p*)**			**3-year ANCOVA (*p*)**		
MD+Nuts associated metabolites		**N vs. E**	**N vs. L**	**E vs. L**	**N vs. E**	**N vs. L**	**E vs. L**
PAGN[8]	1.93 (m) 2.25 (t) 3.67 (m) 4.18 (m) 7.36** 7.42 (t)	↑*	↑*	↑*	-	↑*	↑*
N-AGN	2.08 (m) 2.26 (m) 4.18 (m)	↑*	↑*	↑*	↑	↑*	↑*
Creatine	3.04 (s) 3.93 (s)	-	-	-	↑*	↑*	-
U5.02	5.02 (t)	↓*	↓*	-	-	-	-
U5.15	5.15 (t)	↓*	↓*	-	-	↑*	↑*
U6.76	6.76 (m)	-	↑*	-	↑*	↑	-
Metabolite ID	**δ**(multiplicity)	**1-year ANCOVA (*p*)**			**3-year ANCOVA (*p*)**		

MD+EVOO associated metabolites		**E vs. N**	**E vs. L**	**N vs. L**	**E vs. N**	**E vs. L**	**N vs. L**
Creatinine	3.05 (s) 4.06 (s)	↑	↑	-	↑*	↑*	-
Citrate	2.55 (dd) 2.69 (dd)	↑*	↑*	-	-	↑*	-
cis-aconitate	5.74 (s) 3.12 (s)	↑*	-	↑*	-	-	-
U7.50	7.50 (d)	↓*	↓*	-	-	-	-
U8.49	8.49 (s)	-	↓	-	-	↓*	-
Metabolite ID	**δ**(multiplicity)	**1-year ANCOVA (*p*)**			**3-year ANCOVA (*p*)**		
LFD associated metabolites		**L vs. E**	**L vs. N**	**E vs. N**	**L vs. E**	**L vs. N**	**E vs. N**
Hippurate	3.97 (d) 7.54 (t) 7.64 (t) 7.83 (d) 8.54 (bb)	↑*	↑*	-	↑*	↑*	↓*
TMAO	3.28 (s)	-	-	↓	↑*	-	↓*
Anserine	3.78 (s) 8.28 (s)	↑*	↑	-	↓*	-	-
Histidine[7]	7.08 (s) 7.93 (s)	↑*	↑*	-	-	-	-
3-MH[7]	7.05 (s) 7.95(s)	↑*	-	↓*	-	-	-
1-MH[7]	7.73 (s) 7.01 (s)	-	↑	↓	-	-	-
Carnosine	8.11 (s) 7.18 (s)	-	↑	-	↑*	-	-
Proline betaine[6]	3.11 (s) 3.30 (s)	-	-	-	↑*	↑*	↑*
Xanthosine	5.87 (d)	-	↑*	↑*	↑*	-	-

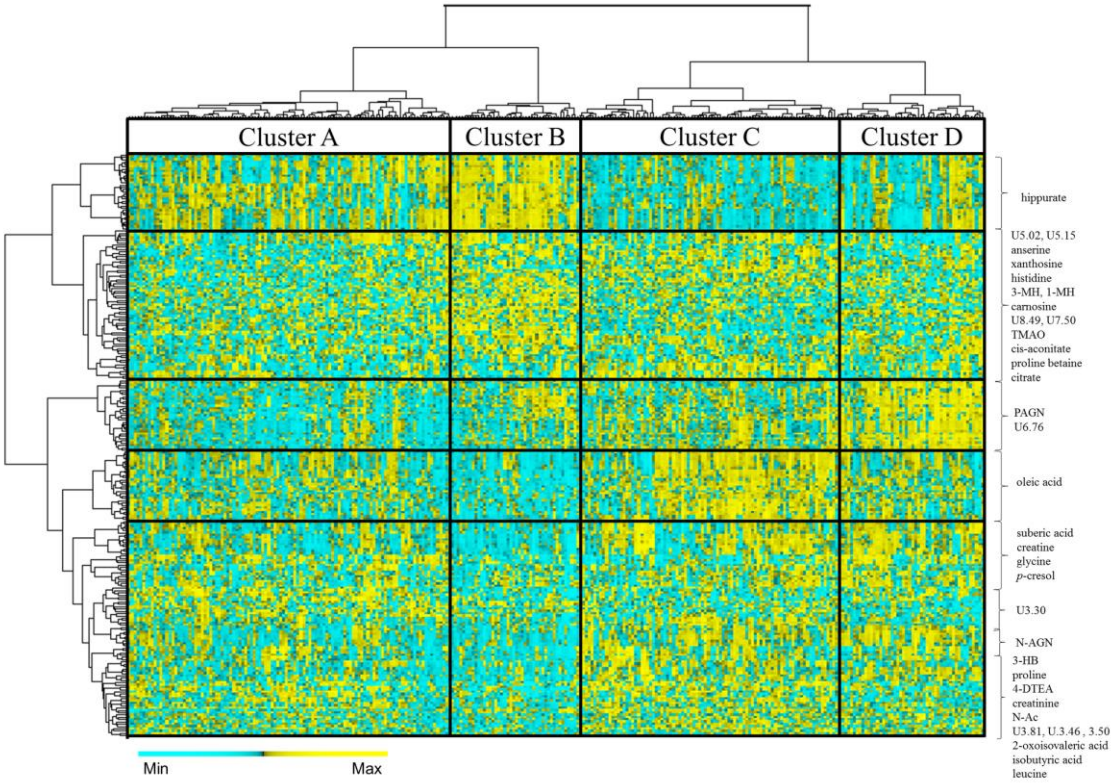
Footnotes: 3-HB: 3-hydroxybutyrate; 4-DTEA: 4-deoxythreonic acid; bb: broad band; d: doublet; E: MD+EVOO; L: LFD; N: MD+Nuts; N-Ac: N-acetylglycoproteins; N-AGN: N-acetylglutamine; L: LFD; bb: broad band; d: doublet; m: multiplet; MH: methylhistidine; N-Ac: N-acetylglycoproteins; PAGN: phenylacetylglutamine; s: singlet; t: triplet; TMAO: trimethylamine-N-oxide; U: unassigned signals. **signal excluded from the analysis because it overlapped with a signal of acetaminophen. Identifications agree with [1](76), [2](71), [3](77), [4](78), [5](79, 80), [6](63), [7](81), [8](16), [9](82), HMDB and Chenomx NMR Suite software.

Two-way hierarchical cluster analysis (HCA) and heat map visualization

For HCA, the samples were classified into two main groups corresponding with MD diets against baseline and LFD samples. Subsequently, these clusters were divided into two cluster levels: on the one hand, clusters A and B, and on the other hand, clusters C and D (Figure 1). Cluster A was predominantly formed by baseline individuals. Cluster B exhibited LFD individuals (1- and 3-year follow-up). The other two clusters corresponded to MD groups: cluster C was the MD+EVOO (1- and -3 year follow-up) and cluster D was the MD+Nuts (1- and 3-year follow-up) group. The classification

table of the individuals in the corresponding cluster for the HCA is shown in Supporting Information (Table S4).

Figure 1. Two-way hierarchical clustering analysis (processed with PermutMatrix according to the Euclidean distance and Ward's aggregation method). Heat map representation of the clustered data matrix in which each coloured cell represents the intensity of appropriate NMR signals, according to the colour scale at the bottom of the figure. Rows: NMR signals (253, VIP>1.5). Columns: urine samples baseline and LFD (1y+3y); MD+EVOO (1y+3y), MD+Nuts (1y+3y).



Footnote: Cluster A: including predominantly baseline individuals; cluster B: including predominantly low-fat-diet individuals; Cluster C: including predominantly Mediterranean diet supplemented with extra-virgin olive oil group of individuals; Cluster D: predominantly Mediterranean diet supplemented with nuts group of individuals; U: unidentified compound; N-ac: N-acetylglycoproteins; 4-DTEA: 4-deoxythreonic acid; 3-HB: 3-hydroxybutyrate; NAG: N-acetylglutamine; PAGN: phenylacetylglutamine; TMAO: trimethylamine-N-oxide; 1MH: 1-methylhistidine; 3-MH: 3-methylhistidine.

ANCOVA and t-test analysis to assess urinary metabolomic differences

After the identification procedure, metabolites were submitted to statistical analysis comparing their urinary excretion between interventions (using ANCOVA analysis) and between time periods (using *t*-test analysis). The significant metabolites were divided according to their excretion into: MD-associated metabolites (observed in both MD groups), MD+Nuts-associated metabolites, MD+EVOO-associated metabolites and LFD-associated metabolites (Table 1). The MD-associated metabolites group concerned ketone bodies such as 3-hydroxybutyrate (3-HB), amino acids such as proline, glycine, the branched-chain amino acid (BCAA) leucine and its derived metabolites (isobutyric acid, 2-oxoisovaleric acid), the threonine metabolite 4-deoxythreonic acid (4-DTEA), the N-acetyl groups of glycoproteins (N–Ac), gut microbiota co-metabolite *p*-cresol, and also the fatty acid (oleic acid) and its breakdown product (suberic acid), along with the unidentified metabolites U3.46, U3.50 and U3.81. The MD+Nuts-associated metabolites compress metabolites of the amino acid glutamine (phenylacetylglutamine [PAGN] and n-acetylglutamine [N-AGN]), creatine and four unknown metabolites. PAGN and N-AGN were statistically significantly more highly excreted in both MD groups than LFD, however they were also higher in MD+Nuts *versus* MD+EVOO. The MD+EVOO-related metabolites included creatinine and two intermediates of the tricarboxylic acid cycle (TCA), citrate and cis-aconitate, with higher excretion in MD+EVOO than in the other groups. Finally, LFD-associated metabolites involved a higher excretion in particular of hippurate (in both time periods), but also of Trimethyl-N-oxide (TMAO) and histidine and its derived metabolites (3-methylhistidine [3-MH], 1-methylhistidine [1-MH], carnosine, anserine), as well as proline-betaine and xanthosine.

In general, results showed more differences between the MD groups than the LFD group, particularly comparing the MD+EVOO and LFD groups, which had the most predominant changes in statistical analysis (lower *p*-values; see Supporting Information, Table S5). Moreover, most MD-associated metabolites were excreted in 1 and 3 years with the same trend. However, some metabolites, particularly from LFD, are characteristic of one of the two time points evaluated.

DISCUSSION

The present results showed that the 1- and 3-year intervention follow-ups presented a marked effect on urinary metabolomic phenotype in the volunteers.

Metabolites from protein, lipid and carbohydrate metabolic pathways in MD groups

BCAA leucine and the products of BCAA reflect differences in gluconeogenesis (GNG) (16) in MD compared to the control diet (LFD). Moreover, these results could suggest a particular importance of BCAAs in lipid oxidation and ketone body synthesis. 4-DTEA is a diastereoisomer of 2,3-dihydroxybutanoic acid. 4-DTEA is a breakdown product of threonine also involved in amino acid catabolism. Threonine deaminase converts threonine to α-ketobutyrate, a precursor of isoleucine (a BCAA), and this is further modified by reductases to 4-deoxyerythronicacid and its diastereoisomer 4-DTEA (39).

The similar results with 3-HB excretion could suggest a possible relationship between these threonine catabolic products and ketone body production, consistent with the ketogenic nature of the amino acid threonine (40).

The increase in urinary excretion of the ketone body 3-HB in MD could suggest an increase in fatty acid oxidation (FAO) (41). FAO is indispensable for the conversion of lipids (dietary or from lipid storage) to ketone bodies (42). Moreover, PAGN is endogenously formed in humans by phenylacetate, which could be related to oxidation of phenyl containing fatty acids (43) or through the exogenous intake contained in plant food sources (44). Differences in excretion of PAGN are described that reflect changes in GNG and the tricarboxylic acid (TCA) cycle (45).

Higher levels of creatine and creatinine present in MD groups are observed. The high excretion of creatine reflects increased turnover in the creatine/creatinine pathway (46). Furthermore, changes in citrate and cis-aconitate suggest a different modulation of the TCA cycle (47). Also, proline is the catabolite of peptide degradation and is a precursor of pyruvate. Pyruvate can be converted into acetyl-CoA, which is the main input for a series of reactions such as the TCA cycle. Additionally, decreases in levels of proline and citrate were observed in diet-induced hyperlipidemia in rats (48).

Food intake metabolites (food metabolome) particular for each intervention group

PAGN and NAG, both derived from the amino acid glutamine, were found associated within the MD groups, particularly in the MD-Nuts group, suggesting an important implication of glutamine metabolism in this group of individuals. Additionally, glutamic acid, whose conversion into glutamine takes place in tissues such as the liver, skeletal muscle and brain (49), is the major amino acid from walnuts (50), hazelnuts (51) and almonds (52), in consonance with the higher excretion in the MD+Nuts group from our results.

Oleic acid (highly excreted in the MD groups, particularly in MD-EVOO) is the major fatty acid in olive oil (75.0% ± 0.8) and is also highly present in nuts (hazelnuts 72.1% ± 0.2, almonds 61.2% ± 0.4, walnuts 14.0% ± 0.3 (data extracted from the composition of virgin olive oil and nuts used in the present trial (21)). Oleic acid is mainly metabolized in the body by FAO; however, amounts of it could be excreted (53) and detected (54) in urine. It is reported that the upregulated short-chain dicarboxylic acids and long-chain fatty acids (such as oleic acid) in the urine of rats showed an increasing pressure on energy utilization from the catalysis of the fatty acid pathway in fasting (55). Further, suberic acid is a dicarboxylic acid, which is a metabolic breakdown product derived from oleic acid (56). The increased intake of PUFAs ((polyunsaturated fatty acids, contained within nuts) and MUFAs (monounsaturated fatty acids, contained in EVOO and nuts) resulted in greater excretion of FAO products in the urine. Thus, it is suggested that a supplementation with EVOO and nuts in the MD groups manifested an increase in their FAO metabolism and turnover. These results are in line with other results published after 12 weeks of nut consumption in an interventional study (57). Similarly, it is described that MUFA and PUFA upregulate FAO (4) by activating ligands for peroxisome proliferator-activated receptors (PPARs), as fatty acids are natural PPAR ligands (58). Activation of PPARs by dietary fatty acids such as oleic acid makes tissues more dependent on FAO by stimulating fatty acid utilization pathways including transport, esterification and oxidation (59), thereby improving fatty acid utilization.

3-MH, 1-M, carnosine and anserine are compounds derived from the histidine metabolic pathway related to muscle protein breakdown (60) and also biomarkers of meat consumption (8). Likewise, in the LFD group, a moderate association ($r>0.4$, $p<0.05$) was found with reported red meat intake from the FFQ for histidine, 3-MH and carnosine. Some studies exhibited metabolites being discriminant among lactovegetarian and omnivorous people, showing a decreased excretion of them in people following vegetarian diets, while an increase of N-Ac, citrate, glycine and PAGN is evidenced (18, 19) in vegetarian diets. Similarly, a moderate association ($r>0.3$, $p<0.05$) was found with some MD-associated metabolites (3-HB, leucine, isobutyric, 2-oxoisovaleric acid, N-Ac, proline, glycine *p*-cresol and PAGN) with vegetable, legume and fruit intake in MD groups from the FFQ.

Finally, xanthosine is a metabolite involved in purine metabolism. Decreased levels of urinary xanthosine are found in rats with myocardial infarction compared with controls (61). Also, xanthosine is a discriminant compound indicating different diets found in the urine of rats fed by a normal diet and a turkey-based diet (62). On the other hand, proline betaine is a marker of citrus consumption based on orange juice intake (63) . Moreover, the proline betaine signals of the present results correlate with the orange consumption($r>0.5$, $p<0.0001$) reported in the FFQ in the LFD group, but also in the MD groups, exhibiting a moderate association of citrus fruit consumption by all the population.

Metabolites derived from microbial metabolism

A number of studies have demonstrated the importance of the gut microbiota in contributing to the excretion of metabolites such as PAGN and TMAO, hippurate and *p*-cresol, which are often referred to as urinary microbial co-metabolites (64-66). Overall, our results suggest an increase in the activity of microbiota in an MD associated with PAGN and *p*-cresol, while, on the other hand, in the LFD group an increase in the gut microbiota-related pathway associated with hippurate and TMAO is shown. Gut microbiota also extensively catabolize protein and aromatic amino acids including phenylalanine and tyrosine, to form PAGN and *p*-cresol (67, 68). Gut microbiota facilitate host energy recovery from dietary sources (which suggests that gut microbiota are an important environmental factor that affects energy harvest from the diet and energy storage in the host) (69). *p*-Cresol is a microbial metabolite associated with both dietary polyphenols and aromatic amino acids (from dietary proteins) (70). The presence of PAGN and *p*-cresol in MD groups may highlight a relationship between microbiota activity and the host metabolism of aromatic compounds (71).

TMAO was also associated with fish intake; however, no correlation in our samples has been shown with any kind of fish or seafood intake in the LFD group. In addition, TMAO has recently been proposed as a pro-atherogenic species (72); in this regard, low levels of this metabolite in MD+EVOO present after 3 years of intervention are an interesting focus to explore. Hippurate is a gut microbial mammalian co-metabolite of benzoic acid that can be generated by a range of gut microbes from low-molecular-weight aromatic compounds and polyphenols in the gut (73). Urinary levels of hippurate have been shown to correlate with the obese phenotype in different animal models (74). Hippurate has also been inversely linked to blood pressure, suggesting a further connection with diet and obesity (17). However, no correlation with body mass index, hypercholesterolemia or hypertension has been associated with these signals in the present results. We conclude that differences in hippurate in the LFD group suggest a

disturbance of the intestinal microbiota associated with hippurate metabolism. Likewise, higher urinary excretion of metabolites such as hippurate and TMAO was observed in heart failure patients, whereas metabolites such as creatinine, citrate and cis-aconitate (higher in MD groups) were relatively lower (75).

The present results show important changes in urinary metabolome predominantly at 1-year follow-up for all three groups. Some urinary excretion changes are maintained at 3 years of follow-up. The marked changes are a predominant enhancement in an MD of catabolic-associated pathways. Results suggest a persistent effect of an MD, particularly MD+EVOO, on urinary metabolome for the 3-year follow-up (see significance in Supporting Information Table S1). Further, MD+Nuts also had the same excretion pattern tendency as the MD+EVOO group. Likewise, compliance with both Mediterranean diets was assessed on the whole population of the PREDIMED study with compliance biomarkers for both MD groups (3), exhibiting an overall good compliance in the interventions. In addition, future steps along similar lines will be taken to evaluate changes in metabolome in a diabetic population linked to Mediterranean diets.

CONCLUSIONS

These results have shown that the interventions monitored after 3 years of follow-up had an observed effect on human urinary metabolome. MD groups had a different metabolic fingerprinting compared to baseline and the control (LFD) groups. The most prominent hallmarks of these changes concerning MD groups were related to carbohydrate and lipid metabolism, amino acids (and derived metabolites) and also microbial co-metabolites (PAGN, *p*-cresol). Finally, LFD-associated metabolites were hippurate and metabolites related to histidine metabolism, as well as xanthosine. Furthermore, food metabolome metabolites have enabled to associate intervention groups with particular food intakes. MD groups exhibited a moderate association with vegetable and fruit intake. Additionally, a moderate association with meat intake biomarkers was observed in the LFD group. The application of NMR-based metabolomics enabled the classification of individuals regarding their dietary pattern and highlighted the potential of this approach in evaluating changes in the urinary metabolomic fingerprinting of these individuals at different time points of follow-up in response to a particular intervention.

ASSOCIATED CONTENT

Supporting Information: Quantitative Score of Adherence to the Mediterranean Diet (Table S1). Baseline characteristics of the participants (Table S2). Summary of parameters for assessing OSC-PLS-DA modelling quality in the training and validation set (Table S3). *P*-values in the ANCOVA test of metabolites derived from multivariate analysis (Table S4). Flow chart of participants evaluated in the subsample of the PREDIMED trial (Figure S1). PCA exploratory data analysis: (A) 3D score plot from individuals, and (B) 3D loading plot from the NMR signals before removing acetaminophen signals; (C) 3D score plot from the individuals, and (D) 3D loading plot from the NMR signals after removing the acetaminophen signals. Red spheres from (B) indicate the acetaminophen signals (Figure S2). 2D and 3D OSC-PLS-DA score plots of NMR signals deriving from the urine samples collected at 0 days (baseline)

MD+EVOO (1-year and 3-year), MD+Nuts (1-year and 3-year) and LFD (1-year and 3-year) (Figure S3). **This material is available free of charge via the Internet at http://pubs.acs.org.**

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NOTES

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ABBREVIATIONS

1-MH, 1-methylhistidine; 3-HB, 3-hydroxybutyrate; 3-MH, 3-methylhistidine; 4-DTEA, 4-deoxythreonic acid; ANCOVA, analysis of covariance; BCAA, branched-chain amino acids; CVD, cardiovascular disease; FAO, fatty acid oxidation; EVOO, extra-virgin olive oil; FFQ, food frequency questionnaire; GNG, gluconeogenesis; HCA, hierarchical clustering analysis; IQR, interquartile range; LFD, low-fat diet; MD, Mediterranean diet; MUFA, monounsaturated fatty acids; N-Ac, N-acetylglycoproteins; OSC-PLS-DA, partial least-squares discriminant analysis with orthogonal signal correction; PAGN, phenylacetylglutamine; PCA, principal component analysis; PPPAR, proliferator-activated receptor; PUFA, polyunsaturated fatty acids; TCA, tricarboxylic acids; TMAO, trimethylamine-N-oxide; U, unidentified; VIP, variable importance in projection.

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