

1 **Microbial metabolites are associated with a high adherence to a Mediterranean**
2 **dietary pattern using a ¹H-NMR-based untargeted metabolomics approach[★]**

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19 **Abstract:** The study of biomarkers of dietary patterns including the Mediterranean diet (MedDiet)
20 is scarce and could improve the assessment of these patterns. Moreover, it could provide a better
21 understanding of health benefits of dietary patterns in nutritional epidemiology. We aimed to
22 determine a robust and accurate biomarker associated with a high adherence to a MedDiet pattern
23 that included dietary assessment and its biological effect. In this cross-sectional study, we included
24 56 and 63 individuals with high (H-MDA) and low (L-MDA) MedDiet adherence categories,
25 respectively, all from the Prevención con Dieta Mediterránea trial. A ¹H-NMR-based untargeted
26 metabolomics approach was applied to urine samples. Multivariate statistical analyses were
27 conducted to determine the metabolite differences between groups. A stepwise logistic regression
28 and receiver operating characteristic curves were used to build and evaluate the prediction model for
29 H-MDA. Thirty-four metabolites were identified as discriminant between H-MDA and L-MDA. The
30 fingerprint associated with H-MDA included higher excretion of proline betaine and
31 phenylacetylglutamine, among others, and decreased amounts of metabolites related to glucose
32 metabolism. Three microbial metabolites — phenylacetylglutamine, p-cresol and 4-
33 hydroxyphenylacetate — were included in the prediction model of H-MDA (95% specificity, 95%
34 sensitivity and 97% area under the curve). The model composed of microbial metabolites was the
35 biomarker that defined high adherence to a Mediterranean dietary pattern. The overall metabolite
36 profiling identified reflects the metabolic modulation produced by H-MDA. The proposed biomarker
37 may be a better tool for assessing and aiding nutritional epidemiology in future associations between
38 H-MDA and the prevention or amelioration of chronic diseases.

39 **Keywords:** Microbiota; Biomarkers; Metabolomics; Dietary patterns; Mediterranean diet; High
40 adherence

41 **Abbreviations:** AUC, area under the curve; CVD, cardiovascular disease; FFQ, food frequency
42 questionnaire; ¹H-NMR, proton nuclear magnetic resonance; HMDA, high Mediterranean diet
43 adherence; HPPA, 3-(3-hydroxyphenyl)-3-hydroxypropanoate; L-MDA, low Mediterranean diet
44 adherence; MedDiet, Mediterranean diet; MetS, metabolic syndrome; PREDIMED, Prevención con
45 Dieta Mediterránea; OSC-PLS-DA, orthogonal signal correction–partial least squares discriminant

46 analysis; PAGN, phenylacetylglutamine; ROC, receiver operating characteristic; VIP, variable
47 importance in projection.

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61 **1. Introduction**

62 High adherence to healthy dietary patterns is associated with lower risk of chronic diseases [1]. The
63 measurement of dietary intake is an essential component in studies attempting to establish links
64 between dietary exposure and health outcomes [1]. Currently, the quality and adherence of dietary
65 patterns in nutritional epidemiology are measured by the use of self-reported questionnaires such as
66 the dietary indexes/scores [2]. A healthy dietary score is based on dietary recommendations as a result
67 of scientific consensus or as proposed by investigators using an evidence-based approach [3]. Several
68 dietary scores have been developed and applied to populations to evaluate the role of diet in a more
69 holistic perspective in the risk of mortality, cardiovascular disease (CVD) and cancer [4]. However,
70 because dietary patterns are complex not only in terms of composition but also in terms of amounts

71 and frequency of food intake, it is well recognized that, in addition to the conventional methods, the
72 emergence of novel biomarkers of food exposure may help to improve the accuracy of the assessment
73 of compliance and adherence [5]. In this regard, metabolomics has emerged as a valuable tool in
74 nutrition research for the discovery of novel dietary biomarkers for both single foods [6,7] and food
75 patterns [5,8] and, in addition, is able to evaluate their effect in the organism [9]. The use of these
76 biomarkers is a more specific tool and complementary to traditional indexes/scores. Moreover,
77 although progress has been made in the metabolite characterization of dietary patterns, most of the
78 metabolomic studies have applied approaches based on multivariate analyses of food and nutritional
79 data intake [10,11], leaving the complementary use of dietary scores and metabolomics as a new field
80 to explore in dietary patterns characterization. Up to now, several Mediterranean diet (MedDiet)
81 adherence scores have been described in the literature, such as the 9-item MedDiet score [12] or the
82 Prevención con Dieta Mediterránea (PREDIMED) 14-item MedDiet score [13]. These scores were
83 developed with the aim of appraising the adherence to a traditional MedDiet of several populations
84 as well as to evaluate the effect of adherence to a MedDiet on microbiota composition [14], CVD risk
85 factors [15], aging diseases [16] or total mortality [12]. The application of targeted and untargeted
86 metabolomics approaches in the study of the effects of the protective mechanisms of a MedDiet on
87 CVD has been poorly studied but is now beginning to attract more interest [9,17]. Furthermore,
88 currently, there are some reports about metabolic profiling in biological samples (feces or urine) that
89 enable the characterization of high adherence to a MedDiet pattern [14,18], but to our knowledge,
90 there are no reports calculating a prediction model of MedDiet adherence. In addition, the study of
91 biomarkers to explain the assessment of the pattern and gain a better understanding of its health
92 benefits in nutritional epidemiology is limited. The characterization of dietary patterns by using
93 metabolomic approaches is important because it would allow insights into the relationship between
94 diet, taking into account the bioavailability of MedDiet bioactives, and the risk of chronic diseases
95 [14,19]. In the current study, we aimed to determine a robust and accurate urinary biomarker
96 associated with a high adherence to MedDiet pattern that included dietary assessment and its

97 biological effect on the organism by using a 1H-NMR-based untargeted metabolomics approach
98 which can be usefully applied in nutritional epidemiology.

99 **2. Subjects and methods**

100 *2.1. Study population and study design*

101 The PREDIMED study is a multicenter, randomized, parallel and controlled clinical trial conducted
102 in Spain and aimed at assessing the effects of a MedDiet on primary prevention of CVD. Full details
103 of the design and methods have been published elsewhere [20,21]. Briefly, the study population
104 included men (55–80 years) and women (60–80 years) without a previous history of CVD at
105 enrolment but with either type 2 diabetes mellitus or at least three or more of the following CVD risk
106 factors: current smoking, hypertension, high low-density lipoprotein cholesterol, low high-density
107 lipoprotein cholesterol, overweight/ obesity or family history of premature CVD. Exclusion criteria
108 were the presence of any severe chronic illness, alcohol or drug abuse, body mass index (BMI; in
109 kg/m²) ≥ 40 , and allergy or intolerance to olive oil or nuts. The trial was registered at
110 <http://www.controlledtrials.com> (ISRCTN35739639). For the current work, we conducted a cross-
111 sectional study with baseline dietary data and urine samples of 119 individuals recruited in 2
112 PREDIMED trial centers (Hospital Clinic of Barcelona and University of Valencia). At baseline, one
113 morning urine sample was collected from all participants and immediately aliquoted and stored at –
114 80°C until the day of analysis. *2.2. Assessment of Mediterranean diet adherence and other*
115 *parameters* In order to appraise the adherence to a MedDiet among participants, the validated 14-
116 item PREDIMED MedDiet score questionnaire was administered. In detail, the MedDiet score
117 questionnaire consists of 12 questions on food consumption frequency and 2 questions on food intake
118 habits considered characteristic of the Spanish MedDiet [13]. Each item/question is scored as 1 or 0
119 according to whether it is met or not, respectively (Supplementary Table 1). Thus, the total MedDiet
120 score ranges from 0 to 14 points, meaning that the higher the score, the higher the adherence to a
121 MedDiet. The MedDiet adherence score was calculated for all participants and used for their
122 subsequent stratification, which was done by using the proposed cutoff values previously reported in

123 the PREDIMED study. [15]: MedDiet score ≤ 7 indicated low MedDiet adherence (L-MDA) (n=63),
124 and MedDiet score ≥ 10 (n=56) indicated high MDA (H-MDA). All participants were also asked to
125 complete a validated semiquantitative 137-item food frequency questionnaire (FFQ) [22] and the
126 Spanish version of the Minnesota Leisure-Time Physical Activity Questionnaire [23]. Trained
127 dieticians in the PREDIMED study administered all questionnaires, including the PREDIMED
128 MedDiet score questionnaire. The nutrient composition and energy intakes were calculated from the
129 FFQ data by using Spanish food composition tables [24]. Also, anthropometrical measurements were
130 taken directly by qualified nurses.

131 *2.3. Metabolite profiling*

132 ¹H-NMR analysis and spectra processing were performed by following previous methodology [8].
133 Briefly, the urine samples were thawed, vortexed and centrifuged at 13,200 rpm for 5 min. From each
134 supernatant, a volume of 300 μ l was taken and diluted with 200 μ l of H₂O/D₂O and mixed with a
135 buffer solution [8]. The optimized pH of the buffer was set at 7.0, with a potassium deuterioxide
136 solution, to minimize variations in the chemical shifts of the ¹H-NMR resonances. This mixture was
137 transferred to a 5-mm NMR tube. ¹H-NMR spectra acquisition was performed using a Varian-Inova
138 500-MHz NMR spectrometer with presaturation of the water resonance using a NOESYPRESAT
139 pulse sequence. The spectra data processed were binned to 1165 variables with bin widths of 0.005
140 ppm and integrated with ACD/NMR Processor 12.0 software (Advanced Chemistry Development,
141 Toronto, Canada). The spectral region containing water (δ 4.75–5.00 ppm) was excluded before
142 normalization to avoid spectral interference. Integrated spectra were row-wise normalized by sum
143 using MetaboAnalyst 3.0 (www.metaboanalyst.ca), a web server designed to permit comprehensive
144 metabolomics data analysis. Metabolites were identified following a multistep procedure as
145 previously reported [8]. This multistep includes (a) comparison of the experimental NMR spectra with
146 those in the library of ChenomxSuite 8.1 profiler software (Chenomx, Inc., Edmonton, Canada),
147 which includes access to the Human Metabolome Database (HMDB) library [25]. Other databases
148 such as the Biological Magnetic Resonance Data Bank and the Madison Metabolomics

149 ConsortiumDatabase were also consulted along with existing NMR-basedmetabolomics literature.

150 (b) At the same time, the NMR peak assignments were correlated using Pearson's correlation

151 coefficient ($r \geq 0.7$, $P \leq 0.05$) to confirmthemultiplicity and identify clusters of metabolites which were

152 then compared to databases with NMR data [8,25]. Biological interpretation was made by consulting

153 the HMDB and Kyoto Encyclopedia of Genes and Genomes databases. *2.4. Statistical analyses*

154 Demographic characteristics, medication usage and dietary intake were compared between groups by

155 conducting Student's t tests and χ^2 analyses for continuous and categorical variables, respectively.

156 Variables with a non-normal distribution were log transformed before analyses. Multivariate data

157 analysis was performed using SIMCAP+ 13.0 (Umetrics, Umeå, Sweden) software. Data sets

158 containing the integrated NMR spectral bins were log transformed and Pareto scaled before

159 performing a principal component analysis to explore the quality of data acquisition. An orthogonal

160 signal correction (OSC) filter was applied to the data sets in order to reduce the variability not

161 associated with the dietary classification [8], in this case, the category of adherence to a MedDiet.

162 Afterward, a partial least squares discriminant analysis (PLS-DA) was performed to examine the

163 difference in metabolite profile between subjects at H-MDA and L-MDA. The quality and validation

164 of resultant model were also appraised, the first through the R²Y (cum) and Q² (cum) parameters,

165 and the second with a permutation test (n=200). Discriminant variables between groups were selected

166 based on their variable importance in projection (VIP) value ≥ 1.0 , which is a generally accepted

167 threshold [26]. The normality of discriminant variables was assessed using a Kolmogorov–Smirnov

168 testwith Lilliefors significance correction, and additionally Mann–Whitney or independent Student's

169 t tests were performed according to the normality of the data. To account for multiple comparisons

170 in the metabolomic analysis, we used a corrected P value with the Benjamini–Hochberg procedure.

171 Aiming to identify the metabolites with the best discriminant capability between H-MDA and L-

172 MDA, we designed and assessed the performance of a prediction model with the H-MDA as

173 dependent variable and the discriminant metabolites identified from the OSCPLS- DA model as

174 independent variables [6]. To this end, first the data set of individuals (n=119) was randomly split

175 into two thirds to build one training set (n=79) and one third for a validation set (n=40). A stepwise

176 binary logistic regression analysis was performed in the training set in order to identify the
177 metabolites with the most significant predictive capacity, and from these, a combined model was
178 built. Subsequently, a receiver operating characteristic (ROC) curve analysis was performed, first in
179 the training set and then in the validation set, to evaluate the obtained model, as well as the individual
180 metabolites included. Then the performance of both the combined model and the individual
181 metabolites was determined by the area under the curve (AUC) of the ROC curves, as well as by the
182 sensitivity and specificity at the optimal cutoff point defined as the minimum distance to the top-left
183 corner in the ROC curve. Finally, the association between the combined multimetabolite model and
184 individual metabolites with food groups was tested by a Spearman's rank correlation analysis with
185 correction of P value using Benjamini–Hochberg procedure. All univariate analyses, including
186 normality, Student's t tests, Spearman's rank correlation, logistic regression and ROC curve analyses,
187 were performed on IBM SPSS 21 statistics software (IBM Corp., Armonk, NY, USA).

188 **3. Results**

189 *3.1. Demographic and dietary intake measurements*

190 For the current study, we included individuals who, according to their individual MedDiet score, were
191 assigned to L-MDA (≤ 7 points, $n=63$) or H-MDA (≥ 10 points, $n=56$). Our population had a mean
192 (\pm S.D.) age of 67 ± 6 years and a mean (\pm S.D.) BMI of 30.3 ± 4.5 kg/m², and 68.9% of the participants
193 were women (Table 1). Cardiovascular risk factors as well as medication use were similar between
194 both groups (Table 1). With regard to food and nutrient intake (Table 2), individuals in H-MDA
195 consumed higher amounts of olive oil, nuts, vegetables, fruits, legumes and fish, and total dietary
196 fiber than L-MDA participants ($P<0.05$). However, no statistically significant differences were found
197 for total energy, total fat, carbohydrates and protein intakes. *3.2. Discriminant metabolite profile of*
198 *high adherence to a Mediterranean diet pattern* The OSC-PLS-DA analysis resulted in one latent
199 component model with R²Y (cum) and Q²Y (cum) values of 0.913 and 0.764, respectively, indicating
200 a good ability to classify individuals according to their MedDiet adherence. In addition, a permutation
201 test ($n=200$), with intercept R² and Q² values of 0.346 and -0.154 , respectively, showed the validity

202 of the model. Table 3 shows a list of 34 metabolites that were identified after the selection of
203 discriminant variables from the OSC-PLS-DA (based on VIP N1.0 values). Individuals in H-MDA
204 group had a marked excretion of metabolites involved in protein/amino acid metabolism
205 characterized by a higher excretion of anserine, carnosine, creatine, creatinine, guanidoacetate,
206 histidine and N-acetylglutamine, as well as a lower excretion of 3-methylhistidine, alanine, glycine
207 and lysine, than those in L-MDA. Similarly, another major group of metabolites derived from gut
208 microbiota was identified. Concretely, H-MDA was characterized by a higher urinary amount of 4-
209 hydroxyhippurate, 4-hydroxyphenylacetate, dimethylsulfone, 3-(3-hydroxyphenyl)-3-
210 hydroxypropanoate (HPHPA), p-cresol and phenylacetylglutamine (PAGN) and lower urinary
211 amounts of 3-indoxyl sulfate, hippurate and isobutyrate compared to the L-MDA. Furthermore,
212 participants in the H-MDA group excreted lower levels of metabolites involved in the energy
213 pathway, and in the propanoate and purine and caffeine pathways, than those in L-MDA. In addition,
214 participants in the H-MDA group had higher levels of metabolites involved in the choline pathway
215 (except betaine), as well as the inositol, niconitate, nicotidamide and pyrimidine pathways (Table 3).

216 3.3. Prediction model for high adherence to Mediterranean dietary pattern We performed a model
217 based on a stepwise binary logistic regression analysis including the previous 34 metabolites
218 identified from the OSC-PLS-DA model and further ROC curve analyses to evaluate the resulting
219 model and the individual metabolites included in this. To this end, the data set of individuals from the
220 H-MDA and L-MDA groups was divided into training and validation sets, as indicated above. The
221 resulting model included three metabolites derived from gut microbiota, namely, PAGN, p-cresol and
222 4-hydroxyphenylacetate (Supplementary Table 2). The specificity and sensitivity of the model were
223 higher than 90% in both the training and validation sets. The PAGN had 85.7% and 68.4% specificity
224 and sensitivity, respectively, in the validation set, while p-cresol and 4-hydroxyphenylacetate showed
225 values between 66% and 84% in these parameters (Table 4) for the validation set. Furthermore, the
226 global performance of the combined model in terms of AUC was 97.7% for the training and 97.0%
227 for the validation set, while individually, each metabolite had values of AUC between 59% and 86%
228 (Table 4). Fig. 1 illustrates that the model improves the classification of MedDiet adherence (H-MDA

229 and L-MDA) in comparison with the use of each metabolite individually. In the analysis of
230 correlation, the combined metabolite model showed a strong correlation with the MedDiet score
231 ($r=0.7$; $Pb.001$), as well as with the intake of vegetables, fruits, legumes, fish ($r=0.2-0.3$; $Pb.01$)
232 (Supplementary Table 3) and dietary fiber ($r=0.3$; $Pb.01$). Otherwise, the three individual metabolites
233 had good correlation with the MedDiet score ($r=0.3-0.6$; $Pb.001$) but weaker or not significant
234 correlations with the intake of individual foods.

235 **4. Discussion**

236 In the present metabolomic study, we identified the urinary metabolite profile consisting of 34
237 metabolites that enable discrimination between 2 groups of individuals with high or low adherence
238 to the score of Mediterranean diet adherence validated in the PREDIMED study [13]. Participants in
239 the L-MDA group had a cutoff of ≤ 7 points and those in the H-MDA group ≥ 10 points, as previously
240 proposed [15]. The set of metabolites that discriminated between H-MDA and LMDA suggested the
241 metabolic modulation of the MedDiet. These metabolites are involved in multiple molecular
242 mechanisms and metabolic pathways, which together provide a holistic view of variations in the urine
243 metabolome due to the effect of following this dietary pattern. Some of these metabolites have previously
244 been proposed as putative biomarkers of single food intake and also related to foods included in
245 MedDiet, as well as up- and down-regulated endogenous metabolites. We found that the higher
246 excretion of proline betaine and scyllo-inositol in H-MDA correlated significantly with the intake of
247 citrus fruits ($r=0.36$ and $r=0.35$; $Pb.001$, respectively), which is consistent with previous studies that
248 have proposed them as markers of citrus fruit intake [27]. Recently, in our previous work, we also
249 found positive correlations of proline betaine signals with orange consumption in long-term MedDiet
250 intervention and low-fat diet groups in a subsample of nondiabetic participants of the PREDIMED
251 study [8]. In this regard, in the H-MDA group, we also identified other metabolites derived from the
252 microbial metabolism: 4-hydroxyhippurate, 3-(3-hydroxyphenyl)-3-hydroxypropanoate and 4-
253 hydroxyphenylacetate. These metabolites have been described after interventions with mixed red
254 wine/grape juice extracts [28], as well as linked to the intake of other polyphenol-rich foods such as
255 cocoa and almond, among others (<http://phenol-explorer.eu/>). It is interesting to note that these

256 microbial metabolites were not found in our previous work evaluating the intervention with MedDiet
257 [8]. Other metabolites have been related to the intake of meat or fish. Both dimethylamine and
258 trimethylamine-N-oxide (TMAO), which were higher in the H-MDA group than in the L-MDA, have
259 been related to the intake of fish and seafood [29,30]. Nevertheless, only TMAO had a significant
260 correlation with the intake of total fish ($r=0.24$, $Pb.01$) in our study sample, and this correlation was
261 not previously observed in the subset of samples of nondiabetics in the PREDIMED study [8]. In
262 addition, we identified some compounds related to the histidine pathway: carnosine, anserine and 3-
263 methylhistidine. While 3- methylhistidine and carnosine are proposed as good biomarkers of red meat
264 intake [31–33], anserine has alternatively been proposed as a marker of white meat (i.e., poultry) [34]
265 or salmon intake [35]. In our study, subjects in the H-MDA group excreted lower amounts of 3-
266 methylhistidine and higher amounts of carnosine and anserine than those in the L-MDA group. We
267 found significant correlations between anserine, with antioxidant properties, and the intake of white
268 meats ($r=0.231$, $Pb.01$) but not with fish ($PN.05$). In addition, we did not find significant correlations
269 between methylhistidine or carnosine and foods. Nevertheless, it should be noted that carnosine is a
270 normal constituent in human urine, which occurs naturally in the skeletal muscle of mammals and
271 has antioxidant properties and therapeutic potential against numerous diseases [36]. To the best of
272 our knowledge, there is limited information about which mechanisms could increase these
273 antioxidants in the organism, but a recent study has shown that dietary vitamin B6 could determine
274 the carnosine concentration in the skeletal muscle of rats [37]. In this regard, we observed
275 significantly higher ingested concentrations of vitamin B6 in subjects in the H-MDA group (2.9 ± 0.7
276 mg/d) than in those in the LMDA group (2.4 ± 0.5 mg/d , $Pb.001$). Moreover, 3-methylhistidine is a
277 metabolite of anserine and carnosine in the histidine pathway. 3- Methylhistidine is also found in
278 urine as anserine and carnosine, and its concentration was inversely correlated with them in our study
279 ($r=-0.32$, $Pb.05$; $r=-0.27$; $Pb.05$, respectively). Thus, the contribution of a MedDiet in the modulation
280 of the histidine pathway could be an interesting field to further explore. Besides the metabolites
281 related to the intake of foods, we also identified other endogenousmetabolites involved in
282 energymetabolism. In particular, individuals in the H-MDA group showed lower excretion of

283 glucose, lactate and succinate than those in the L-MDA group, whose metabolites are related to
284 pathways affected in diabetic patients and other diseases [38]. This finding is interesting because
285 although our population included diabetic individuals, their distribution and the use of medication
286 between the H-MDA and L-MDA groups were well balanced (Table 1). This fact suggested therefore
287 that, in comparison to the L-MDA group, individuals in the H-MDA group could show relatively
288 better glycemic control. Supporting this notion, several studies have previously found an inverse
289 association between adherence to a MedDiet and indices of glucose homeostasis in the general
290 population, including elderly people, and high-risk patients [39]. Previous works comparing
291 intervention with MedDiet and low-fat diet in nondiabetic PREDIMED participants did not find
292 changes in these metabolites [8]. After identifying 34 metabolites in the H-MDA pattern, we studied
293 the prediction of high adherence to MedDiet pattern. To improve the prediction of H-MDA, a model
294 with a combination of more than one discriminatory metabolite was developed. For this purpose,
295 population was split into training and validation sets. The model included PAGN, 4-
296 hydroxyphenylacetate and p-cresol. Interestingly and in accordance with this finding, in our previous
297 study, we also reported associations of PAGN and p-cresol with long-term interventions with
298 MedDiet [8]. Currently, the use of multimetabolite biomarkers is still limited. In a recent study,
299 Marklund et al. (2014) combined six serum metabolites to create a dietary biomarker score [19]. This
300 score was aimed at assessing the compliance with a healthy Nordic diet in a population with metabolic
301 syndrome (MetS) and subsequently used to analyze the effects of diet on cardiometabolic risk factors
302 among those individuals with highest compliance to this diet [19]. In our study, the inclusion of
303 PAGN, 4-hydroxyphenylacetate and p-cresol in the model, as well as the identification of other
304 metabolites derived from gut microbiota in the previous set of discriminant metabolites, highlights
305 the role of a MedDiet in the modulation of gut microbiota. Supporting this notion, De Filippis et al.
306 (2015) demonstrated the relationship between the level of MedDiet adherence, gut microbiota and
307 microbial metabolites [18]. Later, Haro et al. (2016) demonstrated that a long-term intervention with
308 a MedDiet partially restores the alteration in the gut microbiota composition in individuals with MetS
309 [40]. Interestingly, by using the same PREDIMED MedDiet score, authors found a weak but

310 significant correlation between this score and the abundance of *Faecalibacterium prausnitzii* and
311 *Bifidobacterium adolescentis*, which were observed to be decreased in MetS patients compared with
312 non-MetS patients [40]. More recently, Gutierrez-Díaz et al. (2017) found increased fecal
313 concentrations of benzoic and 3- hydroxyphenylacetic acids in individuals with higher adherence to
314 MedDiet [14] and Vazquez-Fresno et al. (2016) found that 4- hydroxyphenylacetate significantly
315 increased in urine in a “healthier” cluster of participants compared to “obese and diabetic” cluster
316 after intervention with wine polyphenol intake, exhibiting a distinct postintervention metabolic
317 response between groups possibly associated with differences/alteration in gut microbiota
318 metabolism [41]. Interestingly and in accordance with our results, a hydroxyphenylacetic acid
319 metabolite entered in our multimetabolite model, thus supporting the notion that H-MDA modulates
320 gut microbiota metabolism. Nowadays, there exists an increasing interest in the study of the
321 relationship between MedDiet adherence and food-derived alterations of the gut microbiota in order
322 to use these data in the prevention of food-related diseases [42]. The three metabolites included in
323 our multimetabolite model may arise from several food sources. PAGN is formed by the conjugation
324 of glutamine with phenylacetate, which can arise from endogenous β - oxidation of phenyl-containing
325 fatty acids or phenylalanine metabolism [43], or be obtained through the exogenous intake contained
326 in plant-food sources [44]. In line with our data, O’Sullivan et al. (2011) found a positive association
327 between the urinary concentration of PAGN and vegetable intake, suggesting that PAGN in urine
328 may be a useful biomarker of vegetable intake [11]. p-Cresol is a product of microbial tyrosine
329 breakdown via 4-hydroxyphenylacetate [45]. Moreover, 4-hydroxyphenylacetate has been found to
330 be related to the intake of vegetarian diets [31] and polyphenol-rich foods, including red wine [46]
331 and dark chocolate [47]. A further correlation analysis showed that although we found weak or
332 nonsignificant correlations between individual metabolites and some food groups, the combined
333 multimetabolite model was significantly associated with the intake of vegetables, fruits, legumes, fish
334 and total fiber (Supplementary Table 3). The main limitation of this cross-sectional study is that our
335 results may not be generalized or extrapolated to other populations, mainly because of the age of our
336 population as well as their high risk of CVD. However, the validity of the PREDIMEDMedDiet score

337 in distinguishing between individuals at high or low MedDiet adherence has been ascertained within
338 the prominent PREDIMED study [15]. Moreover, the current complementary use of this score with
339 an NMR-based untargeted metabolomics approach deserves mention, first, because dietary scores are
340 still the main tool to assess dietary patterns adherence and, second, because this robust analytical
341 platform allows us to identify differences in urinary metabolome at micro- to millimolar levels [48].
342 In conclusion, the model composed of microbial metabolites was the biomarker that defined high
343 adherence to a Mediterranean dietary pattern. This fact highlights the role of microbiota in the study
344 of the biomarkers associated to the MedDiet pattern. The effect of the MedDiet involves several
345 interconnected molecular mechanisms through complex regulatory networks, which are reflected in
346 the microbiota metabolism as the metabolic modulation of H-MDA. Future studies in nutritional
347 research should have to include the measurement of these dietary biomarkers in order not only to
348 improve the assessment of dietary intake but also to understand in-depth the molecular mechanisms
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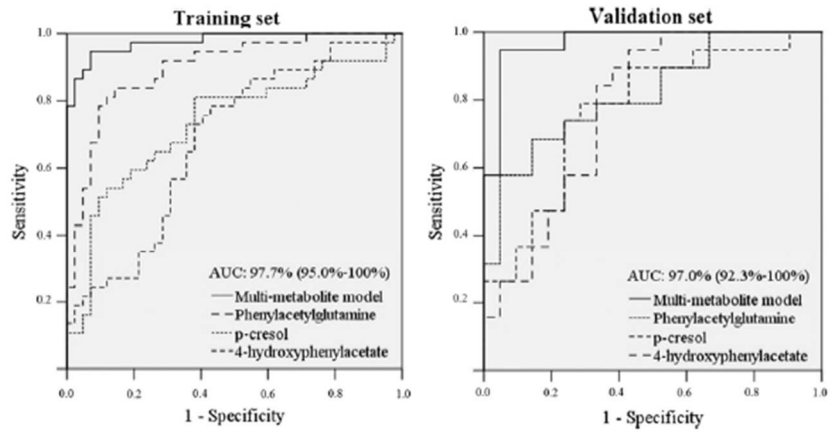


Fig. 1. ROC curves of the combined multimetabolite model and individual metabolites included for discrimination between high and low Mediterranean diet adherence in both training and validation sets.

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TABLES

Table 1
Demographic characteristics and medication usage of 119 participants classified in low and high Mediterranean diet adherence at baseline

	Total	L-MDA (≤ 7 MedDiet score)	H-MDA (≥ 10 MedDiet score)	P value
Participants, n	119	63	56	
Age, y	66.7 \pm 5.9	66.8 \pm 6.1	66.6 \pm 5.7	.83
Women, n (%)	82 (68.9)	42 (66.7)	40 (71.4)	.58
BMI, kg/m ²	30.3 \pm 4.5	31.0 \pm 4.1	29.5 \pm 4.7	.08
Current smoker, n (%)	12 (10.1)	7 (11.1)	5 (8.9)	.69
Family history of CHD, n (%)	39 (32.8)	23 (36.5)	16 (28.6)	.34
Diabetes, n (%)	63 (52.9)	36 (57.1)	26 (46.4)	.24
Hypertension, n (%)	102 (85.7)	51 (81.0)	51 (91.1)	.12
Hypercholesterolemia, n (%)	85 (71.4)	44 (69.8)	41 (73.2)	.68
<i>Medication use</i>				
Oral antidiabetic agents, n (%)	46 (38.7)	26 (41.3)	20 (35.7)	.53
Insulin, n (%)	18 (15.1)	10 (16.0)	8 (14.3)	.81
Antihypertensive agents, n (%)	56 (47.1)	27 (42.9)	29 (51.8)	.33
Lipid-lowering medication, n (%)	58 (48.7)	29 (46.0)	29 (51.8)	.53

Values are mean \pm S.D. or n (%) as appropriate. Differences between low and high Mediterranean diet adherence groups were tested by Student's *t* test and χ^2 test for continuous and categorical variables, respectively ($P < .05$). Abbreviation: CHD, coronary heart disease.

Table 2
 Dietary intake of 119 participants with low and high adherence to Mediterranean diet

Food and nutrient intake	Total	L-MDA (≤ 7 MedDiet score)	H-MDA (≥ 10 MedDiet score)	<i>P</i> value
Participants, <i>n</i>	119	63	56	
<i>Foods</i>				
Olive oil, g/d	38 \pm 15	35 \pm 15	41 \pm 13	.024
Nuts, g/d	12 \pm 12	9 \pm 12	15 \pm 12	.018
Vegetables, g/d	402 \pm 192	377 \pm 215	430 \pm 160	.032
Fruits, g/d	480 \pm 242	419 \pm 235	550 \pm 233	.003
Legumes, g/d	20 \pm 10	17 \pm 9	22 \pm 10	.004
Cereals, g/d	269 \pm 111	275 \pm 112	263 \pm 111	.56
Fish, g/d	103 \pm 50	84 \pm 47	124 \pm 45	<.001
Meat, g/d	147 \pm 69	152 \pm 81	142 \pm 52	.41
Dairy, g/d	358 \pm 213	366 \pm 207	350 \pm 220	.70
Pastries, g/d	18 \pm 21	19 \pm 22	16 \pm 20	.44
Wine, ml/d	56 \pm 116	54 \pm 4	47 \pm 3	.71
<i>Nutrients</i>				
Total energy intake, kcal/d	2397 \pm 569	2399 \pm 635	2394 \pm 490	.96
Carbohydrates, % of total energy	43.3 \pm 6.8	43.4 \pm 6.6	43.2 \pm 7.0	.87
Proteins, % of total energy	16.7 \pm 2.6	16.4 \pm 2.8	17.1 \pm 2.4	.13
Total fat, % of total energy	38.0 \pm 6.2	37.8 \pm 6.5	38.2 \pm 5.8	.71
Dietary fiber, g/d	28.4 \pm 8.0	26.2 \pm 7.0	31.0 \pm 8.3	.001
MedDiet score	8.3 \pm 2.2	6.4 \pm 0.9	10.5 \pm 0.6	<.001

Values are mean \pm S.D. Differences between low and high Mediterranean diet adherence groups were tested by Student's *t* test (*P*<.05).

Table 3
List of discriminant urinary metabolites between individuals with low and high Mediterranean diet adherence^a

Metabolite	¹ H δ , ppm (multiplicity)	Excretion in H-MDA	P value ^b
<i>Protein/amino acid metabolism</i>			
3-Methylhistidine	7.95 (s)	↓	2.9×10 ⁻²
Alanine	1.49 (d)	↓	9.4×10 ⁻³
Anserine	3.79 (s), 8.29 (s)	↑	7.8×10 ⁻⁴
Carnosine	7.18 (s), 8.15 (s)	↑	3.8×10 ⁻²
Creatine	3.94 (s)	↑	7.6×10 ⁻³
Creatinine	3.05 (s), 4.07 (s)	↑	2.3×10 ⁻³
Glycine	3.57 (s)	↓	9.1×10 ⁻³
Guanidoacetate	3.78 (s)	↑	6.7×10 ⁻³
Histidine	7.09 (s), 7.93 (s)	↑	4.8×10 ⁻⁴
Lysine	1.72 (m)	↓	2.1×10 ⁻²
N-Acetylglutamine	2.08 (m), 2.30 (m)	↑	6.0×10 ⁻³
Proline betaine	3.11 (s), 3.30 (s), 4.08 (m)	↑	6.2×10 ⁻⁷
<i>Gut microbiota metabolites</i>			
3-Indoxyl sulfate	7.27 (t), 7.51 (d), 7.71 (d)	↓	1.6×10 ⁻⁴
4-Hydroxyhippurate	6.98 (d), 7.75 (d)	↑	5.3×10 ⁻⁴
4-Hydroxyphenylacetate	6.88 (d),	↑	5.0×10 ⁻⁴
	7.16 (s)		
Dimethylsulfone	3.16 (s)	↑	6.7×10 ⁻³
Hippurate	7.55 (tt), 7.82 (dd)	↓	2.0×10 ⁻³
HPPHA	5.02 (t), 6.86 (d),	↑	3.4×10 ⁻²
	6.93 (br s), 7.30 (t)		
Isobutyrate	1.07 (d)	↓	2.2×10 ⁻⁴
p-Cresol	2.34 (s), 7.22 (d), 7.28 (d)	↑	9.6×10 ⁻⁵
Phenylacetylglutamine	1.93 (m), 2.27 (m), 3.66 (m),	↑	1.5×10 ⁻¹¹
	4.18 (m), 7.36 (t),		
	7.43 (t), 8.0 (d)		
<i>Energy metabolism (glycolysis/gluconeogenesis, TCA and ketone bodies)</i>			
β -Glucose	3.39 (m), 3.44 (m), 3.49 (m),	↓	1.9×10 ⁻⁴
	3.90 (m), 4.66 (d)		
Lactate	1.35 (d)	↓	1.1×10 ⁻²
Succinate	2.42 (s)	↓	4.7×10 ⁻³
<i>Choline metabolism</i>			
Dimethylamine	2.72 (s)	↑	5.2×10 ⁻⁴
Betaine	3.27 (s), 3.91 (s)	↓	1.2×10 ⁻²
TMAO	3.28 (s)	↑	2.6×10 ⁻³
<i>Inositol phosphate metabolism</i>			
Scyllo-inositol	3.36 (s)	↑	7.8×10 ⁻⁴
<i>Nicotinate and nicotinamide metabolism</i>			
N-Methylnicotinamide	4.49 (s), 8.85 (m), 9.13 (s)	↑	9.3×10 ⁻³
<i>Propanoate metabolism</i>			
Isopropanol	1.15 (d)	↓	1.9×10 ⁻²
<i>Purine and caffeine metabolism</i>			
Xanthosine	7.86 (s)	↓	1.1×10 ⁻²
<i>Pyrimidine metabolism</i>			
Urea	5.82 (br s)	↑	3.8×10 ⁻³
Methylguanidine	2.82 (s)	↑	2.0×10 ⁻²
Malonate	3.12 (s)	↑	2.7×10 ⁻²

^a Obtained from the OSC-PLS-DA model (VIP>1.0) and using the high Mediterranean diet adherence group as reference: (↑) and (↓) indicate a relatively higher and lower excretion, respectively, in H-MDA with respect to L-MDA.

^b Differences between NMR-signals were tested by Student's *t* test or Mann-Whitney *U* tests according to their normal distribution. Significance was corrected by the Benjamini-Hochberg procedure. Abbreviations: multiplicity (s, singlet; br, s, broad singlet; d, doublet; dd, doublet of doublets; t, triplet; tt, triplet of triplets; m, multiplet).

Table 4

ROC curve parameters of combined multimetabolite model and individual metabolites for prediction of high Mediterranean diet adherence

	ROC curve parameters							
	Training set				Validation set			
	Specificity (%)	Sensitivity (%)	AUC (95% CI)	<i>P</i> value	Specificity (%)	Sensitivity (%)	AUC (95% CI)	<i>P</i> value
Multimetabolite model	93.0	94.6	97.7 (95.0–100)	<.001	95.2	94.7	97.0 (92.3–100)	<.001
Phenylacetylglutamine	85.7	83.8	89.7 (82.7–96.7)	<.001	85.7	68.4	81.7 (68.5–94.9)	.001
<i>p</i> -Cresol	59.5	81.1	73.2 (61.7–84.8)	<.001	76.2	73.7	77.2 (62.4–92.0)	.003
4-Hydroxyphenylacetate	62.0	73.0	68.1 (56.4–79.9)	.006	66.7	84.2	77.7 (63.1–92.3)	.003

