Clinical phenotype clustering in cardiovascular risk patients for the identification of responsive metabotypes after red wine polyphenol intake

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25 ABSTRACT

26 Metabolic phenotypes of individuals are the result of genes, environment, lifestyle, diet and gut

27 microbiota interactions. The aim of this study is to evaluate the robustness of clinical and

28 metabolic phenotyping in identifying differential responsiveness to dietary strategies in the

29 improvement of cardiometabolic status. Clinical phenotyping of 57 male volunteers with high

30 cardiovascular risk factors was performed using K-means cluster analysis based on 69

31 anthropometric and plasma biochemical parameters. Cluster validation analysis based on Dunn

32 analysis for internal coherence and FOM analysis for external homogeneity was applied. The K-

33 means analysis produced four clusters with particularly significant clinical profiles. Basal

34 differences on the urine metabolomic profiles among clinical phenotypes were explored and

35 validated by OSC-PLS-DA models. Multivariate analysis (OSC-PLS-DA) of ^{1}H-NMR spectra

36 revealed that the model comparing the "obese and diabetic cluster" (OD-c) against the

37 "healthier cluster" (H-c) showed the best predictability and robustness in terms of explaining the

38 pairwise differences between clusters. When considering these two clusters, two different

39 groups of metabolites were observed after following an intervention with wine polyphenol

40 intake (WPI, 733 Equivalents of Gallic Acid [GAE/day] per 28 days). Cluster differences

41 between baseline and post-intervention values of 24h-urine NMR metabolomic data were

42 analyzed by ANOVA. Those associated to a specific metabotype (OD-c), glucose as the

43 significantly characteristic of the group (FDR correction, $p<0.01$,) and lactate, betaine and

44 dimethylamine with a trend; and those associated to wine polyphenol intervention (OD-c_WPI

45 and H-c_WPI), tartrate (FDR correction $p<0.001$), and mannitol, threonine methanol, fucose

46 and 3-hydroxyphenylacetate in a trending profile. On the other hand, 4-HPA (metabolite derived

47 from gut microbial metabolism after wine polyphenol intake) significantly increased (FDR

48 correction, $p<0.05$) for H-c_WPI compared to OD-c_WPI and basal periods (H-c_BAS and

49 OD-c_BAS), exhibiting a metabotypic intervention effect. This study provides efficient

50 strategies for targeting the heterogeneity in individual's responsiveness to dietary intervention

51 and the identification of health benefits in specific population groups.

52

1 INTRODUCTION

Metabolic phenotypes (metabotypes) are the result of interactions among several different factors (diet, lifestyle, gut microbiota, genetics, etc.), and describe characteristic metabolic profiles reflecting the biochemistry, the physiological status, and the environmental exposure in a population (Rezzi, Ramadan et al. 2007; Holmes, Wilson et al. 2008). Applications of metabolic phenotyping in nutrition research could be very useful in terms of assigning individuals to a particular metabolic phenotype. This could help improve our understanding of the linkage between both diet and disease with the different individual metabotypes (McNiven, German et al. 2011; Kinross, Li et al. 2014).

Metabolomic technologies permit the characterization of large numbers of small molecules in human biofluids. ^{1}H-NMR-based metabolomics is a very robust technique for performing metabolomic studies, enabling the simultaneous detection and quantification of a wide range of different metabolites. Because of this, NMR-based metabolomics has been applied in a variety of disciplines. In the field of nutrition, NMR-based metabolomics has been used to identify the most significant changes in a metabolic profile arising from dietary intervention studies, dietary biomarker studies and diet related disease studies (Brennan 2014). It can also be used to identify new small molecule candidates for disease biomarkers (Rupérez, Ramos-Mozo et al. 2012; Yang, Wang et al. 2013)

Cardiovascular disease (CVD) is the leading cause of mortality worldwide, with CVD-associated deaths rising very quickly in low-to-middle income countries. Modifiable risk factors for CVD—which include hypertension, smoking, abdominal obesity, abnormal lipids, diabetes mellitus, stress, low consumption of fruits and vegetables, and lack of regular physical activity—are the major contributors to CVD morbidity and mortality (Dahlöf 2010). Additionally, reduced plasma HDL levels and elevated plasma TAG concentrations are known to be significant risk factors for ischemic heart disease (IHD) (Lewington, Whitlock et al. 2007; Frikke-Schmidt, Nordestgaard et al. 2008). C-reactive protein (CRP) is another CVD risk marker (Ridker, Danielson et al. 2009). In addition, high plasma levels of homocysteine are

80   considered to be a risk factor for vascular disease, heart failure and strokes (Kaptoge, Di

81   Angelantonio et al. 2010). Another important risk factor is type 2 diabetes mellitus (T2D). The

82   prevalence of T2D is increasing rapidly around the world. Clinical predictors such as body mass

83   index (BMI), fat distribution measured by Waist-hip ratio (WHR), CRP and fasting blood

84   glucose levels can be helpful in measuring diabetes risk (Pradhan, Manson et al. 2001; Wilson,

85   Meigs et al. 2007; Wannamethee, Papacosta et al. 2010; Wang, Larson et al. 2011).

86   The low incidence of coronary heart disease (CHD) in Mediterranean countries has been partly

87   assigned to their distinct dietary habits (Dauchet, Amouyel et al. 2009). Several studies have

88   shown an inverse association between the Mediterranean diet and the incidence of CVD

89   (Estruch, Ros et al. 2013). As one of the main constituents of Mediterranean diet, wine and its

90   components, especially polyphenols, may provide additional health benefits (Chiva-Blanch,

91   Urpi-Sarda et al. 2013). In particular, the regular consumption of wine polyphenols used in this

92   study appears to mitigate CVD risk factors, leading to reduced blood pressure (Chiva-Blanch,

93   Urpi-Sarda et al. 2012) and inflammatory parameters (Chiva-Blanch, Urpi-Sarda et al. 2012).

94   The health benefits of polyphenols provided by wine intake are of particular interest. In the

95   present study, a long-term feeding trial was performed to determine changes in urinary

96   metabolites between different metabotypes. Therefore, the aim of the present work was to

97   classify a specific population into phenotypic groups according to their biochemical

98   characteristics, and then to use ^{1}H-NMR-based urinary metabolomics to observe the different

99   metabolic responses after red wine polyphenols intake.

100 2 MATERIAL AND METHODS

101 **2. 1 Subjects and Study Design**

102 The study was a prospective, randomized, crossover, and controlled trial (Chiva-Blanch, Urpi-

103 Sarda et al. 2012). High-risk subjects aged ≥55 years without documented CHD (CHD:

104 ischemic heart disease—angina/recent or past myocardial infarction/previous or cerebral

105 vascular accident, peripheral vascular disease) were recruited for the study. The subjects

4

106 included had diabetes mellitus or more than three of the following CHD risk factors: tobacco

107 smoking, hypertension, hypercholesterolemia, plasma LDL cholesterol $\geq$160 mg/dL, plasma

108 HDL cholesterol <40 mg/dL, obesity (BMI (in kg/m2) $\geq$30), and/or a family history of

109 premature CHD (first-line male relatives <55 years or females <65 years). Participants had to

110 voluntarily give signed informed consent. Subjects with a previous history of CVD, any severe

111 chronic disease, alcoholism, or other toxic substance abuse were excluded.

112 To fulfil the objectives of the present study, we used ^{1}H-NMR spectroscopy to evaluate the

113 urinary metabolomes from 57 participants between baseline and after 28 days of red wine

114 polyphenols intake (WPI, polyphenol content:733 Equivalents of Gallic Acid [EGA/day]) in

115 form of dealcoholized wine from a Merlot grape variety. Results of polyphenol composition

116 analysis of the beverages are shown in Supplemental material, Table S1. The Institutional

117 Review Board of the hospital approved the study protocol, and all participants gave written

118 consent before participation in the study. The trial has been registered in the Current Controlled

119 Trials in London, International Standard Randomized Controlled Trial Number

120 (ISRCTN88720134).

121 **2.2 Anthropometric Measurements and Biochemical Analyses**

122 Anthropometric measurements and biochemical analyses were performed using standardized

123 methods (Estruch, Martínez-González et al. 2006). BMI and WHR were measured in all the

124 participants to evaluate their obesity status. Systolic and diastolic blood pressures as well as

125 heart rate were also measured. Clinical parameters were tested in the blood and urine of

126 participants at the beginning of the study (baseline) in order to characterize the biochemical

127 status of each participant. Blood glucose levels, total cholesterol, high-density lipoprotein

128 cholesterol (HDL), low-density lipoprotein cholesterol (LDL), LDL/HDL ratio,

129 triacylglycerides (TAG), 24h-diuresis, plasmatic creatine, uric acid, aminotransferases,

130 bilirubin, ferritin, C-reactive protein, albumin, enzymes (alkaline phosphatase, lactate

131 dehydrogenase), ions (Na^+, K^+), as well as globulins, apolipoprotein levels, hemoglobin and red

132   blood cell count; with several coagulation parameters (prothrombin, thrombin, fibrinogen) were

133   measured. In total, 69 anthropometric and biochemical baseline parameters were evaluated.

134   These are shown in Table 1.

135   **2.3 Biochemical Biomarkers and Clinical Phenotype by a *k*-means Algorithm**

136   The final data set contained 69 variables from 57 samples (of the initial set of 61 individuals, 4

137   were excluded because of incomplete data regarding clinical and anthropometric parameters).

138   Prior to *k*-means analysis all variables were typified. All cluster metrics were computed with

139   1000 different random initializations of the *k*-means algorithm in order to avoid local minima. A

140   maximum number of 100 iterations were allowed in the *k*-means calculations. All computations

141   were carried out using the R package for Statistical Computing v. 2.14.1. This included the

142   statistics package for the *k*-means algorithm and the clValid package for the cluster validation

143   analysis. Dunn analysis for internal coherence and FOM analysis for external homogeneity were

144   applied to the dataset employing Euclidean distances and a *k*-means clustering algorithm. Our

145   results suggest that a cluster solution consisting of 4 centers or groups (4 clusters) showed the

146   optimal properties of internal coherence and grouping stability (the detailed methodology and

147   the validation procedure are in the supplemental material).

148   **2. 4 Metabolomic NMR Spectroscopy**

149   *2.4.1 ^{1}H-NMR sample preparation, data acquisition and processing*

150   The protocols used for this work were based on previously published methodology (Vázquez-

151   Fresno, Llorach et al. 2012). The urine samples were thawed, vortexed, and centrifuged at

152   13,200 rpm for 5 min. The supernatant (600 μL) from each urine sample was mixed with an

153   internal standard solution (120 μL, consisting of 0.1 % TSP (3-(trimethylsilyl)-proprionate-

154   2,2,3,3-d4, chemical shift reference), 2 mM of sodium azide (NaN_3, bacteriostatic agent), and

155   1.5 M KH_2PO_4 in 99 % deuterium water (D_2O)). The optimized pH of the buffer was set at 7.0,

156   with a potassium deuteroxide (KOD) solution, to minimize variations in the chemical shifts of

157   the NMR resonances. The mixture was transferred to a 5-mm NMR tube. The processed

158   spectral data were bucketed in domains of 0.005 ppm and integrated using ACD/NMR

159   Processor 12.0 software (Advanced Chemistry Development, Inc.). The spectral region between

160   4.75 and 5.00 ppm was excluded from the data set to avoid spectral interference from residual

161   water.

162   **2.5 Statistical Analysis**

163   *2.5.1 Biochemical biomarkers and phenotyping cluster differences*

164   Clusters were performed using *k*-means cluster analysis as described previously. A

165   Kolmogorov-Smirnov test ($p<0.05$) was used to test the normality of the all variables using

166   SPSS, version 18.0 for Windows (SPSS[®], Chicago, IL, USA). ANOVA analysis was performed

167   to evaluate differences in the mean biochemical measurements across clusters where statistical

168   differences were analyzed ($p<0.05$). Comparisons between clusters were assessed using a

169   Tukey post-hoc multiple comparison test. In the case of non-parametric variables, a Kruskall

170   Wallis test was used to test significant differences. Additionally, a Mann-Whitney test was used

171   to detect significances between clusters. All these tests were performed by SPSS, version 18.0

172   for Windows (SPSS[®], Chicago, IL, USA).

173   *2.5.2 Metabolomic cluster analysis- OSC-PLS-DA multivariate analysis*

174   Data generated from the NMR spectral integration were submitted to MetaboAnalyst (Xia,

175   Mandal et al. 2012). Data were normalized using the sum of the spectral intensities, then log

176   transformed and Pareto scaled. Data were then analyzed using the SIMCA-P+ 13 software

177   (Umetrics, Umea, Sweden) by multivariate discriminant analysis OSC-PLS-DA. A pairwise

178   comparison analysis between the four clusters was carried out. The quality of the models was

179   evaluated by the goodness-of-fit parameter (R^2X), the proportion of the variance of the response

180   variable that is explained by the model (R^2Y) and the predictive ability parameter (Q), which

181   was calculated using seven-fold internal cross-validation (Vázquez-Fresno, Llorach et al. 2014).

182   Validation of the OSC-PLS-DA models was carried out by a permutation test (n=200).

183   Additional information about the methodology is provided in the supplementary data. After

184   untargeted analysis with baseline samples which characterized two most discriminant clusters,

185   then, the quantification of the samples was performed for these two clusters.

186   *2.5. 3 Metabolomic phenotype analysis by ANOVA*

187   Quantified data were submitted to MetaboAnalyst in order to find possible differences between

188   clusters after WPI and normalized (24-h urine volume normalization, cube root transformed and

189   Pareto scaled) (Xia, Mandal et al. 2012) before further analysis. Metabolites were analyzed by a

190   one-way ANOVA test followed by Fisher's LSD test for multiple comparisons. The false

191   discovery rate (FDR) test, a statistical approach to the problem of multiple comparisons, was

192   used in this study to counter the effect of multiple testing and verify the most discriminating

193   metabolites (Benjamini and Hochberg 1995). Box-plots were used to show the statistical

194   differences between treatments with *P* values <0.05 being considered significant. **Figure 1**

195   displays a summary of the steps followed in this study.

196   **2.6 Metabolite Identification and Quantification**

197   The methyl singlet produced by a known quantity of TSP (0.97mM) was used as an internal

198   standard for chemical shift referencing (set to 0 ppm) and for quantification. The ^{1}H-NMR

199   spectra were analyzed using the Chenomx NMR Suite Professional Software package (version

200   7.8; Chenomx Inc, Edmonton, ALB, Canada), which permitted both identification and

201   quantification by manually fitting the NMR spectra to an internal metabolite database.

202

203   3 RESULTS AND DISCUSSION

204   **3. 1 Characterization of Clinical Phenotypes.**

205   61 participants were initially recruited into this study; of these, 57 participants were included in

206   the final cluster analysis (4 were removed from the study because an incomplete biochemical

207   profile). Of the 69 baseline biochemical parameters, *k*-means cluster analysis classified 4

208   distinct phenotypic groups: cluster 1 (n= 12), cluster 2 (n= 13), cluster 3 (n= 14) and cluster 4

209   (n= 18). Age, smoking habits, mean dietary intake, mean concentrations of biochemical

210   parameters and statistical tests for each cluster are presented in **Table 1**. Cluster 1 was defined

211   by a significantly lower systolic blood pressure (mmHg), α2-globulin (%) and neutrophil levels

212   (%); higher total cholesterol (mg/dL), LDL cholesterol (mg/dL), apolipoprotein B (mg/dL), and

213   apolipoprotein B/apolipoprotein A ratio (APOB/APOA), compared with all other clusters.

214   Cluster 2 showed lower LDL/HDL ratio compared with all other clusters and significantly

215   higher blood glucose levels compared to cluster 4. Cluster 3 was characterized by significantly

216   higher BMI values, α2-globulin (%), β-globulin (%),albumin/globulin ratio, and homocysteine

217   (μmol/L) levels and a lower albumin percentage (%) compared with the other clusters. In

218   addition, CRP values were the highest in cluster 3 and statistically significant compared with

219   clusters 1 and 4. Furthermore, glucose levels were significantly higher in cluster 3 (>126mg/dL)

220   compared with cluster 4 (<110mg/dL). More than >126mg/dL is diagnostic of T2D following

221   the American Diabetes Association (ADA) criteria. Cluster 3 had the highest but not statistically

222   significant values of the WHR index, a measure of fat distribution and also a BMI>30 indicating

223   an obese participants cluster (Apovian and Gokce 2012). There is a strong positive association

224   between obesity (measured by BMI) and risk of T2D in men (Wannamethee, Papacosta et al.

225   2010). In epidemiological studies, high plasma levels of homocysteine (hyperhomocysteinemia)

226   are considered to be a risk factor for vascular disease (Welch and Loscalzo 1998), heart failure

227   and strokes (Collaboration 2002). Among persons with T2D, the association between

228   homocysteine levels and cardiovascular disease may be stronger than that in non-diabetic

229   individuals (Ndrepepa, Kastrati et al. 2008). Moreover, cluster 3 showed the lowest HDL-

230   cholesterol levels and the highest TAG levels compared to the other clusters. These features are

231   considered risk factors for IHD (Lewington, Whitlock et al. 2007; Frikke-Schmidt,

232   Nordestgaard et al. 2008). Finally, cluster 4 showed significantly lower concentrations of TAG

233   (mg/dL), leucocyte count ($x10^9$/L), neutrophils ($x10^9$/L), lymphocytes ($x10^9$/L) and erythrocyte

234   sedimentation rate (mm/h) than cluster 3; also K (mEq/L) presented lower levels compared to

235   other clusters. The elevated circulating white blood cell count (neutrophils, lymphocytes and

236 monocytes) has been proposed as one of a few biomarkers of potential utility for cardiovascular

237 disease risk prediction (Horne, Anderson et al. 2005). Moreover, the erythrocyte sedimentation

238 rate (<10 mm/h) may be indicative of inflammation and a useful additional diagnostic criterion

239 for coronary heart disease (Yayan 2012). On the other hand, low serum potassium levels (<4

240 mEq/L) in a propensity-matched study was associated with higher mortality and chronic heart

241 failure (Ahmed, Zannad et al. 2007). Overall, cluster 4 had lower levels of cardiovascular

242 disease biomarkers than all other clusters (Table 1).

243 **3.2 Clinical Phenotypes and NMR-based Metabolomic Profiles**

244 After separation of the participants into 4 biochemically distinct clusters, an OSC-PLS-DA

245 analysis was performed to discriminate the clusters by their NMR-derived urinary profiles. The

246 results obtained by OSC-PLS-DA showed that the most strongly discriminated clusters were

247 cluster 3 *versus* cluster 4 (see Supplemental Material); for this reason, all subsequent analyses

248 were focused on further characterizing cluster 3 which was named the "obese and diabetic

249 cluster" (OD-c), versus cluster 4 or named the "healthier cluster" (H-c).

250 **3. 3 Metabolomic Phenotype Analysis to Responses to Wine Polyphenol Intake**

251 Table 2 presents the results from the multiple comparison ANOVA analysis comparing cluster

252 OD-c and H-c before and after wine polyphenols intake (WPI). Several metabolites exhibited to

253 be associated to wine polyphenols intervention including tartrate, 4-hydroxyphenylacetate (4-

254 HPA), 3-hydroxyphenylacetate (3-HPA), mannitol, methanol threonine and fucose. Further, 4

255 metabolites presented an association to metabolic phenotype (OD-c) which includes glucose,

256 lactate betaine and dimethylamine, associated to obesity and TD2 (Xie, Waters et al. 2012;

257 Menni, Fauman et al. 2013).

258 After false discovery rate (FDR) correction, tartrate, glucose and 4-hydroxyphenylacetate (4-

259 HPA) exhibited significant results. Different patterns of response were observed for these 3

260 metabolites: Tartrate was higher for both clusters after WPI (OD-c_WPI and H-c_WPI) (*wine*

261 *polyphenols intervention metabolite*). Glucose was higher in the baseline group and after

262   intervention in cluster OD-c (OD-c_BAS, OD-c_WPI) compared with cluster H-c (H-c_BAS

263   and H-c_WPI) (*metabolic phenotype related metabolite*) characteristic for cluster OD-c. Finally,

264   4-HPA showed higher urinary excretion after WPI among subjects H-c_WPI than those in OD-

265   c_WPI and at baseline (OD-c_BAS, H-c_BAS), exhibiting a distinct post-intervention

266   metabolic response in individuals for different clusters (*metabotypic intervention effect)*. Box-

267   plots show the statistical differences observed for these metabolites by multiple comparison

268   ANOVA analysis (**Figure 2**).

269   Tartrate is the major organic acid in grapes and so it is also present in wine (Son, Kim et al.

270   2008; Son, Hwang et al. 2009). Recently, it has been proposed to be a biomarker of wine

271   consumption for both interventional and epidemiological studies (Vázquez-Fresno, Llorach et

272   al. 2014). The tartrate urinary excreted amounts for OD-c_WPI were 1.06±0.19 mmols

273   (84.57±14.58 µM/mM creatinine), as well as H-c_WPI 1.29±0.29 µmols (107.89±16.69

274   µM/mM creatinine) in 24h-urine samples (**Table 2**). Similar to our results, a recent study

275   reported a tartrate concentration of 91.8µg/mg creatinine (73.69 µM/mM creatinine) measured

276   after 10h of acute wine intake (200 ml) (Regueiro, Vallverdú-Queralt et al. 2013). Additionally,

277   the presence of this metabolite at statistically significant levels in both groups after WPI

278   demonstrated a global compliance by all individuals in this intervention study.

279   The presence of glucose in urine has long been used as an indicator of diabetes mellitus

280   (Urakami, Kubota et al. 2005). T2D is characterized by the presence of glucose in urine,

281   obesity, high levels of homocysteine and CRP, which are all characteristics of cluster OD-c

282   subjects (Table 1). Moreover, there is a strong positive association between obesity (measured

283   by BMI) and T2D risk (Wannamethee, Papacosta et al. 2010). Glucose excretion amounts for

284   volunteers corresponding to the OD-c were 14.04±7.56 mmols (2157.79±1108.56 µM/mM

285   creatinine) and 13.78±7.85 mmols (1613.70±1042.12 µM/mM creatinine) in 24h-urine samples

286   for the OD-c_BAS and OD-c_WPI groups, respectively. These values were significantly higher

287   than concentrations found in normal urine (12.5 - 58.4 µM/mM creatinine) (Bouatra, Aziat et al.

288   2013). When reported values of glucose in urine are ≥100 mg/dl (5.5 mM) it is considered to be

289   a positive test for diabetes (Urakami, Kubota et al. 2005).

290   Lastly, 4-hydroxyphenylacetate (4-HPA) is a metabolite involved in tyrosine and phenylalanine

291   metabolism. Also, 4-HPA is a compound that is known to be increased in urine after

292   consumption of wine (Vázquez-Fresno, Llorach et al. 2012), chocolate (Martin, Rezzi et al.

293   2009), or cranberries (Prior, Rogers et al. 2010). This is because it is also a metabolic byproduct

294   of polyphenol degradation by gut microbiota (Moco, Martin et al. 2012), particularly *F.*

295   *prausnitzii, Bifidobacterium, Clostridium difficile, Subdoligranulum, Lactobacillus sp.* are

296   described to be responsible of metabolism of 4-HPA (Nicholson, Holmes et al. 2012). The 4-

297   HPA excretion amount for the differential response cluster (H-c_WPI) was 0.28±0.03 mmols

298   (25.35±1.48 µM/mM creatinine), significantly higher than excretion values described in the

299   literature in normal conditions (1.4-14.6 µM/mM creatinine) (Bouatra, Aziat et al. 2013). It has

300   been described that obese and diabetic people experience changes in gut microbial metabolites

301   as a result of these cardiovascular related pathologies (Shen, Obin et al. 2013). Some studies

302   found a decrease of *Bifidobacterium, F. prausnitzii,* and some species of *Clostridium and*

303   *Lactobacillus* in obese (Tagliabue and Elli 2013) and diabetic subjects (Everard and Cani 2013).

304   These findings are in agreement with our results, as lower levels of 4-HPA were found in cluster

305   OD-c than in cluster H-c after wine polyphehols intake.

306

307   4 CONCLUSIONS

308   The present study has shown that phenotypic analysis using an unsupervised clustering

309   technique (*k-means* analysis) can identify clusters according with their biochemical profiles.

310   The two most discriminating clusters were named according to their clinical parameters and

311   identified as the "obese and diabetic cluster" (OD-c) and the "healthier cluster" (H-c).

312   Moreover, metabolomic phenotyping using NMR detected a distinct metabolic response

313   between individuals grouped in these phenotypic clusters. In particular, comparisons between

314   OD-c and H-c exhibited different levels of excretion of 4-HPA after wine polyphenols intake.

315   Likewise, a metabolite linked with a specific metabotype (glucose) and another metabolite

316   linked with dietary intervention (tartrate) were also observed. According to our results subjects

317   in OD-c could have altered the gut metabolism compared to individuals of H-c. Lastly, this

318   approach showed that clinical phenotyping combined with metabolomic analysis can produce

319   interesting quantitative results, providing new insights about the relationship between diet, gut

320   microbiota and health.

321

333

334   Abbreviations: WPI: wine polyphenols intake; BAS: baseline period; H-c: Healthier cluster;

335   OD-c: obese and diabetic cluster; CVD: cardiovascular disease; CHD: coronary heart disease;

336   T2D: type 2 Diabetes Mellitus; LDL: low density lipoprotein; HDL: high density lipoprotein;

337   BMI: body mass index; TAG: triacylglycerides; WHR: waist-to-hip index; OSC-PLS-DA:

338   Orthogonal signal correction partial least-squares discriminant analysis; 4-HPA: 4-

339   hydroxyphenylacetate; FDR: false discovery rate

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Table 1. Biochemical and anthropometrical parameters of subjects and mean baseline
concentrations of individuals clusters. *P*-values of ANOVA test ($*$) for parametric variables and
Kruskall Wallis test ($\blacklozenge$) for non-parametric variables, ($p<0.05$) for both tests. Superscript
numbers ([1,2,3,4]) indicate differences between number cluster shown, in Tukey post-hoc test (for
parametrical variables) and Mann-Whitney test.(for non-parametrical variables).

	Cluster 1 (n=12)	Cluster 2 (n=13)	Cluster 3 (n=14)	Cluster 4 (n=18)	*p*-value
Characteristics					
Age (y)	59.83 ±8.62	62.08 ± 10.84	61.21 ± 5.98	59.78 ± 8.13	0.87[+]
Current smokers (%)	1.75 ± 0.45	1.85 ± 0.38	1.86 ± 0.36	1.72 ± 0.46	0.14[$\blacklozenge$]
Dietary Data					
Energy (MJ)	8074.70 ± 211.65	8046.42±1738.63	8347.50±1909.31	7387.47 ± 1838.73	0.54[+]
TE protein (%)	21.37 ± 1.97	20.55 ± 3.17	19.86 ± 4.04	21.12 ± 3.94	0.70[+]
TE carbohydrates (%)	43.19 ± 7.02	40.57 ± 8.58	43.05 ± 8.27	41.90 ±7.03	0.83[+]
TE fat (%)	33.68 ± 6.60	37.31 ± 7.36	36.44 ± 6.26	36.22 ± 4.54	0.56[+]
Anthropometrical and biochemical parameters					
BMI (kg/m^2)	28.17 ± 2.62	27.33 ± 2.78	33.56 ± 4.01[1,2,4]	29.02 ± 3.86	<0.001[+]
Waist-hip ratio	0.94 ± 0.05	0.98 ± 0.03	0.99 ± 0.05[1]	0.96 ± 0.04	0.01[+]
Heart Rate (beats/min)	72.17 ± 11.85	69.69 ± 7.09	68.71 ± 10.71	68.78 ± 8.94	0.78[+]
Systolic blood pressure (mm Hg)	124.58 ± 15.08[2,3,4]	153.54 ± 17.58	143.14 ± 13.79	141.22 ± 12.06	<0.001[+]
Diastolic blood pressure (mm Hg)	81.58 ± 9.1	79.23 ± 7.36	77.00 ± 9.14	84.50 ± 8.59	0.098[+]
CRP (mg/dL)	0.15 ± 0.09	0.27 ± 0.31	0.37 ± 0.29[1,4]	0.14 ± 0.18[2,3]	0.028[$\blacklozenge$]
Glucose (mg/dL)	101.25 ± 20.87	119.92 ± 28.62	132.07 ± 53.84[1,4]	95.22 ± 18.10[2,3]	0.01[$\blacklozenge$]
Diuresi 24h (mL)	1587.50 ± 621.35	1946.15 ±753.45[4]	1470.00 ± 441.46	1339.56±425.43	0.03[+]

Plasmatic creatinine (mg/dL)	1.01 ± 0.17	0.96 ± 0.19	1.02 ± 0.11	0.96 ± 0.11	0.52[+]
Uric acid (mg/dL)	6.58 ± 1.47	6.10 ± 0.85	6.89 ± 1.38	6.11 ± 0.78	0.19[+]
Na (mEq/L)	141.75 ± 2.14	141.15 ± 1.77	141.36 ± 2.06	140.72 ± 1.49	0.50[+]
K (mEq/L)	4.28 ± 0.23	4.19 ± 0.36	4.24 ± 0.33	3.90 ± 0.24[1,2,3]	0.002[+]
P (mEq/L)	3.66 ± 0.61[2]	3.02 ± 0.49	3.40 ± 0.58	3.09 ± 0.67	0.03[+]
Mg (mEq/L)	2.07 ± 0.19	2.21 ± 0.31	2.02 ± 0.15	2.12 ± 0.16	0.13[+]
Fe (mEq/L)	85.00 ± 23.65	99.38 ± 26.96	75.43 ± 21.18	94.89 ± 30.19	0.084[+]
Total cholesterol (mg/dL)	243.83±36.28[2,3,4]	189.69 ± 14.68	184.79 ± 20.79	203.28 ± 40.97	<0.001[+]
LDL cholesterol (mg/dL)	167.33 ±32.82[2,3,4]	116.92 ± 14.14	114.71 ± 17.87	137.67 ± 33.57	<0.001[+]
HDL cholesterol (mg/dL)	48.92 ± 13.14	52.46 ± 9.98	37.86 ± 5.07[1,2]	46.61 ± 10.09	0.003[+]
LDL cholesterol:HDL cholesterol ratio	3.61 ± 1.06	2.31 ± 0.54[1,3,4]	3.06 ± 0.50	2.98 ± 0.53	<0.001[+]
Triglycerides (mg/dL)	138.17 ± 59.29	101.69 ± 33.27	160.71 ± 73.14[2,4]	95.61 ± 35.28	0.03[+]
Apolipoprotein APOA1 (mg/dL)	146.58 ± 23.36	157.92 ± 14.83[3]	138.43 ± 15.16	147.33 ± 17.28	0.05[+]
Apolipoprotein APOB (mg/dL)	136.42 ± 24.98[2,3,4]	98.85 ± 13.37	103.36 ± 13.89	106.50 ± 19.96	<0.001[+]
APOB/A ratio	0.95 ± 0.23[2,3,4]	0.63 ± 0.095	0.76 ± 0.15	0.72 ± 0.086	<0.001[+]
ASAT (UI/L)	30.08 ± 13.56	23.23 ± 4.92	28.14 ± 10.6	24.39 ± 6.05	0.203[♦]
ALAT (UI/L)	44.42 ± 41.39	26.15 ± 8.97	31.21 ± 16.33	28.83 ± 15.03	0.230[♦]
GGT (UI/L)	42.00 ± 33.68	26.08 ± 7.95	36.71 ± 25.78	27.22 ± 17.36	0.450[♦]
Bilirubin (mg/dL)	0.77 ± 0.31	0.85 ± 0.26[3]	0.56 ± 0.12[2]	0.73 ± 0.25	0.024[♦]
Alkaline phosphatase (UI/L)	135.00 ± 20.69	162.54 ± 53.67	127.29 ± 31.30	135.28±34.03	0.082[+]
Lactate DH (UI/L)	327.83 ± 64.77	305.31 ± 16.25	340.50 ± 63.66	357.39±46.26	0.104[+]
Total proteins (g/L)	74.08 ± 2.64	73.46 ± 3.69	72.14 ± 2.69	69.94±2.75[1,2]	0.001[+]
Albumin (g/L)	47.33 ± 1.92	47.31 ± 1.88[3,4]	44.00 ± 2.77	43.39 ± 1.82	<0.001[+]
Albumin (%)	65.87 ± 2.11	64.79 ± 2.49	60.32 ± 3.82[1,2,4]	63.36 ± 2.53	<0.001[+]

α1 globulin (%)	2.76 ± 0.22[3,4]	3.10 ± 0.46	3.53 ± 0.48	3.27 ± 0.62	0.002[+]
α2 globulin (%)	5.93 ± 0.74[2,3,4]	7.39 ± 1.14	9.10 ± 1.62[1,2,4]	7.25 ± 1.49	<0.001[+]
B globulin (%)	11.19 ± 0.76	11.21 ± 1.28	12.80 ± 1.83[1,2,4]	11.02 ± 0.87	0.001[+]
Γ globulin (%)	14.25 ± 1.99	13.52 ± 2.04	14.25 ± 2.68	15.10 ± 1.77	0.25[+]
Albumin:globulin ratio	1.94 ± 0.18	1.85 ± 0.21	1.54 ± 0.24[1,2,4]	1.74 ± 0.19	<0.001[+]
Ferritine(ng/mL)	167.92 ± 102.02	187.85 ± 166.93	179.07 ± 144.77	208.56±152.61	0.89[+]
Folic acid (serum) (ng/mL)	9.50 ± 3.43	10.98 ± 2.79	8.51 ± 3.52	10.27 ± 4.65	0.36[+]
Intraerythrocytary folic acid (ng/mL)	333.27 ± 79.13	401.54 ± 69.95	417.57 ± 107.39	374.38 ± 80.27	0.08[+]
Vitamin B-12 (pg/mL)	525.58 ± 368.70	428.85 ± 145.05	377.07 ± 139.37	380.44 ±110.63	0.29[♦]
Homocysteine (µmol /L)	11.07 ± 2.60	11.17 ± 1.24[1,2,4]	14.31 ± 4.28	11.26 ± 1.79	0.005[+]
Hemoglobin concentration (g/L)	152.75 ± 9.92	151.00 ± 11.77	143.57 ± 9.15	147.33 ± 8.09	0.08[+]
Hematocrit (L/L)	0.45 ± 0.03	0.45 ± 0.03	0.43 ± 0.03	0.44 ± 0.025	0.16[+]
Erythrocyte mean corpuscular volume (fL)	89.80 ± 2.59	91.05 ± 3.73	90.35 ± 5.30	88.78 ± 3.35	0.42[+]
Mean corpuscular hemoglobin (pg)	30.20 ± 0.93	30.82 ± 0.98	30.31 ± 1.89	29.88 ± 1.16	0.28[+]
Mean corpuscular hemoglobin concentration (g/L)	336.25 ± 5.15	338.45 ± 4.74	335.57 ± 7.44	336.56 ± 9.99	0.78[+]
Erytrocyte sedimentation rate (mm/h)	7.08 ± 4.44	8.08 ± 3.86	11.07 ± 5.26[1,4]	5.44 ± 2.31[2,3]	0.003[♦]
Reed distribution width (%)	13.06 ± 0.58[4]	13.17 ± 0.59	13.59 ± 0.66	13.68 ± 0.53	0.01[+]
Hemoglobin distribution width (g/L)	25.74 ± 1.46	24.49 ± 1.96	25.51 ± 2.65	26.03 ± 2.18	0.24[+]
Platelet count (x109/L)	282.17 ± 56.36[4]	233.54 ± 45.98	264.64 ± 63.99	220.17 ± 33.78	0.006[+]

Mean platelet volume (fL)	8.27 ± 0.47	9.11 ± 0.74	8.85 ± 0.72	8.89 ± 0.99	0.05[+]
Leucocyte count (x10^9/L)	6.84 ± 1.73	7.09 ± 0.88	7.96 ± 1.23[4]	6.15 ± 1.30	0.004[+]
Neutrophils (%)	50.45 ± 5.82[2,3,4]	63.84 ± 8.78	58.26 ± 4.43	58.21 ± 6.32	<0.001[+]
Lymphocytes (%)	35.83 ± 4.42[2,4]	24.90 ± 7.07	29.90 ± 5.06	29.83 ± 5.87	<0.001[+]
Monocytes (%)	6.58 ± 1.07	5.85 ± 1.09	6.44 ± 0.88	7.47 ± 0.63[2,3]	<0.001[+]
Eosinophils (%)	4.12 ± 2.04[2,4]	2.65 ± 1.23	2.77 ± 1.19	2.15 ± 0.72	0.002[+]
Basophils (%)	0.71 ± 0.25	0.79 ± 0.39[4]	0.57 ± 0.2	0.49 ± 0.19	0.01[+]
Unclassified cells (%)	2.16 ± 0.95	1.99 ± 0.93	2.05 ± 0.50	1.86 ± 0.56	0.726[+]
Neutrophils (x10^9/L)	3.49 ± 1.11	4.55 ± 0.89[1,4]	4.63 ± 0.79[1,4]	3.56 ± 0.84	0.001[+]
Lymphocytes (x10^9/L)	2.43 ± 0.61[2,4]	1.75 ± 0.46[1,3]	2.39 ± 0.56[2,4]	1.85 ± 0.57	0.002[+]
Monocytes (x10^9/L)	0.45 ± 0.12	0.42 ± 0.10	0.52 ± 0.13	0.45 ± 0.10	0.24[♦]
Eosinophil (x10^9/L)	0.27 ± 0.15	0.19 ± 0.086	0.21 ± 0.09	0.13 ± 0.05[1,2,3]	0.001[♦]
Basophils (x10^9/L)	0.05 ± 0.03	0.05 ± 0.04	0.03 ± 0.04	0.08 ± 0.01[1,2]	0.002[♦]
Unclassified cells (x10^9/L)	0.16 ± 0.06	0.15 ± 0.06	0.17 ± 0.05[4]	0.11 ± 0.05[1,3]	0.029[♦]
Prothrombin time (%)	97.58 ± 3.68[4]	95.54 ± 5.72	96.00 ± 4.93	92.50 ± 5.83	0.126[♦]
Prothrombin time (seg)	13.07 ± 0.45	13.23 ± 0.78	13.29 ± 0.47	13.56 ± 0.67	0.186[+]
Thromboplastin partial time (seg)	28.48 ± 0.89	26.65 ± 7.97	26.38 ± 7.55	29.35 ± 2.11	0.386[+]
Fibrinogen (g/L)	3.18 ± 0.51	4.08 ± 0.82[1,4]	3.73 ± 0.62	3.18 ± 0.37	<0.001[+]

477   All values are mean ± SD, *p*-values are based on simple ANOVA test ([+]) for parametric variables and
478   Kruskall Wallis test ([♦]) for non-parametric variables, (*p*<0.05 for both tests); Superscript numbers
479   adjacent to values from each cluster denote a significant difference between indicated cluster number
480   based on Tukey post-hoc test (for parametrical variables) and Mann-Whitney test.(for non-parametrical
481   variables). TE protein %: protein as percentage of total energy intake; TE carbohydrate%: carbohydrate as
482   percentage of total energy intake; TE fat %: fat as percentage of total energy intake. PCR: c-reactive
483   protein; BMI: body mass index; ASAT: aspartate aminotranspherase; ALAT: alanine aminotransferase;
484   GGT:gamma-glutamyl transpeptidase; Na: sodium; K: potassium; P: phosphorous; Mg: Magnessium; Fe:
485   iron. DH: dehydrogenase;

486

487   **Table 2**. Urinary excretion amounts [mmols, 24h-urine] of metabolites after ANOVA analysis, in parenthesis μM/mM creatinine. OD-c_BAS: obese and

488   diabetic cluster in basal period; H-c_BAS: healthier cluster in basal period; OD-c_WPI: obese and diabetic cluster after wine polyphenols intake; H-c_WPI:

489   healthier cluster after wine polyphenol intake. WPI: wine polyphenols intake; MTP: metabotype.4-HPA: 4-hydroxyphenylacetate; 3-HPA: 3-

490   hydroxyphenylacetate. DMA: dimethylamine.

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| Metabolite	MEAN ± SEM; mmols, 24h (μM/mM creatinine)				*p*-value	*FDR p-value*	Metabolite information
	OD-c_BAS	H-c_BAS	OD-c_WPI	H-c_WPI			
Tartrate	0.140 ± 0.052 (14.16 ± 5.78)	0.451 ± 0.019 (39.83 ± 14.74)	1.065 ± 0.19 (84.57 ± 14.58)	1.292 ± 0.285 (107.89 ± 16.69)	7.09E-07	6.02E-05	WPI
Glucose	14.044 ± 7.562 (2157.79 ± 1108.56)	0.210 ± 0.019 (19.95 ± 1.61)	13.786± 7.850 (1613.70 ± 1042.12)	0.188± 0.038 (16.53 ± 2.08)	0.0001	0.005	MTP
4-HPA	0.170 ± 0.025 (17.23 ± 2.04)	0.163 ± 0.013 (15.52 ± 1.13)	0.184 ± 0.022 (16.23 ± 1.95)	0.280 ± 0.029 (25.35 ± 1.48)	0.0008	0.02	WPI-MTP
3-HPA	0.056 ± 0.01 (40.71± 8.34)	0.062 ± 0.005 (48.39± 3.99)	0.0856 ± 0.016 (56.04± 10.97)	0.101 ± 0.012 (75.7± 10.43)	0.01	0.18	WPI
Mannitol	0.556 ± 0.112 (462.62+142.13)	0.782 ± 0.144 (646.87± 132.08)	1.223 ± 0.157 (804.66± 117.30)	1.312 ± 0.255 (957.04± 210.15)	0.005	0.99	WPI
Threonine	0.094 ± 0.014 (9.07 ± 1.17)	0.107 ± 0.013 (10.33 ± 1.51)	0.141 ± 0.013 (12.64 ± 2.19)	0.173 ± 0.032 (15.00 ± 1.99)	0.02	0.18	WPI
Methanol	0.473 ± 0.077 (46.83 ± 6.80)	0.436 ± 0.050 (39.99 ± 3.72)	0.682 ± 0.133 (51.02 ± 5.07)	0.676 ± 0.076 (59.91 ± 3.82)	0.03	0.30	WPI
Fucose	0.32 ± 0.04 (30.58 ± 2.36)	0.327 ± 0.023 (30.55 ± 1.81)	0.503 ± 0.069 (39.01 ± 4.56)	0.495 ± 0.081 (42.84 ± 3.87)	0.04	0.31	WPI
Lactate	0.516 ± 0.19 (65.56 ± 34.17)	0.212 ± 0.075 (19.77 ± 1.37)	0.432 ± 0.087 (37.83 ± 11.02)	0.294 ± 0.030 (26.09 ± 1.80)	0.04	0.33	MTP
Betaine	0.549 ± 0.217 (56.01 ± 21.61)	0.207 ± 0.030 (19.36 ± 2.64)	0.676 ± 0.245 (53.77 ± 18.74)	0.214 ± 0.037 (18.83 ± 2.28)	0.02	0.18	MTP
DMA	0.469 ± 0.021 (51.68 ± 7.82)	0.503 ± 0.048 (45.89 ± 3.20)	0.905 ± 0.230 (65.36 ± 8.11)	0.568 ± 0.078 (51.21 ± 4.62)	0.02	0.18	MTP

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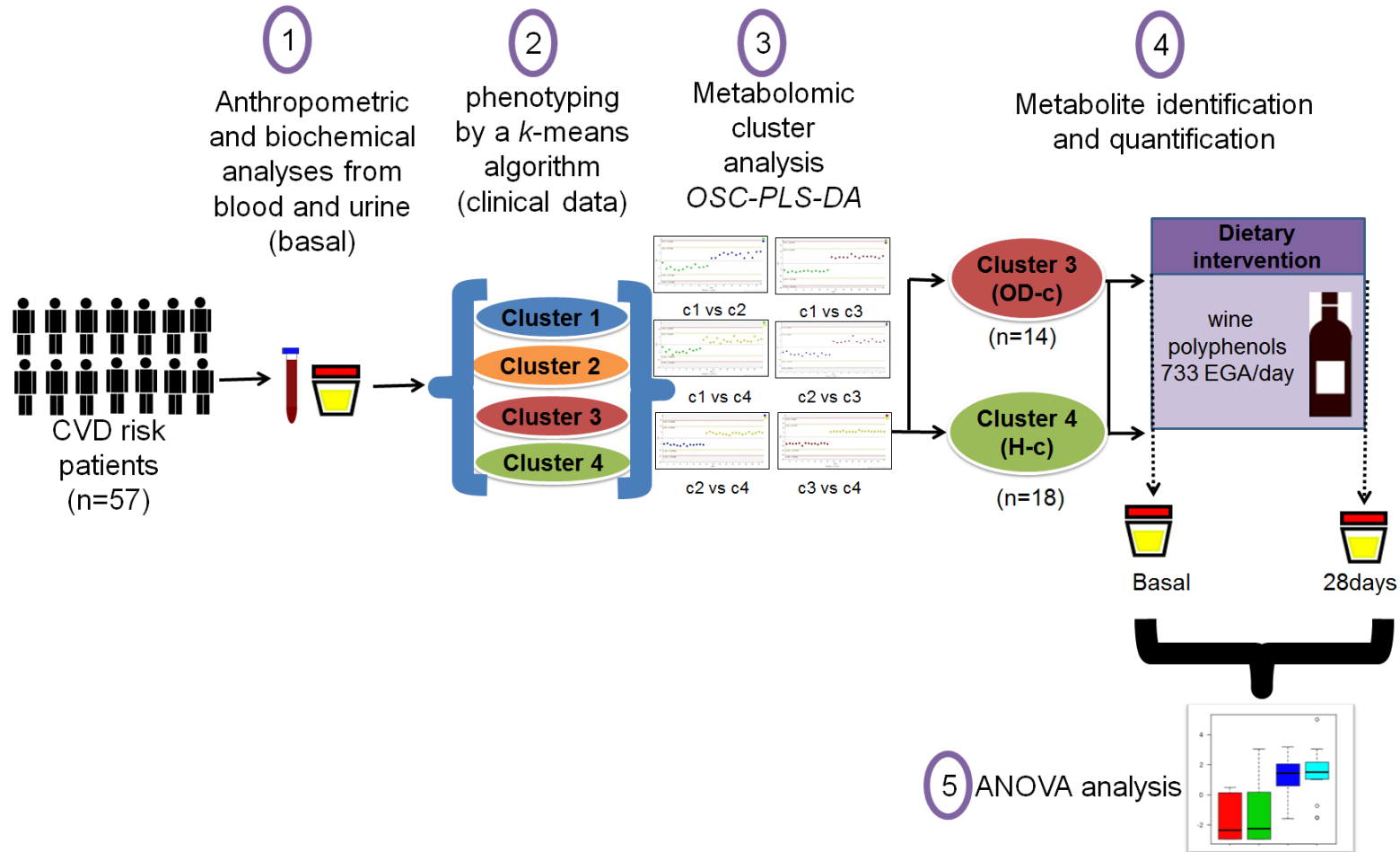
494
495   **Figure 1.** Methodological strategy steps followed in the present study. CVD: cardiovascular disease; c: cluster; OD-c: obese and diabetic cluster; H-c:

496   healthier cluster. OSC-PLS-DA: partial least-squares discriminant analysis with orthogonal signal correction. EGA: equivalents of gallic acid
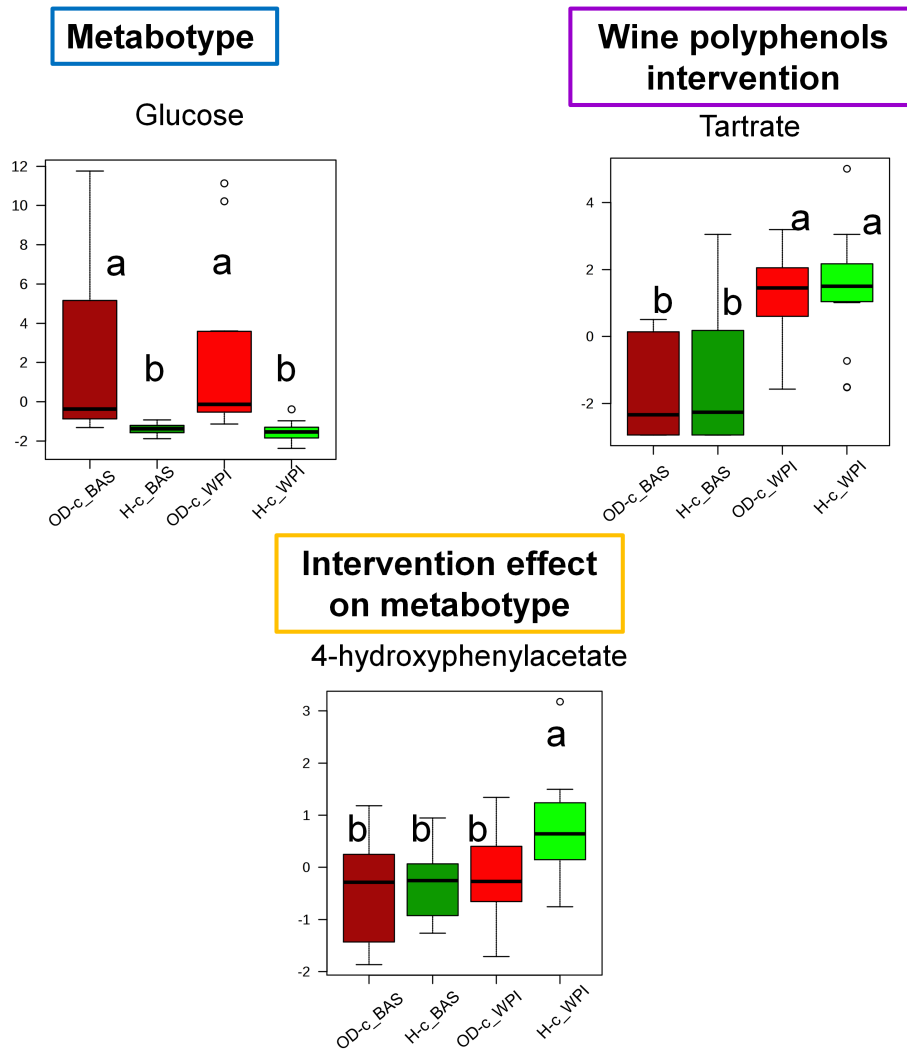
Figure 2. Box-plots of the metabolites derived from ANOVA test ($p<0.05$ after FDR correction). Different letters indicate significant differences between interventions. OD-c_BAS: obese and diabetic cluster in basal period; H-c_BAS: healthier cluster in basal period; OD-c_WPI: obese and diabetic cluster after wine polyphenols intake; H-c_WPI: healthier cluster after wine polyphenols intake.