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

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ABSTRACT

Metabolic phenotypes of individuals are the result of genes, environment, lifestyle, diet and gut microbiota interactions. The aim of this study is to evaluate the robustness of clinical and metabolic phenotyping in identifying differential responsiveness to dietary strategies in the improvement of cardiometabolic status. Clinical phenotyping of 57 male volunteers with high cardiovascular risk factors was performed using *K*-means cluster analysis based on 69 anthropometric and plasma biochemical parameters. Cluster validation analysis based on Dunn analysis for internal coherence and FOM analysis for external homogeneity was applied. The *K*-means analysis produced four clusters with particularly significant clinical profiles. Basal differences on the urine metabolomic profiles among clinical phenotypes were explored and validated by OSC-PLS-DA models. Multivariate analysis (OSC-PLS-DA) of ^{1}H-NMR spectra revealed that the model comparing the "obese and diabetic cluster" (OD-c) against the "healthier cluster" (H-c) showed the best predictability and robustness in terms of explaining the pairwise differences between clusters. When considering these two clusters, two different groups of metabolites were observed after following an intervention with wine polyphenol intake (WPI, 733 Equivalents of Gallic Acid [GAE/day] per 28 days). Cluster differences between baseline and post-intervention values of 24h-urine NMR metabolomic data were analyzed by ANOVA. Those associated to a specific metabotype (OD-c), glucose as the significantly characteristic of the group (FDR correction, $p<0.01$,) and lactate, betaine and dimethylamine with a trend; and those associated to wine polyphenol intervention (OD-c_WPI and H-c_WPI), tartrate (FDR correction $p<0.001$), and mannitol, threonine methanol, fucose and 3-hydroxyphenylacetate in a trending profile. On the other hand, 4-HPA (metabolite derived from gut microbial metabolism after wine polyphenol intake) significantly increased (FDR correction, $p<0.05$) for H-c_WPI compared to OD-c_WPI and basal periods (H-c_BAS and OD-c_BAS), exhibiting a metabotypic intervention effect. This study provides efficient strategies for targeting the heterogeneity in individual's responsiveness to dietary intervention and the identification of health benefits in specific population groups.

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

106 included had diabetes mellitus or more than three of the following CHD risk factors: tobacco
107 smoking, hypertension, hypercholesterolemia, plasma LDL cholesterol ≥160 mg/dL, plasma
108 HDL cholesterol <40 mg/dL, obesity (BMI (in kg/m2) ≥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

distinct phenotypic groups: cluster 1 (n= 12), cluster 2 (n= 13), cluster 3 (n= 14) and cluster 4 (n= 18). Age, smoking habits, mean dietary intake, mean concentrations of biochemical parameters and statistical tests for each cluster are presented in **Table 1**. Cluster 1 was defined by a significantly lower systolic blood pressure (mmHg), α2-globulin (%) and neutrophil levels (%); higher total cholesterol (mg/dL), LDL cholesterol (mg/dL), apolipoprotein B (mg/dL), and apolipoprotein B/apolipoprotein A ratio (APOB/APOA), compared with all other clusters. Cluster 2 showed lower LDL/HDL ratio compared with all other clusters and significantly higher blood glucose levels compared to cluster 4. Cluster 3 was characterized by significantly higher BMI values, α2-globulin (%), β-globulin (%),albumin/globulin ratio, and homocysteine (µmol/L) levels and a lower albumin percentage (%) compared with the other clusters. In addition, CRP values were the highest in cluster 3 and statistically significant compared with clusters 1 and 4. Furthermore, glucose levels were significantly higher in cluster 3 (>126mg/dL) compared with cluster 4 (<110mg/dL). More than >126mg/dL is diagnostic of T2D following the American Diabetes Association (ADA) criteria. Cluster 3 had the highest but not statistically significant values of the WHR index, a measure of fat distribution and also a BMI>30 indicating an obese participants cluster (Apovian and Gokce 2012). There is a strong positive association between obesity (measured by BMI) and risk of T2D in men (Wannamethee, Papacosta et al. 2010). In epidemiological studies, high plasma levels of homocysteine (hyperhomocysteinemia) are considered to be a risk factor for vascular disease (Welch and Loscalzo 1998), heart failure and strokes (Collaboration 2002). Among persons with T2D, the association between homocysteine levels and cardiovascular disease may be stronger than that in non-diabetic individuals (Ndrepepa, Kastrati et al. 2008). Moreover, cluster 3 showed the lowest HDL-cholesterol levels and the highest TAG levels compared to the other clusters. These features are considered risk factors for IHD (Lewington, Whitlock et al. 2007; Frikke-Schmidt, Nordestgaard et al. 2008). Finally, cluster 4 showed significantly lower concentrations of TAG (mg/dL), leucocyte count ($x10^9$/L), neutrophils ($x10^9$/L), lymphocytes ($x10^9$/L) and erythrocyte sedimentation rate (mm/h) than cluster 3; also K (mEq/L) presented lower levels compared to other clusters. The elevated circulating white blood cell count (neutrophils, lymphocytes and

monocytes) has been proposed as one of a few biomarkers of potential utility for cardiovascular disease risk prediction (Horne, Anderson et al. 2005). Moreover, the erythrocyte sedimentation rate (<10 mm/h) may be indicative of inflammation and a useful additional diagnostic criterion for coronary heart disease (Yayan 2012). On the other hand, low serum potassium levels (<4 mEq/L) in a propensity-matched study was associated with higher mortality and chronic heart failure (Ahmed, Zannad et al. 2007). Overall, cluster 4 had lower levels of cardiovascular disease biomarkers than all other clusters (Table 1).

3.2 Clinical Phenotypes and NMR-based Metabolomic Profiles

After separation of the participants into 4 biochemically distinct clusters, an OSC-PLS-DA analysis was performed to discriminate the clusters by their NMR-derived urinary profiles. The results obtained by OSC-PLS-DA showed that the most strongly discriminated clusters were cluster 3 *versus* cluster 4 (see Supplemental Material); for this reason, all subsequent analyses were focused on further characterizing cluster 3 which was named the "obese and diabetic cluster" (OD-c), versus cluster 4 or named the "healthier cluster" (H-c).

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

Table 2 presents the results from the multiple comparison ANOVA analysis comparing cluster OD-c and H-c before and after wine polyphenols intake (WPI). Several metabolites exhibited to be associated to wine polyphenols intervention including tartrate, 4-hydroxyphenylacetate (4-HPA), 3-hydroxyphenylacetate (3-HPA), mannitol, methanol threonine and fucose. Further, 4 metabolites presented an association to metabolic phenotype (OD-c) which includes glucose, lactate betaine and dimethylamine, associated to obesity and TD2 (Xie, Waters et al. 2012; Menni, Fauman et al. 2013).

After false discovery rate (FDR) correction, tartrate, glucose and 4-hydroxyphenylacetate (4-HPA) exhibited significant results. Different patterns of response were observed for these 3 metabolites: Tartrate was higher for both clusters after WPI (OD-c_WPI and H-c_WPI) (*wine 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

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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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469

470

471 TABLES

472 **Table 1.** Biochemical and anthropometrical parameters of subjects and mean baseline
473 concentrations of individuals clusters. *P*-values of ANOVA test (*) for parametric variables and
474 Kruskall Wallis test (♦) for non-parametric variables, (p<0.05) for both tests. Superscript
475 numbers (^{1,2,3,4}) indicate differences between number cluster shown, in Tukey post-hoc test (for
476 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♦
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♦
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♦
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$^{\blacklozenge}$
ALAT (UI/L)	44.42 ± 41.39	26.15 ± 8.97	31.21 ± 16.33	28.83 ± 15.03	0.230$^{\blacklozenge}$
GGT (UI/L)	42.00 ± 33.68	26.08 ± 7.95	36.71 ± 25.78	27.22 ± 17.36	0.450$^{\blacklozenge}$
Bilirubin (mg/dL)	0.77 ± 0.31	0.85 ± 0.26[3]	0.56 ± 0.12[2]	0.73 ± 0.25	0.024$^{\blacklozenge}$
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^{+}
Β 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$^{\blacklozenge}$
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$^{\blacklozenge}$
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$^{\blacklozenge}$
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$^{\blacklozenge}$
Basophils ($x10^9$/L)	0.05 ± 0.03	0.05 ± 0.04	0.03 ± 0.04	0.08 ± 0.01[1,2]	0.002$^{\blacklozenge}$
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$^{\blacklozenge}$
Prothrombin time (%)	97.58 ± 3.68[4]	95.54 ± 5.72	96.00 ± 4.93	92.50 ± 5.83	0.126$^{\blacklozenge}$
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^{+}

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

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-

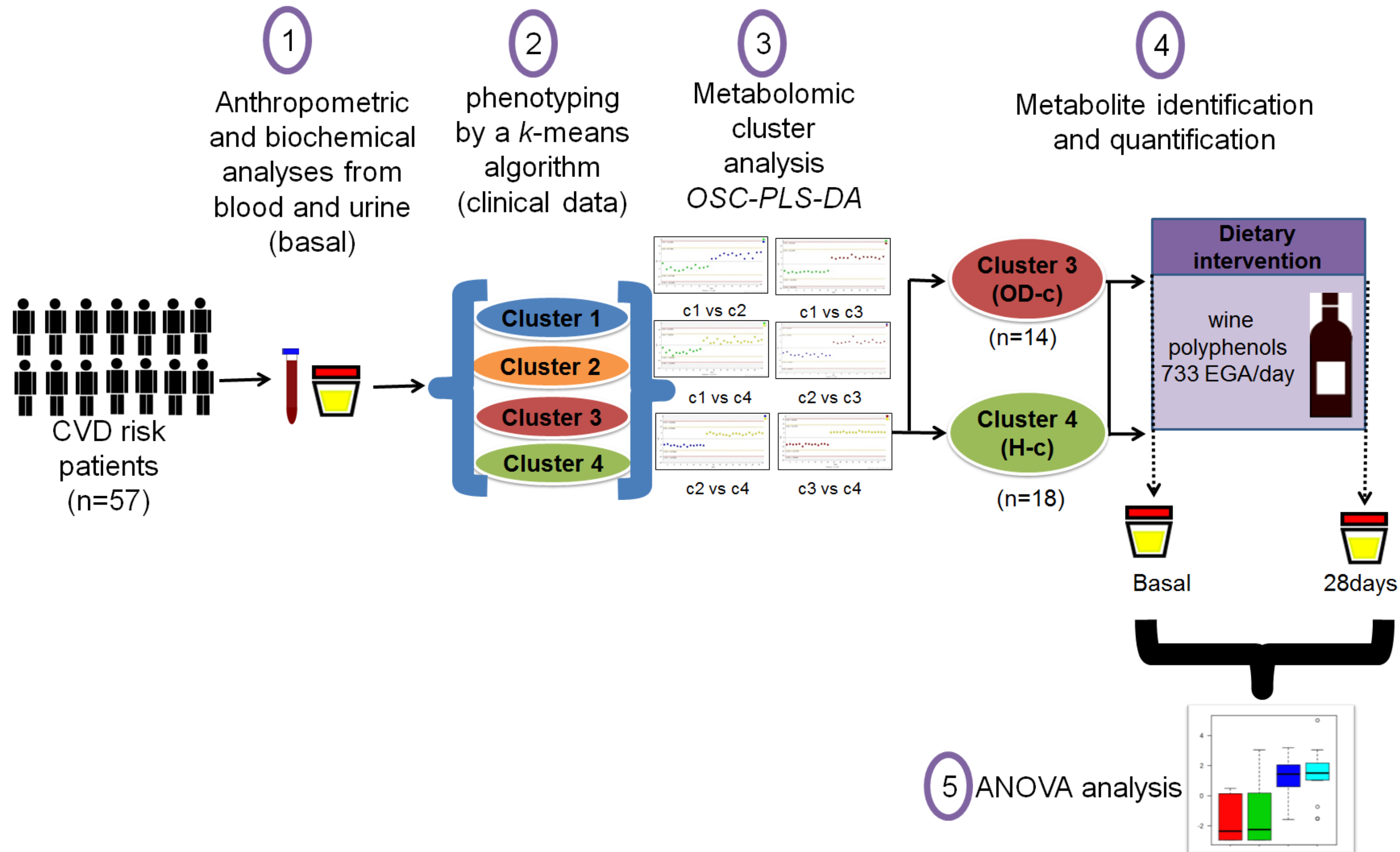
490 hydroxyphenylacetate. DMA: dimethylamine.

491

	MEAN ± SEM; mmols, 24h (µM/mM creatinine)						
Metabolite	**OD-c_BAS**	**H-c_BAS**	**OD-c_WPI**	**H-c_WPI**	***p*-value**	***FDR p-value***	**Metabolite information**
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

492

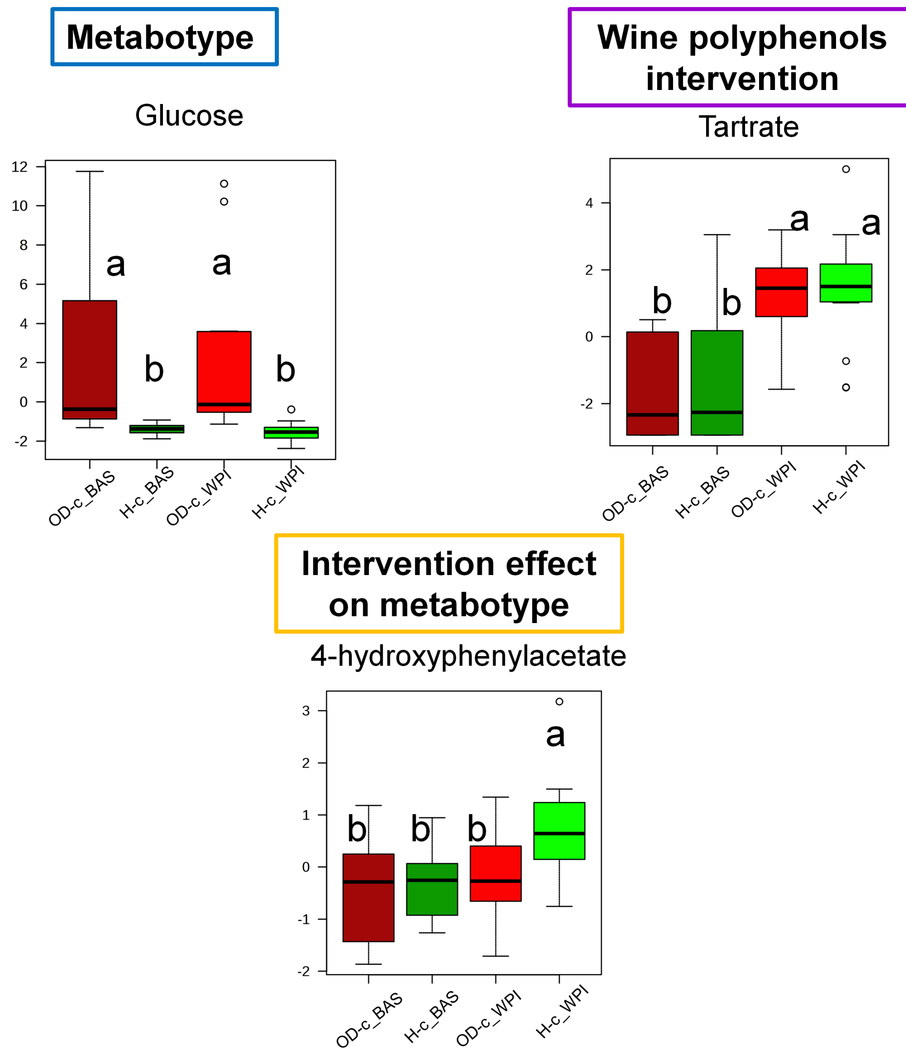
493 **FIGURE LEGEND**



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



497

498 **Figure 2**. Box-plots of the metabolites derived from ANOVA test (*p*<0.05 after FDR

499 correction). Different letters indicate significant differences between interventions. OD-c_BAS:

500 obese and diabetic cluster in basal period; H-c_BAS: healthier cluster in basal period; OD-

501 c_WPI: obese and diabetic cluster after wine polyphenols intake; H-c_WPI: healthier cluster

502 after wine polyphenols intake.