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# Metabolome-based clustering after moderate wine consumption

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**ABSTRACT**

Grouping individuals according to their metabolic capacities (metabotyping) has caused a shift from individualised to grouped treatments for the optimisation of nutritional interventions. Several studies have reported a stratification of patients into metabolic clusters after the intake of certain foods, of which polyphenols seem to be mostly associated with metabotypes. Despite this, there is a lack of metabotyping studies regarding wine consumption. In this context, the human urinary metabolome of healthy volunteers (n=41) was explored by means of a non-targeted metabolomic approach after an intervention with red wine (250 mL/day, 28 days). Three clusters of volunteers based on their relative production of phenolic metabolites were perceived, and the compounds responsible for this clustering were identified. To our knowledge, this is the first time that different urinary metabotypes have been described in healthy volunteers after moderate red wine consumption. Our findings suggest that stratification of individuals in clinical trials according to their metabotype is necessary to fully understand the health effects of wine polyphenols.

**KEYWORDS**

wine, polyphenols, urinary metabolome, metabotypes clustering

**Supplementary data can be downloaded through: https://oeno-one.eu/article/view/2983**

## INTRODUCTION

Excessive alcohol consumption increases the risk of liver cirrhosis and cancers (mostly those of the upper digestive and respiratory tract), while low to moderate red wine consumption has been associated with health-promoting properties (Artero *et al.*, 2015). Red wine is different from other alcoholic beverages due to its content in various phenolic compounds. Moderate consumption of red wine has been linked to a lower risk of cardiovascular disease (CVD), diabetes, osteoporosis, and maybe neurological diseases (Artero *et al.*, 2015; Karatzi *et al.*, 2004; Iriti and Varoni, 2014). Recent studies have suggested that the positive health effects of wine, particularly red wine, cannot be merely attributed to its ethanol content (Karatzi *et al.*, 2004; Iriti and Varoni, 2014). Wine is a complex matrix that contains many compounds of biological interest, of which phenolic compounds are some of the most interesting, as they are all biologically active and may play a role in the health benefitsof wine (Waterhouse, 2002; Nash *et al.*, 2018). With the growing interest in this area, understanding the absorption, bioavailability, and metabolism of phenolic compounds from wine in humans is of utmost importance (Cueva *et al.*, 2017; Fernandes *et al.*, 2017; Mosele *et al.*, 2015; Nash *et al.*, 2018).

In general terms, the human metabolome is defined as the complete set of small metabolites found in a biological sample (i.e., cells, tissue, organ, biological fluids), and is influenced by gut microbiota and genetics, as well as by environmental factors (diet, contaminants, pharmaceuticals, and other lifestyle factors) (Riedl *et al.*, 2017). As a consequence, all these factors contribute to interindividual differences in the metabolome, which translate into distinct nutritional requirements and diverse responses to nutritional or medical interventions (Riedl *et al.*, 2017; Morand and Tomás-Barberán, 2019). An emerging idea derived from this issue consists in studying these metabolic differences in order to group subjects with common metabolic profiles when studying the effects of a specific intervention. This has led to the definition of the metabotype, which refers to a group of individuals with similar metabolic profiles (Tomás-Barberán *et al.*, 2016; Cueva *et al.*, 2017). Although there is no global consensus regarding the parametres for the definition of metabotypes, three different approaches can be applied depending on the objective of the study: general fasting metabotypes (including all the possible biochemical parametres); specific fasting metabotypes (searching for differences only in certain parametres, such as lipid profile); or response clustering, which is defined after a previously designed intervention has been carried out. Metabolomics has emerged as a tool for classification of individuals in metabotytes, and can therefore be used to predict physiological response in a dietary intervention. A recent metabolomic study has revealed two main clusters of postmenopausal women after bread intake, based on the correlation of data obtained from fasting metabotyping and insulin plasma levels (Moazzami *et al.*, 2014). In the same way, a metabolomic approach applied to major metabolites (bile acids, fatty acids, amino acids, carboxy acids, hydroxylic acids, and aromatic derivatives) has led to individual stratification into two clusters corresponding to contrasting dietary patterns (high meat and low vegetable intake; low meat and high vegetable intake) (Wei *et al.*, 2018).

Dietary polyphenols belong to one of the compound classes in which metabolism seems to be associated with individual metabotypes (Selma *et al.*, 2009; Bolca *et al.*, 2013; Manach *et al.*, 2017; Mena *et al.*, 2018). For instance, there is evidence of stratification in ellagitannin-metabolizing phenotypes (urolithin-producer metabotypes) or isoflavone-metabolizing phenotypes (equol-producer metabotypes). In previous work, three different urolithin-producing metabotypes have been described (Tomás-Barberán *et al.*, 2014): after a 6 months intervention with pomegranate capsules enriched in phenolics, a reduction in different blood lipid biomarkers - including total cholesterol, LDL-cholesterol or non-HDL cholesterol among others - was only perceived in metabotype B obese patients (González-Sarrias *et al.*, 2017). In a further study, a consistent relationship between urolithin metabotypes and cardiometabolic risk biomarkers was reported; results demonstrated that overweight metabotype B patients are at risk of cardiovascular disease, whereas metabotype A patients are protected from these disorders (Selma *et al.*, 2017). In those previous studies, the term 'individual stratification' was used to classify patients prior to an intervention and predict their response in terms of level of responsiveness/non-responsiveness.

Several studies on wine have applied metabolomics to study changes in metabolites after moderate wine consumption (Vázquez-Fresno *et al.*, 2015; Boto-Ordonez *et al.*, 2013; Urpi-Sarda *et al.*, 2015; Muñoz-González *et al.*, 2013). In particular, 1H-NMR and UHPLC-MS/MS approaches have

been used to evaluate differences in the urinary metabolome of cardiovascular risk patients after moderate intake of red wine and dealcoholized red wine (Vázquez-Fresno *et al.*, 2012; Boto-Ordoñez *et al.*, 2013). More specifically, Vázquez-Fresno *et al.* (2016) clustered a specific population into four phenotypic groups according to their biochemical characteristics. The two most discriminating clusters ("obese and diabetic" metabotype and "healthier" metabotype) showed different metabolic responses to a wine polyphenol intervention.

The present study aims to investigate the differential responsiveness to moderate wine consumption in healthy subjects. For this purpose, urinary metabolomics of samples collected before and after a red wine intervention period were studied, and differences in the observed metabotypes were explored.

## MATERIALS AND METHODS

### 1. Chemicals

All chemicals were of analytical grade. Formic acid was purchased from Riedel-de Haën (Seelze, Germany). Acetonitrile and water were of MS grade (Labscan, Gliwice, Poland). A commercial standard mixture consisting of 42 low-molecular weight compounds (including acidic, basic and neutral metabolites) was obtained from Sigma-Aldrich (St. Louis, MO, USA) and was used in order to assess instrument variability during the study. Commercial standards of citric acid, 4-hydroxyhippuric acid, quinic acid and epicatechin were purchased from Panreac (Barcelona, Spain), Phytolab (Madrid, Spain), Extrasynthese (Genay, France) and Sigma-Aldrich (St. Louis, MO, USA), respectively.

### 2. Red wine intervention

During the intervention study, healthy subjects were given a young red wine from a Pinot Noir grape variety (2010) kindly provided by Bodegas Miguel Torres S.A. (Spain) with a total phenolic content of 1758 mg of gallic acid equivalents/L (Muñoz-González *et al.*, 2013). Among them, total anthocyanins comprised 447 mg of malvidin-3-*O*-glucoside equivalents/L; meanwhile total catechins content was 1612 mg, expressed as mg of (+)-catechin equivalents/L (Muñoz-González *et al.*, 2013). The composition of individual phenolic compounds, including the content of specific compounds such as resveratrol (7.12 mg/L), has also been previously reported in Muñoz-González *et al.* (2013). Other wine properties were: ethanol content (13.8 % v/v), pH (3.52), total acidity (6.45 g/L tartaric acid) and volatile acidity (0.56 g/L acetic acid), determined according to OIV (International Organization of Vine and Wine, 1990) procedures.

### 3. Human intervention study

A randomised and controlled 4-week intervention study was carried out involving 41 healthy 22-65 year-old volunteers (8 control and 33 case subjects; age mean: 36 ± 11 years) (Muñoz-González *et al.*, 2013; Esteban-Fernández *et al.*, 2018). The participants (22 women and 19 men) were not suffering from any disease or intestinal disorder, and had not taken antibiotics or any other medical treatment for at least 6 months before the start of the study or during the study (including the washout period). All the participants had been fully informed about the study and had given written informed consent. The study was approved by the Ethics Committee from CSIC (Madrid, Spain). Each participant underwent a 2-week washout period during which they avoided rich polyphenol-food, wine and other alcoholic beverages. After the washout period, subjects (n=33) underwent an intervention period of 4 weeks, during which they daily ingested 250 mL of the red wine, divided into two doses (439.5 mg of equivalents of polyphenols per day). A control group (n=8) followed the same pattern with the difference that no red wine was consumed. 24h-urine samples were collected after the wash-out period (labeled as "pre-wine" samples) and after the intervention period (labeled as "post-wine" samples). Samples were stored at -80 ºC until analysis.

### 4. Metabolomic analysis

An MS-based metabolomic analysis was performed using the previously described method after slight modification (Jiménez-Girón *et al.*, 2015). Urine samples were thawed at room temperature. 500 µL of the urine were centrifuged at 20,000 x g for 10 min at 6 °C. Supernatants were directly analysed using an ultra-high performance liquid chromatography (UHPLC) system, 1290 Infinity from Agilent (Santa Clara, CA, USA), coupled to a quadrupole-time-of-flight mass spectrometer (Q/TOF MS), Agilent 6540, equipped with an orthogonal electrospray ionisation (ESI) source (Agilent Jet Stream, AJS), and an acquisition set in negative ion mode. Chromatographic separation was performed on an Agilent ZORBAX Eclipse Plus C18 column (2.1 x 100 mm, 1.8 µm) held at 40 ºC. The mobile phases consisted of (A) 0.01 %

formic acid in water and (B) 0.1 % formic acid in acetonitrile. A linear gradient was applied as follows: 0-30 % B in 7 min, 30-100 % B in 2 min, 100 % B in 2 min; and the column was equilibrated for 4 min. The flow rate was 0.5 mL/min, and the injection volume was 2 µL. A sample of pooled urine samples was also prepared for quality control and was injected several times into each sample batch. To avoid possible bias, the sequences of the injections (samples and controls) were randomised.

## 5. Data processing

Raw UHPLC-MS data were pre-processed by MassHunter v7.0 software from Agilent Technlogies, and a selection of peaks with an absolute height ≥ 5000 counts was applied. Archives with a proper format were validated and filtered, and peaks were aligned (Mass Profiler Professional software v14.0, Agilent technologies). After confirming the quality of the analysis, the data was filtered to obtain high quality ions, resulting in a high quality time-aligned data set of detected metabolites with their corresponding retention time, m/z, and peak area. Data was then submitted for statistical analysis.

## 6. Statistical analysis and data interpretation

A principal component analysis (PCA) provided an overview of data distribution (Mass Profiler Professional software v14.0, Agilent technologies) (Figure 1).

The metabolites that had been significantly altered after wine consumption [26] were then selected and an unsupervised Hierarchical Clustering Analysis (HCA) using SIMCA-P software v.14 was carried out. Based on the classification, a non-parametric Kruskall-Wallis analysis was performed (Mass Profiler Professional software v14.0, Agilent Technologies) and a list of the metabolites ($p<0.05$) responsible for the stratification of the volunteers into the clusters was obtained. Only those with a Coefficient of Variance (CV)<20 % were selected for further analysis. After that, statistical differences between the stratified clusters, determined using Orthogonal Partial Least-Squares (OPLS) discriminant analysis (DA) was performed and metabolites with Variable Importance in Projection values (VIP)>1 were selected. Finally, the models were validated by the goodness-of-fit parameter ($R^2X$), the proportion of the variance of the response variable that is explained by the model ($R^2Y$) and the predictive ability parametre ($Q^2$), calculated by seven-fold internal cross-validation. The validation of the OPLS-DA models was carried out with a permutation test (n=200).

A tentative identification of statistically different ($p<0.05$) metabolites was performed by carrying out a mass search in the HMDB within a mass accuracy window of 15 ppm. When several candidates were found for a given m/z, metabolite identification was sorted giving preference to metabolites normally found in urine or human

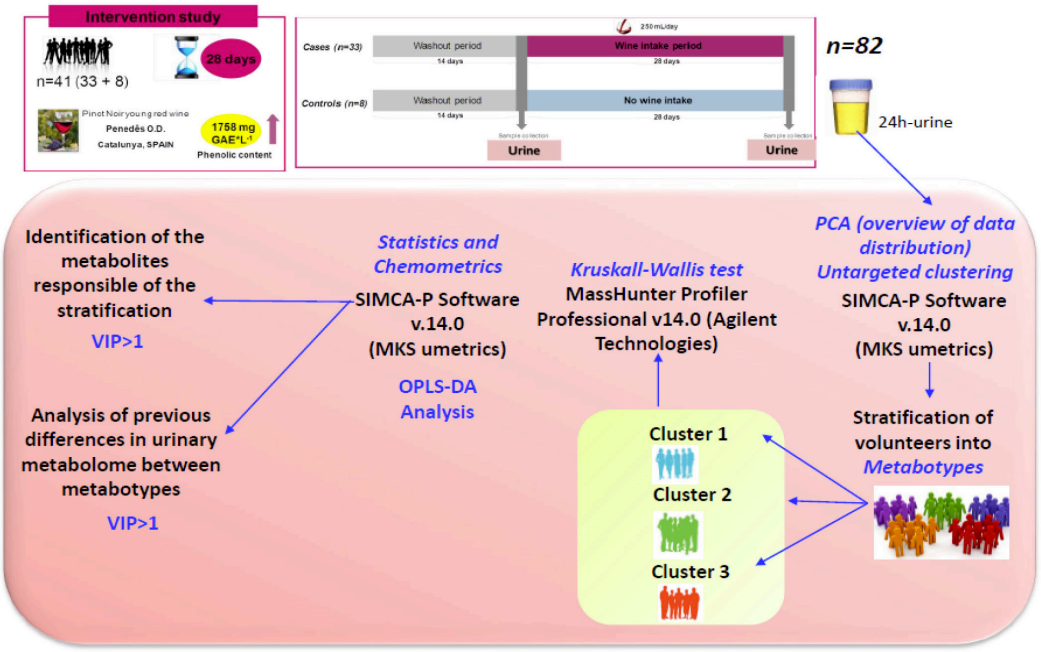


**FIGURE 1.** Workflow and statistical approach.

  

fluids after wine intake. When available, co-injection of standards with urine samples was carried out for further confirmation.

## RESULTS

### 1. Urine metabotypes associated with red wine consumption

A total of 33 healthy subjects were accepted for the trial for a 4-week intervention study during which subjects daily ingested 250 mL of red wine. A control group (n=8) followed the same pattern with the difference that no red wine was consumed. 24h-urine samples after a wash-out period (labeled as "pre-wine" samples), and after the intervention period (labeled as "post-wine" samples) were analysed by UHPLC-MS using a non-targeted metabolomic approach. After UHPLC-MS analysis and data pretreatment, 1825 entities were obtained after deconvolution, alignment and filtering. This data set was used for PCA analysis to evaluate the differences in the urinary metabolome between the experimental groups (Figure 1). PCA scores plot (Figure 2) shows a clear separation

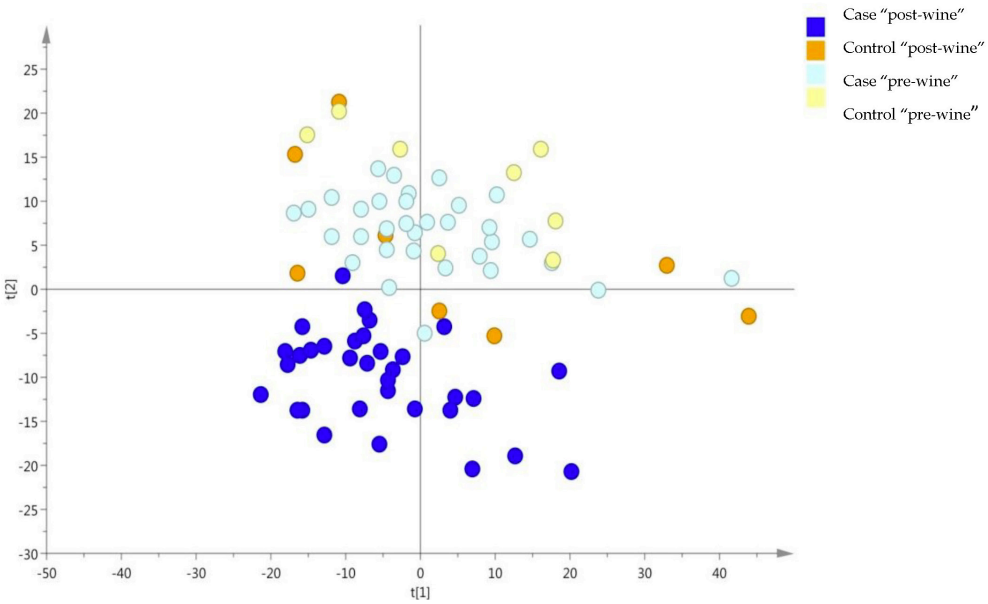


**FIGURE 2.** PCA score plot of the first two components from non-targeted metabolomics data from urine belonging to all studied groups: "post-wine" case, "post-wine" control, "pre-wine" case and "pre-wine" control. The first component explains 10.1 % of variance and the second component explains 5.7 % of variance.

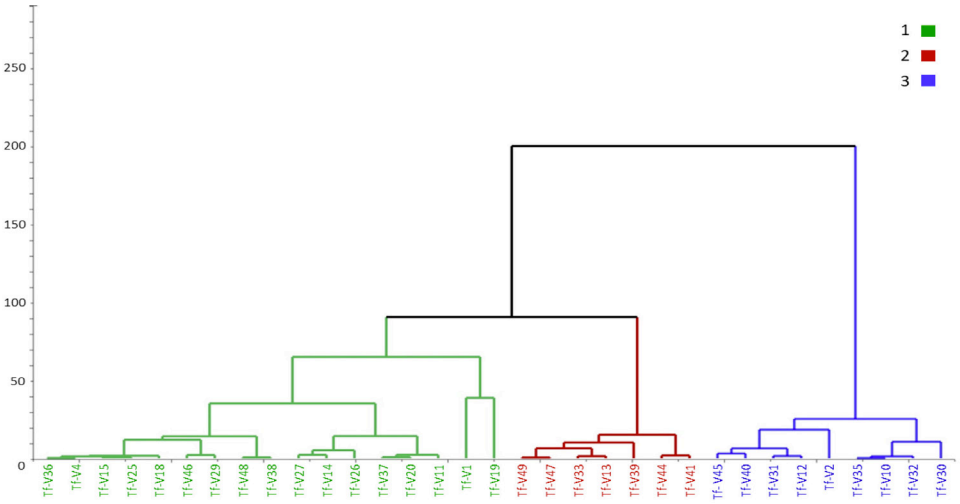


**FIGURE 3.** Dendrogram of HCA shows case "post-wine"samples clustered into three groups: cluster 1 (green), cluster 2 (red), cluster 3 (blue).

**TABLE 1.** Metabolites identified or tentatively identified with VIP>1 and associated with principal component 1 and principal component 2 of OPLS-DA analysis of post-consumption samples.

| Retention time | Detected m/z | Assigned ion | Molecular Formula | Error (ppm) | Tentative ID | Level of annotation according to MSI | Group | VIP value | | Relative response order |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | PC1 | PC2 | |
| 2.29 | 147.07 | M-H | $C_6H_{12}O_4$ | 3.52 | 2,3-Dihydroxy-3-methylvalerate | | Wine | 1.87 | 1.64 | 3>2>1 |
| 6.86 | 613.11 | M+Cl | $C_{30}H_{26}O_{12}$ | 2.24 | Procyanidin B-type dimer | | Microbial | 1.71 | 1.61 | 3>2>1 |
| 8.38 | 293.10 | 3M-H | $C_5H_6O_2$ | 0.47 | 2-Furanmethanol | | Wine | 1.64 | 1.38 | 3>2>1 |
| 1.99 | 161.05 | M-H | $C_6H_{10}O_5$ | 3.43 | 3-Hydroxymethylglutaric acid | | Wine | 1.60 | 1.32 | 3>2>1 |
| 3.47 | 175.06 | M-H | $C_7H_{12}O_5$ | 6.30 | 2,3-Dimethyl-3-hydroxyglutaric acid | | Wine | 1.57 | 1.32 | 3>2>1 |
| 3.47 | 175.06 | M-H | $C_7H_{12}O_5$ | 6.30 | Isopropylmalic acid | | Wine | 1.57 | 1.31 | 3>2>1 |
| 1.05 | 129.02 | M-H | $C_5H_6O_4$ | 8.27 | γ-delta-Dioxovaleric acid | | Wine | 1.54 | 1.27 | 3>2>1 |
| 1.03 | 147.03 | M-H | $C_5H_8O_5$ | 7.50 | Hydroxyglutaric acid | | Wine | 1.48 | 1.32 | 3>2>1 |
| 1.05 | 111.01 | M-3H | $C_{12}H_{16}O_9S$ | 0.83 | 4-Hydroxy-5-(dihydroxyphenyl)-valeric acid-O-methyl-O-sulphate | | Microbial | 1.46 | 1.20 | 3>2>1 |
| 2.09 | 144.07 | M-2H | $C_{15}H_{14}O_6$ | 5.16 | Epicatechin/Catechin | | Wine | 1.46 | 1.21 | 3>2>1 |
| 1.06 | 191.02 | M-H | $C_6H_8O_7$ | 5.09 | Citric acid | 1 | Wine | 1.44 | 1.19 | 3>2>1 |
| 0.96 | 264.99 | M+K-2H | $C_{13}H_8O_4$ | 4.40 | Urolithin A | | Microbial | 1.37 | 1.27 | 1>2>3 |
| 3.48 | 263.95 | M+Br | $C_7H_7NO_5$ | 12.91 | 2-Amino-3-carboxymuconic acid semialdehyde | | endogenous | 1.34 | 1.20 | 3>2>1 |
| 0.76 | 193.03 | M+Cl | $C_7H_{10}O_4$ | 4.72 | Isopropylmaleate | | Wine | 1.30 | 1.07 | 3>2>1 |
| 4.37 | 277.00 | M+Hac-H | $C_7H_6O_6S$ | 0.51 | Sulfosalicylic acid | | Microbial | 1.24 | 1.13 | 2>3>1 |
| 0.78 | 144.03 | M-2H | $C_{15}H_{14}O_6$ | 5.16 | Epicatechin/Catechin | 1 | Wine | 1.11 | 1.04 | 2>3>1 |
| 0.75 | 191.02 | M-H | C7H12O6 | 8.84 | Quinic acid | | Wine | 1.09 | >1 | 3>2>1 |
| 2.66 | 161.05 | M+FA-H | $C_5H_8O_3$ | 0.90 | 2-Oxovaleric acid | | Wine | 1.09 | >1 | 3>2>1 |
| 1.52 | 424.05 | M+Cl | $C_{12}H_{23}NO_9S_2$ | 4.78 | 3-Methylbutyl glucosinolate | | Diet | 1.06 | >1 | 3>2>1 |
| 0.75 | 227.00 | M-H | $C_{13}H_8S_2$ | 3.82 | 5-Ethynyl-5'-(1-propynyl)-2,2'-bithiophene | | Diet | 1.03 | >1 | 3>2>1 |
| 2.07 | 641.21 | M+TFA-H | $C_{25}H_{36}O_{12}$ | 1.97 | 16,17-Dihydro-16a,17-dihydroxygibberellin A4 17-glucoside | | Microbial | 1.02 | >1 | 3>2>1 |
| 2.77 | 269.11 | M-H | $C_{12}H_{18}N_2O_5$ | 0.39 | Hypoglycin B | | Diet | >1 | 1.52 | 2>3>1 |
| 3.67 | 204.03 | M-H20-H | $C_{10}H_9NO_5$ | 2.05 | (R)-2,3-Dihydro-3,5-dihydroxy-2-oxo-3-indoleacetic acid | | Wine | >1 | 1.39 | 2>1>3 |
| 1.32 | 442.08 | M+TFA-H | $C_{17}H_{15}NO_6$ | 13.07 | (Z)-N-Feruloyl-5-hydroxyanthranilic acid | | Microbial | >1 | 1.30 | 2>1>3 |
| 2.20 | 365.14 | M+Na-2H | $C_{20}H_{24}O_5$ | 3.12 | 5-(6-Hydroxy-3,7-dimethyl-2,7-octadienyloxy)-7-methoxycoumarin | | Wine | >1 | 1.27 | 2>3>1 |
| 9.30 | 233.15 | M+Hac-H | $C_{13}H_{18}$ | 0.01 | 1-Methyl-4-(1-methyl-2-propenyl)-benzene | | Diet | >1 | 1.14 | 1>3>2 |
| 2.29 | 245.11 | M-H | $C_{10}H_{18}N_2O_5$ | 1.65 | Aspartyl-Leucine/Leucyl-Aspartate | | endogenous | >1 | 1.13 | 2>3>1 |
| 2.68 | 194.05 | M-H | $C_9H_9NO_4$ | 1.64 | 4-Hydroxyhippuric acid | 1 | Microbial | >1 | 1.12 | 3>1>2 |
| 1.35 | 293.03 | M-H20-H | $C_{13}H_{12}O_9$ | 12.82 | Cis-Caffeoyl tartaric acid/Caftaric acid | | Wine | >1 | 1.12 | 3>2>1 |

  

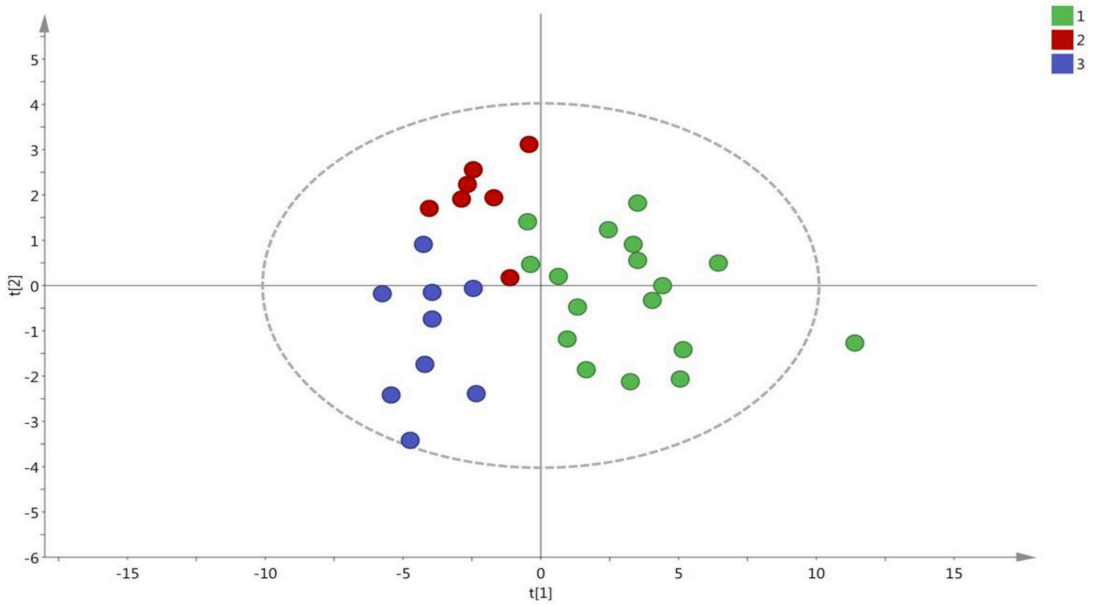


**FIGURE 4.** OPLS-DA score plot of "post-wine" urine samples. Cluster 1 is represented by green circles, cluster 2 by red circles and cluster 3 by blue circles. The ellipse shown in the model represents the Hotelling T2 with 95 % confidence.

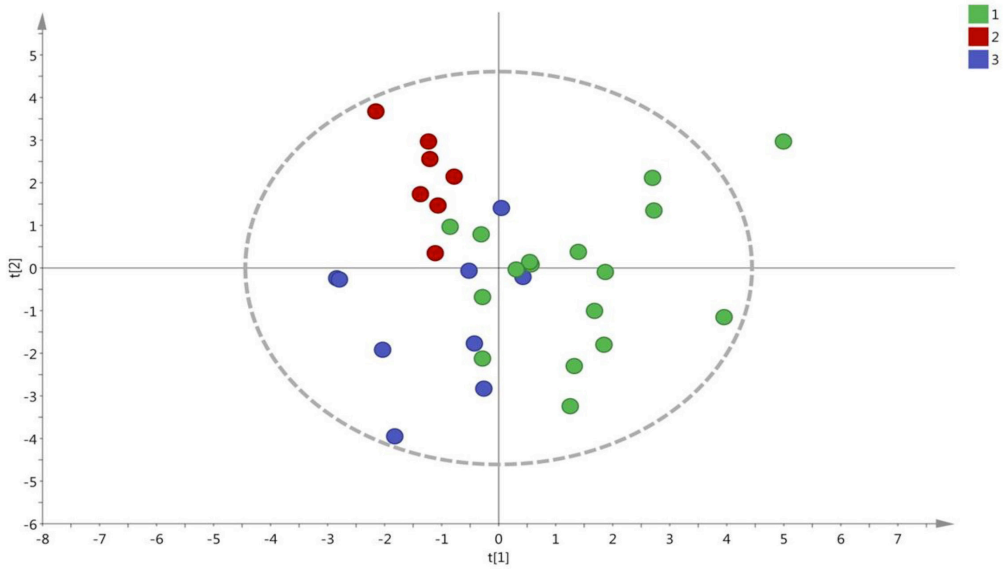


**FIGURE 5.** OPLS-DA score plot of "pre-wine" urine samples. Cluster 1 is represented by green circles, cluster 2 by red circles and cluster 3 by blue circles. The ellipse shown in the model represents the Hotelling T2 with 95 % confidence.

of subjects after intervention ("post-wine"), indicating that red wine consumption clearl induced perturbations in the urine metabolome. Moreover, as might be expected, an overlapping of samples from control subjects and case subjects before wine intervention ("pre-wine") was observed. Hierarchical clustering analysis (HCA) was then performed with those metabolites whose responses were significantly altered ($p<0.05$) after wine intervention (n=182) (Esteban-Fernández *et al.*, 2018; Jiménez-Girón *et al.*, 2015). A dendrogram showing the overall structural similarity determined by Ward's clustering based on Euclidean distance is represented in Figure 3. The clustering of individuals by HCA suggests the existence of three clusters: Cluster 1

  

(metabotype 1) was the most abundant (n=17) and showed a close link with cluster 2 (metabotype 2, n=7), while cluster 3 (metabotype 3, n=9) was clearly differentiated from clusters 1 and 2. With this new classification of volunteers, data of samples after wine intervention were subjected to a Kruskal-Wallis statistical analysis and a new set of 1460 significant signals ($p<0.05$) were generated. Only those signals with a Coefficient of Variance (CV) <20 % (n=59) were selected for further OPLS-DA analysis. Score plots from the supervised OPLS-DA (Figure 4) showed distinguishable separation between clusters 1, 2 and 3. To select the metabolites that were important for this model, VIP scores were used. VIP scores > 1 were considered as relevant and 29 metabolites were therefore considered significant in differentiating the three metabotypes (Table 1).

The model parametres were: R2X=0.39, R2Y=0.67 and Q2=0.51. The response permutation test (with n=200) was used to validate the predictive capability of the computed OPLS-DA model (Figure S1 of the Supporting Information). With the aim of detecting possible underlying determinants leading to the 3 metabotype clusters, we analysed the urinary metabolomic profile of samples collected before the dietary intervention. 328 metabolites were detected after Kruskall-Wallis analysis ($p<0.05$) and those with CV<20 % were selected for further identification. From them, 28 metabolites were tentatively identified and submitted to OPLS-DA analysis. When OPLS-DA was performed for the metabolomic data set obtained from urine samples before wine intervention, a limited separation between previously found clusters was observed (Figure 5). A value of VIP (Variable Important in Projection) $\geq 1$ was considered to identify the variables most important to group separation (Table 2). The OPLS-DA model parametres were: R2X=0.26, R2Y=0.44 and Q2=0.27. The volunteers' metabolic phenotypes before wine intervention were more similar and thus appeared closer in the OPLS-DA. PC1 (13 %) differentiated between cluster 1 and cluster 3, whereas PC2 (13 %) separated Cluster 2.

## 2. Metabotypes separation based on significant metabolites

Individuals in the metabotype 3 group had higher urinary levels of valeric acid derivatives, including 2,3-dihydroxy-3-methylvalerate, γ-delta-dioxo-valeric acid, oxovaleric acid, and 4-hydroxy-5-(dihydroxyphenyl)-valeric acid-O-methyl-O-sulfate. Other short-chain hydroxy acids, such as 3-hydroxymethylglutaric acid, 2,3-dimethyl-3-hydroxyglutaric acid, isopropylmalic acid, quinic and citric acid, as well as hydroxyhippuric and caffeoyl tartaric/caftaric acids, also differed depending on the metabotypes. Metabotypes 2 and 3, however, exhibited the highest concentrations of epicatechin/catechin, and they could be slightly differentiated from metabotype 1 due to its relative levels of microbially-derived metabolite sulfosalicylic acid. Metabotype 1 volunteers displayed the highest production of urolithin A,

**TABLE 2.** Metabolites with VIP values >1 and associated with PC1 and PC2 of OPLS-DA analysis in pre-consumption urine samples.

| Retention time | Detected m/z | Assigned ion | Molecular Formula | Error (ppm) | Tentative ID | Group | VIP value | | Relative response order |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | PC1 | PC2 | |
| 1.98 | 385.10 | M+Br | $C_{18}H_{26}O_4$ | 3.13 | Capsiate | Diet | 1.83 | 1.42 | 2>3>1 |
| 1.32 | 160.06 | M-H | $C_6H_{11}NO_4$ | 0.42 | Acetylhomoserine | Diet | 1.79 | 1.63 | 3>2>1 |
| 0.69 | 177.04 | M-H | $C_7H_8N_4O_3$ | 3.67 | Dimethyluric acid | Endogenous | 1.67 | 1.83 | 1>3>2 |
| 2.15 | 328.05 | M-H | $C_{10}H_{12}N_5O_6P$ | 2.91 | Adenosine 2',3'-cyclic phosphate | Endogenous | 1.63 | 1.36 | 3>2>1 |
| 2.20 | 365.14 | M-H | $C_{17}H_{22}N_2O_7$ | 1.30 | Tetrahydropentoxyline | Diet | 1.60 | 1.29 | 3>2>1 |
| 2.31 | 317.06 | M+TFA-H | $C_7H_{12}N_2O_5$ | 3.44 | Aspartyl-Alanine/Alanyl-Aspartate | Endogenous | 1.31 | 1.01 | 3>2>1 |
| 9.00 | 194.08 | M+Hac-H | $C_8H_9NO$ | 1.37 | 2,3-Dihydro-1H-pyrrolizine-5-carboxaldehyde | Diet | 1.21 | 1.41 | 2>3>1 |
| 2.30 | 317.06 | M+TFA-H | $C_7H_{12}N_2O_5$ | 2.81 | Alanyl-Aspartate/Aspartyl-Alanine | Endogenous | 1.15 | >1 | 3>2>1 |
| 3.61 | 439.14 | M+FA-H | $C_{18}H_{22}N_2O_8$ | 0.27 | N-Acetylserotonin glucuronide | Endogenous | 1.05 | >1 | 2>3>1 |
| 0.93 | 248.03 | M+Hac-H | $C_6H_7NO_4S$ | 10.37 | 2-aminophenol sulphate | Diet | 1.05 | >1 | 2>3>1 |
| 3.99 | 263.10 | M-H | $C_{13}H_{16}N_2O_4$ | 1.78 | di-Hydroxymelatonin | Endogenous | 1.02 | 1.76 | 2>3>1 |
| 3.99 | 835.29 | 2M-H | $C_{18}H_{26}O_{11}$ | 1,98 | Oleoside dimethyl ester | Diet | >1 | 1.13 | 2>1>3 |
| 0.71 | 147.03 | M+H | $C_6H_{12}S_2$ | 2,27 | Propenyl propyl disulfide | Diet | >1 | 1.07 | 1>2>3 |

  

a microbial metabolite produced from ellagic acid. Other compounds which are unrelated to wine and which affected volunteer stratification were also observed: 3-methylbutyl glucosinolate (from brassica vegetables), 5-ethynyl-5'-(1-propynyl)-2,2'-bithiophene (from sunflowers), and 16,17-dihydro-16a,17-dihydroxygibberellin A4 17-glucoside (found in rice) (Jiménez-Girón *et al.*, 2015; Vázquez-Fresno *et al.*, 2016; Esteban -Fernández *et al.*, 2018). Only one endogenous metabolite, 2-amino-3-carboxymuconic acid semialdehyde was found to be significant to cluster differentiation before wine intervention.

The metabolite differences found among the volunteers from all three metabotype groups before the intervention were found to be endogenous or acquired by means of diet (mainly from vegetables). Larger quantities of metabolites were identified in metabotype 2 or 3 subjects, while metabotype 1 subjects displayed higher levels of dimethyluric acid and propenyl propyl disulfide. The highest levels of acetylhomoserine, an amino acid normally derived from peas, and tetrahydropentoxyline, an amino acid associated with fruit juices, were found in metabotype 3 volunteers. Metabotype 2 subjects produced capsiate, a methoxyphenol contained in peppers, 2,3-dihydro-1H-pyrrolizine-5-carboxaldehyde, a Maillard reaction product, and 2-aminophenol sulphate, a phenylsulfate previously used as a urinary biomarker of rye bread intake (Bondia-Pons *et al.*, 2013). Metabotype 3 subjects had increased levels of endogenous adenosine 2',3'-cyclic phosphate, related to purine metabolism, and aspartyl-alanine/alanyl-asparte dipeptide.

## DISCUSSION

Polyphenol-rich dietary interventions in humans have demonstrated high interindividual variability in polyphenol degradation patterns. A number of factors contribute to interindividual differences in the human metabolome (gut microbiota, and genetic and environmental factors). Metabotyping has been proposed to overcome the confounding effects of variation, and is becoming accepted for studying the effects of a specific intervention. In the nutritional field, metabotyping is closely linked to the concept of "personalised nutrition", which has evolved from a nutrigenomic concept to an approach involving dietary, phenotypic and genotypic features (O'Donovan *et al.*, 2016). Nowadays, nutritional issues haveprogressed from individual to group guidelines, optimising dietary interventions and reducing healthcare costs and diagnosis time (O'Donovan *et al.*, 2016; Toro-Martín *et al.*, 2016). In agreement with this, the "Food4me" study has shown that personalised nutrition can cause positive changes in the nutritional behaviour of patients (Celis-Morales *et al.*, 2017). However, in order to deliver targeted dietary advice, the identification of metabolic phenotypes or "metabotypes" is of key importance.

The definition of robust metabotypes is a difficult task, since it involves taking into account a variety of parametres (Toro-Martín *et al.*, 2017; Brennan, 2017). Nevertheless, the study of responses to a dietary intervention is of great interest since the differences between metabolic activities become more obvious. In this context, metabolomics has been successfully applied in the development of novel population classification methods after the consumption of a specific food, particularly polyphenol-rich foods, such as strawberry, pomegranate or walnuts (Bolca *et al.*, 2007; González-Sarria *et al.*, 2017; Spencer *et al.*, 2008; Truchado *et al.*, 2012). Regarding wine or grape-derived products, metabolomics has been mainly applied to the discovery of biomarkers associated with their intake (Vázquez-Fresno *et al.*, 2015; Van Dorsten *et al.*, 2010). Only Vazquez *et al.* have determined changes in urinary metabolites among different clinic phenotypes in an intervention study with dealcoholized red wine (Vázquez-Fresno *et al.*, 2016). In the present study, after carrying out an unsupervised HCA, healthy volunteers were clustered into three different groups after moderate red wine consumption for a 4-week period. Since we did not use a quantitative approach, it is not possible to assume that these clusters correspond to individuals with great, moderate and low metabolic capacity. However, they are in good agreement with the results of a previous study in which we identified three distinct metabotypes (low, moderate and high metaboliser phenotypes), based on the total phenolic content of faeces from the same volunteers (Muñoz-González *et al.*, 2013). Metabotypes observed in this previous study were evidenced after a dietary intervention, which, to our knowledge, has not been previously reported in relation to moderate red wine consumption. Regarding beneficial health outcomes, it is assumed that wine should only be consumed in a moderate and regular way by healthy people, within a framework of balanced habits. Therefore, the categorisation of individuals demonstrated in this work has revealed the relevance of wine moderate intake in a real and everyday nutritional intervention/situation.

Metabotype 3 subjects produced the highest levels of the most detected metabolites, particularly valeric acid and valerolactones derivatives, which have already been proposed as urinary biomarkers associated with wine consumption (Urpi-Sarda *et al.*, 2015; Vázquez-Fresno *et al.*, 2012). Valeric acids are short-chain fatty acids (SCFA) produced by the microbial catabolism of protein-derived branched chain amino acids. They have been linked to several health benefits, including anti-inflammatory (Huda-Faujan *et al.*, 2010), anti-tumorigenic and antimicrobial effects (Tan *et al.*, 2014). This group of individuals also showed a metabotype marked with increased SCFAs production, highlighting the effects of moderate wine consumption produced on the gut microbial communities, as already observed for gut bacteria, such as *Flavonifractor plautii (*Navarro-Peran *et al.*, 2008*)*. Metabotype 3 individuals showed the highest levels of procyanidins and 4-hydroxy-5-(dihydroxyphenyl)-valeric acid-O-methyl-O-sulphate, the latter being a phenolic conjugate previously observed in human urine after tea intake. Metabotype 3 subjects had the largest increase in epicatechin/catechin oligomers (procyanidin B-type dimers). The oligomers and monomers of epicatechin and catechin and their metabolites exert several health-promoter activities including antioxidant, antibacterial, antiadhesive and cardio protective effects (Navarro-Peran *et al.*, 2008; Escandón *et al.*, 2016). The metabotype 3 group was also characterised by significantly higher 2-amino-3-carboxymuconic acid semialdehyde, an intermediate on the tryptophan-niacin catabolic pathway; it is therefore related to quinic acid, which is also produced by the different metabotypes. In agreement with our observations, an increase of urine nicotinic acid, as well as changes in amino acids related to the tryptophan metabolic pathway, have been previously observed in healthy volunteers after short-term intake of red wine and grape polyphenol extract (Jacobs *et al.*, 2012). Moreover, quinic acid is metabolised by microbiota into hippuric acid, and it has been used as a dietary supplement due to its ability to increase nicotinamide and tryptophan in urine, suggesting a positive effect of this acid in nutrient bioavailability. Hydroxyhippuric acid, its product, has been previously reported in metabolomics studies concerning moderate wine consumption (Vázquez-Fresno *et al.*, 2012; van Dorsten *et al.*, 2010).

Interestingly, metabotype 1 subjects produced the highest levels of urolithin A, a metabolite produced in the gut following consumption of ellagitannin. This type of compound is not only known for its cardioprotective (Graf *et al.*, 2015) and anti inflammatory (David *et al.*, 2014) properties, but also for its protective effects on the ageing process (Ryu *et al.*, 2016). The benefits implied from this metabolite seems to be structure-dependent and its production has been associated with the composition of colonic microbiota, concretely with the presence of *Gordinobacter spp.* (González-Sarrias *et al.*, 2017).

In site of all the above-mentioned advantages of personalised nutrition and metabotyping, there are some limiting factors. An important challenge is the translation of basic research into clinically relevant dietary guidelines, which could be achieved by establishing a common framework for scientist, clinicians and health professionals. It would be necessary to properly define the metabotypes and to select the metabolites to be analysed for population stratification in wine intervention studies. This selection should be wide enough to be representative of a physiological condition and be meaningful for the appropriate clustering of individuals/patients. However, the most notable bottleneck is the scale of these observations regarding the overall population, since there is huge diversity in the parametres and observations of each study, thus making it difficult to establish universal criteria.

## CONCLUSIONS

Urine, an easily accessible biofluid, can be used to reveal different metabotypes associated with moderate wine consumption. This study has successfully demonstrated the stratification of a population into three different clusters of individuals/metabotypes after moderate and regular wine consumption. This novel clustering has been performed on healthy volunteers, highlighting the potential applications of tailored nutrition, not only for patients at risk of disease, but also as a promoter/indicator of healthy life habits. Metabolomics constitutes a useful tool in metabotyping; however, in order to optimise dietary interventions or medical treatments, more effort should be made to homogenise the studies and facilitate access to these tools by general population.

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