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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

This study aims to evaluate the robustness of clinical and metabolic phenotyping through, for the first time, the identification of differential responsiveness to dietary strategies in the improvement of cardiometabolic risk conditions. Clinical phenotyping of 57 volunteers with cardiovascular risk factors was achieved using *k*-means cluster analysis based on 69 biochemical and anthropometric parameters. Cluster validation based on Dunn and Figure of Merit analysis for internal coherence and external homogeneity were employed. *k*-Means produced four clusters with particular clinical profiles. Differences on urine metabolomic profiles among clinical phenotypes were explored and validated by multivariate orthogonal signal correction partial least-squares discriminant analysis (OSC-PLS-DA) models. OSC-PLS-DA of ^{1}H-NMR data revealed that model comparing "obese and diabetic cluster" (OD-c) against "healthier cluster" (H-c) showed the best predictability and robustness in terms of explaining the pairwise differences between clusters. Considering these two clusters, distinct groups of metabolites were observed following an intervention with wine polyphenol intake (WPI; 733 equivalents of gallic acid/day) per 28 days. Glucose was significantly linked to OD-c metabotype ($P<.01$), and lactate, betaine and dimethylamine showed a significant trend. Tartrate ($P<.001$) was associated with wine polyphenol intervention (OD-c_WPI and H-c_WPI), whereas mannitol, threonine methanol, fucose and 3-hydroxyphenylacetate showed a significant trend. Interestingly, 4-hydroxyphenylacetate significantly increased in H-c_WPI compared to OD-c_WPI and to basal groups ($P<.05$)-gut microbial-derived metabolite after polyphenol intake-, thereby exhibiting a clear metabotypic intervention effect. Results revealed gut microbiota responsive phenotypes to wine polyphenols intervention. Overall, this study illustrates a novel metabolomic strategy for characterizing interindividual responsiveness to dietary intervention and identification of health benefits.

Keyword: Metabolic phenotype; Metabolomics; Wine polyphenols; NMR; 4-Hydroxyphenylacetate; Gut microbiota; Metabotype; Cardiovascular disease

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. There have been many reports of direct applications of metabolic phenotyping in a clinical setting [1]. Diet is

Abbreviations: 4-HPA, 4-hydroxyphenylacetate; BAS, baseline period; CHD, coronary heart disease; CVD, cardiovascular disease; FDR, false discovery rate; H-c, healthier cluster; OD-c, obese and diabetic cluster; OSC-PLS-DA, orthogonal signal correction partial least-squares discriminant analysis; T2D, type 2 diabetes mellitus; TAG, triacylglycerides; WHR, waist-to-hip index; WPI, wine polyphenol intake

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an important modulator of the human metabolic phenotype. The study of metabolic phenotyping will drive to a deeper analysis of the heterogeneous metabolic response to apparently homogeneous dietary interventions [2], improving our understanding upon distinct individual metabotypes and the linkage between diet and disease [3].

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 [4,5]. It can also be used to identify new small molecule candidates for disease biomarkers, including conditions such as cardiovascular disease [6].

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 [7]. Additionally, reduced plasma high-density lipoprotein (HDL) levels and elevated plasma triacylglyceride (TAG) concentrations are known to be significant risk factors for ischemic heart disease (IHD) [8]. C-reactive protein (CRP) is another CVD risk marker, and high plasma levels of homocysteine are considered to be a risk factor for vascular disease, heart failure and strokes [9]. Another important risk factor is type 2 diabetes mellitus (T2D). The prevalence of T2D is increasing rapidly around the world. Clinical predictors such as body mass index (BMI), fat distribution measured by waist-hip ratio (WHR), CRP and fasting blood glucose levels can be helpful in measuring diabetes risk [10–13]; however, the integration of high-throughput technologies as metabolomics can incorporate novel biomarkers with the potential to shift the research paradigm from the traditional "black-box" strategy to a systems approach [14].

The low incidence of CVD and coronary heart disease (CHD) in Mediterranean countries has been partly assigned to their distinct dietary habits [15]. As one of the main constituents of Mediterranean diet, wine and its components, especially polyphenols, may provide additional health benefits [16]. In particular, the regular consumption of wine polyphenols used in this study appears to mitigate CVD risk factors, leading to reduced blood pressure [17] and inflammatory parameters [18]. Furthermore, the health benefits of polyphenols provided by wine intake on gut microbiota are of particular interest [19]. In the present study, a long-term feeding trial was performed to determine changes in urinary metabolites between different metabotypes. Therefore, the aim of the present work was to classify a specific population into phenotypic groups according to their biochemical characteristics, and then to use ^{1}H-NMR-based urinary metabolomics to observe the different metabolic responses after red wine polyphenol intake (WPI).

2. Materials and methods

2.1. Subjects and study design

The study was a prospective, randomized, crossover and controlled trial [17]. High-risk subjects aged ≥55 years without documented CHD (CHD: IHD — angina/recent or past myocardial infarction/previous or cerebral vascular accident, peripheral vascular disease) were recruited for the study. The subjects included had diabetes mellitus or more than three of the following CVD risk factors: tobacco smoking, hypertension, hypercholesterolemia, plasma low-density lipoprotein (LDL) cholesterol ≥160 mg/dl, plasma HDL cholesterol <40 mg/dl, obesity [BMI (in kg/m^2) ≥30] and/or a family history of premature CHD (first-line male relatives <55 years or females <65 years). Participants had to voluntarily give signed informed consent. Subjects with a previous history of CVD, any severe chronic disease, alcoholism or other toxic substance abuse were excluded.

To fulfil the objectives of the present study, we used ^{1}H-NMR spectroscopy to evaluate the urinary metabolomes from 57 participants between baseline and after 28 days of red WPI (polyphenol content: 733 equivalents of gallic acid/day) in the form of dealcoholized wine from a Merlot grape variety. Results of polyphenol composition analysis of the beverages are shown in Supplementary Table 1. Twenty-four-hour urine samples were collected at baseline and the day after the last day of the intervention. Aliquots were immediately stored at −80°C until analysis. The institutional review board of the hospital approved the study protocol, and all participants gave written consent before participation in the study. The trial has been registered in the Current Controlled Trials in London, International Standard Randomized Controlled Trial Number (ISRCTN88720134).

2.2. Anthropometric measurements and biochemical analyses

Anthropometric measurements and biochemical analyses were performed using standardized methods [20]. BMI and WHR were measured in all the participants to evaluate their obesity status. Systolic and diastolic blood pressures as well as heart rate were also measured. Clinical parameters were tested in the blood and urine of participants at the beginning of the study (baseline) in order to characterize the biochemical status of each participant. Blood glucose levels, total cholesterol, HDL cholesterol, LDL cholesterol, LDL/HDL ratio, TAGs, 24-h diuresis, plasmatic creatine, uric acid, aminotransferases, bilirubin, ferritin, CRP, albumin, enzymes (alkaline phosphatase, lactate dehydrogenase) and ions (Na^+, K^+), as well as globulins, apolipoprotein levels, hemoglobin and red blood cell count, with several coagulation parameters (prothrombin, thrombin, fibrinogen) were measured. In total, 69 anthropometric and biochemical baseline parameters were evaluated. These are shown in Table 1.

2.3. Biochemical biomarkers and clinical phenotype by a k-means algorithm

The final data set contained 69 variables from 57 samples (of the initial set of 61 individuals, 4 were excluded because of incomplete data regarding clinical and anthropometric parameters). Prior to *k*-means analysis, all variables were typified. All cluster metrics were computed with 1000 different random initializations of the *k*-means algorithm in order to avoid local minima. A maximum number of 100 iterations were allowed in the *k*-means calculations. All computations were carried out using the R package for Statistical Computing v. 2.14.1. This included the statistics package for the *k*-means algorithm and the clValid package for the cluster validation analysis. Dunn analysis for internal coherence and Figure of Merit analysis for external homogeneity were applied to the data set employing Euclidean distances and a *k*-means clustering algorithm. Our results suggest that a cluster solution consisting of four centers or groups (four clusters) showed the optimal properties of internal coherence and grouping stability (the detailed methodology and the validation procedure are in the Online Supplementary Material; Supplementary Methodology).

2.4. Metabolomic NMR spectroscopy

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

The protocols used for this work were based on previously published methodology [21]. The urine samples were thawed, vortexed and centrifuged at 13,200 rpm for 5 min. The supernatant (600 μl) from each urine sample was mixed with an internal standard solution [120 μl, consisting of 0.1% 3-(trimethylsilyl)-proprionate-2,2,3,3-d4 (TSP), chemical shift reference, 2 mM of sodium azide (NaN_3, bacteriostatic agent) and 1.5 M KH_2PO_4 in 99% deuterium water (D_2O)]. The optimized pH of the buffer was set at 7.0, with a potassium deuteroxide (KOD) solution, to minimize variations in the chemical shifts of the NMR resonances. The mixture was transferred to a 5-mm NMR tube. The processed spectral data were bucketed in domains of 0.005 ppm and integrated using ACD/NMR Processor 12.0 software (Advanced Chemistry Development, Inc.). The spectral region between 4.75 and 5.00 ppm was excluded from the data set to avoid spectral interference from residual water.

2.5. Statistical analysis

2.5.1. Biochemical biomarkers and phenotyping cluster differences

Clusters were performed using *k*-means cluster analysis as described previously. A Kolmogorov–Smirnov test (P<.05) was used to test the normality of the all variables using SPSS, version 18.0 for Windows (SPSS, Chicago, IL, USA). Analysis of variance (ANOVA) was performed to evaluate differences in the mean biochemical measurements across clusters where statistical differences were analyzed (P<.05). Comparisons between clusters were assessed using a Tukey post hoc multiple comparison test. In the case of nonparametric variables, a Kruskal–Wallis test was used to test significant differences. Additionally, a Mann–Whitney test was used to detect significances between clusters. All these tests were performed by SPSS, version 18.0 for Windows (SPSS, Chicago, IL, USA).

2.5.2. Metabolomic cluster analysis — OSC-PLS-DA multivariate analysis

Data generated from the NMR spectral integration were submitted to MetaboAnalyst (www.metaboanalyst.ca). Data were normalized using the sum of the spectral intensities, then log transformed and Pareto scaled. Data were then analyzed using the SIMCA-P+ 13 software (Umetrics, Umea, Sweden) by multivariate discriminant analysis OSC-PLS-DA (orthogonal signal correction partial least-squares discriminant analysis). A pairwise comparison analysis between the four clusters was carried out. The