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Phenolic metabolites as compliance biomarker for polyphenol intake in a randomized controlled human intervention

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ABSTRACT

Clinical trials are needed to confirm the association between polyphenol consumption and lower incidence of chronic diseases observed in epidemiological studies. However, a reliable evaluation of polyphenol intake is complicated.
An 8-week randomized controlled trial (78 subjects) was performed using two isoenergetic diets differing only for polyphenol contents. Then, urinary phenolic metabolite profiles were analysed using gas-chromatography with mass detection. Phenolic metabolites detected in 24-h-urine were hydroxylated phenolic acids with C1–C3 side chain in the group consuming polyphenol-rich diet. The intake of polyphenol-rich foods increases the excretion of phenolic metabolites in urine providing a profile, which may serve as compliance biomarker of polyphenol-rich diet.

1. Introduction

Epidemiological studies suggest that polyphenol intake is associated with lower incidence of chronic diseases, especially cardiovascular diseases (CVDs) and type 2 diabetes mellitus (T2DM) (Arts & Hollman, 2005; Hollman, Geelen, & Kromhout, 2010; Jing et al., 2009; Knekt et al., 2002; van Dieren et al., 2009). Several trials have been carried out to assess whether dietary polyphenols can improve metabolic parameters (Bladé, Arola, & Salvadó, 2010; Grassi et al., 2008; Tsuda, Ueno, Yoshikawa, Kojo, & Osawa, 2006), but the evidence is still not convincing, due to the lack of correct and reliable detection of biomarkers of polyphenol intake. Thus, an accurate and objective measure of dietary intake of phenolic compounds is mandatory.

Polyphenols are plant-derived compounds and they are identified as part of dietary fibre (DF) according to the definition of the European Commission (The Commission of the European Communities, 2008). In the colon, microbial community degrades the DF matrix, releases the phytochemicals and converts them to microbial metabolites (Anson et al., 2009; Aura et al., 2013; Bazzocco, Mattila, Guyot, Renard, & Aura, 2008). Surprisingly, it has also been shown that phenolic compounds from beverages undergo the same structural transformations as those enclosed in the DF matrix before excretion to urine (Mateo Anson et al., 2011; Olthof, Hollman, Buijsman, van Amelsvoort, & Katan, 2003).

After dietary intake, polyphenols are deglycosylated and conjugated by reactions such as methylation, sulphation and glucuronidation in the mouth, stomach, upper intestinal epithelial cells and liver (Day, Bao, Morgan, & Williamson, 2000; Németh et al., 2003; Scalbert, Morand, Manach, & Rémésy, 2002; Walle, Browning, Steed, Reed, & Walle, 2005). The free part of non-conjugated phenolic compounds is absorbed early in the stomach but the rest of the phenolic compounds are absorbed from the colon after the transformations by microbiota (Azzini et al., 2010; Del Rio et al., 2010; Vitaglione et al., 2007).

In the colon structural conversions are even more pronounced by gut microbiota, because phenolic compounds are deconjugated (deglycosylated, deglucuronidated), C-ring of flavonoids is cleaved, and double-bonds are reduced. In addition oxidation, demethylation and dehydroxylation further diversify the metabolite profile from the colon. As a consequence phenolic compounds with varying initial chemical structure (flavonoids, hydroxycinnamic acids and polymeric proanthocyanins) share the same metabolites, namely hydroxylated phenylpropionic acids, phenylacetic and benzoic acids (Aura et al., 2005; Hein, Rose, van't Slot, Friedrich, & Humpf, 2008; Mateo Anson et al., 2011; Moco, Martin, & Rezzi, 2012; Rechner et al., 2004).

Phenolic metabolites have a long residence time in plasma (up to 24–48 h after a single dose of the precursor) (Kuijsten, Arts, Vree, &

Abbreviations: 3,4-diMeBA, 3,4-dimethoxybenzoic acid; 3,4-diOHBA, 3,4-dihydroxybenzoic acid; 3,4-diOHPAc, 2-(3′,4′-dihydroxyphenyl)acetic acid; 3,4-diOHPPr, 3-(3′,4′-dihydroxyphenyl)propionic acid; 3,5-diOHBA, 3,5-dihydroxybenzoic acid; 3-OHBA, 3-hydroxybenzoic acid; 3-OHPAc, 2-(3′-hydroxyphenyl)acetic acid; 3-OHPPr, 3-(3′-hydroxyphenyl)propionic acid; 3-PPr, 3-phenylpropionic acid; 4-OHBA, 4-hydroxybenzoic acid; 4-mecatechol, 4-methylcatechol; 4-OHPPr, 3-(4′-hydroxyphenyl) propionic acid.

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Hollman, 2005; Sawai, Kohsaka, Nishiyama, & Ando, 1987). Finally, phenolic acid derivatives are excreted in urine mainly as free non-conjugated acids (Gross et al., 1996; Sawai et al., 1987), whereas lactone structures, such as urolithins and enterolignans, are excreted as conjugated glucuronides and sulphates metabolized in the liver and intestinal epithelia (Adlercreutz et al., 1995; Cerdá, Periago, Espín, & Tomás-Barberán, 2005; Seeram et al., 2006). The effect of hepatic metabolism on microbial phenolic acid structure requires further investigation, because shortening of the chain length and possible further modification may take place to the colonic metabolites, because of entero-hepatic re-circulation.

Linseisen and Rohrmann (2008) suggested that dietary phenolic compounds could be used as biomarkers of the intake of plant foods. Therefore, the aim of the present study is to analyse the specific microbial metabolite profile in urine to obtain better compliance biomarkers for long-term polyphenol-rich food intake.

2. Materials and methods

2.1. Participants

Subjects who were enrolled to this study participated in a clinical trial, the primary outcome of which was lipid metabolism in subjects at high risk of cardiovascular diseases. In order to detect a 30% difference between treatments in triglyceride levels with an 80% power at 5% significance level, 80 patients had to be studied. The trial was registered to www.clinicaltrials.gov (number NCT01154478).

Briefly, 86 overweight/obese subjects with a high cardiovascular and metabolic risk profile were enrolled in the study. They had a waist circumference above 102 cm, for men, and above 88 cm for women and at least one of the characteristics of the metabolic syndrome according to the NCEP/ATP III criteria (2002): fasting triglycerides ≥150 mg/dl, high-density lipoprotein cholesterol <40 mg/dl (men)/ <50 mg/dl (women), blood pressure ≥130/85 mm Hg, and fasting glucose ≥100 mg/dl. Exclusion criteria were: age <35 and >70 years, fasting triglycerides ≥400 mg/dl, fasting cholesterol >270 mg/dl, cardiovascular events (myocardial attack or stroke) during the 6 months prior to the study, established diabetes mellitus, intensive exercise activity, renal and liver failure (creatinine >1.7 mg/dl and ALT/AST >2 times than normal values, respectively), any chronic disease, anaemia (Hb <12 g/dl), anti-inflammatory and lipid lowering drugs. They had stable food habits and were not vegetarians. The participants were asked to refrain from any dietary supplements for 1 month prior to and during the study. The trial was carried out in Naples area, Italy, from January 2010 to June 2012. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the "Federico II" University Ethics Committee. Written informed consent was obtained from all subjects.

2.2. Study design

The intervention was headed by a run-in period of 3 weeks during which participants were exhorted to follow their habitual diet. According to a randomised parallel-group design, participants were randomly assigned to one of the following diets for an 8-week period: a) low-polyphenol diet, used as control diet, or b) high-polyphenol diet. The two diets were isoenergetic (2500 Kcal) and had the same composition for nutrients, but different in content of polyphenols (Table 1). This difference between the two diets was obtained through the selection of natural foods and beverages as follows: for polyphenol-rich diet artichokes, onions, spinaches, rocket salad (*rucola*), extra-virgin olive oil, dark chocolate, oranges, blackberry jam, and green tea were served, whereas for polyphenol-poor diet cauliflowers, gourd, peppers, mixed vegetables-soup, virgin olive oil, white chocolate, kiwis, orange jam, pineapple juice and skimmed milk were consumed. Furthermore, only one cup of decaffeinated coffee per day was allowed for the low polyphenol diet, while subjects who followed the high-polyphenol diet were encouraged to drink four cups of decaffeinated coffee daily. The intake of alcoholic beverages was not allowed. All foods and beverages were provided to the subjects during the intervention.

Table 1
Composition of the experimental diets.

Nutrients	Low-polyphenol diet	High-polyphenol diet
Energy (*kcal*)	2500	2500
Proteins (%)	16	16
Total fats (%)	34	34
SAFA (%)	7	7
MUFA (%)	20	19
PUFA (%)	4	3.9
Cholesterol (*mg*)	195	187
Carbohydrates (%)	51	51
Sugars (%)	20	20
Fibre (*g*)	29	28
Total polyphenols (*mg*)	363	2868
Flavonoids:		
Flavones (*mg*)	2.3	7.6
Flavonols (*mg*)	6.4	223
Flavanols (*mg*)	0.2	1194
Flavanones (*mg*)	9.3	102
Anthocyanidines (*mg*)	34	111
Isoflavones (*mg*)	0.0	0.02
Phenolic acids (*mg*)	311	1245

MUFA: monounsaturated fatty acids; SFA: saturated fatty acids; PUFA: polyunsaturated fatty acids.

Calories, nutrients and non-nutrients of the diets were calculated from tables of food composition of the Italian Institute of Nutrition using the MetaDieta software (Meteda s.r.l., Ascoli-Piceno, Italy). Intake of polyphenols was assessed through USDA database (2007, 2008).

To improve dietary compliance, patients were seen weekly by an experienced nutritionist. Moreover, most of all foods were bought or cooked, frozen, if necessary, and given weekly to the subjects for the whole period of the trial. The random allocation to the intervention, stratified for sex, age, BMI, and plasma triglycerides was performed by the minimization method utilizing MINIM software (www.users.york.ac.uk). Adherence to the dietary treatments was evaluated by a 7-day-food record filled in by the participants at run-in-period and then every 15 days (total 4 times) until the end of the study. 24-h-urine collection was performed to assess urinary metabolite profile, during the 3-week-run-in period and after 8 weeks of dietary treatment.

2.3. Urinary metabolite profile

All urine samples were collected in 3000 ml plastic urine containers (SARSTEDT s.r.l. Verona, Italy). The volunteers received careful instructions and motivation for the collection of the urine to complete 24-h-urine collection. The volume of the 24-h-urine samples was recorded and 1.5 ml aliquots were stored at −80 °C until the analysis.

In order to normalize the excretion rate of urinary metabolites, additional 1 ml aliquots were stored at −20 °C until urinary creatinine concentration was analysed with ABX Pentra 400 (HORIBA Medical, Montpellier, France).

2.4. Analysis of the samples

All urine samples were hydrolysed using β-glucuronidase enzyme from *Helix pomatia* (10 mg; SIGMA G0751-500KU, St. Louis, USA) at 37 °C for 16 h and an internal standard (123 ppm 2-hydroxycinnamic acid, Aldrich, St Louis, USA). All samples were extracted on OASIS® HLB 1 cc Extraction Cartridge (Waters Corp., Milford, MA, USA) with methanol. 400 μl of methanol extract was evaporated to dryness under nitrogen flow.

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