Urolithins Are the Main Urinary Microbial-Derived Phenolic Metabolites Discriminating a Moderate Consumption of Nuts in Free-Living Subjects with Diagnosed Metabolic Syndrome

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ABSTRACT: Walnuts (Juglans regia L.), hazelnuts (Corylus avellana L.), and almonds (Prunus dulcis Mill.) are rich sources of ellagitannins and proanthocyanidins. Gut microbiota plays a crucial role in modulating the bioavailability of these high molecular weight polyphenols. However, to date there are no studies evaluating the capacity to produce nut phenolic metabolites in subjects with metabolic syndrome (MetS), a pathology associated with an altered gut bacterial diversity. This study applied a LC-MS targeted approach to analyze the urinary excretion of nut phenolic metabolites in MetS subjects following 12 weeks of nut consumption, compared to sex- and age-matched individuals given a nut-free control diet. Metabolites were targeted in both hydrolyzed and nonhydrolyzed urine by LC-ESI-ITD-FT-MS. Ellagitannin-derived urolithins A and B significantly increased after the nut-enriched-diet, urolithins C and D were also detected, and a complex combination of urolithin-conjugated forms was observed in nonhydrolyzed urine, confirming an extensive phase II metabolism after absorption. In contrast, no significant increases in proanthocyanidin microbial metabolites were observed in urine following nut consumption. Because the intestinal microbiota of the subjects in this study could catabolize ellagitannins into a wide range of urolithins, further research is strongly warranted on the in vivo potential of these microbial metabolites in reducing cardiometabolic risk.

KEYWORDS: metabolic syndrome, nuts, polyphenols, microbiota, urolithins, hydroxyphenylvalerolactones

INTRODUCTION

Diet seems to play a pivotal role in the prevention, reversion, and management of the metabolic syndrome (MetS), defined as a constellation of co-occurring metabolic disturbances including abdominal obesity, hyperglycemia, arterial hypertension, and dyslipidemia, which determine an increased risk for coronary artery disease, stroke, and type 2 diabetes. Tree nuts have been recently proposed among the most promising functional foods for managing both long- and short-term cardiometabolic risk factors, especially walnuts (Juglans regia L.), almonds (Prunus dulcis (Mill.) D.A. Webb), and hazelnuts (Corylus avellana L.) traditionally associated with the Mediterranean diet. Spain is particularly the southern European country with the highest percentage of habitual nut consumers and the greatest average portion sizes, and results from the Spanish PREDIMED large clinical trial confirmed that, when the Mediterranean diet is enriched with a moderate intake of mixed nuts, a more incisive reduction in the prevalence of the MetS is observed. The beneficial effects of nuts already described range from the reduction of insulin resistance and risk of type 2 diabetes to the improvement of blood lipid profile, the reduction of adiposity, low-grade systemic inflammation, and oxidative damage to the overall protection from fatal and nonfatal cardiovascular disease (CVD) outcomes. These health-promoting effects have been traditionally explained as the sum of small positive actions of nut macronutrients and micronutrients on the...
individual significant components of the MetS. However, non-nutritive phytochemicals contained in nuts, such as phytosterols/phytostanols and polyphenols, seem to also contribute to the protection against metabolic alterations.

Nut polyphenols are mainly located in the skin covering the kernels and strongly vary depending on the nut species. Tannin structures are the most abundant polyphenolic constituents, with high molecular weight nonflavonoid hydrolyzable tannins (ellagitannins, ETs) prevailing in walnuts, whereas flavonoid condensed tannins (proanthocyanidins, PAs) represent the major polyphenols described in almonds and hazelnuts.

Although both in vitro and in vivo studies have recently furnished new evidence on the anti-inflammatory, antiadipogenic, and anti-diabetic potential of nut polyphenols, the biological properties of these high molecular weight phytochemicals are greatly dependent on their bioavailability. As a common characteristic, ETs and PAs are not absorbed as such and, once in the colon, they become fermentable substrates for bacterial enzymes, leading to the extensive breakdown of their original tannin structures into a number of smaller absorbable metabolites. Within the large intestine, the ellagic acid (EA) released from ET hydrolysis undergoes microbial enzymatic transformation to produce a series of metabolites characterized by a common 6H-dibenzo[a,d]pyran-6-one nucleus and a decreasing number of phenolic hydroxyl groups (urolithin D, ETs) prevailing in walnuts, whereas urolithin A glucuronide was synthesized as previously described.

Phenylacetic acid, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxyphenylethylamine (homoprotocatechic acid), 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid), 3,4-dihydroxyphenylpropionic acid, 4-hydroxyphenylpropionic acid (phloretic acid), 3,4-dihydroxyphenylpropionic acid (homocaffeic acid), 3-(4-hydroxy-3-methoxyphenyl)propionic acid (hydroferulic acid), 2,4-dihydroxybenzyl (cinnamic) acid, 4,5-dihydroxybenzyl (protocatechuic) acid, 3,4-dihydroxybenzyl (salicylic) acid, 3- and 4-hydroxybenzoic acids, 2,5-dihydroxybenzoic (gentisic) acid, 3,4-dihydroxybenzoic acid (protocatechueinic acid), 2,6-dihydroxybenzoic (γ-resorcyclic acid), 3,5-dihydroxybenzoic (α-resorcyclic acid), 3,4,5-trihydroxybenzoic (gallic) acid, 4-O-methylgallic acid, ellagic acid, (→)-epicatechin, (→)-catechin, ethyl gallate, and β-glucuronidase/sulfatase from Helix pomatia were purchased from Sigma-Aldrich (St. Louis, MO, USA). 4-Hydroxybenzoylglycerol (4-hydroxyhippuric) acid was purchased from PhytoLab (GmbH & Co. KG). 4-Hydroxy-3-methoxybenzoic acid (vanillic acid), 3,4-dihydroxybenzoic acid (m-coumaric acid), and taxifolin were purchased from Extrasynthese (Genay, France). HPLC-grade solvents methanol, acetonitrile, glacial acetic acid, and formic acid were purchased from Panreac (Barcelona, Spain).

Subjects. Forty-one volunteers with diagnosed MetS (23 men and 18 women, ages between 31 and 63 years) participated in a prospective, randomized, controlled, parallel-designed, 12 week interventional feeding trial (see Supplemental Table 1 in the Supporting Information). The design of the trial has been reported in detail elsewhere.

Briefly, subjects were screened from Primary Care Centers affiliated with the Sant Joan University Hospital, Reus (Spain), and recruited if meeting at least three of the updated criteria for the diagnosis of MetS according to the National Cholesterol Education Program’s Adult Treatment Panel III (ATP III): (1) abdominal obesity, waist circumference ≥102 cm in men and ≥88 cm in women; (2) hypertriglyceridemia, triglycerides ≥2.3 mmol/L or drug treatment for elevated triglycerides; (3) low HDL cholesterol, HDL cholesterol <0.9 mmol/L in men and <1.1 mmol/L in women, or drug treatment; (4) hypertension, systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg and/or antihypertensive drug treatment; and (5) impaired fasting glucose, fasting blood glucose ≥5.5 mmol/L. Exclusion criteria were a history of nut allergy; established type 2 diabetes; body mass index (BMI) ≥35 kg/m²; acute or chronic infection, inflammatory disease or endocrine disorders; history of cancer; leukocytosis (>10 × 10⁹ cells/L); anti-inflammatory, corticosteroid, hormonal, or antibiotic drug treatment; a history of alcohol abuse or drug dependence; and a restrictive diet or a weight change ≥5 kg during the 3 months prior to the study. The protocol was approved by the institutional review board, and all participants provided written informed consent. This clinical trial was registered as the International Standard Randomized Controlled Trial Number (ISRCTN36468613).

Study Design. Subjects were stratified by sex and age (≤50 or >50 years) and subsequently randomly assigned to one of two 12 week parallel intervention groups, the control (CT, n = 20) and the nuts (NU, n = 22) groups. Participants of both groups were only advised to follow the same qualitative dietary recommendations, according to the American Heart Association dietary guidelines in the absence of any low-in-polyphenols washout diet, to mimic their normal free-living conditions as much as possible. The NU group also received a supplement of 30 g/day of raw unpeeled mixed nuts (15 g of walnuts, 7.5 g of almonds, and 7.5 g of hazelnuts), whereas participants of the CT group were advised against nut or peanut intake for the duration of the study. Extra packages of nuts were distributed to the NU group participants for family consumption, to increase compliance. Participants were also instructed to maintain the same level of physical activity throughout the study.

MATERIALS AND METHODS

Standards and Reagents. Urolithin A (3,8-dihydroxy-6H-dibenzo[a,d]pyran-6-one, 95% purity), urolithin B (3-hydroxy-6H-dibenzo[b,d]pyran-6-one, 98% purity), and urolithin B glucuronide were synthesized according to the method of Lucas and coworkers, whereas urolithin A glucuronide was synthesized as previously described. Phenylacetic acid, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxyphenylethylamine (homoprotocatechic acid), 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid), 3,4-dihydroxyphenylpropionic acid, 4-hydroxyphenylpropionic acid (phloretic acid), 3,4-dihydroxyphenylpropionic acid (homocaffeic acid), 3-(4-hydroxy-3-methoxyphenyl)propionic acid (hydroferulic acid), 2,4-dihydroxybenzyl (cinnamic) acid, 4,5-dihydroxybenzyl (protocatechuic) acid, 3,4-dihydroxybenzyl (salicylic) acid, 3- and 4-hydroxybenzoic acids, 2,5-dihydroxybenzoic (gentisic) acid, 3,4-dihydroxybenzoic acid (protocatechueinic acid), 2,6-dihydroxybenzoic (γ-resorcyclic acid), 3,5-dihydroxybenzoic (α-resorcyclic acid), 3,4,5-trihydroxybenzoic (gallic) acid, 4-O-methylgallic acid, ellagic acid, (→)-epicatechin, (→)-catechin, ethyl gallate, and β-glucuronidase/sulfatase from Helix pomatia were purchased from Sigma-Aldrich (St. Louis, MO, USA). 4-Hydroxybenzoylglycerol (4-hydroxyhippuric) acid was purchased from PhytoLab (GmbH & Co. KG). 4-Hydroxy-3-methoxybenzoic acid (vanillic acid), 3,4-dihydroxybenzoic acid (m-coumaric acid), and taxifolin were purchased from Extrasynthese (Genay, France). HPLC-grade solvents methanol, acetonitrile, glacial acetic acid, and formic acid were purchased from Panreac (Barcelona, Spain). Hydrochloric acid was purchased from Panreac (Barcelona, Spain).
Twenty-four hour urine samples were collected at baseline and at the end of the 12 week intervention, starting in the morning after an overnight fast. The 24 h total excreted volume of urine of each volunteer was recorded, and aliquots were stored at –80 °C until further analysis.

**Dietary Information.** Information on the food intake of the subjects and on their adherence to the interventions was collected by 3 day food records at baseline and control visits, namely, every 4 weeks during the trial. Recount of empty nut packages was an additional criterion of adherence to the dietary intervention. The dietary intake of flavonoids of the subjects was estimated by using the recently expanded USDA databases, which are the most complete, updated, and used databases in the estimation of flavonoid intake. Because exhaustive and updated data on the ET content in food are still not accessible, the total dietary intake of ellagic acid equivalents was estimated by using the information available from the web-based database Phenol-Explorer, version 1.5.3, as a measure of the intake of both ellagic acid monomers (i.e., ellagic acid glycosides) and ETs.

**Enzymatic Hydrolysis and Extraction of Phenolic Metabolites from Urine Samples.** Samples were subjected to enzymatic hydrolysis through a previously validated methodology, to release the aglycone phenolic metabolites from the conjugated forms and easily identify and quantify the unconjugated forms through the help of the available commercial standards. Briefly, the day of the analysis, urine samples (500 μL) were thawed on ice in the dark, spiked with 100 μL of 2.02 nmol/L (400 pph) ethyl gallate as internal standard, and then vortexed, acidified with 25 μL of 0.58 mol/L acetic acid to pH 4.9, and subsequently incubated with β-glucuronidase/sulfatase at 37 °C for 45 min. Immediately afterward, samples were acidified to pH 2 with 6 mol/L HCl, and the extraction of the unconjugated phenolic metabolites was carried out using solid-phase extraction (SPE) Oasis MCX 96-well plates (Waters) as previously described. The extracts were reconstituted with 100 μL of initial mobile phase, vortexed for 5 min, and then transferred into a 96-well plate suitable for LC-MS/MS analysis. For calibration purposes, an aqueous pool of commercially available phenolic standards was serially diluted in synthetic urine samples (500 μL) were thawed on ice in the dark, spiked with 100 μL of 0.58 mol/L acetic acid to pH 4.9, and subsequently incubated with β-glucuronidase/sulfatase at 37 °C for 45 min. Immediately afterward, samples were acidified to pH 2 with 6 mol/L HCl, and the extraction of the unconjugated phenolic metabolites was carried out using solid-phase extraction (SPE) Oasis MCX 96-well plates (Waters) as previously described. The extracts were reconstituted with 100 μL of initial mobile phase, vortexed for 5 min, and then transferred into a 96-well plate suitable for LC-MS/MS analysis. For calibration purposes, an aqueous pool of commercially available phenolic standards was serially diluted in synthetic urine.

**LC-PDA/ESI-QqQ-MS/MS Analysis of Phenolic Metabolites in Hydrolyzed Urine.** The analysis of phenolic metabolites in urine samples was carried out by LC-PDA/ESI-QqQ-MS/MS on an API 3000 triple-quadrupole mass spectrometer (ABSciex, Concord, ON, Canada) equipped with a Turbo Ionspray negative ionization mode, coupled to an Acquity UPLC system equipped with a Waters binary pump system (Milford, MA, USA) and a refrigerated autosampler. UV spectral data were acquired in the range of 200–400 nm. Separation was achieved on a Phenomenex Luna C18 analytical column (50 × 2.0 mm i.d., 5 μm) temperature controlled at 40 °C, at the chromatographic elution conditions previously described, with some modifications. Turbo Ionspray source settings were as follows: capillary voltage, –3700 V; nebulizer gas (N2), 10 (arbitrary units); curtain gas (N2), 12 (arbitrary units); collision gas (N2), 5 (arbitrary units); entrance potential, –10 V; drying gas (N2) heated to 400 °C and introduced at a flow rate of 6000 cm3/min. The system was controlled by software Analyst 1.4.2 supplied by ABSciex (Foster City, CA, USA).

Prior to analysis, direct infusion experiments were carried out by infusing individual solutions of the standards available (10 μg/mL) into the mass spectrometer using a model syringe pump (Harvard Apparatus, Holliston, MA, USA), at a constant flow rate of 5 μL/min, and manual tuning was performed. For each standard compound, the molecular ion [M − H]− in full scan mode and at least the two most abundant fragment ions in product ion scan mode (m/z) were detected. The optimal focusing potential (–200 → –80 V), declustering potential (–70 → –20 V), and collision energy (–30 → –10 V) for the fragmentation of the molecule ion were recorded. For quantification, data were collected in the multiple-reaction monitoring (MRM) mode, tracking the transition of the molecule to the main product ion specific for each compound (Table 2), with a dwell time of 40 ms. Metabolites lacking the commercial standards were tentatively identified by matching the mass spectral data obtained by targeted collision-induced fragmentation experiments (m/z 100−700) with those previously reported, and the MRM transitions suitable for quantification were recorded. Accuracy and precision met the acceptance criteria.

**Phase II Conjugated Metabolites of Ellagic Acid and Urolithins in Nonhydrolyzed Urine.** In nonhydrolyzed urine, phase II phenolic metabolites originated from ET microbial metabolism (methyl and dimethyl ethers, glucuronic acid, and sulfate esters or their combined forms) were then investigated in their actual conjugated form by LC-PDA-MS/MS. The chromatographic, PDA, and ESI operating conditions were the same as previously described, with some modifications. A first screening profile of the possible conjugated forms occurring in the samples was obtained by full-scan data acquisition, followed by targeted collision-induced fragmentation experiments (m/z 75–1000). Urolithins A and B and their corresponding monoglucuronides were identified through the available standards, and quantified through their corresponding calibration curves, in the MRM mode (MRM transitions 403/227 and 387/211 for urolithin A glucuronic acid and urolithin B glucuronic acid, respectively). The identification of ET and PA metabolites lacking the commercial standards was tentatively achieved by matching the experimental UV and MS/MS data with those previously reported in the literature. When literature information on the metabolite MS spectral patterns was poorly available, the compound’s identity was confirmed by multiple MS fragmentation experiments (see below). However, the lack of suitable standards did not allow the quantification of these conjugated metabolites.

**LC-ESI-QTD-FT-MS.** An LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Hemel Hempstead, U.K.) equipped with an ESI source was used for multiple MS fragmentation experiments, coupled to an Accela system (Thermo Scientific) equipped with a quaternary pump and a thermostated autosampler. The column and chromatographic conditions were the same as employed in the LC-ESI-QqQ-MS/MS targeted analysis. The ES-FT-MS data were acquired in FT MS mode (scan range from m/z 100−1000) and in MS mode (Orbitrap resolution range from 13000 to 30000 fwhm). Operation parameters were as follows: source voltage, 4 kV; source current, 100 μA; S-Lens RF levels, 94%; sheath gas, 70 (arbitrary units); auxiliary gas, 20 (arbitrary units); sweep gas, 0 (arbitrary units); and capillary temperature, 325 °C. The instrument was calibrated with the Pierce LTQ Velos ESI Positive Ion Calibration commercial solution to match the mass accuracy equipment specifications (RMS error < 2 ppm), and then the tune method was optimized by direct infusion of a standard solution of ellagic acid (5 ng/μL) at a constant flow rate of 5 μL/min (ions at m/z 300.9989, 257.00915, 229.01424, and 185.02441). Mass chromatograms and spectral data were acquired using XCalibur software 2.0 (Thermo Scientific, San Jose, CA, USA).

**Statistical Analysis.** The concentration of the phenolic compounds quantified in urine was expressed as μmol/24 h of urine collection, and the eventual variation in their excretion following the dietary intervention was reported as changes (Δ) between baseline and 12 week urinary concentrations (Δ ± SEM). Statistical analyses were carried out through SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). The nonparametric nature of the data was confirmed by the Kolmogorov test, and then two distinct statistical tests were used to jointly evaluate the effects of two factors. Within-group differences between baseline and postintervention data were analyzed by the Wilcoxon test for related samples (changes due to time factor), whereas-between-group differences (NU versus CT) were assessed by the Kruskal–Wallis test for comparison between unpaired samples (changes due to diet factor). To investigate the relationship between the excretion of the total aglycone and monoglucuronide conjugated forms of urolithins quantified in hydrolyzed and nonhydrolyzed urine, respectively, correlation analysis (Spearman correlation coefficient) were used. Differences were considered to be significant at p < 0.05.
### RESULTS AND DISCUSSION

Changes in the Dietary Intake of Flavonoids and Ellagic Acid Equivalents. Adherence to the general dietary recommendations was good, as evaluated from the 3 day food records, as was compliance with nut ingestion in the NU group (94%). At baseline, no between-group differences existed in the daily intake of flavonoid and nonflavonoid polyphenolic compounds (Table 1). In contrast, the nut-enriched diet globally resulted in a significantly increased intake of EA equivalents ($p < 0.001$) and PAs ($p = 0.01$) compared to the control diet, consistent with the high content of ETs and PAs in the mixed nuts administered (Table 1). The daily intake of PAs was particularly the highest among all flavonoid subclasses, already at baseline (98.8 and 125.0 mg/day in the NU and CT groups, respectively, Table 1), in line with their ubiquitous occurrence and intake in the human diet. The basal intake of EA equivalents was relatively low in both groups, in agreement with the limited occurrence of EA and ETs in edible plants and their average intake in distinct populations.  

Gut Microbial Phenolic Metabolites of ETs in Hydrolyzed and Nonhydrolyzed Urine. Table 2 shows all of the unconjugated phenolic metabolites targeted in hydrolyzed urine, including potential gut microbial-derived phenolic metabolites of ETs, flavan-3-ol oligomers, and PAs, as well as flavan-3-ol monomers potentially excreted after the mixed-nut consumption.

Three of the seven targeted ET-derived metabolites were detected and quantified in hydrolyzed urine after the 12 week nut consumption, namely, unconjugated EA and its main microbial-derived dihydroxy- (urolothin A) and monohydroxy-6H-dibenzo[b,d]pyran-6-one (urolothin B) metabolites. The excretion of EA aglycone did not increase significantly in the NU group at the end of the nut intervention (Table 2) suggesting that, after the intraluminal hydrolysis of ETs, the released EA is subjected to extensive microbial metabolism to 6H-dibenzo[b,d]pyran-6-one structures, as the preferential catabolic route prior to absorption. According to the current knowledge, the tri- and tetrahydroxylated 6H-dibenzo[b,d]-pyran-6-one metabolites (urolothins C and D, respectively) would be the first to be produced from the distal part of the small intestine, where anaerobic bacteria responsible for the EA metabolism seem to be already present. Their metabolism would then continue along the colon, with the sequential removal of hydroxyl groups, leading to the more lipophilic and easily absorbed urolithins A and B. In line with previous findings, the moderate daily consumption of nuts in our study induced an increased excretion of walnut ET microbial-derived urolithins. The concentration of urolithins A (MRM transition 227/198) and B (MRM transition 211/167) significantly increased, in hydrolyzed urine, compared to the CT group (Table 2), confirming them as the most representative microbial metabolites of ETs. Unconjugated urolithins C (MRM transition 243/199) and D (MRM transition 259/215) were also detected (Figure 1), and their identification was tentatively confirmed through LC-ESI-ITD-FTMS fragmentation experiments, although the lack of suitable commercial standards did not allow their quantitation. The early production of urolithins C and D as products of ET transformation was recently confirmed in vitro in the time course profile of culture fermentation by human fecal microbiota. In keeping with our results, the occurrence of urolithin C and D conjugated metabolites was previously detected in bile and plasma of Iberian pigs fed fresh acorns, and traces of free urolithin C were also reported in urine, suggesting that these less lipophilic urolithins may be also absorbed and subjected to extensive enterohepatic circulation. The tentative identification of urolithin D derivatives in human urine was not reported previously.

In the present study, the targeted analysis of nonhydrolyzed urine showed a complex combination of conjugated derivatives of EA (EA glucuronide) and urolithins (Table 3). A total of nine mono- (i.e., glucuronides), di- (i.e., sulfoglucuronides, diglucuronides), and triconjugated forms of urolithins (i.e., traces of a methyl ether sulfoglucuronide conjugate of urolithin C) predominated in urine after the nut intervention, although free forms of urolithins A, B, and C were also detected. These findings confirmed that, once absorbed, all of these low molecular weight microbial-derived metabolites are further subjected to first-pass phase II metabolism (glucuronidation, methylation, sulfation, or combination of them) within the enterocytes and/or hepatocytes, to finally enter the systemic circulation, accumulate in tissues, or be eliminated in urine. In general, conjugated forms with glucuronic acid predominated. Thanks to the standards’ availability, urolithin A and urolithin B glucuronide derivatives were identified and quantified. The

### Table 1. Estimated Intake (Milligrams per Day) of Flavonoids and Ellagic Acid Equivalents by the Daily Serving of Mixed Nuts, through the Baseline Diet and as 12 Week Changes in the Two Interventions$^a$

| compound                  | daily serving | NU group ($n = 22$) | CT group ($n = 20$) | treatment effect ($p^a$) |
|---------------------------|---------------|----------------------|----------------------|--------------------------|
|                           | baseline diet$^b$ | change after 12 weeks | baseline diet$^b$ | change after 12 weeks |
|                           | total (30 g)   |                       | total (30 g)   |                       |
| total flavonoids          | 15.32         | 21.50                | 20.41 ± 25.97      | −16.16 ± 6.64           | 0.01 |
| flavan-3-ols equiv        | 0.36          | 0.42                 | 20.64 ± 2.89       | −3.61 ± 1.21            | 0.61 |
| proanthocyanidins equiv   | 14.1          | 39.27                | 125.04 ± 19.82     | −16.16 ± 4.93           | 0.01 |
| flavones equiv            | 1.77±0.54     | 1.71 ± 0.54          | 2.56 ± 0.54       | 0.74 ± 0.15             | 0.29 |
| flavonols equiv           | 1.77±0.54     | 1.71 ± 0.54          | 2.56 ± 0.54       | 0.74 ± 0.15             | 0.29 |
| flavonones equiv          | 1.77±0.54     | 1.71 ± 0.54          | 2.56 ± 0.54       | 0.74 ± 0.15             | 0.29 |
| isoflavones equiv         | 1.77±0.54     | 1.71 ± 0.54          | 2.56 ± 0.54       | 0.74 ± 0.15             | 0.29 |
| anthocyanidins equiv      | 1.77±0.54     | 1.71 ± 0.54          | 2.56 ± 0.54       | 0.74 ± 0.15             | 0.29 |
| ellagic acid equiv        | 0             | 37.88                | 7.51 ± 3.21        | −4.35 ± 0.85            | <0.001 |

$^a$The total daily intakes through the diet are expressed as the mean ± SEM. $^b$No significant between-group differences at baseline. $^c$Significance ($p < 0.05$) of the difference in changes between groups (nuts diet versus control diet), calculated by the unpaired Mann–Whitney U test. $^d$Significantly different from baseline ($p < 0.05$) by paired samples test. $e$ $p < 0.001$.  

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Table 2. Changes in the 24 h Urinary Excretion of Flavan-3-ol Monomers and Gut Microbial-Derived Phenolic Metabolites of PAs and ETs following the 12 Week Intervention (NU versus CT) in Hydrolyzed Urine

| Phenolic metabolite | MRM transition | Δ ± SEM (µmol, 24 h) |
|---------------------|----------------|---------------------|
|                     |                | NU group | CT group       |
| Ellagic acid        | 301/257        | 3.14 ± 2.98 | 0.24 ± 3.60   |
| Ellagic acid methyl ether | 315/300       | nd | nd             |
| Ellagic acid dimethyl ether | 329/300      | nd | nd             |
| Urolithin A         | 227/198        | 49.97 ± 1.93b,c | -1.33 ± 0.97  |
| Urolithin B         | 211/167        | 23.20 ± 2.09b,c | -2.74 ± 1.42  |
| Urolithin C         | 243/199        | nq | nq             |
| Urolithin D         | 259/215        | nq | nq             |
| Hydroxyphenyl-γ-valerolactones |         |         |
| 5-(dihydroxyphenyl)-γ-valerolactone isomer 1 | 207/163 | -31.60 ± 6.17 | 55.65 ± 7.62 |
| 5-(dihydroxyphenyl)-γ-valerolactone isomer 2 | 207/163 | 132.86 ± 30.46 | 60.81 ± 23.69 |
| 5-(trihydroxyphenyl)-γ-valerolactone | 223/179 | 5.22 ± 1.59 | -0.96 ± 0.25 |
| Hydroxyphenylpropionic acids |            |         |
| 3-hydroxyphenylpropionic acid | 165/121 | 2.03 ± 0.60 | -4.40 ± 1.33 |
| 4-hydroxyphenylpropionic acid | 165/121 | 0.28 ± 1.15 | 6.58 ± 1.34 |
| 3,4-dihydroxyphenylpropionic acid | 181/137 | 0.68 ± 0.36 | 1.05 ± 0.49 |
| 3-(4-hydroxy-3-methoxyphenyl)propionic acid | 195/136 | -2.19 ± 1.80 | 2.14 ± 3.35 |
| Hydroxyphenylacetic acids |            |         |
| Phenylacetic acid   | 135/91         | 2.28 ± 0.37 | -0.25 ± 0.68 |
| 3-hydroxyphenylacetic acid | 151/107 | 2.02 ± 0.75 | 3.54 ± 1.61 |
| 4-hydroxyphenylacetic acid | 151/107 | 5.92 ± 5.98 | 6.92 ± 6.36 |
| 3,4-dihydroxyphenylacetic acid | 167/123 | 9.20 ± 3.83 | 8.36 ± 4.98 |
| 3-methoxy-4-hydroxyphenylacetic acid | 181/137 | -10.39 ± 4.54 | 38.97 ± 8.02 |
| Hydroxyaspartic acids |            |         |
| O-coumaric acid     | 163/119        | 0.48 ± 0.15 | 0.33 ± 0.05  |
| M-coumaric acid     | 163/119        | -1.93 ± 0.57 | 1.55 ± 0.28 |
| P-coumaric acid     | 163/119        | -1.73 ± 0.55 | 0.93 ± 0.27  |
| Caffeic acid        | 179/135        | 3.78 ± 2.33 | 10.82 ± 1.17 |
| Ferulic acid        | 193/134        | 29.28 ± 13.25 | 27.64 ± 3.62 |
| Sinapinic acid       | 223/164        | 0.06 ± 0.06 | 0.69 ± 0.11  |
| Hydroxycinnamic acids |            |         |
| 2-Hydroxybenzoic acid | 137/93        | -1.80 ± 0.44 | -1.45 ± 0.3  |
| 3-Hydroxybenzoic acid | 137/93        | nd | nd             |
| 4-Hydroxybenzoic acid | 137/93        | 5.13 ± 6.39 | 71.05 ± 15.1 |
| 2,5-Dihydroxybenzoic acid | 153/109       | 0.46 ± 0.34 | -0.27 ± 0.32 |
| 2,6-Dihydroxybenzoic acid | 153/109       | -4.22 ± 0.65 | -0.010 ± 0.002 |
| 3,5-Dihydroxybenzoic acid | 153/109       | -0.35 ± 0.78 | -2.56 ± 1.08 |
| Procochatechic acid  | 153/109        | -0.93 ± 1.05 | -3.09 ± 1.45 |
| Vanillic acid       | 167/152        | -233.76 ± 39.14 | 119.98 ± 50.96 |
| Gallic acid         | 169/125        | -0.85 ± 0.22 | -0.09 ± 0.15 |
| 4-O-Methylgallic acid | 167/108       | nd | nd             |
| Hydroxybutyric acids |            |         |
| 3-Hydroxybutyric acid | 194/150       | 4.69 ± 3.98 | 1.24 ± 4.77  |
| 4-Hydroxybutyric acid | 194/100       | 2.09 ± 1.23 | -19.33 ± 4.22 |
| Flavan-3-ols        |            |         |
| (+)-Catechin        | 289/245        | 0.11 ± 0.01 | 0.01 ± 0.04  |
| (+)-Epicatechin     | 289/245        | nd | nd             |
| O-Methylepicatechin | 303/137        | nd | nd             |
| (+)-Epicatechin-3-O-gallate | 441/289 | nd | nd             |

Data are the mean ± SEM. nd, not detected; nq, not quantified due to the lack of suitable standards. Significant difference from baseline (p < 0.05) by paired sample t test. Significance (p < 0.05) of the difference in changes between groups (NU group versus CT group), calculated by the unpaired Mann–Whitney U test.

Main urolithin A monoglucuronide isomer represented the most ubiquitously excreted urolithin following the 12 week intervention (51.09 ± 9.41 µmol/24 h in the NU group), accounting alone for the almost total urolithin A excretion (Spearman coefficient = 0.845, p < 0.01) and suggesting an eligible marker of ET-rich food intake not only in healthy but also in MetS-diagnosed subjects. Also, the concentration of urolithin B glucuronide (14.18 ± 6.44 µmol/24 h in the NU group, following nut consumption) positively correlated with the total excretion of urolithin B aglycone measured in
hydrolyzed urine (Spearman coefficient = 0.834, \( p < 0.01 \)), although other undetected urolithin B conjugates probably contributed to the total excretion of type B urolithins.

Finally, two urolithin A sulfoglucuronide isomers and a urolithin C methyl ether sulfoglucuronide were the only sulfate conjugates detected in this study, the latter mainly found in traces. The urolithin A sulfoglucuronide isomers were characterized by similar MS² and MS³ daughter spectra (at \( m/z \ 483 \) and 403, respectively) but distinct MS⁴ spectra at \( m/z \ 227 \) (Figure 2).

**Interindividual Variability and Urolithin-Excreting Capacity of MetS subjects.** Interindividual differences in the excretion rates of total urolithin A and B aglycones were observed in hydrolyzed urine, as a typical characteristic of microbial metabolism. Distinct combinations of phase II conjugated forms of urolithins were also observed among subjects after the nut intervention (Table 3). Nevertheless, the lack of detection of urolithin A aglycone occurred in only two subjects (\(~9\%) of the NU group following the nut intake, unlike the strong interindividual variability previously observed among healthy subjects ascribed to differences in their gut microbiota.\(^{10,37}\) These findings demonstrated that, despite the alterations in the gut microbiota recently associated with obesity and MetS, the urolithin-producing and -excreting capacity of the MetS-diagnosed subjects did not seem to be compromised. Although interpersonal differences were observed, in fact, the urinary excretion of urolithins A and B clearly discriminated the subjects consuming nuts from those abstaining and confirmed these compounds to be the most characteristic microbial-derived metabolites of walnut ETs and potentially eligible biomarkers of ET-rich food intake. Interestingly, urolithin A and B aglycones were also detected in some baseline or control urines after enzyme treatment (data not shown), reflecting the punctual consumption of other nonforbidden ET-rich foods (i.e., strawberries), as confirmed by the 3 day food records.

The urolithin-excreting capacity in the subjects in the study could be the consequence of a “urolithin prone-to-produce” gut microbiota, potentially in accordance with the association of obesity and related metabolic perturbations with a specific and altered gut microbial community.\(^{24}\) However, it may also result from a nut-induced gradual change in the colonic microflora of the subjects in the study, along the 12 week intervention, in keeping with the confirmed role of diet and, specifically, nut polyphenols in modulating the colonic microbiota through selective prebiotic effects.\(^{38−40}\) The lack of feces collection in the present study did not allow verifying the fecal bacterial composition of the subjects and the eventual nut-dependent
Table 3. Profiling of the Microbial-Derived Phenolic Metabolites of Ellagitannins Detected in the Nonhydrolyzed Urine of the NU Group after 12 Weeks of Nut Consumption

| Assignment                        | $t_a$ (min) | MS [M-H] | MS/MS<sup>b</sup> | 1 | 2 | 3 | 4 | 5 | 7 | 8 | 9 | 10 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
|-----------------------------------|-------------|----------|-------------------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|
| urolithin A diglucuronide         | 13.8        | 579      | 533, 403, 227, 175|   |   |   |   |   |   |   | X  | X  |    |    |    |    |    |    |    |    |    |    |    |    |
| urolithin D glucuronide           | 14.5        | 435      | 417, 329, 259, 241, 175|   |   |   |   |   |   |   |   |   | X  | X  |    |    |    |    |    |    |    |    |    |    |    |
| urolithin A sulfoglucuronide      | 17.2        | 483      | 403, 307, 227, 175|   |   |   |   |   |   |   | X  | X  |    | X  |    |    |    |    |    |    |    |    |    |    |    |
| urolithin A sulfoglucuronide isomer | 18.4     | 483      | 403, 307, 227, 175|   |   |   |   |   |   |   |   |   |   |   | X  | X  |    |    |    |    |    |    |    |    |    |
| urolithin A glucuronide           | 21.2        | 403      | 227, 175, 113 | X  | X  | X  | X  | X  | X  | X  |   |   | X  | X  |    |    |    |    |    |    |    |    |    |    |    |
| isourolithin A glucuronide        | 21.8        | 403      | 227, 175, 113 | X  |   |   |   |   |   |   | X  |   |   | X  |   |    |    |    |    |    |    |    |    |    |    |
| urolithin D methyl ether glucuronide | 22.9    | 449      | 273, 175 |   | X  | X  | X  | X  |   |   | X  |   |   | X  |   |    |    |    |    |    |    |    |    |    |    |
| urolithin C                        | 25.3        | 243      | 239, 227, 209, 165, 157| X  | X  | X  | X  | X  | X  | X  |   |   | X  | X  |    |    |    |    |    |    |    |    |    |    |
| urolithin C methyl ether sulfoglucuronide | 27.7    | 513      | 467, 337, 241, 175, 157 |   |   |   |   | X  |   |   |   |   |   |   |   | X  |   |    |    |    |    |    |    |    |    |
| urolithin B glucuronide           | 28          | 387      | 211, 175, 113 | X  |   |   |   |   |   |   | X  |   |   |   |   |   | X  |   |    |    |    |    |    |    |    |
| urolithin C isomer<sup>c</sup>    | 28.4        | 243      | 229, 199, 163, 148| X  |   |   |   |   | X  |   |   |   |   |   |   |   |   |   | X  |   |    |    |    |    |    |
| ellagic acid glucuronide          | 29.7        | 477      | 459, 433, 301, 227, 175, 157| X  | X  | X  | X  | X  | X  | X  | X  | X  | X  |   |   |   |   |   |   | X  |   |    |    |    |    |
| urolithin A                        | 31.1        | 227      | 198, 168, 179, 163|   |   |   |   |   | X  |   |   |   |   |   |   |   |   |   |   |   |   | X  |   |    |    |
| urolithin B<sup>d</sup>           | 34.2        | 211      | 167, 139, 117 |   |   |   |   | X  |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | X  |    |

<sup>a</sup>The compound assignation was confirmed by the MS/MS and UV spectral data, which have been reported in previous papers (Cerdà et al., 2005; Espín et al., 2007; González-Sarrías et al., 2010; González-Barrio et al., 2011) or by comparison with the available standard (in the case of urolithins A and B as well as their corresponding glucuronides). Information from two volunteers (NU 6 and NU 11) is missing due to lack of urine samples available for this analysis. <sup>b</sup>MS/MS spectra resulting from product ion scan (PIS) experiments. <sup>c</sup>Traces. <sup>d</sup>Highest excretor for each metabolite.
changes. For these reasons, further studies to identify the gut bacterial strains responsible for the ET catalysis, to investigate their relative occurrence in subjects with high versus low cardiometabolic risk, and to compare the effects of polyphenolic constituents in subjects with or without metabolic disturbances, in the presence or absence of a compromised microbial community, are strongly warranted. As well, the prebiotic role of nut polyphenols in reverting Bacteroides/Firmicutes balance and metabolic alterations should be further investigated.14,36 These issues are relevant to establish the bioefficacy of ETs in reducing cardiometabolic risks if urolithins, besides being much more bioavailable than the precursor compounds, could also be shown to have higher in vivo bioactivities.12,37,41

Gut Microbial-Derived Phenolic Metabolites of PAs in Hydrolyzed Urine. In hydrolyzed urine, 34 compounds represented the targeted phenolic metabolites potentially resulting from the complex microbial catabolism of flavan-3-ol structures (dimers, oligomers, and PAs). Among them, 30 metabolites belonged to the classes of hydroxyphenylvalerolactones and hydroxyphenylpropionic, hydroxyphenylacetic, hydroxycinnamic, hydroxybenzoic, and hydroxyhippuric acids (Table 2) and are produced by sequential biotransformations starting with the flavonoid C-ring-opening and followed by lactonization, decarboxylation, dehydroxylation, and α- or β-oxidation reactions.21,42 Four intact flavan-3-ol monomers were also investigated in urine (Table 2) because their excretion may result from the direct absorption of native monomeric flavan-3-ols contained in almond and hazelnut skins,11,20 or from the depolymerization of oligomeric and polymeric forms by the interflavan cleavage, previously described as an alternative microbial first step in the PA catabolic pathway.22

With the exception of 3-hydroxybenzoic acid and 4-O-methylgallic acid, which were not detected, all of the targeted hydroxyphenylvalerolactones and phenolic acids were already excreted at baseline, in a wide range of concentrations. Among the flavan-3-ol monomers, only (+)-catechin was detected and quantified in hydrolyzed urine. The excretion of hydroxyphenylvalerolactones was of particular interest because, originating from the initial and more specific reactions of flavonoid C-ring-opening followed by lactonization, they are currently considered to be the most eligible candidate biomarkers of flavan-3-ol intake;19,20 5-(dihydroxyphenyl)-γ-valerolactones and 5-(trihydroxyphenyl)-γ-valerolactones were specifically targeted, because of the previous association with the microbial catabolism of (epi)-catechin and (epi)gallocatechin moieties, respectively.19,43,44 Two mass signals were detected at the MRM transition 207/163 (Figure 3A), indicating the possible occurrence of two isomers of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone. The MS/MS spectra of both metabolites were in line with that previously proposed for 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone through similar LC-ESI-QqQ-MS/MS analysis,53 and their LC-ESI-ITD-FTMS fragmentation patterns (fragment ions at m/z 163.07575 and 122.03675) were tentatively confirmed to be in the presence of two dihydroxyphenyl-γ-valerolactone isomers, with the hydroxyl groups on distinct positions of the aromatic ring. As well, a signal detected at the MRM transition 223/179 was assigned to 5-(triisoxyphenyl)-γ-valerolactone (Figure 3B), and structural information obtained by MS experiments

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**Figure 2.** LC-ESI-ITD-FTMS spectra of the two urolithin A sulfoglucuronide isomers detected in nonhydrolyzed urine following the 12 weeks of nut consumption: (A) MS2 daughter spectrum at m/z 483 shared by the two isomers; (B) MS3 daughter spectrum from the common fragment ion at m/z 403; (C, D) distinct MS4 daughter spectra of the two urolithin A sulfoglucuronide isomers from the fragment ion at m/z 227.
tentatively confirmed the compound identification (Figure 3C,D). However, although an apparent increased excretion of dihydroxyphenyl-γ-valerolactone isomer 2 and trihydroxyphenyl-γ-valerolactone was observed in the NU group following nut consumption (Table 2), all changes lacked statistical significance, independent from the intervention group. In general, a higher interindividual variation was observed in the excretion of all the flavan-3-ol and PA potential phenolic metabolites, with respect to urolithins.

Several factors probably played a role in compromising the observation of significant changes in the urinary excretion of phenolic derivatives of nut PAs. First, to mimic the normal free-living conditions of the subjects in the study and not perturb their gut microbial community, the study design lacked a washout period based on a low-in-polyphenols diet, generally used to reduce and standardize the baseline excretion of phenolic metabolites. Consequently, the intake of PAs was already high at baseline and, therefore, able to mask the increased intake obtained through the moderate daily serving of nuts (30 g). As well, besides the prohibition against eating nuts or peanuts for the participants of the CT group, no other flavonoid- or ET-rich foods were forbidden in both intervention groups during the study, thus furnishing an uncontrolled consumption of rich sources of all flavonoids subclasses and particularly of PAs, which are ubiquitous in the human diet.

**ASSOCIATED CONTENT**

Supporting Information
Supplemental Table 1. This material is available free of charge via the Internet at http://pubs.acs.org.

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