

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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Supporting Information

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-PDA-QqQ-MS/MS analysis, and identification of metabolites lacking available standards was confirmed 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.¹ 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.² Spain is particularly the southern European country with the highest percentage of habitual nut consumers and the greatest average portion sizes,³ 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.⁴ The beneficial effects of nuts already described range from the reduction of insulin resistance and risk of type 2 diabetes⁵ to the improvement of blood lipid profile,⁶ the reduction of adiposity,⁷ low-grade systemic inflammation, and oxidative damage⁸ to the overall protection from fatal and nonfatal cardiovascular disease (CVD) outcomes.^{5,9} These health-promoting effects have been traditionally explained as the sum of small positive actions of nut macronutrients and micronutrients on the

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individual diagnostic components of the MetS.² However, nonnutritive 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,¹⁰ whereas flavonoid condensed tannins (proanthocyanidins, PAs) represent the major polyphenols described in almonds and hazelnuts.¹¹ Although both in vitro and in vivo studies have recently furnished new evidence on the anti-inflammatory, antiadipogenic, and antidiabetic potential of nut polyphenols,^{12–14} 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.¹⁵ Within the large intestine, the ellagic acid (EA) released from ET hydrolysis undergoes microbial enzymatic transformation^{10,13,16,17} to produce a series of metabolites called urolithins, characterized by a common 6*H*-dibenzo[*b,d*]-pyran-6-one nucleus and a decreasing number of phenolic hydroxyl groups (urolithin D → C → A → B).¹⁸ Also, aromatic and phenolic structures including phenylvalerolactones and phenylvaleric, phenylpropionic, phenylacetic, hippuric, and benzoic acids, with different hydroxylation patterns, have been already proposed as main products of the microbial biotransformation of flavan-3-ol dimers, oligomers (DP > 3), and PAs (flavan-3-ol polymers).^{19–22}

The colonic bacteria responsible for ET and PA gut transformation are only partly known,²³ but do not seem to be ubiquitous in the gut. In contrast, it is currently accepted that obesity and MetS are associated with phylum-level changes in the gut microbiota, reduced bacterial diversity, and altered representation of bacterial genes and metabolic pathways, including those involved in nutrient harvest.²⁴ Despite the potential contribution of bioactive metabolites bioavailable from nut polyphenols in reducing cardiometabolic risk, to the best of our knowledge no previous studies investigated the gut microbial-derived metabolism of nut polyphenols in subjects with diagnosed MetS or other metabolic disturbances associated with an altered gut bacterial diversity.

A randomized parallel-group feeding trial was recently carried out to evaluate the clinical effects on the MetS phenotype of a 12 week mixed-nut-enriched diet (30 g/day), compared to a control diet.^{25–27} As previously published, a decreased insulin resistance, a remarkably significant improvement in 8-oxo-7,8-dihydro-20-deoxyguanosine urinary excretion, and a borderline improvement in inflammatory markers was associated with the MetS patients who consumed the mixed-nut supplement for 12 weeks, compared to those who abstained from nuts.^{25,26} In the present study, we applied a liquid chromatography–mass spectrometry (LC-MS) targeted approach to determine the eventual concomitant changes in the urinary excretion of microbial-derived phenolic metabolites of nut flavan-3-ols, PAs, and ETs, following the nut intervention.

MATERIALS AND METHODS

Standards and Reagents. Urolithin A (3,8-dihydroxy-6*H*-dibenzo[*b,d*]pyran-6-one, 95% purity), urolithin B (3-hydroxy-6*H*-

dibenzo[*b,d*]pyran-6-one, 98% purity), and urolithin B glucuronide were synthesized according to the method of Lucas and co-workers,²⁸ whereas urolithin A glucuronide was synthesized as previously described.²⁹ Phenylacetic acid, 3-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic (homoprotocatechuic) acid, 3-methoxy-4-hydroxyphenylacetic (homovanillic) acid, 3-hydroxyphenylpropionic acid, 4-hydroxyphenylpropionic (phloretic) acid, 3,4-dihydroxyphenylpropionic (hydrocaffeic) acid, 3-(4-hydroxy-3-methoxyphenyl)-propionic (hydroferulic) acid, 2-hydroxycinnamic (*o*-coumaric) acid, 4-hydroxycinnamic (*p*-coumaric) acid, 3,4-dihydroxycinnamic (caffeic) acid, 3-(4-hydroxy-3-methoxyphenyl)prop-2-enoic (ferulic) acid, 3-(4-hydroxy-3,5-dimethoxyphenyl)prop-2-enoic (sinapinic) acid, 2-hydroxybenzoic (salicylic) acid, 3- and 4-hydroxybenzoic acids, 2,5-dihydroxybenzoic (gentisic) acid, 3,4-dihydroxybenzoic (protocatechuic) acid, 2,6-dihydroxybenzoic (γ -resorcylic) acid, 3,5-dihydroxybenzoic (α -resorcylic) 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-Hydroxybenzoylglycine (4-hydroxyhippuric) acid was purchased from PhytoLab (GmbH & Co. KG). 4-Hydroxy-3-methoxybenzoic (vanillic) acid, 3-hydroxycinnamic (*m*-coumaric) acid, and taxifolin were purchased from Extrasynthèse (Genay, France). HPLC-grade solvents methanol, acetonitrile, glacial acetic acid, and formic acid were purchased from Chemie S.A. (Barcelona, Spain). Hydrochloric acid was 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.^{25,26} 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 (1) abdominal obesity, waist circumference ≥ 102 cm in men and ≥ 88 cm in women; (2) hypertriglyceridemia, triglycerides ≥ 1.7 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 ≥ 130 mmHg and/or diastolic blood pressure ≥ 85 mmHg 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 ($> 10 \times 10^9$ 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 guidelines³⁰ 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.

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,³¹ 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 1.5.2,³² 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,³³ 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 ppb) 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.³³ 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 prepared as previously described³⁴ and subjected to the extraction procedure, as for the samples. Sample and standard preparation was carried out in a dark room with a red safety light to avoid oxidation of the analytes.

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-MS/MS on an API 3000 triple-quadrupole mass spectrometer (ABSciex, Concord, ON, Canada) equipped with a Turbo Ionspray source operating in negative 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 \times 2.0 mm i.d., 5 μm) temperature controlled at 40°C , at the chromatographic elution conditions previously described, with some modifications.³³ Turbo Ionspray source settings were as follows: capillary voltage, -3700 V ; nebulizer gas (N_2), 10 (arbitrary units); curtain gas (N_2), 12 (arbitrary units); collision gas (N_2), 5 (arbitrary units); entrance potential, -10 V ; drying gas (N_2), heated to 400°C and introduced at a flow rate of $6000\text{ cm}^3/\text{min}$. 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 $\mu\text{g}/\text{mL}$) into the mass spectrometer using a model syringe pump (Harvard Apparatus, Holliston, MA, USA), at a constant flow rate of 5 $\mu\text{L}/\text{min}$, and manual tuning was performed. For each standard compound, the molecular ion $[\text{M} - \text{H}]^-$ 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 ($-200 \rightarrow -80\text{ V}$), declustering potential ($-70 \rightarrow -20\text{ V}$), and collision energy ($-30 \rightarrow -10\text{ V}$) 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 molecular 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,^{18,33} and the MRM transitions suitable for quantification were recorded. Accuracy and precision met the acceptance criteria.³³

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.¹⁸ 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 glucuronide and urolithin B glucuronide, 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-ITD-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 ESI-FT-MS data were acquired in FT MS mode (scan range from m/z 100–1000) and in MSⁿ mode (Orbitrap resolution range from 15000 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 reach the mass accuracy equipment specifications (RMS error $< 2\text{ ppm}$), 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 $\mu\text{L}/\text{min}$ (ions at m/z 300.99898, 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 $\mu\text{mol}/24\text{ h}$ 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 ($\Delta \pm \text{SEM}$). 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 $p < 0.05$.

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 (<i>n</i> = 22)		CT group (<i>n</i> = 20)		treatment effect (<i>p</i>) ^c
	almonds (7.5 g)	hazelnuts (7.5 g)	walnuts (15 g)	total (30 g)	baseline diet ^b	change after 12 weeks	baseline diet ^b	change after 12 weeks	
total flavonoids	15.32	40.24	9.46	65.02	188.21 ± 21.50	88.48 ± 6.01 ^d	201.41 ± 25.97	−16.16 ± 6.64	0.01
flavan-3-ols equiv	0.36	0.42	0	0.78	21.38 ± 3.54	−1.92 ± 0.87	20.64 ± 2.89	−3.61 ± 1.21	0.61
proanthocyanidins equiv	14.1	39.27	9.05	62.41	98.84 ± 15.03	77.80 ± 4.12 ^{d,e}	125.04 ± 19.82	−16.16 ± 4.93	0.01
flavones equiv	0	0	0	0	3.20 ± 0.54	−0.37 ± 0.09	3.12 ± 0.75	−0.65 ± 0.18	0.98
flavanols equiv	0.63	0	0	0.63	23.37 ± 2.78	2.01 ± 0.57	21.75 ± 2.59	−2.56 ± 0.70	0.37
flavanones equiv	0.03	0.01	0	0.04	26.04 ± 6.82	6.66 ± 2.06	21.47 ± 6.14	3.10 ± 1.2	0.33
isoflavones equiv	0	0	0.01	0.01	1.03 ± 0.54	0.25 ± 0.14	0.42 ± 0.06	0.74 ± 0.15	0.29
anthocyanidins equiv	0.2	0.54	0.41	1.14	14.35 ± 2.19	4.54 ± 0.73	17.97 ± 3.87	9.19 ± 1.48	0.98
ellagic acid equiv	0	0	37.88	37.88	3.59 ± 1.54	32.70 ± 0.77 ^{d,e}	7.51 ± 3.21	−4.35 ± 0.85	<0.001

^aThe total daily intakes through the diet are expressed as the mean ± SEM. ^bNo significant between-group differences at baseline. ^cSignificance (*p* < 0.05) of the difference in changes between groups (nuts diet versus control diet), calculated by the unpaired Mann–Whitney *U* test. ^dSignificantly different from baseline (*p* < 0.05) by paired samples test. ^e*p* < 0.001.

■ 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.³⁵ 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.¹⁵

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- (uroolithin A) and monohydroxy-6*H*-dibenzo[*b,d*]pyran-6-one (uroolithin 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 6*H*-dibenzo[*b,d*]pyran-6-one structures, as the preferential catabolic route prior to absorption. According to the current knowledge, the tri- and tetrahydroxylated 6*H*-dibenzo[*b,d*]pyran-6-one metabolites (uroolithins 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.^{18,36} 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.^{10,16,37} 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 quantification. 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.³⁶ 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.¹⁸ 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.¹⁸ 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 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^a

phenolic metabolite	MRM transition	$\Delta \pm \text{SEM}$ (μmol , 24 h)	
		NU group	CT group
ETs derivatives			
ellagic acid	301/257	3.14 \pm 2.98	0.24 \pm 3.60
ellagic acid methyl ether	315/300	nd	nd
ellagic acid dimethyl ether	329/300	nd	nd
uroolithin A	227/198	49.97 \pm 1.93 ^{b,c}	−1.33 \pm 0.97
uroolithin B	211/167	23.20 \pm 2.09 ^{b,c}	−2.74 \pm 1.42
uroolithin C	243/199	nq	nq
uroolithin D	259/215	nq	nq
hydroxyphenyl- γ -valerolactones			
5-(dihydroxyphenyl)- γ -valerolactone <i>isomer 1</i>	207/163	−31.60 \pm 6.17	55.65 \pm 7.62
5-(dihydroxyphenyl)- γ -valerolactone <i>isomer 2</i>	207/163	132.86 \pm 30.46	60.81 \pm 23.69
5-(trihydroxyphenyl)- γ -valerolactone	223/179	5.22 \pm 1.59	−0.96 \pm 0.25
hydroxyphenylpropionic acids			
3-hydroxyphenylpropionic acid	165/121	2.03 \pm 0.60	−4.40 \pm 1.33
4-hydroxyphenylpropionic acid	165/121	0.28 \pm 1.15	6.58 \pm 1.34
3,4-dihydroxyphenylpropionic acid	181/137	0.68 \pm 0.36	1.05 \pm 0.49
3-(4-hydroxy-3-methoxyphenyl)propionic acid	195/136	−2.19 \pm 1.80	2.14 \pm 3.35
hydroxyphenylacetic acids			
phenylacetic acid	135/91	2.28 \pm 0.37	−0.25 \pm 0.68
3-hydroxyphenylacetic acid	151/107	2.02 \pm 0.75	3.54 \pm 1.61
4-hydroxyphenylacetic acid	151/107	5.92 \pm 5.98	6.92 \pm 6.36
3,4-dihydroxyphenylacetic acid	167/123	9.20 \pm 3.83	8.36 \pm 4.98
3-methoxy-4-hydroxyphenylacetic acid	181/137	−10.39 \pm 4.54	38.97 \pm 8.02
hydroxycinnamic acids			
<i>o</i> -coumaric acid	163/119	0.48 \pm 0.15	0.33 \pm 0.05
<i>m</i> -coumaric acid	163/119	−1.93 \pm 0.57	1.55 \pm 0.28
<i>p</i> -coumaric acid	163/119	−1.73 \pm 0.55	0.93 \pm 0.27
caffeic acid	179/135	3.78 \pm 2.33	10.82 \pm 1.17
ferulic acid	193/134	29.28 \pm 13.25	27.64 \pm 3.62
sinapinic acid	223/164	0.06 \pm 0.06	0.69 \pm 0.11
hydroxybenzoic acids			
2-hydroxybenzoic acid	137/93	−1.80 \pm 0.44	−1.45 \pm 0.3
3-hydroxybenzoic acid	137/93	nd	nd
4-hydroxybenzoic acid	137/93	5.13 \pm 6.39	71.05 \pm 15.1
2,5-dihydroxybenzoic acid	153/109	0.46 \pm 0.34	−0.27 \pm 0.32
2,6-dihydroxybenzoic acid	153/109	−4.22 \pm 0.65	−0.010 \pm 0.002
3,5-dihydroxybenzoic acid	153/109	−0.35 \pm 0.78	−2.56 \pm 1.08
protocatechuic acid	153/109	−0.93 \pm 1.05	−3.09 \pm 1.45
vanillic acid	167/152	−233.76 \pm 39.14	119.98 \pm 50.96
gallic acid	169/125	−0.85 \pm 0.22	−0.09 \pm 0.15
4- <i>O</i> -methylgallic acid	167/108	nd	nd
hydroxyhippuric acids			
3-hydroxyhippuric acid	194/150	4.69 \pm 3.98	1.24 \pm 4.77
4-hydroxyhippuric acid	194/100	2.09 \pm 1.25	−19.33 \pm 4.22
flavan-3-ols			
(+)-catechin	289/245	0.11 \pm 0.01	0.01 \pm 0.04
(−)-epicatechin	289/245	nd	nd
<i>O</i> -methylepicatechin	303/137	nd	nd
(−)-epicatechin-3- <i>O</i> -gallate	441/289	nd	nd

^aData are the mean \pm SEM. nd, not detected; nq, not quantified due to the lack of suitable standards. ^bSignificant difference from baseline ($p < 0.05$) by paired samples test. ^cSignificance ($p < 0.05$) 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 ($51.09 \pm 9.41 \mu\text{mol}/24 \text{ h}$ 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 healthy¹⁰ but also in MetS-diagnosed subjects. Also, the concentration of urolithin B glucuronide ($14.18 \pm 6.44 \mu\text{mol}/24 \text{ h}$ in the NU group, following nut consumption) positively correlated with the total excretion of urolithin B aglycone measured in

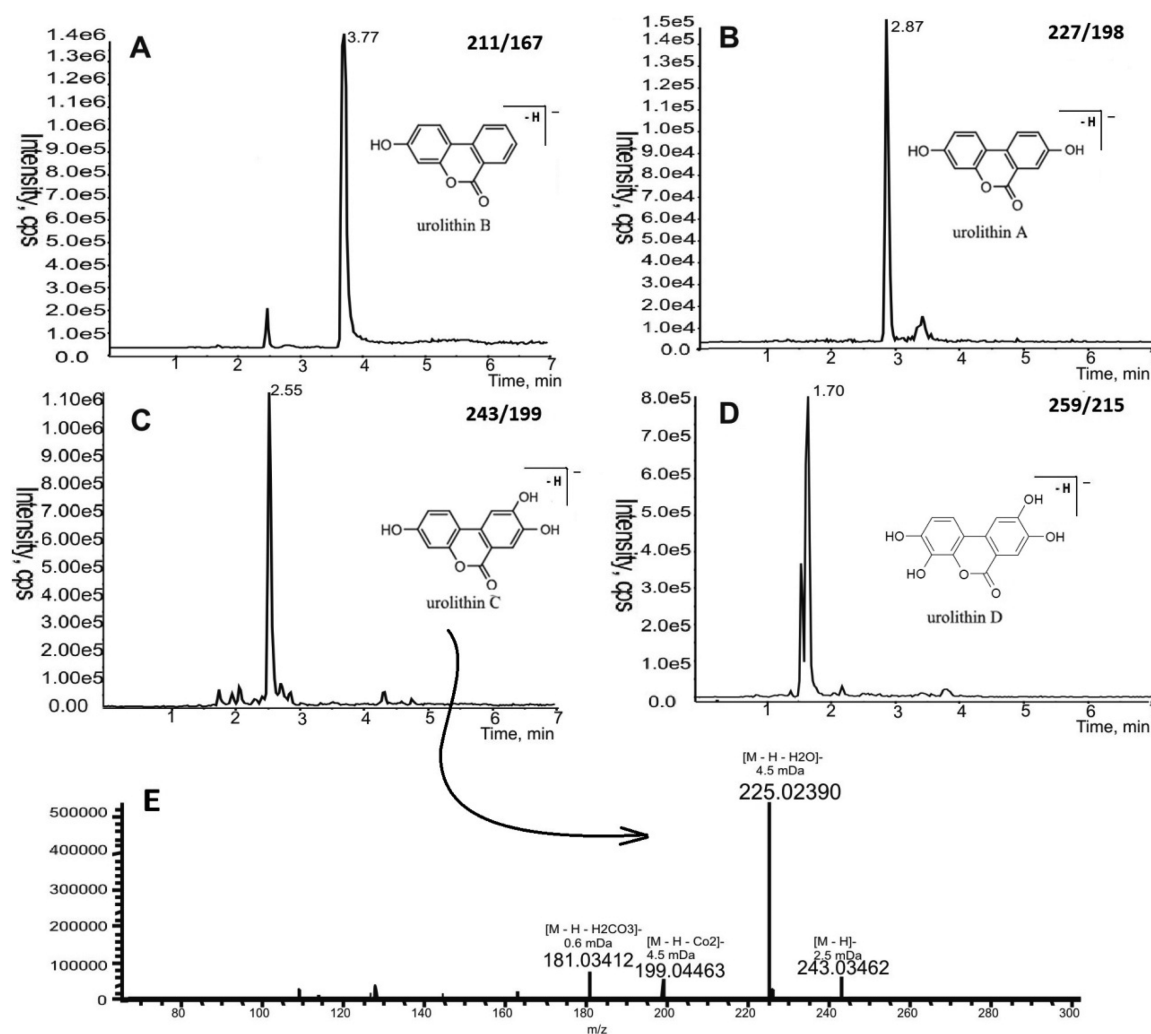


Figure 1. MRM trace chromatograms of (A) monohydroxy- (urolithin B), (B) dihydroxy- (urolithin A), (C) trihydroxy- (urolithin C), and (D) tetrahydroxydibenzopyran-6-ones (urolithin D). (E) MS² spectra of urolithin C by LC-ESI-ITD-FTMS.

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^a

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

^aThe compound assignment was confirmed by the MS/MS and UV spectral data, which have been reported in previous papers (Cerdá et al., 2005; Espín et al., 2007; González-Sarrias 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. ^bMS/MS spectra resulting from product ion scan (PIS) experiments. ^cTraces. ^dHighest excreter for each metabolite.

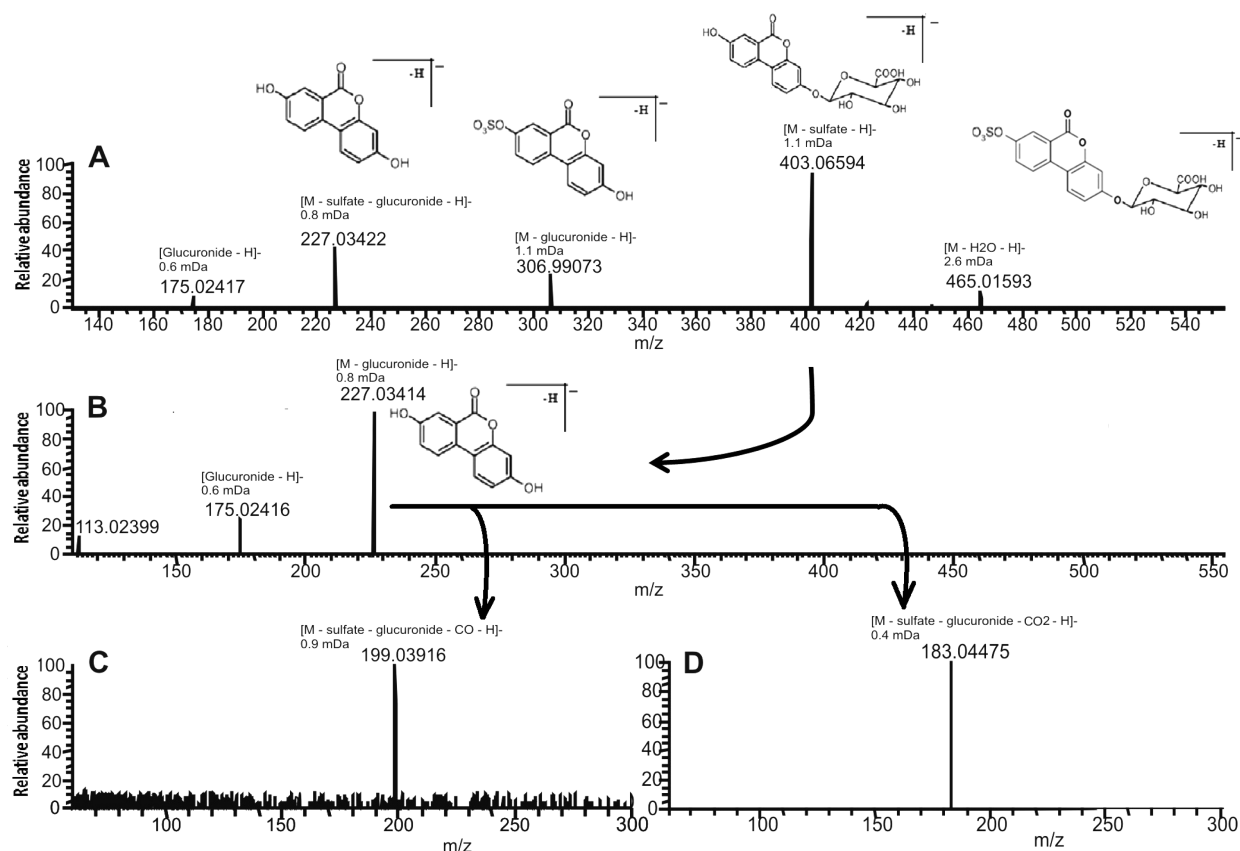


Figure 2. LC-ESI-ITD-FTMS spectra of the two urolithin A sulfoglucuronide isomers detected in nonhydrolyzed urine following the 12 weeks of nut consumption: (A) MS² daughter spectrum at m/z 483 shared by the two isomers; (B) MS³ daughter spectrum from the common fragment ion at m/z 403; (C, D) distinct MS⁴ daughter spectra of the two urolithin A sulfoglucuronide isomers from the fragment ion at m/z 227.

changes. For these reasons, further studies to identify the gut bacterial strains responsible for the ET catabolism, to investigate their relative occurrence in subjects with high versus low cardiometabolic risk, and to compare the effects of nut 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-(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,³³ 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-(trihydroxyphenyl)- γ -valerolactone (Figure 3B), and structural information obtained by MSⁿ experiments

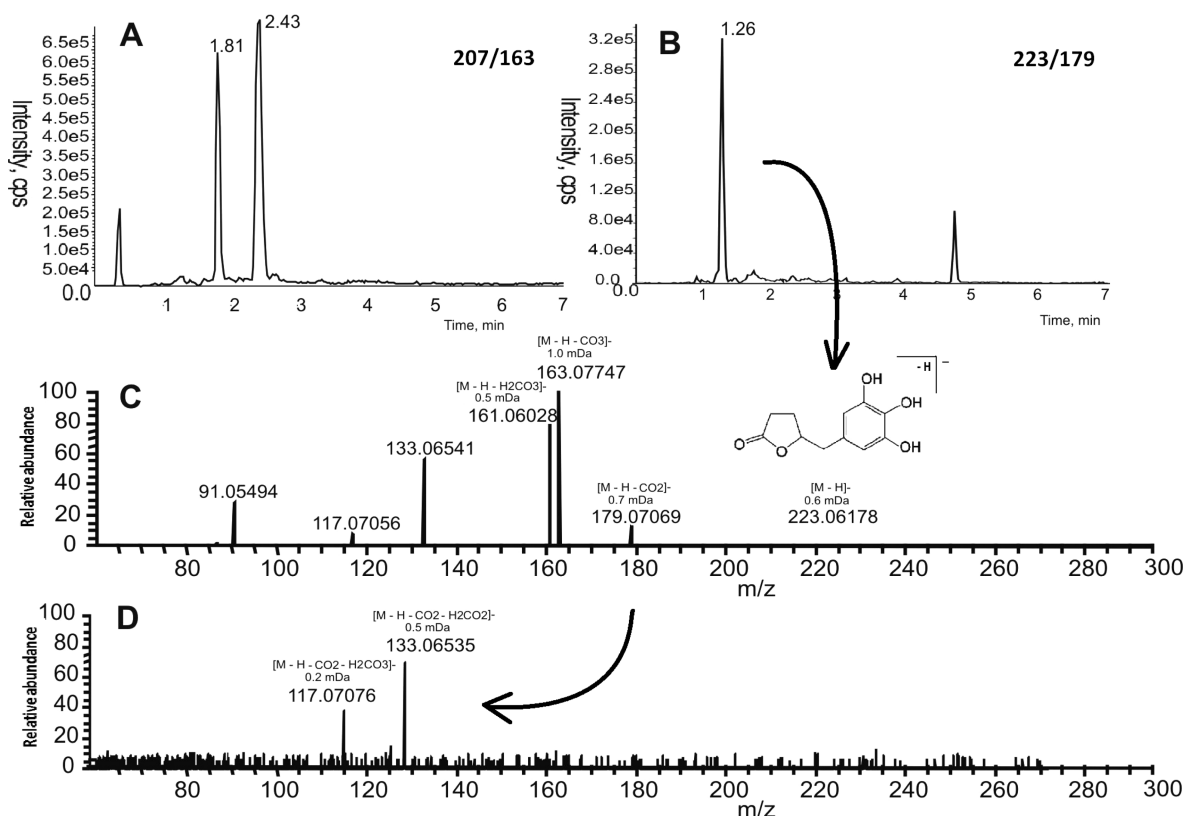


Figure 3. MRM trace chromatograms of (A) 5-(dihydroxyphenyl)- γ -valerolactone and (B) 5-(trihydroxyphenyl)- γ -valerolactone in hydrolyzed urine, following the 12 weeks of nut consumption. LC-ESI-ITD-FTMS spectra of 5-(trihydroxyphenyl)- γ -valerolactone: (C) MS² daughter spectra from the molecular ion at m/z 223; (D) MS³ daughter spectra from the fragment ion at m/z 179.

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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Notes

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■ REFERENCES

- (1) Grundy, S. M.; Cleeman, J. I.; Daniels, S. R.; Donato, K. A.; Eckel, R. H.; Franklin, B. A.; Gordon, D. J.; Krauss, R. M.; Savage, P. J.;

Smith, S. C.; Spertus, J. A.; Costa, F. Diagnosis and management of the metabolic syndrome: an American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement. *Circulation* **2005**, *112* (17), 2735–2752.

(2) Ros, E. Nuts and novel biomarkers of cardiovascular disease. *Am. J. Clin. Nutr.* **2009**, *89* (5), 1649S–1656S.

(3) Jenab, M.; Sabaté, J.; Slimani, N.; Ferrari, P.; Mazuir, M.; Casagrande, C.; Deharven, G.; Tjønneland, A.; Olsen, A.; Overvad, K.; Boutron-Ruault, M.-C.; Clavel-Chapelon, F.; Boeing, H.; Weikert, C.; Linseisen, J.; Rohrmann, S.; Trichopoulou, A.; Naska, A.; Palli, D.; Sacerdote, C.; Tumino, R.; Mattiello, A.; Pala, V.; Bueno-de-Mesquita, H. B.; Ocké, M. C.; Peeters, P. H.; Engeset, D.; Skeie, G.; Jakšzyn, P.; Ardanaz, E.; Quirós, J. R.; Chirlaque, M. D.; Martínez, C.; Amiano, P.; Berglund, G.; Palmqvist, R.; van Guelpen, B.; Bingham, S.; Key, T.; Riboli, E. Consumption and portion sizes of tree nuts, peanuts and seeds in the European Prospective Investigation into Cancer and Nutrition (EPIC) cohorts from 10 European countries. *Br. J. Nutr.* **2006**, *96* (Suppl. S2), S12–S23.

(4) Salas-Salvado, J.; Fernandez-Ballart, J.; Ros, E.; Martinez-Gonzalez, M. A.; Fito, M.; Estruch, R.; Corella, D.; Fiol, M.; Gomez-Gracia, E.; Aros, F.; Flores, G.; Lapetra, J.; Lamuela-Raventos, R.; Ruiz-Gutierrez, V.; Bullo, M.; Basora, J.; Covas, M. I. Effect of a Mediterranean diet supplemented with nuts on metabolic syndrome status: one-year results of the PREDIMED randomized trial. *Arch. Intern. Med.* **2008**, *168* (22), 2449–2458.

(5) Li, T. Y.; Brennan, A. M.; Wedick, N. M.; Mantzoros, C.; Rifai, N.; Hu, F. B. Regular consumption of nuts is associated with a lower risk of cardiovascular disease in women with type 2 diabetes. *J. Nutr.* **2009**, *139* (7), 1333–1338.

(6) Berryman, C. E.; Preston, A. G.; Karmally, W.; Deckelbaum, R. J.; Kris-Etherton, P. M. Effects of almond consumption on the reduction of LDL-cholesterol: a discussion of potential mechanisms and future research directions. *Nutr. Rev.* **2011**, *69*, 171–185.

(7) Casas-Agustench, P.; Bulló, M.; Ros, E.; Basora, J.; Salas-Salvado, J. Nureta-PREDIMED investigators. Cross-sectional association of nut intake with adiposity in a Mediterranean population. *Nutr. Metab. Cardiovasc. Dis.* **2011**, *21* (7), 518–525.

(8) Rajaram, S.; Connell, K. M.; Sabaté, J. Effect of almond-enriched high-monounsaturated fat diet on selected markers of inflammation: a randomised, controlled, crossover study. *Br. J. Nutr.* **2010**, *103* (6), 907–912.

(9) Sabaté, J.; Ang, Y. Nuts and health outcomes: new epidemiologic evidence. *Am. J. Clin. Nutr.* **2009**, *89* (5), 1643S–1648S.

(10) Cerda, B.; Tomas-Barberan, F. A.; Espin, J. C. Metabolism of antioxidant and chemopreventive ellagitannins from strawberries, raspberries, walnuts, and oak-aged wine in humans: identification of biomarkers and individual variability. *J. Agric. Food Chem.* **2005**, *53* (2), 227–235.

(11) Monagas, M.; Garrido, I.; Lebrón-Aguilar, R.; Gómez-Cordovés, M. C.; Rybarczyk, A.; Amarowicz, R.; Bartolomé, B. Comparative flavan-3-ol profile and antioxidant capacity of roasted peanut, hazelnut, and almond skins. *J. Agric. Food Chem.* **2009**, *57* (22), 10590–10599.

(12) Bai, N.; He, K.; Roller, M.; Zheng, B.; Chen, X.; Shao, Z.; Peng, T.; Zheng, Q. Active compounds from *Lagerstroemia speciosa*, insulin-like glucose uptake-stimulatory/inhibitory and adipocyte differentiation-inhibitory activities in 3T3-L1 cells. *J. Agric. Food Chem.* **2008**, *56* (24), 11668–11674.

(13) Larrosa, M.; García-Conesa, M. T.; Espín, J. C.; Tomás-Barberán, F. A. Ellagitannins, ellagic acid and vascular health. *Mol. Aspects Med.* **2010**, *31* (6), 513–539.

(14) Rastmanesh, R. High polyphenol, low probiotic diet for weight loss because of intestinal microbiota interaction. *Chem.-Biol. Interact.* **2011**, *189* (1–2), 1–8.

(15) Serrano, J.; Puupponen-Pimiä, R.; Dauer, A.; Aura, A.-M.; Saura-Calixto, F. Tannins: current knowledge of food sources, intake, bioavailability and biological effects. *Mol. Nutr. Food Res.* **2009**, *53*, S310–S329.

(16) Cerdá, B.; Espín, J. C.; Parra, S.; Martínez, P.; Tomás-Barberán, F. A. The potent in vitro antioxidant ellagitannins from pomegranate

juice are metabolised into bioavailable but poor antioxidant hydroxy-6H-dibenzopyran-6-one derivatives by the colonic microflora of healthy humans. *Eur. J. Nutr.* **2004**, *43* (4), 205–220.

(17) González-Barrio, R. T. P.; Ito, H.; Espín, J. C.; Tomás-Barberán, F. A. UV and MS identification of urolithins and nasutins, the bioavailable metabolites of ellagitannins and ellagic acid in different mammals. *J. Agric. Food Chem.* **2011**, *59* (4), 1152–1162.

(18) Espín, J. C.; González-Barrio, R.; Cerdá, B.; López-Bote, C.; Rey, A. I.; Tomás-Barberán, F. A. Iberian pig as a model to clarify obscure points in the bioavailability and metabolism of ellagitannins in humans. *J. Agric. Food Chem.* **2007**, *55* (25), 10476–10485.

(19) Appeldoorn, M. M.; Vincken, J. P.; Aura, A. M.; Hollman, P. C.; Gruppen, H. Procyanidin dimers are metabolized by human microbiota with 2-(3,4-dihydroxyphenyl)acetic acid and 5-(3,4-dihydroxyphenyl)- γ -valerolactone as the major metabolites. *J. Agric. Food Chem.* **2009**, *57* (3), 1084–1092.

(20) Urpi-Sarda, M.; Garrido, I.; Monagas, M.; Gómez-Cordovés, C.; Medina-Remón, A.; Andres-Lacueva, C.; Bartolomé, B. Profile of plasma and urine metabolites after the intake of almond [*Prunus dulcis* (Mill.) D.A. Webb] polyphenols in humans. *J. Agric. Food Chem.* **2009**, *57* (21), 10134–10142.

(21) Monagas, M.; Urpi Sarda, M.; Sánchez-Patán, F.; Llorach, R.; Garrido, I.; Gómez-Cordovés, C.; Andres-Lacueva, C.; Bartolomé, B. Insights into the metabolism and microbial biotransformation of dietary flavan-3-ols and the bioactivity of their metabolites. *Food Funct.* **2010**, *1*, 233–253.

(22) Stoupi, S.; Williamson, G.; Drynan, J. W.; Barron, D.; Clifford, M. N. A comparison of the in vitro biotransformation of (–)-epicatechin and procyanidin B2 by human faecal microbiota. *Mol. Nutr. Food Res.* **2010**, *54* (6), 747–759.

(23) Kutschera, M.; Engst, W.; Blaut, M.; Braune, A. Isolation of catechin-converting human intestinal bacteria. *J. Appl. Microbiol.* **2011**, *111*, 165–175.

(24) Ley, R. E. Obesity and the human microbiome. *Curr. Opin. Gastroenterol.* **2010**, *26* (1), 5–11.

(25) Casas-Agustench, P.; Lopez-Uriarte, P.; Bullo, M.; Ros, E.; Cabre-Vila, J. J.; Salas-Salvado, J. Effects of one serving of mixed nuts on serum lipids, insulin resistance and inflammatory markers in patients with the metabolic syndrome. *Nutr. Metab. Cardiovasc. Dis.* **2011**, *21* (2), 126–135.

(26) Lopez-Uriarte, P.; Nogues, R.; Saez, G.; Bullo, M.; Romeu, M.; Masana, L.; Tormos, C.; Casas-Agustench, P.; Salas-Salvado, J. Effect of nut consumption on oxidative stress and the endothelial function in metabolic syndrome. *Clin. Nutr.* **2010**, *29* (3), 373–380.

(27) Tulipani, S.; Llorach, R.; Jáuregui, O.; López-Uriarte, P.; García-Aloy, M.; Bullo, M.; Salas-Salvado, J.; Andrés-Lacueva, C. Metabolomics unveils urinary changes in subjects with metabolic syndrome following 12-week nut consumption. *J. Proteome Res.* **2011**, *10* (11), 5047–5058.

(28) Lucas, R.; Alcantara, D.; Morales, J. C. A concise synthesis of glucuronide metabolites of urolithin-B, resveratrol, and hydroxytyrosol. *Carbohydr. Res.* **2009**, *344* (11), 1340–1346.

(29) Giménez-Bastida, J. A.; González-Sarrías, A.; Larrosa, M.; Tomás-Barberán, F. A.; Espín, J. C.; García-Conesa, M. T. Ellagitannins metabolites, urolithin-A-glucuronide and its aglycone urolithin-A, ameliorate TNF- α induced inflammation and associated molecular markers in human aortic endothelial cells. *Mol. Nutr. Food Res.* **2012**, DOI: 10.1002/mnfr.201100677.

(30) Krauss, R. M.; Eckel, R. H.; Howard, B.; Appel, L. J.; Daniels, S. R.; Deckelbaum, R. J.; Erdman, J. W.; Kris-Etherton, P.; Goldberg, I. J.; Kotchen, T. A.; Lichtenstein, A. H.; Mitch, W. E.; Mullis, R.; Robinson, K.; Wylie-Rosett, J.; St. Jeor, S.; Suttie, J.; Tribble, D. L.; Bazzarre, T. L. AHA Dietary Guidelines: Revision 2000: a statement for healthcare professionals from the Nutrition Committee of the American Heart Association. *Circulation* **2000**, *102* (18), 2284–2299.

(31) Zamora-Ros, R.; Andres-Lacueva, C.; Lamuela-Raventós, R. M.; Berenguer, T.; Jakšzyn, P.; Barricarte, A.; Ardanaz, E.; Amiano, P.; Dorronsoro, M.; Larrañaga, N.; Martínez, C.; Sánchez, M. J.; Navarro, C.; Chirlaque, M. D.; Tormo, M. J.; Quirós, J. R.; González, C. A.

Estimation of dietary sources and flavonoid intake in a Spanish adult population (EPIC-Spain). *J. Am. Diet. Assoc.* **2010**, *110* (3), 390–398.

(32) Neveu, V.; Perez-Jiménez, J.; Vos, F.; Crespy, V.; du Chaffaut, L.; Mennen, L.; Knox, C.; Eisner, R.; Cruz, J.; Wishart, D.; Scalbert, A. Phenol-Explorer: an online comprehensive database on polyphenol contents in foods. *Database* **2010**, *2010*.

(33) Urpi-Sarda, M.; Monagas, M.; Khan, N.; Lamuela-Raventos, R. M.; Santos-Buelga, C.; Sacanella, E.; Castell, M.; Permanyer, J.; Andres-Lacueva, C. Epicatechin, procyanidins, and phenolic microbial metabolites after cocoa intake in humans and rats. *Anal. Bioanal. Chem.* **2009**, *394* (6), 1545–1556.

(34) Miro-Casas, E. F. A.; Covas, M. I.; Rodriguez, J. O.; Menoyo, C. E.; Lamuela Raventos, R. M. d. I. T. R Capillary gas chromatography-mass spectrometry quantitative determination of hydroxytyrosol and tyrosol in human urine after olive oil intake. *Anal. Biochem.* **2001**, *294*, 63–72.

(35) Gu, L.; Kelm, M. A.; Hammerstone, J. F.; Beecher, G.; Holden, J.; Haytowitz, D.; Gebhardt, S.; Prior, R. L. Concentrations of proanthocyanidins in common foods and estimations of normal consumption. *J. Nutr.* **2004**, *134* (3), 613–617.

(36) Bialonska, D.; Ramnani, P.; Kasimsetty, S. G.; Muntha, K. R.; Gibson, G. R.; Ferreira, D. The influence of pomegranate by-product and punicalagins on selected groups of human intestinal microbiota. *Int. J. Food Microbiol.* **2010**, *140* (2–3), 175–182.

(37) González-Sarriás, A.; Giménez-Bastida, J. A.; García-Conesa, M. T.; Gómez-Sánchez, M. B.; García-Talavera, N. V.; Gil-Izquierdo, A.; Sánchez-Álvarez, C.; Fontana-Compiano, L. O.; Morga-Egea, J. P.; Pastor-Quirante, F. A.; Martínez-Díaz, F.; Tomás-Barberán, F. A.; Espín, J. C. Occurrence of urolithins, gut microbiota ellagic acid metabolites and proliferation markers expression response in the human prostate gland upon consumption of walnuts and pomegranate juice. *Mol. Nutr. Food Res.* **2010**, *54*, 311–322.

(38) Mandalari, G.; Bisignano, C.; D'Arrigo, M.; Ginestra, G.; Arena, A.; Tomaino, A.; Wickham, M. S. Antimicrobial potential of polyphenols extracted from almond skins. *Lett. Appl. Microbiol.* **2010**, *51* (1), 83–89.

(39) Tzounis, X.; Rodriguez-Mateos, A.; Vulevic, J.; Gibson, G. R.; Kwik-Urbe, C.; Spencer, J. P. Prebiotic evaluation of cocoa-derived flavanols in healthy humans by using a randomized, controlled, double-blind, crossover intervention study. *Am. J. Clin. Nutr.* **2011**, *93* (1), 62–72.

(40) Queipo-Ortuño, M. I.; Boto-Ordóñez, M.; Murri Perr, M.; Gomez Zumaquero, J. M.; Clemente-Postigo, M.; Estruch, R.; Cardona Diaz, F.; Andrés-Lacueva, C.; Tinahones, F. J. Influence of red wine polyphenols and ethanol on the gut microbiota ecology and biochemical biomarkers. *Am. J. Clin. Nutr.* **2012**.

(41) Bialonska, D.; Kasimsetty, S. G.; Khan, S. I.; Ferreira, D. Urolithins, intestinal microbial metabolites of pomegranate ellagitannins, exhibit potent antioxidant activity in a cell-based assay. *J. Agric. Food Chem.* **2009**, *57* (21), 10181–10186.

(42) Selma, M. V.; Espín, J. C.; Tomas-Barberan, F. A. Interaction between phenolics and gut microbiota: role in human health. *J. Agric. Food Chem.* **2009**, *57* (15), 6485–6501.

(43) Roowi, S.; Stalmach, A.; Mullen, W.; Lean, M. E.; Edwards, C. A.; Crozier, A. Green tea flavan-3-ols: colonic degradation and urinary excretion of catabolites by humans. *J. Agric. Food Chem.* **2010**, *58* (2), 1296–1304.

(44) Llorach, R.; Garrido, I.; Monagas, M. A.; Urpi-Sarda, M.; Tulipani, S.; Bartolome, B. A.; Andres-Lacueva, C. Metabolomics study of human urinary metabolome modifications after intake of almond (*Prunus dulcis* (Mill.) D.A. Webb) skin polyphenols. *J. Proteome Res.* **2010**, *9* (11), 5859–5867.