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Metabolomics Unveils Urinary Changes in Subjects with Metabolic Syndrome following 12-Week Nut Consumption

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S Supporting Information

ABSTRACT: Through an HPLC-Q-TOF-MS-driven nontargeted metabolomics approach, we aimed to discriminate changes in the urinary metabolome of subjects with metabolic syndrome (MetS), following 12 weeks of mixed nuts consumption (30 g/day), compared to sex- and age-matched individuals given a control diet. The urinary metabolome corresponding to the nut-enriched diet clearly clustered in a distinct group, and the multivariate data analysis discriminated relevant mass features in this separation. Metabolites corresponding to the discriminating ions (MS features) were then subjected to multiple tandem mass spectrometry experiments using LC-ITD-FT-MS, to confirm their putative identification. The metabolomics approach revealed 20 potential markers of nut intake, including fatty acid conjugated metabolites, phase II and microbial-derived phenolic metabolites, and serotonin metabolites. An increased excretion of serotonin metabolites was associated for the first time with nut consumption. Additionally, the detection of urinary markers of gut microbial and phase II metabolism of nut polyphenols confirmed the understanding of their bioavailability and bioactivity as a priority area of research in the determination of the health effects derived from nut consumption. The results confirmed how a nontargeted metabolomics strategy may help to access unexplored metabolic pathways impacted by diet, thereby raising prospects for new intervention targets.

KEYWORDS: metabolomics, nuts, metabolic syndrome, urolithins, serotonin, pyrogallol, food biomarkers, multivariate analysis

INTRODUCTION

Metabolic syndrome (MetS) is defined as a constellation of metabolic disturbances including abdominal obesity, hyperglycemia, arterial hypertension, and dyslipidemia.^{1,2} Although not included in the current diagnostic criteria of MetS, a low-grade chronic inflammation is considered as a causative and contributing factor for the etiology and development of the syndrome, and a dysregulation of tryptophan metabolism seems to be strongly involved in its aetiology and progression.³ The syndrome is a serious threat to public health worldwide, not only because of its epidemic proportions⁴ but also because of its unequivocal association with an increased risk of developing type 2 diabetes, cardiovascular disorders and all-cause morbidity and mortality.⁵

Diet seems to play a pivotal role in the development of MetS, within a complex interaction between genetic determinants and environmental factors.⁶ Thus, dietary approaches are considered first-line interventions for the prevention, reversion and treatment of the syndrome. Nuts have been recently proposed as promising functional foods for managing both long- and short-term cardiometabolic risk factors.^{7,8} Among them, walnuts (*Juglans regia* L.), almonds (*Prunus dulcis* (Mill.) D.A. Webb) and hazelnuts

(*Corylus avellana* L.) are commonly consumed within the Mediterranean diet⁹ and are now considered intrinsic to several dietary guidelines worldwide.¹⁰ The beneficial effects of frequent nut consumption are reasonably attributed to the synergistic action of several constituents taking part in their complex composition and range from the reduction of insulin resistance, adiposity and low-grade systemic inflammation^{11–13} to the improvement of blood lipid profile¹⁴ and the overall protection from fatal and nonfatal CVD outcomes and diabetes.^{15,16} Besides having a low glycaemic index, nuts are rich sources of unsaturated fatty acids (mainly MUFA in almonds and hazelnuts, and n-3/n-6 PUFA in walnuts), proteins unusually rich in essential amino acids (such as L-arginine and tryptophan), biogenic amines (i.e., serotonin in walnuts), vitamins, minerals, fiber, and phytochemicals, such as phytosterols and polyphenols.¹⁷ The skin covering the nut kernels is particularly rich in polyphenols, including both flavonoid and nonflavonoid compounds, whose composition, extensively characterized, varies greatly depending on the nuts. Specifically, nut skins are very rich in tannins, including both

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condensed tannins, such as proanthocyanidins (in almonds and hazelnuts)^{18,19} and hydrolyzable tannins, namely ellagitannins (ETs) (in walnuts and hazelnuts),²⁰ whose consumption has recently been associated with a reduced risk of cardiovascular and obesity-related disorders.^{20,21}

The traditional method used to assess the clinical effects of nut consumption is based on the measurement of a single or several biochemical markers. However, these individual clinical parameters do not necessarily reflect the overall physiological status of the patients and the subtle changes that occur in their metabolism. Metabolomics is the rapidly evolving field of the comprehensive measurement of ideally all endogenous and exogenous metabolites in a biological fluid.²² With the help of advanced multivariate statistical and bioinformatic tools, the metabolomics approach has the power to identify slight modifications in the body's metabolic homeostasis. These modifications may be related to the presence of exogenous metabolites coming from the absorption, digestion and metabolism of nut components (biomarkers of intake),^{23,24} but may also depict diet-induced changes in the endogenous metabolism (biomarkers of effect), potentially offering access to unexplored metabolic pathways that are affected by diet.^{25–27}

To date, no previously published studies have explored, from a metabolomics perspective, the effects of a long-term consumption of mixed nuts on subjects with MetS.

A randomized parallel-group feeding trial was recently carried out to evaluate the clinical effects on the MetS phenotype of a 12-week (12-w) mixed nuts enriched diet (30 g/day), compared to a control diet.^{28,29} As previously published, there was 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 associated to the MetS patients who consumed the 12-w mixed nuts supplement, compared to those who did not consume it.^{28,29} In the present study, we applied a liquid chromatography–mass spectrometry (LC–MS) nontargeted approach to analyze the urinary metabolome of the MetS-diagnosed patients involved in the trial, to find new reliable indicators of nut intake and effect, and to offer new insights into the potential role of nuts in regulating metabolic homeostasis in the presence of diagnosed perturbations.

EXPERIMENTAL SECTION

Chemicals

The following chemicals were obtained commercially: D-L-carnitine, L-citrulline, L-phenylalanine, L-methyl-L-histidine, L-tryptophan, 2'-deoxyadenosine, 2'-deoxyguanosine, glycochenodeoxycholic acid, gallic acid, syringic acid, (–)-epicatechin and procyanidin B2, 5-hydroxyindoleacetic acid, N-acetylserotonin (Sigma-Aldrich, St Louis, MO); 4-hydroxyhippuric acid (PhytoLab GmbH & Co. KG); naringenin (Extrasynthèse, Genay, France). Urolithin A (3,8-dihydroxy-6H-dibenzo(b,d)pyran-6-one, 95% purity), Urolithin B (3-hydroxy-6H-dibenzo(b,d)pyran-6-one, 98% purity), Urolithin A glucuronide and Urolithin B glucuronide were chemically synthesized by Kylolab S.A. (Murcia, Spain). HPLC-grade methanol, acetonitrile and formic acid were purchased from Scharlau Chemie S. A. (Barcelona, Spain). Ultrapure water (Milli-Q) was obtained from a Milli-Q Gradient A10 system (Millipore, Bedford, MA).

Subjects

Forty-two male and female volunteers aged between 31 and 63 (52 ± 8) y with MetS participated in a prospective, randomized,

controlled, parallel-designed, 12-w interventional feeding trial. The design of the trial has been reported in detail elsewhere.^{28,29} Briefly, subjects were screened by Primary Care Centers affiliated with the Sant Joan University Hospital, Reus (Spain), and recruited if meeting at least 3 of the updated ATP III criteria for the diagnosis of MetS² (Supplemental Table 1, in Supporting Information). Exclusion criteria were a history of nut allergy; established type 2 diabetes; body mass index (BMI) $>35 \text{ kg/m}^2$; acute or chronic infection, inflammatory disease or endocrine disorders; a history of cancer; leukocytosis ($>10 \times 10^9 \text{ cells/L}$); anti-inflammatory, corticosteroid, hormonal or antibiotic drug treatment; a history of alcohol abuse or drug dependence; and a restrictive diet or a weight change $\geq 5 \text{ kg}$ 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 at the International Standard Randomized Controlled Trial Number (ISRCTN36468613).

Study Design

Study subjects were stratified by sex and age (≤ 50 or >50 y) and subsequently randomly assigned to one of two 12-w parallel intervention groups, the Control (CT) and the Nuts (NU) groups. At the beginning of the study, participants of both groups received the same qualitative dietary recommendations, according to the American Heart Association dietary guidelines.³⁰ In addition, the NU group 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), while participants in 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. The common dietary recommendations consisted of following a fruit- and vegetables-rich diet, preferring whole-grain high-fiber foods and fat-free or low-fat dairy products, eating fish at least twice a week, controlling the intake of cholesterol and SFA by replacing red meat with white meats, limiting the use of hydrogenated vegetable oils and salt and alcohol intake, and curtailing sweetened soft drinks and snacks. Additionally, participants were instructed to maintain the same level of physical activity throughout the study. To facilitate adherence to the interventions, individual nutritional counseling was guaranteed to all subjects at the beginning of the study and every 4 weeks during the trial.

Twenty-four hour urine samples were collected at baseline (V0) and at the end of the 12-w intervention (V3). 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. Energy and nutrient intakes were calculated using Spanish food composition databases, while information from the USDA databases was used to assess the intake of the essential amino acids L-arginine and tryptophan. SPSS 17.0 software (SPSS Inc., Chicago, IL) was used for statistical analyses, with statistical significance set at $p < 0.05$. With the exception of L-arginine and tryptophan, the dietary variables followed normal distribution (Kolmogorov-Smirnov test). Within-group differences between baseline and postintervention data were analyzed by the paired *t* test or Wilcoxon test (nonparametric variables), while between-group

differences were assessed with the unpaired *t* test or Mann–Whitney *U* test (nonparametric variables).

Sample Treatment

The urine samples were thawed on ice before analysis and centrifuged for 5 min at 12 000 × *g*. A 50 μ L aliquot of the supernatant was mixed with an equal volume of Milli-Q water, and the resulting solution was vortexed and transferred into a 96-well plate for HPLC–Q-TOF analysis. The following quality control (QC) samples were prepared: QC1, Milli-Q water samples; QC2, aqueous standard solution of a mixture of phenolic compounds (5 ng/ μ L) consisting of gallic acid, 4-hydroxyhippuric acid, syringic acid, (–)-epicatechin, procyanidin B2, and naringenin; QC3, aqueous standard solution of a mixture of endogenous urinary metabolites (5 ng/ μ L) consisting of D-L-carnitine, L-citrulline, L-phenylalanine, 1-methyl-L-histidine, L-tryptophan, 2'-deoxyadenosine, 2'-deoxyguanosine and glycochenodeoxycholic acid. The QC4 consisted of reinjecting of urine samples in opposite positions along the sequence of analysis.

LC–ESI–Q-TOF Mass Spectrometry

Liquid chromatography (LC) was performed on an HPLC Agilent series 1200RR system using a Phenomenex RP 18 Luna column (50 × 2.0 mm, 5 μ m), with a sample injection volume of 15 μ L for both urine and QC samples. A linear gradient elution was performed with a binary system consisting of [A] Milli-Q water 0.1% HCOOH (v/v) and [B] acetonitrile 0.1% HCOOH (v/v), at a constant flow rate of 600 μ L min^{–1}. The gradient elution (v/v) of [B] used was as follows (time, min; B, %): (0, 1), (4, 20), (6, 95), (7.5, 95), (8, 1), (12, 1). The HPLC system was online coupled with a hybrid quadrupole time-of-flight (Q-TOF) QSTAR Elite (Applied Biosystems/MDS Sciex) equipped with a TurboIonSpray source operating in positive or negative ion modes. Full data acquisition was performed scanning from 70 to 700 *m/z* in both ionization modes. Spray parameters were set as previously described.^{24,31} The TOF was calibrated with taurocholic acid (ions at *m/z* 79.9568 and *m/z* 514.2844) (1 pmol/ μ L) and reserpine (ions at *m/z* 195.1651 and *m/z* 609.2812) (1 pmol/ μ L) for negative and positive mode calibration, respectively. LC–MS data were acquired in 2 successive batches of analysis and the sequences of injections were randomized in order to avoid possible bias. The QC samples were analyzed throughout the run, every 15 injections, to provide a measurement of the stability and performance of the system and evaluate the quality of the acquired data.³²

Data Processing

LC–MS data were analyzed using MarkerView TM 1.2.1. software (Applied Biosystems, MDS Sciex, Toronto, Ontario, Canada), which performs feature extraction by peak finding for each sample and alignment using mass and retention time windows for the peaks. Peak detection was performed using a minimum peak width of 1 ppm, a minimum RT peak width of 3 scans, a noise threshold of 5, and a subtraction multiple factor of 1.5. Alignment used 0.1 min RT tolerance window, and 0.01 and 0.04 Da mass tolerance window for positive and negative ionization mode, respectively. At these feature extraction conditions, data sets containing 3000 mass features were obtained, including redundant mass signals (isotopes, adducts, in-source fragments etc.).

Multivariate Analysis (MVA)

The MVA was performed using SIMCA-P+ 11.5 software (Umetrics, Umeå, Sweden). Unsupervised (PCA) and supervised

statistical models (OSC-PLS-DA) were used. The data set was log transformed and Pareto-scaled (each variable weighted according to 1/ $\sqrt{\text{SD}}$) before principal component analysis (PCA), and log-transformed and mean-centered before partial least-squares discriminant analysis with orthogonal signal correction (OSC-PLS-DA). The PCA was used to have an overview of the quality of the data acquisition step, while the OSC-PLS-DA was employed for the exploration of the differences among the samples groups. Diet (NU versus CT) and time of urine collection (V0 versus V3) were selected as correction factors for the OSC filter.

The quality of the models was evaluated by parameters largely used in metabolomics studies, namely the goodness-of-fit parameter (R^2X), the proportion of the variance of the response variable that is explained by the model (R^2Y) and the predictive ability parameter (Q^2), which was calculated by a seven-round internal cross-validation of the data using a default option of the SIMCA-P+ 11.5 software. The values of $Q^2 < 0$ suggests a model with no predictive ability, and $0 < Q^2 < 1$ suggests some predictive character, with the reliability increasing as Q^2 approaches 1.³¹ Validation of the OSC-PLS-DA models and the evaluation of the degree of over fitting was then crucial to ensure that models were robust and not overfitted, as well as to exclude models that are just due to chance. For this purpose, a response permutation test ($n = 20$) was performed, and the correlation coefficient between the original *Y* and the permuted *Y* plotted against the cumulative R^2 and Q^2 was calculated.

The contribution of ions to the separation of the classes was visualized by using S-plots,³³ which graphically combine the weight (*p*) of each metabolite within the model and its correlation (*p*(corr)) with the modeled class designation, thus helping in the selection of statistically significant and potentially relevant metabolites. The pairwise discrimination between the NU_V3 class and, separately, the CT_V3 and V0 classes was carried out by two distinct S-plots, and information obtained from the two plots was combined. Only mass features showing high correlation coefficients in both the S-plots ($|p(\text{corr})| \geq 0.5$ arbitrary cutoff, previously adopted by our research group^{23,24}) were included into the list of candidate markers explaining the separation between diets, which was then submitted to the metabolite identification procedure.

Metabolite Identification

Markers contributing to the discrimination between diets were identified through a multiple-step procedure. First, to identify clusters of ions originating from the same metabolite (i.e., molecular ions, in-source molecular fragments, adducts and ¹³C isotopes) and all characterized by the same retention time, a two-way HCA was carried out using Pearson correlation.³⁴ Discriminative markers were then compared with the exact mass, chemical structure and LC–MS/MS spectra of metabolites proposed by freely available databases (± 5 mDa of accepted mass difference), namely the Human Metabolome,³⁵ the METLIN Metabolite,³⁶ the MassBank,³⁷ and the LIPID MAPS Structure³⁸ Databases. Information from external databases was also complemented by querying an in-house database mainly focused on the metabolites expected from the intake of dietary phytochemicals. For the final identification, markers were then subjected to further LC–ESI–MSⁿ fragmentation experiments with accurate mass measurements, while MassTRIX database³⁹ and the Kyoto Encyclopedia of Genes and Genomes (KEGG)

Table 1. Energy and Nutrient Intake at Baseline and After the 12-Week Intervention in the Two Intervention Groups^a

variable	NU group (<i>n</i> = 22)			CT group (<i>n</i> = 20)			treatment effect
	baseline ^b	after 12-w	<i>p</i> ^c	baseline ^b	after 12-w	<i>p</i> ^c	<i>p</i> ^d
Energy (kcal/day)	2070 ± 531	1874 ± 525	0.025	1954 ± 450	1596 ± 480	0.001	0.174
Carbohydrate (%)	40.8 ± 7.0	41.4 ± 7.5	0.629	42.4 ± 5.7	45.7 ± 7.3	0.073	0.208
Protein (%)	18.5 ± 3.1	19.0 ± 3.8	0.559	18.5 ± 3.4	20.8 ± 3.1	0.010	0.097
L-arginine (g/day)	4.67 ± 1.36	4.75 ± 1.09	0.770	4.48 ± 0.86	4.40 ± 1.66	0.741	0.199
Tryptophan (g/day)	0.98 ± 0.27	0.92 ± 0.20	0.225	0.97 ± 0.15	0.94 ± 0.36	0.851	0.782
Total Fat (%)	36.4 ± 5.2	36.8 ± 6.5	0.714	36.2 ± 6.3	32.4 ± 6.2	0.018	0.029
Saturated	10.6 ± 2.3	8.5 ± 3.0	0.005	10.7 ± 3.0	8.4 ± 3.0	0.003	0.903
Monounsaturated	17.3 ± 3.9	17.6 ± 3.4	0.591	17.5 ± 3.6	15.7 ± 3.4	0.017	0.026
Polyunsaturated	5.4 ± 1.7	7.4 ± 1.4	0.000	5.0 ± 1.1	4.7 ± 1.9	0.811	0.003
Alcohol	4.3 ± 5.5	2.8 ± 4.1	0.009	2.8 ± 5.8	1.1 ± 2.6	0.215	0.844
Cholesterol (mg/day)	338 ± 86	246 ± 93	0.001	309 ± 81	282 ± 125	0.417	0.121
Fiber (g/day)	20.2 ± 7.8	22.4 ± 7.5	0.270	19.0 ± 8.4	21.1 ± 23.4	0.701	0.998

^a Values are means ± SD. Statistical significance at *p* < 0.05. ^b No significant between-groups differences at baseline. ^c *p*-value for within-group differences (baseline versus postintervention intakes). ^d *p*-value for between-groups differences in changes (Nuts diet versus Control diet).

(<http://www.genome.jp/>) were then queried for the biological interpretation of the results.

LC–ESI–LTQ–Orbitrap Mass Spectrometry

For LC–ESI–MSⁿ experiments, an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Hemel Hempstead, U.K.) equipped with an ESI source (LC–ESI–LTQ–Orbitrap) was used, coupled to an Accela system (Thermo Scientific, Hemel Hempstead, UK) equipped with a quaternary pump and a thermostatted autosampler. The column and chromatographic conditions were the same as those employed in the LC–ESI–Q–TOF–MS experiments. The ESI–LTQ–Orbitrap data were acquired in FT MS mode (scan range from 100 to 1000 *m/z*) and in MSⁿ mode (Orbitrap resolution range from 15 000 to 30 000 fwhm). Operation parameters were as follows: source voltage, 4 (kV); source current, 100 (μA); S-Lens RF levels, 94 (%) in negative mode and 69 (%) in positive mode; sheath gas, 70 (arbitrary units); auxiliary gas, 20 (arbitrary units); sweep gas, 0 (arbitrary units); and capillary temperature, 325 °C. Default values were used for the other acquisition parameters (Fourier transform automatic gain control target 1 × 10⁶ for MS full mode, 1 × 10⁵ for SIM mode and 5 × 10⁴ for MSⁿ mode). The maximum injection time was set to 100 ms with two micro scans for MS mode, and to 1000 ms with one micro scan for MSⁿ mode. To optimize the method, the LTQ–Orbitrap was tuned with dodecanedioic acid (decane-1,10-dicarboxylic acid) (ions at *m/z* 229.14452, *m/z* 211.13395 and *m/z* 167.14412) and 5-hydroxyindoleacetic acid (ions at *m/z* 192.06551, *m/z* 146.06003 and *m/z* 118.06512) (5 ng/μL) for negative and positive mode tuning, respectively. Mass chromatograms and spectral data were acquired using XCalibur software 2.0 (Thermo Scientific, San Jose, CA).

The MSⁿ spectra obtained were then compared with those proposed by the databases and matched with the subsequently available commercial standards.

RESULTS AND DISCUSSION

Subjects

As presented in the Supplemental Table 1 (Supporting Information), no between-group differences existed in the baseline

characteristics of the study subjects. All participants met the criteria for MetS, and variables were homogeneously balanced between groups.

Adherence to Intervention and Diet Information

Adherence to the general dietary recommendations was good, as evaluated from the 3-day food records, as well as compliance with nut ingestion in the NU group (94%). Table 1 shows baseline energy, nutrient and alcohol intake and 12-week changes in the two treatment groups. At baseline, no dietary between-group differences existed. While both the NU and CT diets resulted in decreased energy and SFA intake compared to baseline, only the NU diet was associated with a significantly increased intake of MUFA and PUFA, probably reflecting the lipid composition of walnuts (particularly rich in α -linolenic and linoleic polyunsaturated fatty acids) and hazelnuts and almonds (rich in oleic monounsaturated fatty acid).

Data Acquisition Quality

As shown in the Supplemental Figure 1 (Supporting Information), a PCA analysis of the four classes of QCs revealed that neither carryover nor apparent clustering due to the batch injections occurred. Intra- and interbatch retention time shifts (<0.04 min), mass accuracy deviations (<5 mDa) and peak areas variation coefficients (<20%) in QC2 and QC3 were within the acceptable limits. No artifactual trends in urine samples took place during the data acquisition, since QC4 replicates positioned very closely in the PCA score plots. These data were in agreement with the quality criteria recently proposed for metabolomic studies,^{32,40} suggesting the overall good experimental quality of the study.

Urinary Metabolome Modifications

The LC–MS data sets obtained in negative and positive modes were separately submitted for MVA analysis. To evaluate the diet-dependent changes in the urinary metabolome of the CT and NU groups, an OSC-PLS-DA was applied to the data matrix, thus removing the variation in X (HPLC–MS spectra) unrelated to Y (diet). The OSC filter applied to the data set removed 11 components, maintaining 59% nonorthogonal variation in the original data set. The OSC-PLS-DA analysis resulted in four latent variable models characterized by good robustness and

Table 2. Summary of Parameters for Assessing the OSC-PLS-DA Modeling Quality

models	no. ^a	R ² X(cum) ^b	R ² Y(cum) ^b	Q ² Y(cum) ^b	R intercept ^c	Q intercept ^c
Positive mode data set	4	0.134	0.94	0.651	0.669	−0.456
Negative mode data set	4	0.103	0.972	0.715	0.751	−0.469

^a Number of components. ^b R²X(cum) and R²Y(cum) are the cumulative modeled variation in X and Y matrix, respectively, and Q²Y(cum) is the cumulative predicted variation in Y matrix. ^c Obtained after a permutation test ($n = 20$).

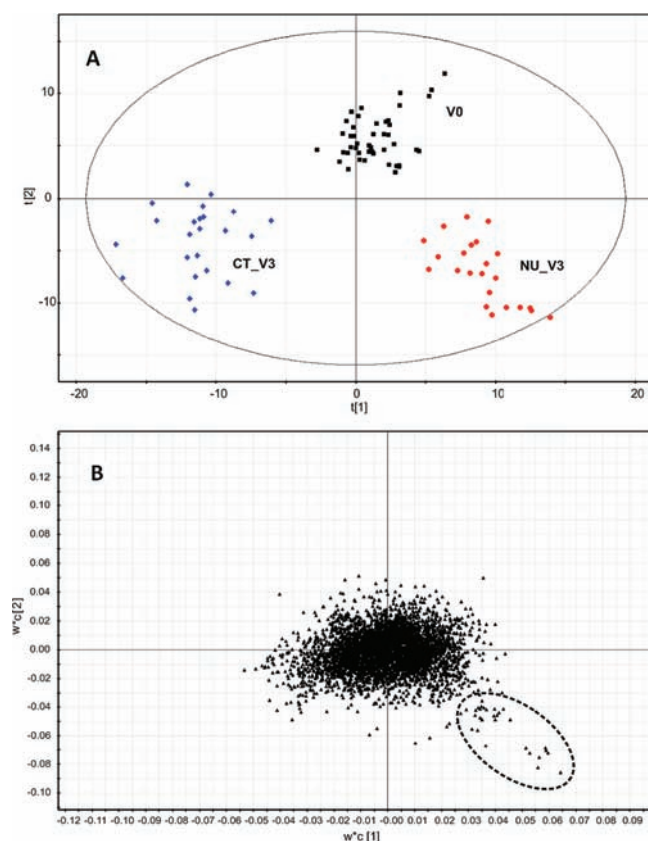


Figure 1. (A) OSC-PLS scores plot deriving from the urine samples collected at baseline (V0, black squares) and after 12-w of control diet (CT_V3, blue diamonds) and nut-enriched diet (NU_V3, red spheres). (B) Corresponding loadings plot. The dotted ellipse highlights markers related to NU diet.

predictability to explain the differences between the three sample classes (V0; CT_V3; NU_V3), as summarized in Table 2.

Following the 12-w of dietary intervention, the urine samples of the Control (CT_V3) and Nuts (NU_V3) groups differed markedly between them and in comparison to the baseline samples (Figure 1A). As observed in the OSC-PLS-DA loadings plot (Figure 1B), the regular administration of mixed nuts in particular perturbed the distribution of the ions in the loading space, by adding a significant number of ions that were deviated in both component 1 (abscissa) and component 2 (ordinate), thus changing the metabolic fingerprints of the NU subjects (Figure 1B, ions within the dotted ellipse).

Detection and Identification of Biomarker Candidates of Nut Consumption

The significant NU diet-discriminating markers responsible for the observed clustering patterns were visualized using S-plots (example in Figure 2). Results are summarized in Table 3, and

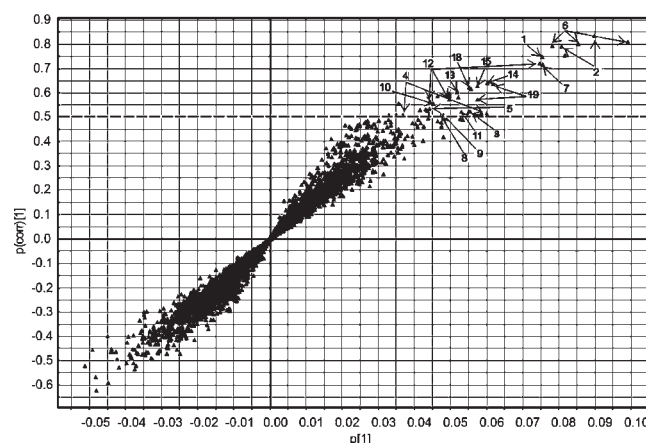


Figure 2. S-plot associated with the OSC-PLS-DA scores plot comparing NU_V3 and CT_V3 samples classes. The higher-right quadrant of the S-plot displays the mass features discriminative of the NU diet, and numbers and arrows correspond to their cluster numbers given in Table 3.

examples of LC–ESI–MSⁿ identification fragmentation patterns are shown in Table 4. A total of 20 significant metabolites were detected (Table 3). The identified biomarker candidates were classified into three groups of compounds, namely fatty acid metabolites (3 clusters), phenolic compound metabolites (including host phase II and microbial metabolites) (6 clusters), and metabolites of the tryptophan/serotonin (methoxyindoles) pathway (2 clusters). The relevant metabolites that remained unidentified have also been included in Table 3, although not discussed. To illustrate the identification procedure, some examples of metabolite clusters from Tables 3 will be described, while the complete explanation of the detected mass signals will be furnished in the Supporting Information.

Twelve of the 20 significant metabolites were detected as clusters composed of different kinds of ions, such as molecular ions, ¹³C isotopes and main in-source fragments related to a loss of water and/or fragments coming from the loss of glucuronide moiety (−176 Da), or even loss of sulfate moiety (−80 Da). As examples, the molecular ions of clusters 1 (m/z^- 257.0085) and 2 (m/z^- 385.1844 and m/z^+ 387.2011) were the most significant markers in discriminating the NU diet (Table 3). The in-source loss of 80 and 176 Da, respectively, allowed hypothesizing the two metabolites as a sulfate and a glucuronide conjugates (Table 3). For the metabolite of cluster 1, the queried databases suggested C₁₀H₁₀O₃ as the possible molecular formula matching the experimental mass of the aglycone metabolite. The further MSⁿ fragmentation of the desulfated ion (at m/z^- 177.0545), resulting in the loss of a water molecule (−18 Da) followed by a carboxylic group (−44 Da) (Table 4), allowed all the candidate molecules which did not present at least a free hydroxyl and a carboxylic group in their structure to be discarded. The experimental

Table 3. Putatively Identified Metabolites Positively Correlated to the Consumption of Mixed Nuts

n. cluster	ion mode	RT	detected mass m/z^a	$p(\text{corr})^b$	$p(\text{corr})^c$	assignment	potential biomarker	theoretical mass m/z
Markers of fatty acids metabolism								
1	—	4.8	257.0085	0.81	0.80	$[\text{M} - \text{H}]^-$	10-Hydroxy-decene-4,6-diynoic acid sulfate	257.0125
	—		177.0545	0.67	0.59	$[\text{M} - \text{H} - \text{sulfate}]^-$		177.0557
2	—	6.25	385.1844	0.82	0.84	$[\text{M} - \text{H}]^-$	Tridecadienoic/tridecynoic acid glucuronide	385.1868
	—		386.188	0.81	0.83	$^{13}\text{C} [\text{M} - \text{H}]^-$		
	+		387.2011	0.76	0.74	$[\text{M} + \text{H}]^+$		387.2013
	+		211.1688	0.78	0.72	$[\text{M} + \text{H} - \text{glucuronide}]^+$		211.1692
	+		193.1576	0.76	0.68	$[\text{M} + \text{H} - \text{glucuronide} - \text{H}_2\text{O}]^+$		193.1587
3	—	6.72	229.1403	0.59	0.55	$[\text{M} - \text{H}]^-$	Dodecanedioic acid	229.1445
	—		230.1441	0.61	0.51	$^{13}\text{C} [\text{M} - \text{H}]^-$		
	—		211.1314	0.61	— ^d	$[\text{M} - \text{H} - \text{H}_2\text{O}]^-$		211.1340
	—		167.1433	0.55	—	$[\text{M} - \text{H} - \text{H}_2\text{O} - \text{CO}_2]^-$		167.1441
Markers of microbial-derived and phase II metabolism of nut phenolics								
4	—	2.55	204.9827		0.56	$[\text{M} - \text{H}]^-$	Pyrogallol sulfate	204.9812
	—		79.9573	0.51	0.59	$[\text{HSO}_3 - \text{H}]^-$		79.9574
5	—	5.1	325.089	0.58	0.52	$[\text{M} - \text{H}]^-$	<i>p</i> -Coumaryl alcohol glucuronide	325.0929
	—		326.0987	0.53	—	$^{13}\text{C} [\text{M} - \text{H}]^-$		326.1001
6	—	5.28	403.0627	0.80	0.79	$[\text{M} - \text{H}]^-$	Urolithin A glucuronide	403.0671
	—		404.0654	0.80	0.80	$^{13}\text{C} [\text{M} - \text{H}]^-$		404.0742
	—		227.0357	0.78	0.79	$[\text{M} - \text{H} - \text{glucuronide}]^-$		227.0350
	+		405.0817	0.75	0.76	$[\text{M} + \text{H}]^+$		405.0816
	+		229.0495	0.76	0.76	$[\text{M} + \text{H} - \text{glucuronide}]^+$		229.0495
7	—	5.3	483.0195	0.77	0.77	$[\text{M} - \text{H}]^-$	Urolithin A sulfoglucuronide	483.0239
8	—	6.55	229.0197	0.58	0.52	$[\text{M} - \text{H}]^-$	<i>p</i> -Coumaryl alcohol sulfate	229.0176
	—		230.0221	0.58	0.51	$^{13}\text{C} [\text{M} - \text{H}]^-$		
	—		149.0615	0.54	—	$[\text{M} - \text{H} - \text{sulfate}]^-$		149.0608
	—		150.0646	0.51	—	$^{13}\text{C} [\text{M} - \text{H} - \text{sulfate}]^-$		150.0676
9	—	6.75	306.9885	0.60	0.60	$[\text{M} - \text{H}]^-$	Urolithin A sulfate	306.9918
Markers of the tryptophan/serotonin metabolic pathway								
10	—	4.3	297.0560	0.53	0.61	$[\text{M} - \text{H}]^-$	<i>N</i> -Acetylserotonin sulfate	297.0551
11	—	4.62	190.0505	0.60	0.60	$[\text{M} - \text{H}]^-$	Hydroxyindoleacetic acid	190.0510
	—		146.0614	0.61	0.55	$[\text{M} - \text{H} - \text{CO}_2]^-$		146.0606
	+		192.0648	0.69	0.57	$[\text{M} + \text{H}]^+$		192.0655
	+		174.0539	0.62	0.51	$[\text{M} + \text{H} - \text{H}_2\text{O}]^+$		174.0550
	+		146.0592	0.70	0.56	$[\text{M} + \text{H} - \text{CH}_2\text{O}_2]^+$		146.0600
Unidentified markers								
12	—	4.77	401.1812	0.60	0.70	$[\text{M} - \text{H}]^-$	Unidentified glucuronide metabolite	
	—		402.1846	0.50	0.64	$^{13}\text{C} [\text{M} - \text{H}]^-$		
	+		403.1962	0.70	0.69	$[\text{M} + \text{H}]^+$		
13	—	4.93	201.1086	0.61	0.62	$[\text{M} - \text{H}]^-$	Unidentified metabolite	
	—		202.1163	0.60	0.63	$^{13}\text{C} [\text{M} - \text{H}]^-$		
	+		203.1275	0.53	0.54	$[\text{M} + \text{H}]^+$		
	+		220.1538	0.55	0.54	$[\text{M} + \text{HNH}_3]^+$		
	+		185.1169	0.50	0.55	$[\text{M} + \text{H} - \text{H}_2\text{O}]^+$		
	+		167.1061	0.50	0.52	$[\text{M} + \text{H} - 2(\text{H}_2\text{O})]^+$		
14	—	5.03	405.2053	0.66	0.68	$[\text{M} - \text{H}]^-$	Unidentified glucuronide metabolite	
	—		5.08	403.1879	0.60	—		$[\text{M} - \text{H} - \text{H}_2]^-$
15	—	5.25	405.043	0.69	0.60		Unidentified metabolite	
16	+	6.1	227.1628	0.67	0.52	$[\text{M} - \text{H}]^-$	Unidentified metabolite	
17	—	6.13	389.2151	0.77	0.80	$[\text{M} - \text{H}]^-$	Unidentified metabolite	
18	—	6.25	401.1509	0.56	0.52		Unidentified metabolite	
19	—	6.37	389.2096	0.59	0.62	$[\text{M} - \text{H}]^-$	Unidentified metabolite	

Table 3. Continued

n. cluster	ion		detected mass		$p(\text{corr})^b$	$p(\text{corr})^c$	assignment	potential biomarker	theoretical mass m/z
	mode	RT	m/z^a						
20	—	6.37	390.2114	0.60	0.69	$^{13}\text{C} [\text{M} - \text{H}]^-$			
	—	6.22	389.1383	−0.69	−0.59		Unidentified metabolite		

^aData obtained by LC–ESI–q-TOF mass spectrometry (see text for details). ^bCalculated from the two-class OSC-PLS-DA model (class FS_V3 versus class CT_V3). ^cCalculated from the two-class OSC-PLS-DA model (class FS_V3 versus class V0). ^dDenotes $p(\text{corr})$ values lower than cutoff ($|0.5|$).

Table 4. Fragmentation Patterns of Candidate Markers of Nut Consumption^a

n. cluster	Potential biomarker	ion mode	Molecular ion m/z	Neutral loss	MS^2	Neutral loss	MS^3	Neutral loss	MS^4
1	10-Hydroxy-decene-4,6-diynoic acid sulfate	—	257.0085	—sulfate→	177.0545	—H ₂ O→	159.04491	—COO→	115.05504
2	Tridecadienoic/tridecynoic acid glucuronide	—	385.1844	—H ₂ O→ —C ₂ H ₄ O ₂ → —glucuronide→	367.17503 325.16444 209.15390	—COO→ —C ₂ H ₂ O ₂ → —H ₂ O→ —COO→	323.18636 267.15957 191.14376 165.12814	—CH ₂ O ₂ →	221.15442
5	4-Coumaryl alcohol glucuronide	—	325.089	—glucuronide→	149.06058	—C ₂ H ₂ O→	107.05014		
7	Urolithin A sulfolglucuronide	—	483.0195	—sulfate→ —glucuronide→ —sulfate/glucuronide→	306.99430 403.06800 227.03520				
8	4-Coumaryl alcohol sulfate	—	229.0197	—SO ₂ → —sulfate→	165.05554 149.0615	—C ₂ H ₂ O→	107.05000		
10	N-Acetylserotonin sulfate	—	297.0560	—sulfate→	217.09776	—C ₂ H ₄ ON→	158.06088	—CO→ —COCH ₂ →	130.06618 116.05053
11	Hydroxyindoleacetic acid	—	190.0505	—COO→	146.0614	—H ₂ → —CH ₃ → —H ₂ O→	144.04523 131.03742 128.05035		
		+	192.0648	—CH ₂ O ₂ →	146.0592	—CO→	118.06523		
12	Unidentified glucuronide metabolite	—	401.1812	—glucuronide→	225.14893				
14	Unidentified glucuronide metabolite	—	405.2053	—glucuronide→	229.18029				

^aData obtained by LC–ESI–LTQ-Orbitrap mass spectrometry (see text for details).

data fitted with the exact mass and fragmentation pattern of a polyunsaturated fatty acid, the hydroxy-decene-4,6-diynoic acid, as proposed by the METLIN and LMSD databases. Several possible structural isomers and stereoisomers of hydroxy-decene-4,6-diynoic acid have already been described (MID 35308–35311; ID LMFA01030710–LMFA01030713), according to the position of the hydroxyl group and the double bond within the molecule. Since the lack of available commercial standards did not allow the isomeric form of the molecule to be elucidated, the metabolite of cluster 1 was generically identified as hydroxy-decene-4,6-diynoic acid. A very similar identification procedure was followed for the metabolite of cluster 2 (Tables 3–4), which was tentatively identified as tridecadienoic/tridecynoic acid glucuronide. In other cases, the availability of the conjugated commercial standard helped the unequivocal metabolites identification. For the metabolite of cluster 6, LC–MS information was available from both negative (m/z^- 403.0627) and positive (m/z^+ 405.0817) ion modes. The in-source fragmentation resulted in the formation of daughter ions (Table 3) corresponding to the aglycone urolithin A (m/z^- 227.0357 and m/z^+ 229.0495) coming from the loss of the glucuronide moiety

(−176 Da). Since the LC and MS/MS behavior of the metabolite matched that of the available standard, the metabolite was identified as urolithin A glucuronide. A sulfolglucuronide (m/z^- 483.0195, cluster 7, Table 3) and a sulfate conjugate (cluster 9, m/z^- 306.9885, Table 3) of urolithin A were also tentatively identified, according to their MS^2 product ions (Table 4) and to the similarity of their elution behavior with what previously reported for urolithin A sulfolglucuronide and sulfate, in similar reverse-phase chromatographic conditions.⁴¹ As a last example, cluster 10 was composed by a single ion (m/z^- 297.0560, Table 3). The MS^2 experiment showed a loss of 80 Da (79.9582) producing a fragment ion at m/z^- 217.09776. As proposed by HMDB and MassTrix databases, the experimental mass of this fragment matched with that of N-acetylserotonin (N-acetyl-5-hydroxytryptamine, N-acetyl-SRT) (metabocard HMDB01238). The MS^3 and MS^4 serial fragmentations produced subsequent losses of C₂H₄ON, CO and CH₂ groups (Table 4), matching with the fragmentation pattern of the N-acetyl-SRT unconjugated commercial standard (Figure 3). Thus, the metabolite of cluster 10 was reasonably identified as N-acetyl-SRT sulfate.

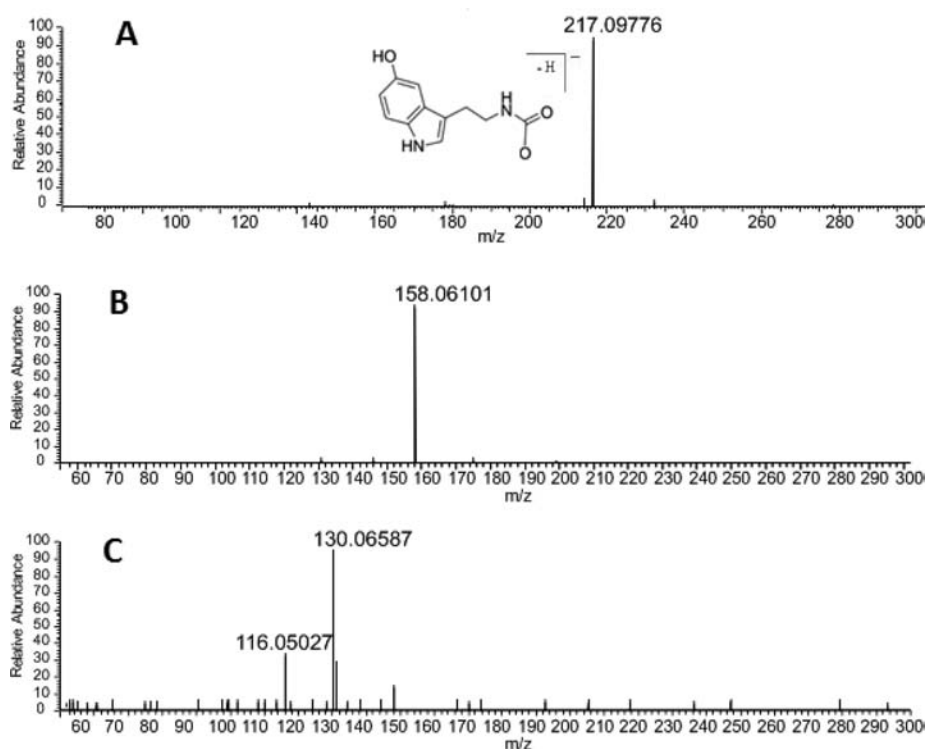


Figure 3. LC–LTQ–Orbitrap MS spectra of commercial standard N-acetylserotonin, in negative ionization mode. (A) MS scan, (B) MS² daughter spectra from the molecular ion at m/z^- 217, and (C) MS³ daughter spectra from the fragment ion at m/z^- 158.

Biological Interpretation

Markers of Fatty Acid Metabolism. Walnuts are the whole food with the highest content of α -linolenic acid (18:3n-3) of all edible plants,¹⁴ and the global PUFA content is consequently high. In our study, phase II metabolites of medium-chain PUFA (C10–C13) were the most significant urinary markers of the 12-w nut consumption, and the urinary excretion of a dicarboxylic acid (dodecanedioic acid, C12) also increased significantly. Our findings seem to suggest that the increased dietary intake of PUFA through nut consumption manifested itself through an increase in their β -oxidative metabolism and turnover. In line with our results, glucuronides of medium-chain and oxidized long-chain fatty acids, as well as sulfate conjugates of bile acids have already been identified as excretion products in human urine.^{42,43} The α -linolenic acid metabolism in humans has almost been elucidated and includes fatty acid β -oxidation, recycling of carbon by fatty acid synthesis *de novo* and conversion to longer-chain n-3 PUFA.⁴⁴ The high rate of oxidation of α -linolenic acid in humans is already known,⁴⁵ and an increased excretion of intermediate products of β -oxidation after increased dietary intake has also been contemplated.⁴⁶ In turn, medium-chain dicarboxylic acids (C6–C12) including dodecanedioic acid are derived in humans from the mitochondrial ω -oxidation of longer-chain dicarboxylic acids. These latter acids are directly ingested through a diet rich in vegetables or are formed *in vivo* by β -oxidation of the correspondent fatty acids.⁴⁷ Interestingly, several Mediterranean nuts, including walnuts, almonds and hazelnuts, have been reported, for the first time, as dietary sources of dicarboxylic acids,⁴⁸ although these have long been considered to be exclusive components of cutin and suberin in vegetables.

Markers of Microbial-Derived and Phase II Metabolism of Nut Phenolics. Other important contributors to the

nut-associated urinary fingerprints were compounds linked to the intake and metabolism of nut polyphenols. In particular, glucuronide and sulfate conjugates of urolithin A (dihydroxydibenzopyran-6-one), the characteristic microbial-derived metabolites of walnuts ETs, constituted the most discriminative group of phenolic metabolites (Table 3). The occurrence of ellagitannins is not so ubiquitous in edible plants, and walnuts are among the highest source of this class of hydrolyzable tannins together with, among others, pecans, cashews, hazelnuts, several varieties of berries and their derivatives such as juices, jams and jellies, pomegranate, oak-aged wines, and muscardine grapes.^{49,50} As recently reviewed, it is known that ellagitannins are not absorbed as such, and the most important site where these complex compounds become available is the large intestine, where they are a fermentable substrate for bacterial microflora.²⁰ The microbial transformation of ellagitannins within the intestinal lumen leads to the formation of a series of derivatives commonly named urolithins, which are then absorbed and become highly bioavailable as phase II conjugates in plasma and urine.⁵¹

The ellagitannins metabolites identified through our nontargeted metabolomics approach were in line with what was previously observed by employing targeted analyses.^{20,41,52} Particularly, the high p and $p(\text{corr})$ values of all the in-source ions originating from urolithin A glucuronide (Table 3) confirmed the compound to be an eligible marker of ET-rich food intake. These findings look interesting, when considering that the anti-inflammatory effects of the microbial-derived urolithin A has already been demonstrated *in vitro*,⁵³ and growing attention is currently being addressed to other potential bioactivities of urolithins, once bioavailable.^{41,54,55} If urolithins are involved in the health-promoting effects of ET-containing foodstuffs, the capacity of the gut microbiota of MetS individuals to produce

these compounds could be critical for the observations of their related biological effects. Furthermore, the pronounced correlation between urolithin excretion and nut consumption observed in this study, suggested that the urolithin producing/excreting capacity of these MetS subjects was quite homogeneous, unlike the strong interindividual variability previously observed among healthy subjects, and ascribed to differences in their gut microbiota.^{41,49} This could be the consequence of a more homogeneous gut microflora among MetS subjects compared to healthy individuals, in accordance with the association of obesity and related metabolic perturbations with a specific gut microbial community.⁵⁶ Interestingly, it may also result from a nut-induced gradual change in the colonic microflora of the subjects in study, due to the well-known role of diet, and specifically nut polyphenols, in modulating the colonic microbiota through selective prebiotic effects.⁵⁷

The increase in urinary pyrogallol sulfate (1,3-dihydroxyphenyl-2-*O*-sulfate) after nut intake could result from two distinct metabolic pathways, both involving the degrading role of the gut microflora. In our study, this compound could represent the only significant indicator of flavan-3-ols microbial metabolism observed (Table 3). In line with this hypothesis, previous targeted analyses have already described pyrogallol as a degradation product of galloylated monomeric flavan-3-ols, namely (–)-epicatechin gallate (ECG) and (–)-epigallocatechin gallate (EGCG), generated by the anaerobic metabolism of human and animal intestinal bacteria and then bioavailable in biological fluids.⁵⁸ Recently, an NMR-based nontargeted approach was also able to discriminate the sulfated form of pyrogallol as a major ECG metabolite in urine, following tea consumption.⁵⁹ In our case, however, the increased excretion of pyrogallol sulfate may also represent a further indicator of the microbial metabolism of hydrolyzable tannins contained in nuts.⁵⁸ Pyrogallol, in fact, has already been described as the last aromatic compound in the microbial degradation of both gallotannins or ellagitannins having a tergalloyl group or a related acyl group in their structure (Mingshu, Kai et al. 2006). Although ellagitannins are considered to be the major hydrolyzable tannins in walnuts, the presence of gallotannins has also been reported.⁶⁰ In any case, our study presented for the first time a positive association between the excretion of pyrogallol sulfate and the consumption of nuts, in free-living subjects and through a nontargeted metabolomics approach. These findings suggest that more attention should probably be focused on this potential marker of intake of nuts and other foods rich in galloylate-containing phytochemicals.

Finally, the presence of two phase II metabolites of *p*-coumaryl alcohol among the metabolic signatures of nut intake was almost unexpected since, until now, the epicuticular wax covering the surface of apples has been considered the main dietary source of *p*-coumaryl alcohol derivatives.⁶¹ Consequently, no exhaustive data are available in literature that could help in the assessment of the dietary intake of monolignols through nut consumption, and further investigations are needed.

Markers of the Tryptophan/Serotonin Metabolic Pathway. Two intermediate metabolites of the tryptophan methoxyindoles pathway, which leads to the biosynthesis of serotonin and melatonin from tryptophan, were identified among the urinary markers of nut intake (Table 3). In line with our results, HIAA and *N*-acetyl-SRT are normally recovered in urine, both in the free and conjugated forms, indicating that they are also substrates for extensive phase II metabolism.⁶² The urinary

excretion of HIAA is also considered a useful marker for the endogenous serotonin turnover.

An increased urinary excretion of 5-HIAA has recently been observed in a series of physiological and pathological conditions. In humans, this has been associated with dietary changes toward a richer-in-protein diet⁶³ and also with autism,⁶⁴ while an increase of urinary 5-HIAA has recently been observed in diabetic rats, compared to control animals.⁶⁵ In our case, no changes were observed either in the total intake of protein, or in the intake of *L*-arginine and tryptophan (the most typical amino acids in nuts), during the study. Furthermore, no differences in the number of subjects with elevated fasting glucose were observed between the two groups (NU and CT) at baseline (Supplemental Table 1, Supporting Information), and a reduction of the fasting insulin and HOMA-insulin resistance was even associated with the nut intervention.²⁸ However, walnuts have long been considered among the richest dietary sources of serotonin.^{62,66} Thus, the increased excretion of HIAA and *N*-acetyl-SRT may reasonably derive from a nut-associated increase in the intake of serotonin and its consequently increased catabolism. Because of the potential role of dietary serotonin in the improvement in oxidation, inflammation, and cardiovascular risk markers on subjects with metabolic perturbations,⁶⁷ our findings would suggest that, until now, the intake of serotonin through walnuts and other serotonin-rich foods has not been sufficiently considered. Certainly, the excreted metabolites deserve further studies, and their exogenous or endogenous nature remains to be elucidated. In fact, it is not possible to exclude that the two serotonin metabolites excreted following nut consumption may represent markers of effect of the dietary intervention, instead of markers of intake.

In any case, our data look interesting, if considering that a dysregulation of tryptophan metabolism, at the expense of serotonin synthesis, seems to be strongly involved in the etiology and progression of MetS.^{3,68} Additionally, a diminished serotonin production and turnover have also been associated with a state of altered food intake and energy expenditure, predisposing subjects to type 2 diabetes.^{69,70}

CONCLUSION

In lieu of the therapeutics, dietary strategies are currently being investigated for their potential role in the prevention and treatment of metabolic alterations. Here, the metabolomics strategy led to the unveiling of the important effects of a 12-w consumption of nuts on the urinary metabolome of MetS subjects, with no dietary restrictions. An increased metabolism and excretion of unsaturated fatty acids was observed, probably attributable to the highly increased intake of PUFA through the consumption of nuts. A urinary increase of microbial and phase II metabolites of nut polyphenols was also detected, particularly of urolithins coming from the metabolism of walnut ellagitannins. Because of the potential role of urolithins in reducing inflammation and improving cardiovascular health, these microbial metabolites may reasonably contribute to the beneficial effects of nut consumption.

Finally, the present work also confirmed how a nontargeted strategy may help to give access to unexplored metabolic pathways impacted by diet and detect unexpected markers of nut intake. For instance, the consumption of nuts was associated for the first time with the urinary excretion of pyrogallol sulfate, which was confirmed to be an eligible marker of intake of

galloylated phytochemicals. Interestingly, urinary markers of serotonin metabolism were also associated, for the first time, to nut consumption, although their endogenous versus exogenous (dietary) origin remains to be elucidated. Because of the important role of serotonin in the regulation of energy balance, metabolism, and glucose homeostasis, attention should also be given in the future to the eventual effects of nut intake on the serotonergic status of subjects with MetS.

■ ASSOCIATED CONTENT

Supporting Information

Supplementary figures and tables. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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