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Urolithins from Walnuts Are Associated with Cognitive Performance: Ancillary Results from the Walnuts and Healthy Aging (WAHA) Randomized Trial.

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ABSTRACT: Urolithins, microbiota-derived metabolites of ellagitannins, phenolic compounds abundant in walnuts, possess anti-inflammatory and antioxidant properties that might mitigate neuronal damage. However, clinical data supporting their effects on cognition are limited. In participants in the Walnuts and Healthy Aging (WAHA) trial, we determined urinary urolithin concentrations and examined their association with cognitive performance. The WAHA study was a parallel-group, 2-center (Barcelona, Spain; Loma Linda, California), randomized controlled trial. Cognitively healthy individuals followed a 2-year walnut-rich diet (15% daily energy) or a control diet excluding walnuts. Baseline and 2-year determinations included urinary urolithins by liquid chromatography-mass spectrometry and a comprehensive neuropsychological battery. The primary outcome was the association between changes in a global cognition composite score and urinary urolithins as the exposure, assessed by multivariable-adjusted regression. Secondary outcomes included associations of urolithins with domain-specific changes in memory, language, perception, and frontal function. A total of 612 participants (66% women, mean age 69 y) were included in analyses. Urolithin A, urolithin C and their glucuronide forms were objective biomarkers of walnut consumption (area under the curve at receiver operator characteristic analysis, 0.83; 95% CI, 0.80-0.86, 0.78; 95% CI, 0.74-0.82, 0.81; 95% CI, 0.77-0.84, and 0.80; 95% CI, 0.76-0.83, respectively). In the WAHA cohort following the walnut diet, changes in urolithin A glucuronide (β = 0.014 [95% CI, 0.003-0.025] per 1-SD) and urolithin C (β = 0.017 [95% CI, 0.001-0.033] per 1-SD) were associated with concurrent changes of the global cognition score. Urinary urolithins, particularly UroA, UroC and their glucuronides, are reliable biomarkers of walnut

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consumption. Changes in urolithin levels after 2 years of daily walnut consumption disclosed a direct association with cognitive performance in cognitively healthy older individuals. These findings provide clinical evidence linking dietary (poly)phenols and cognitive health.

Trial registration: Clinicaltrials.gov as NCT01634841.

Keywords: urolithins, biomarkers, cognition, (poly)phenols, walnuts.

1. Introduction

Cognitive decline and all-cause dementia are leading causes of disability that pose an increasing challenge to health-care systems worldwide [1]. This underscores the urgent need to develop strategies that promote healthy aging and mitigate cognitive impairment, a pre-dementia stage [2]. Oxidative stress and inflammation are key factors in the development and progression of cognitive decline and dementia [3]. There is accumulating evidence that adherence to plant-based dietary patterns, such as the Mediterranean diet, is associated with improved brain health [4]. Among plant-derived components, (poly)phenols stand out as highly bioactive molecules with antioxidant and anti-inflammatory properties that could counteract neuroinflammation [5]. However, (poly)phenol-rich foods and diets have been inconsistently associated with cognitive health in epidemiologic studies and clinical trials [6,7]. Tree nuts and peanuts are particularly rich in (poly)phenols [8], which have been proposed to mediate the beneficial effects of nuts on brain health [9].

Among nuts, walnuts have one of the highest contents of (poly)phenols [10,11]. Observational studies and clinical trials have suggested an association between walnut consumption and cognitive performance [9,12–14]. Yet, these studies have not accounted for the complex metabolic processes that walnut phenolics undergo before entering the bloodstream, crossing the blood brain barrier, and exerting neuroprotective effects. The primary phenolic compounds found in walnuts are ellagitannins and ellagic acid, which have limited bioavailability [15]. These molecules are transformed by the gut microbiota into urolithins, microbial phenolic metabolites that are more readily absorbed [16,17]. The urolithin family includes key end-metabolites, such as urolithin A (UroA), urolithin B (UroB), and urolithin C (UroC) [17]. Among these, UroA and UroB are the most commonly detected in plasma and urine, often in glucuronidated (UroAglu, UroBglu) and sulfated forms [18]. Based on the **gut microbiota's ability** to produce these metabolites, individuals can be classified into distinct urolithin metabotypes: **metabotype 0** (non-producers), **metabotype A** (predominant UroA producers), and **metabotype B** (producers of UroB and/or iso-urolithin A together with UroA) [16,19]. In preclinical studies, urolithins have disclosed a promising potential in neuroprotection and mitigation of neurodegenerative diseases, by modulating oxidative stress, inflammation, and other related mechanisms [20–22]. An overview of the gut–brain axis mechanism of urolithins is presented in **Figure 1**.

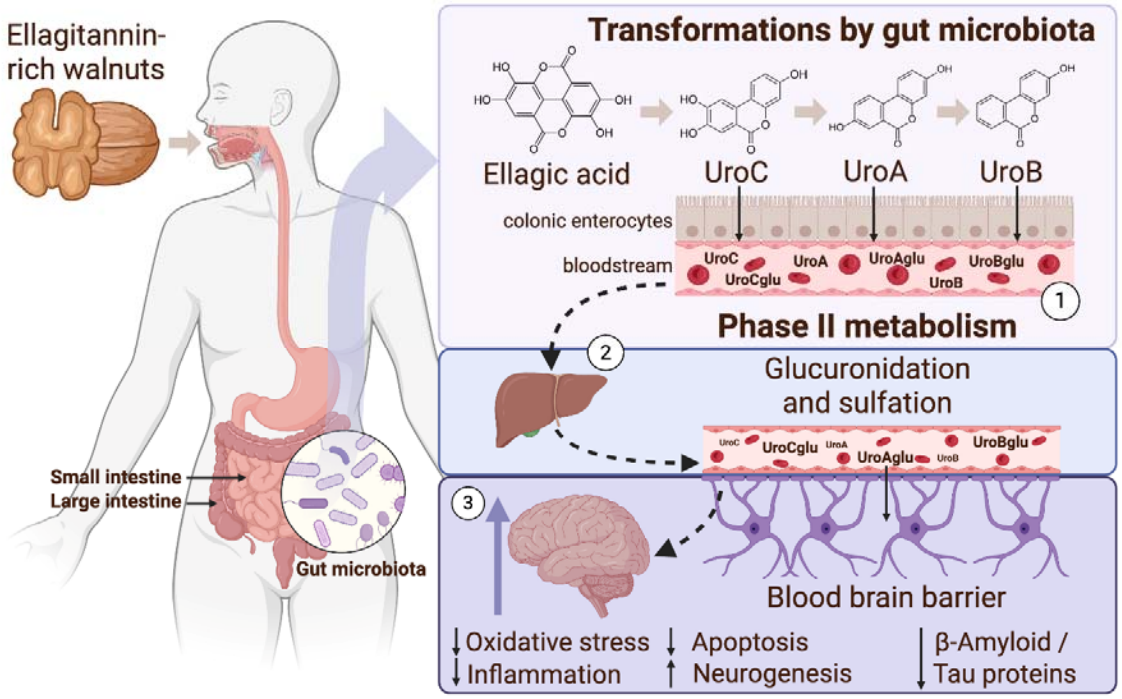

Figure 1. Microbial metabolism of ellagitannins and gut–brain axis mechanisms of action of urolithins.

Following the consumption of ellagitannin-rich walnuts, ellagitannins are hydrolyzed in the small intestine, releasing ellagic acid, which is then metabolized by the gut microbiota in the colon into various urolithins (UroC, UroA, UroB) [23]. These metabolites are absorbed into the blood stream and subsequently undergo phase II metabolism (glucuronidation, sulfation, and methylation) by colonic enterocytes and hepatocytes. Urolithins circulate predominantly in conjugated forms (e.g., glucuronides and sulfates), although unconjugated forms are also present at lower concentrations. They cross the blood–brain barrier and reach the brain, where they exert neuroprotective effects through multiple mechanisms, including the reduction of oxidative stress, inflammation, and neuronal apoptosis; the promotion of neurogenesis; and the inhibition of β-amyloid and tau aggregation. Created with BioRender.com. UroA, urolithin A; UroB, urolithin B; UroC, urolithin C; UroAglu, urolithin A glucuronide; UroBglu, urolithin B glucuronide; UroCglu, urolithin C glucuronide.

Changes in urinary urolithins were a pre-specified secondary outcome of the Walnuts And Healthy Aging (WAHA) randomized controlled trial testing the effects of walnuts on cognitive outcomes in an older population [12,24]. We hypothesized that, in comparison to a control diet, individuals consuming a walnut-rich diet for 2 years would exhibit increased urinary urolithins, which would be associated with cognitive performance outcomes.

2. Methods

2.1 Study design and participants

The WAHA study is a 2-year, parallel-group, randomized controlled trial. Participants were recruited from two sites: Loma Linda University, CA, USA (Loma Linda), and Hospital Clínic, Barcelona, Spain (Barcelona) between May 2012 and May 2014. Eligible participants were healthy men and women, aged 63 to 79 years, with normal cognitive function at enrollment. Further details regarding exclusion criteria are provided in **Methods S1**. The randomization procedure to allocate participants to the walnut or control diets is described in **Methods S2**. The final analytical sample included 612 participants: 315 in the walnut diet group and 297 in the control group.

The study was conducted in accordance with the guidelines of the Declaration of Helsinki and was approved by the Ethics Committee of each recruiting center. All participants provided written informed consent. The study protocol has been published [24].

2.2 Intervention

In the WAHA study, participants assigned to the walnut group were given walnuts for free to incorporate into their daily diet for the entire 2-year period. Those in the control group were instructed to abstain from walnuts and limit the intake of other nuts to no more than two servings per week. The 2-year duration of the intervention was designed to provide adequate time to detect changes in cognitive function while ensuring adherence. Participants in the walnut group consumed ≈45 g of walnuts daily (range, 30 to 60 g), equivalent to 294 kcal or 15% of energy intake for a standard 2000-kcal diet. This dosage was chosen based on evidence suggesting an association with cardioprotective effects, which may also benefit brain health [25].

Participants attended meetings with the study dietitians every 2 months for assessing compliance, providing cues for retention, and delivering the packaged walnuts to those allocated to the walnut diet.

2.3 Measurements

2-year changes in urinary urolithins´ concentrations were the exposure variables. The primary study outcome was the association between urinary urolithins and the 2-year change from baseline in the global cognition composite score. Secondary outcomes included associations with concurrent-domain-specific changes in memory, language, perception, and frontal function [12,24].

Morning urine samples were collected at baseline and end of year 2, and stored frozen at -80 °C. The determination of urolithins in urine was carried out with liquid chromatography-electrospray ionization-high resolution mass spectrometry. Detailed information on the reagents, sample preparation procedures, analytical protocols, and identification and quantification of urolithins in urine samples is provided in **Methods S4 and Table S1**.

A comprehensive battery of neuropsychological tests and a mood assessment (Hamilton Depression Rating Scale) were administered at baseline and 2 years, as reported [12]. All cognitive assessments were administered by trained personnel who were blinded to participants' group allocation. The neuropsychological evaluation is described in **Methods S3**.

Covariates included age, sex, educational attainment (basic/elementary/secondary/post-secondary), smoking status (never/former/current), physical activity, body mass index, diabetes, hypertension, dyslipidemia, APOE ε4 carrier status, total energy intake, and recruiting center. Physical activity was assessed at baseline and study's end using a validated short version of the Minnesota questionnaire [26]. Body mass index was calculated as weight (kilograms) divided by height (meters) squared. Every 6 months trained dietitians measured height and weight using standard procedures. Clinicians administered a general questionnaire at baseline and a follow-up questionnaire at study's end to assess clinical status, medication use, and adverse events. Participants were classified as having diabetes, dyslipidemia, or hypertension if they had a prior medical diagnosis or were receiving treatment with the corresponding medications. The APOE genotype was determined following the method of Hixson and Vernier [27]. Dietary intake was assessed through five unannounced 24-hour diet recalls over 2 years at Loma Linda and through 3-day food records every six months in Barcelona. The nutrient composition of the diets was calculated by using the Food Processor Plus software (ESHA Research, Salem, Oregon, USA), adapted to nutrient databases of local foods when appropriate. Total energy intake (kcal/day) was derived from these dietary assessments.

2.4 Statistical analyses

The present study used the WAHA randomized trial sample, originally designed to evaluate the effects of walnut consumption on cognitive outcomes [12]. Baseline characteristics of participants are presented as percentages for categorical variables and as means and standard deviations (SD) for continuous variables. Physical activity, the Hamilton Depression Rating Scale scores, and raw urolithin concentrations are represented as medians with interquartile ranges.

Between-group differences at baseline and 2-year changes in energy and nutrient intake were evaluated by 1-factor analysis of variance. For statistical analysis, raw urolithin values were transformed using a natural logarithm. Changes in urolithins and changes in cognitive function were calculated as the difference between baseline and follow-up at 2 years.

Multivariable generalized linear regression models were used to estimate β coefficients and 95% confidence intervals (CI) for the associations of allocated diet group (walnut vs control) and time (baseline vs 2 years) with urolithin concentrations. Two models were used: model 1 was adjusted for age (continuous) and sex (male, female), while model 2 was additionally adjusted for educational attainment (4 levels), smoking status (never, former, current), physical activity (METS·min/day, continuous), body mass index (kg/m^2, continuous), diabetes (yes, no), hypertension (yes, no), dyslipidemia (yes, no), *APOE* ε4 carrier status (yes, no), energy intake (kcal/day, continuous), and recruiting center (Barcelona, Loma Linda). Robust variance estimation was applied to account for intracluster correlations.

A receiver operating characteristic curve analysis was conducted using logistic regression, with the assigned group (control/walnuts) as the dichotomous dependent variable and the 2-year change in each urolithin

concentration (based on a 1-SD difference between 2-year and baseline concentrations) as the independent variable. Analyses were performed unadjusted and adjusted for covariates using model 2.

Multivariable linear regression models were also used to examine the associations between changes in urolithins and concurrent changes in cognitive outcomes as the dependent variable. Participants' raw cognition test results were standardized to z-scores to generate cognitive composites. A global cognition composite was calculated as the average of standardized changes across all neuropsychological tests. Analyses of the associations between urolithins and cognitive composites were conducted for each urolithin individually. The same adjusted models were applied, with additional adjustments for baseline concentrations of urolithins, baseline cognitive scores, and Hamilton Depression Rating Scale. All 2-year change analyses were conducted separately within intervention groups.

To account for interindividual variability in the microbiome, sensitivity analyses stratified by urolithin metabotypes were performed using the same linear regression models. To account for multiple testing in the multivariable-adjusted associations with cognitive outcomes, the Simes procedure was applied [28]. *P* values < 0.05 were considered statistically significant. Statistical analyses were performed using Stata 15.1 (Stata-Corp LP, TX, USA) and R software version 4.3.3.

3. Results

3.1 Participants' characteristics

The flowchart of the study is depicted in **Fig. S1**. A total of 708 subjects were randomly assigned to the walnut or control diets and 636 completed the 2-year intervention (90% retention rate). After excluding participants with missing data, the final analysis included 612 participants (315 in the walnut diet group and 297 in the control group) with a mean age of 69 years (66% women). The baseline characteristics of participants were well balanced between treatment groups (**Table 1)**.

Table 1. Baseline characteristics of the study population by allocated group (n = 612)[1]

Characteristics	Walnut diet (n=315)	Control diet (n=297)
Enter with partner	85 (27)	78 (26)
Women	207 (66)	198 (67)
Age, years	69.4 ± 3.8	68.9 ± 3.5
Smoking habit		
Never smoker	264 (84)	249 (84)
Former smoker	37 (12)	44 (15)
Current smoker	14 (4)	4 (1)
Educational level		
Basic (0–4 years)	9 (3)	6 (2)
Elementary (5–8 years)	55 (17)	61 (21)
Secondary (9–12 years)	59 (19)	58 (20)
Post-secondary (>12 years)	192 (61)	172 (58)
Height, cm	164.4 ± 9.3	163.3 ± 9.3
Weight, kg	73.7 ± 15.5	73.5 ± 14.7
Body mass index, kg/m^2	27.1 ± 4.4	27.4 ± 4.4
Hypertension	164 (52)	156 (53)
Type-2 diabetes	33 (10)	23 (8)

Dyslipidemia	170 (54)	155 (52)
Physical activity, METS·min/day[2]	2490 [1282–3846]	2398 [1443–4196]
Total energy intake, kcal/day	1672.4 ± 469.7	1601.7 ± 456.9
APOE ε4 carriers [3]	74 (24)	66 (22)
Hamilton Depression Rating Scale	2 [0, 4]	2 [0, 4]

[1]Values are expressed as *n* (%) for categorical variables and as means ± SD for continuous variables, except for Physical activity and Hamilton Depression Rating Scale score, which are shown as medians [IQR].

[2]METS, metabolic equivalent of task; 1 MET·minute equals approximately 1 kcal of energy expenditure.

[3]Data from 610 participants (2 missing *APOE* genotype).

3.2 Nutrient intake

The nutrient composition of walnuts is described in **Table S2**. Baseline and 2-year changes in nut consumption, energy and nutrient intake are presented by group allocation in **Table S3**. By study design, both intervention groups reported negligible baseline walnut consumption, which increased substantially throughout the trial only in the walnut group, as planned. By the end of the study, participants in the walnut group, compared with those in the control arm, reported changes in nutrient intake consistent with the composition of walnuts: increased intake of energy, total fat, linoleic acid, α-linolenic acid and fiber, and a corresponding decrease in carbohydrate intake.

3.3 Urolithins as biomarkers of walnut consumption

Multivariable adjusted regression analyses (**Table 2**) revealed increases in urinary urolithin concentrations in the walnut group participants, both in comparison to baseline values (within-group changes) and to those of the control group (between-group changes) over the 2-year period. Conversely, no changes in urolithin levels were observed in the control group. These findings were corroborated by the beeswarm plots illustrating urolithins' concentrations in the control and walnut groups at baseline and after 2 years. **Figure 2** highlights UroA and UroAglu, key biomarkers of walnut consumption, while **Fig. S2** provides data on other urolithins.

Table 2. Multivariable adjusted regression between urolithins in each allocated group and 2-year between-group changes

	Walnut diet **(in group changes)**			Control diet **(in group changes)**			**Between-group changes**
	Baseline	2 years	β (CI 95%) per 1-SD	Baseline	2 years	β (CI 95%) per 1-SD	β (CI 95%) per 1-SD
Urolithin A	15.3 [9.1–76.4]	1170.3 [161.8–3096.3]	2.921 (2.606 to 3.236)* **2.921 (2.609 to 3.233)****	12.7 [8.6–60.7]	13.2 [8.6–39.7]	-0.133 (-0.385 to 0.119)* -0.133 (-0.385 to 0.120)**	3.050 (2.661 to 3.439)* **3.074 (2.683 to 3.465)****
Urolithin A glucuronide	122.4 [8.0–824.7]	11009.1 [1603.2–25205.8]	3.503 (3.070 to 3.935)* **3.534 (3.111 to 3.958)****	76.0 [6.4–1203.1]	53.5 [6.9–714.6]	-0.172 (-0.603 to 0.259)* -0.173 (-0.603 to 0.258)**	3.777 (3.209 to 4.344)* **3.840 (3.267 to 4.414)****
Urolithin A sulfate	11.12 [8.24–16.95]	12.23 [8.42–38.76]	0.708 (0.491 to 0.925)* **0.708 (0.494 to 0.921)****	9.8 [7.8–14.1]	9.7 [7.7–14.4]	-0.030 (-0.135 to 0.075)* -0.030 (-0.135 to 0.075)**	0.735 (0.523 to 0.946)* **0.747 (0.537 to 0.957)****
Urolithin B	5.1 [3.6–7.9]	5.8 [3.7–125.7]	0.965 (0.656 to 1.275)* **0.965 (0.658 to 1.272)****	4.4 [3.4–6.8]	4.45 [3.45–7.14]	0.007 (-0.160 to 0.173)* 0.007 (-0.160 to 0.173)**	0.952 (0.663 to 1.242)* **0.964 (0.676 to 1.252)****
Urolithin B glucuronide	10.4 [6.6–47.1]	10.1 [6.5–87.4]	0.469 (0.042 to 0.892)* **0.498 (0.080 to 0.917)****	8.4 [6.4–16.3]	8.0 [6.1–15.4]	-0.116 (-0.426 to 0.193)* -0.114 (-0.419 to 0.192)**	0.827 (0.455 to 1.200)* **0.880 (0.501 to 1.258)****
Urolithin B sulfate	4.74 [3.55–7.07]	5.18 [3.59–11.26]	0.579 (0.391 to 0.766)* **0.579 (0.392 to 0.765)****	4.22 [3.35–6.09]	4.17 [3.33–6.18]	-0.063 (-0.150 to 0.024)* -0.063 (-0.150 to 0.024)**	0.643 (0.447 to 0.839)* **0.647 (0.451 to 0.843)****
Urolithin C	16.2 [12.4–24.7]	54.3 [16.8–282.8]	1.320 (1.121 to 1.518)* **1.320 (1.122 to 1.518)****	15.2 [11.8–22.2]	14.8 [11.9–21.9]	-0.041 (-0.144 to 0.062)* -0.041 (-0.144 to 0.062)**	1.368 (1.149 to 1.587)* **1.403 (1.184 to 1.621)****
Urolithin C glucuronide	16.9 [12.5–25.7]	124.5 [20.4–529.2]	1.699 (1.487 to 1.911)* **1.699 (1.489 to 1.909)****	15.4 [11.8–22.9]	15.0 [11.9–23.2]	-0.009 (-0.130 to 0.113)* 0.009 (-0.130 to 0.112)**	1.720 (1.474 to 1.965)* **1.744 (1.501 to 1.986)****
Urolithin C sulfate	16.0 [12.2–23.6]	20.4 [13.2–93.3]	0.690 (0.532 to 0.849)* **0.690 (0.532 to 0.849)****	14.8 [11.8–20.5]	14.5 [11.8–20.9]	-0.046 (-0.121 to 0.031)* -0.045 (-0.121 to 0.031)**	0.746 (0.572 to 0.920)* **0.771 (0.597 to 0.946)****

Raw urolithin concentrations are expressed as medians (ng/mg creatinine) with interquartile ranges.

A natural logarithmic transformation was applied to the raw values of urolithins to perform multivariable regression analyses.

In each file, the first beta values correspond to Model 1 (*), adjusted for age and sex, and the second beta values correspond to Model 2 (**), which was further adjusted for educational attainment, smoking status, physical activity, body mass index, energy intake, diabetes, hypertension, dyslipidemia, *APOE* ε4 carrier status, and study center.

Analyses were conducted with robust variance estimation to correct for intracluster correlations.

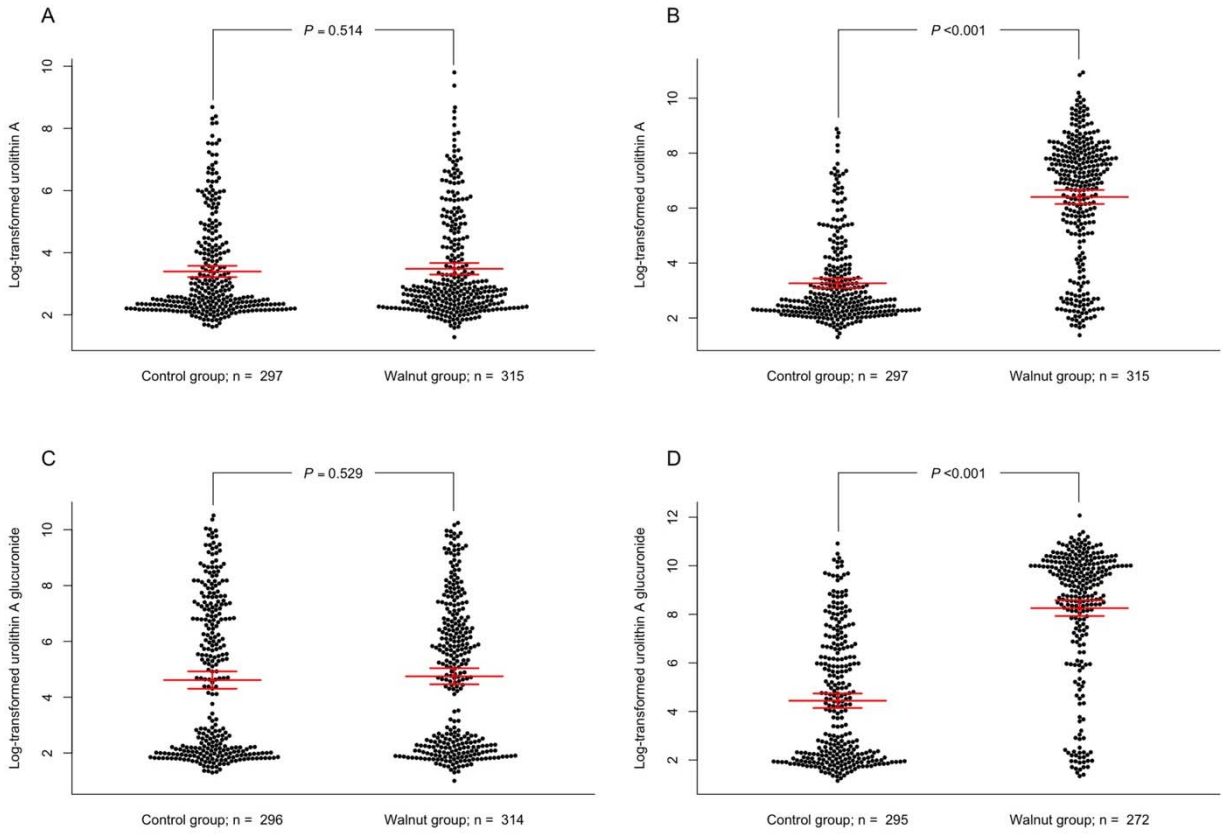

Figure 2. Baseline and 2-year urinary concentrations of urolithin A and urolithin A glucuronide by intervention group.

In red, mean (95% confidence interval). Displayed concentrations are log-transformed.
(A) Baseline Urolithin A concentrations. Values are 3.40 (3.21, 3.58) and 3.48 (3.30, 3.67) for the control and walnut groups, respectively.
(B) 2 years Urolithin A concentrations. Values are 3.26 (3.09, 3.44) and 6.40 (6.15, 6.66) for the control and walnut groups, respectively.
(C) Baseline Urolithin A glucuronide concentrations. Values are 4.61 (4.30, 4.93) and 4.75 (4.46, 5.04) for the control and walnut groups, respectively.
(D) 2 years Urolithin A glucuronide concentrations. Values are 4.44 (4.14, 4.74) and 8.25 (7.93, 8.58) for the control and walnut groups, respectively.
P obtained by 1-way analysis of variance.

Receiver Operating Characteristic (ROC) curve analysis of urolithins with adjustment for potential confounders indicated that UroA, UroAglu, UroC and UroCglu had predictive capability of walnut consumption. The area under the ROC curve (AUC) for UroA was 0.80 (95% CI, 0.77-0.84) and improved to 0.83 (95% CI, 0.80-0.86) after multivariable adjustment (**Figure 3A**). In contrast, UroB and UroBglu exhibited limited predictive capacity, with UroB yielding an AUC of 0.60 (95% CI, 0.56–0.65), slightly improving to 0.68 (95% CI, 0.64–0.72) after adjustment (**Figure 3B**). ROC curves for additional urolithins are provided in **Fig. S3**.

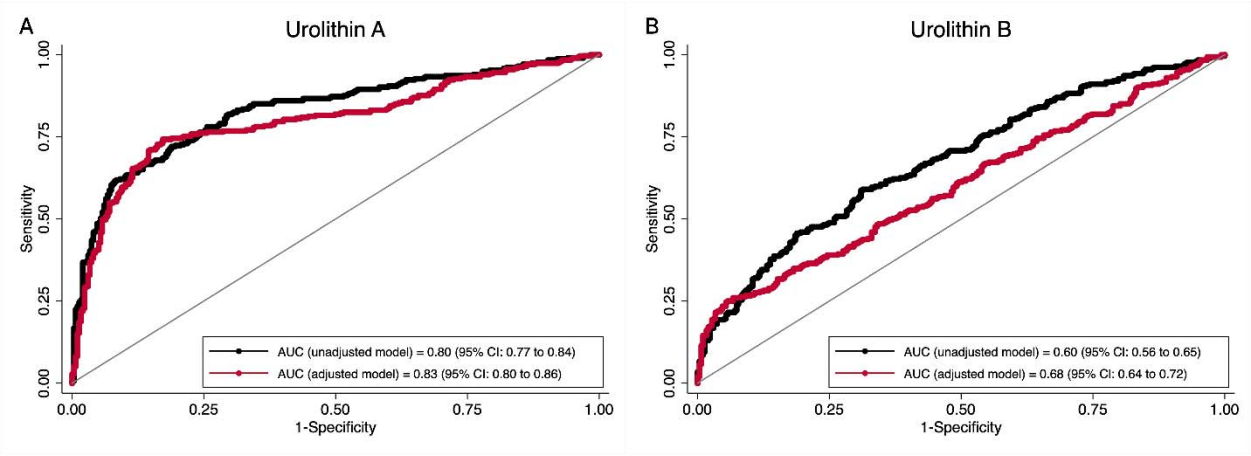

Figure 3. Receiver operating characteristic curve for prediction of the group of intervention (walnut/control) by 2-year changes in urolithin A (A) and urolithin B (B) concentrations.

Difference between 2-year and baseline normalized concentrations unadjusted (red) and adjusted for potential confounders (blue), such as sex, age, educational attainment, smoking status, physical activity, body mass index, energy intake, diabetes, hypertension, dyslipidemia, *APOE* ε4 carrier status, and study center.

3.4 Associations of urolithins with cognitive performance

After intervention for 2 years, increased levels of UroAglu and UroC were associated with global cognitive performance in participants of the walnut diet group (**Table 3**). After multiple testing correction, a trend remained for the association between UroAglu and global cognition (*P* value = 0.066). When stratified by urolithin metabotypes, positive associations were observed by individuals with a metabotype B profile, with positive associations for UroA, UroAglu, UroC, and UroCglu (**Tables S4**). The association between UroA and global cognition remained after correcting for multiple testing (*P* value = 0.050).

For the secondary outcomes, increments of UroC and its glucuronide were linked to frontal function (**Table S5**). Consistent with the findings for global cognition, these associations were driven by participants with a metabotype B profile, showing positive associations for UroA, UroAglu, UroC, and UroCglu (**Table S6**). No associations were observed for other cognitive domains (**Tables S7–S9, S10–S12**).

In the control group, slightly increased levels of UroB were inversely associated with changes in the language domain (**Results S1**), but the association did not persist after stratification by metabotype.

Table 3. Association between 2-year changes in urolithin concentrations and global cognition composite in the walnut group.

Urolithins	**β (95% CI) for perception composite, per 1-SD**	
	No.	
Urolithin A	315	0.0098 (-0.0003 to 0.0200)* 0.010 (-0.003 to 0.0024)**
Urolithin A glucuronide	272	0.011 (0.002 to 0.019)* **0.014 (0.003 to 0.025)****
Urolithin B	315	0.0125 (0.0001 to 0.0248)* 0.007 (-0.007 to 0.020)**

Urolithin B glucuronide	280	0.007 (-0.006 to 0.021)* -0.0003 (-0.0141 to 0.0136)**
Urolithin C	315	0.021 (0.006 to 0.036)* **0.017 (0.001 to 0.033)****
Urolithin C glucuronide	315	0.015 (0.002 to 0.028)* 0.013 (-0.003 to 0.028)**

A natural logarithmic transformation was applied to the raw values of urolithins.
Data from multivariable linear regression models. In each file, the first beta values correspond to Model 1 (*), adjusted for age and sex, and the second beta values correspond to Model 2 (**), which was further adjusted for baseline concentration of urolithins, baseline cognitive outcomes, educational attainment, smoking status, physical activity, body mass index, energy intake, diabetes, hypertension, dyslipidemia, *APOE* ε4 carrier status, Hamilton Depression Rating Scale, and study center. Robust variance estimation was used to correct for intracluster correlations.

4. Discussion

In this pre-specified observational analysis of the WAHA trial conducted in healthy older adults from two different geographical locations (Barcelona in the Mediterranean area and Loma Linda in California), a diet supplemented with walnuts at 15% of energy for 2 years compared with a control diet resulted in sizable increases in urinary urolithins, microbiota-derived metabolites of ellagic acid, a phenolic compound abundant in walnuts. These findings signal urolithins, specifically UroA, UroC and their glucuronide metabolites, as objective biomarkers of walnut consumption. Increased levels of individual urolithins were associated with global cognition after adjusting for potential confounders, including clinical characteristics and risk factors for cognitive decline. Our results provide novel clinical evidence of an association between microbiota-derived (poly)phenol metabolites and brain health and concur with observations of preclinical studies, which support a cognitive benefit of urolithins through the mitigation of neurodegenerative processes [21,22,29].

Our findings revealed direct associations of UroAglu and UroC with global cognitive performance after the 2-year walnut intervention. These observations align with the gut microbiota pathway of urolithin formation, where UroC serves as a precursor to UroA, the most abundant and active urolithin metabolite detected in urine [17,30,31]. When stratifying by urolithin metabotypes, these associations remained consistent and were particularly evident among individuals with a metabotype B profile. While some studies suggest that metabotype B may be associated with cardiovascular risk factors [16,32], currently there is no established link between metabotypes and cognitive health outcomes. Our findings suggest that individuals with this gut microbial profile might experience greater cognitive benefits from urolithins. With respect to UroB, its overall concentrations were low and exhibited only a modest increase after the intervention, which may be ascribed to the fact that UroB results from the microbial conversion of UroA and depends on a specific gut microbiota profile (metabotype B). Also, UroB was unrelated to cognitive changes. In the control group, slightly increased UroB concentrations related inversely to changes in the language domain; however, this association was lost when accounting for metabotypes. Taken together, these findings indicate that interindividual variation in gut microbiota composition may contribute to differential cognitive responses to (poly)phenol intake.

The effect sizes of the observed associations were modest, but consistent with prior evidence on urinary phenolic metabolites and cognitive outcomes in older populations [33]. These modest yet positive associations

may be partly explained by the characteristics of the study population, made up of cognitively healthy individuals. Nonetheless, subtle changes in cognitive performance might yield meaningful public health benefits when considered at the population level, particularly in the context of long-term and broadly implementable dietary strategies [34].

Overall, there is still limited clinical evidence on the potential benefit of (poly)phenol intake on cognitive function. While some observational studies and clinical trials suggest cognitive benefits of (poly)phenol-rich interventions, findings are inconclusive [6,7]. This may be attributed to the limited bioavailability of (poly)phenols, interindividual differences in gut microbiota composition [7], and the lack of mechanistic studies linking specific metabolites to cognitive outcomes. Prior clinical trials that examined the cognitive effects of ellagitannin-rich dietary interventions had mixed results [12–14,35], and the role of urolithins has been little investigated. In an 18-month trial of the effects of exposure to a Mediterranean diet enriched with walnuts and other (poly)phenol-rich foods on brain health outcomes, urinary UroA was associated with attenuated atrophy at the hippocampus, a subcortical brain structure essential for learning and memory [36]. This study focused on brain structure and did not assess the association of UroA with cognitive function. To our knowledge, our study is the first to specifically examine the association of gut microbiota-derived urolithins and cognitive performance. Our findings highlight the role of gut microbiota in generating bioactive metabolites that exert neuroprotective effects. They also support the use of phenolic metabolites as objective biomarkers of consumption of certain foods, offering a promising approach to investigate the relationship between dietary (poly)phenols and cognition.

Recent preclinical evidence supports the neuroprotective potential of gut microbiota-derived urolithins against neurodegenerative diseases [30,37]. Importantly, urolithins have been shown to cross the blood-brain barrier [38,39], enabling their activity in the central nervous system [18]. Key mechanisms involve antioxidant and anti-inflammatory effects, which mitigate oxidative stress by decreasing reactive oxygen species and lipid peroxidation, as demonstrated in neuronal cell models [21,40,41]. These activities are further supported by the suppression of neuroinflammation through the reduction of pro-inflammatory cytokines and nitric oxide production [20,29,42]. Furthermore, urolithins prevent the aggregation and fibrillation of β-amyloid plaques and tau proteins [39], which are pathological markers of Alzheimer's disease [43]. Additionally, their neuroprotective effects have been associated with the capacity to prevent neuronal apoptosis and enhance neurogenesis [22]. Collectively, these mechanisms contribute to the preservation of cognitive function and the attenuation of neurodegenerative disease progression.

Our study has limitations. First, participants were not blinded to the intervention, as it involved consumption of a whole food. Secondly, the WAHA cohort is made up of healthy older adults, therefore the results may not apply to younger individuals or older populations in poor health or with pre-existing cognitive impairment. Age and health status can impact urolithin metabolism, which may limit the generalizability of the findings. Finally, while this study was conducted within a randomized controlled trial, this pre-specified analysis is observational. Therefore, causality cannot be assumed, and there remains the possibility of residual

confounding. There are also strengths to our study. These include the randomized trial design with two geographically dissimilar centers and a large sample size, and the use of a comprehensive neuropsychological battery with composite scores, producing a robust assessment of cognitive changes. Additionally, the use of biological samples provided accurate insights into microbiome conditions, demonstrating that urinary urolithins predict walnut consumption and serve as biomarkers of ellagitannin-containing foods.

5. Conclusions

Urinary urolithins, particularly UroA and UroC in their glucuronidated forms, are reliable biomarkers of walnut consumption. Urolithin changes ensuing a walnut-rich diet for 2 years disclose a direct association with concurrent changes of cognitive function in older, cognitively healthy individuals. Further research is needed to explore biological mechanistic pathways between exposure to urolithins and brain health outcomes.

Conflict of interests

JS reports grants from the California Walnut Commission (CWC) (Folsom, CA) for the conduction of the WAHA study and, outside the submitted work, grants from the Almond Board of California, The Peanut Institute, Egg Nutrition Center and the Hass Avocado Board. AS-V has received research funding through his institutions from the CWC, as well support to attend professional meetings. ER reports grants from the CWC for the conduction of the WAHA study and, outside the submitted work, grants, personal fees, non-financial support and other from Alexion, and speaker fees for lecture presentation and manuscript writing from Sociedad Española de Arteriosclerosis and Fundación Dieta Mediterránea. RL-R reports personal fees from UNIDECO SA, and other contributions including speaking and lecture fees from Wine in Moderation, and funding from Idilia Foods, Oli Migjorn outside the submitted work. All other authors report no conflicts of interest.

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Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used ChatGPT to improve readability and language. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

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