Habitual nut exposure, assessed by dietary and multiple urinary metabolomic markers, and cognitive decline in older adults: the InCHIANTI study

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Abbreviations used: ROC, receiver operating characteristic; MMSE, Mini-Mental State Examination; InCHIANTI, Invecchiare in Chianti (Aging in Chianti); FFQ, food frequency questionnaire; NC, non-nut consumers; OSC-PLS-DA, partial least-squares discriminant analysis with orthogonal signal correction; RC, regular consumer; VIP, variable importance projection; ADL, activities of daily living; IADL, instrumental activities of daily living; CES-D, Center for Epidemiological Studies Depression Scale; IL-6, Interleukin-6; IL-1ra, Interleukin-1 receptor antagonist; hsCRP, high-sensitivity C-reactive protein; TNF-α, tumour necrosis factor-α

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ABSTRACT

Scope

We prospectively evaluated the association between self-reported dietary intake and urinary metabolomic markers of habitual nut exposure with cognitive decline over a 3-year follow-up in an older Italian population.

Methods and results

We selected 119 older participants, from the InCHIANTI cohort based on self referred nut intake: the non-nut consumer (n=72) and the regular nut consumer (≥2.9 g/d, n=47) groups. Nut exposure was measured at baseline either with the use of a validated food frequency questionnaire or with an HPLC-Q-ToF-MS metabolomic approach. Three years after, 28 from non-consumers group and 10 from consumers group experienced cognitive decline. Dietary nut exposure was characterized by urinary metabolites of polyphenols and fatty acids pathways. Nut consumption estimated either by the dietary marker or by the urinary marker model was in both cases associated with less cognitive decline (OR: 0.78, 95% CI: 0.61,0.99; $P = 0.043$ and OR: 0.995, 95% CI: 0.991,0.999; $P = 0.016$, respectively) with AUCs 73.2 (95% CI: 62.9,83.6) and 73.1 (62.5,83.7), respectively.

Conclusions

A high intake of nuts may protect older adults from cognitive decline. The use of a panel of metabolites provides accurate and complementary information of the nut exposure and reinforces the results obtained using dietary information.

1. Introduction

A decline in cognitive function is a core feature of dementia, which represents a growing health problem worldwide [1]. The World Health Organization has predicted that the total number of people with dementia will rise up to 115.4 million by 2050 [2]. Accordingly, identifying effective strategies for preventing dementia or slowing down its progression is becoming a major public health priority. Accumulating evidence suggests an association between healthy cognitive function and dietary factors, in particular high vegetable and fruit intake, low saturated and trans fat intake, high long-chain omega-3 fatty acid intake, and intake of vitamins E and B12 [3, 4]. Recent studies suggested that dietary intake of nuts is protective against cognitive decline [5]. Nuts contain large amounts of plant protein and unsaturated fatty acids, dietary fibre, vitamins (e.g., folic acid, niacin, tocopherols, vitamin B6), minerals (e.g., calcium, magnesium, potassium, zinc), and bioactive compounds such as phytosterols and phenolic compounds [6]. Its peculiar chemical composition is critical for providing their beneficial health effects. Indeed, epidemiological and clinical studies have associated exposure to nuts with a reduced risk of cardiovascular diseases, type 2 diabetes, cancer, metabolic syndrome and total mortality [7, 8]. Because of these data, nut intake is recommended in several dietary guidelines worldwide [9]. However, evidence that nut consumption is protective against cognitive decline is still scant [10].

In addition, a general problem of the studies that evaluated the protective role of nut dietary consumption on cognitive decline is that nut exposure was only based on the assessment of dietary questionnaires, which lack precision and tend to be biased [11]. In order to better evaluate the association between dietary exposure and health outcomes, an accurate and objective assessment of the exposure is needed. The use of food biomarkers may overcome these limitations. For example, untargeted metabolomic

approaches are progressively more used in nutritional studies, alone or in combination to self-reported dietary questionnaires [11].

The aim of this study is to investigate the association between dietary and urinary markers of habitual nut exposure with cognitive decline over a 3-year follow-up in a cohort of Italian participants aged 65 years and older in the InCHIANTI (*Invecchiare in Chianti* (Aging in Chianti)) study. For this propose, we measured the nut exposure either by dietary questionnaire or by applying an untargeted metabolomic approach. We characterized the food metabolome associated with nut intake, and developed a potential model of urinary biomarkers for assessing habitual nut exposure accurately in the InCHIANTI cohort.

2. Experimental Section

STUDY POPULATION

The InCHIANTI study is a population-based prospective cohort study located in two municipalities adjacent to the city of Florence (Italy), and it is described in detail elsewhere [12]. The study randomly sampled 1260 participants aged ≥ 65 years, of whom 1155 agreed to be enrolled. The participation rate was 91.7%. Of these, 82 participants (7.1%) with dementia at baseline, according to criteria set out in the Diagnostic and Statistical Manual of Mental Disorders (Fourth Edition), were excluded. A total of 609 dementia-free participants aged ≥ 65 years were selected based on the availability of baseline 24 h urine samples (collected in 1998–2000), as well as baseline and 3-year follow-up cognitive assessment (administered in 1998–2000 and 2001–2003, respectively) (**Supporting Information Figure 1**). Finally, 119 subjects were selected based on baseline nut intake (see dietary assessment section for details) and randomly selected for metabolomics analysis based on previous experience [13]. The Italian

National Institute of Research and Care of Aging Ethical Committee approved the study protocol, and all participants provided informed consent to participate.

DIETARY ASSESSMENT

Nut intake was assessed at baseline with the use of the Italian version of the food frequency questionnaire (FFQ) developed and validated in the European Prospective Study into Cancer and Nutrition (EPIC) [14]. This questionnaire includes one item regarding the consumption of walnuts, almonds, hazelnuts and peanuts with four categories for the frequency of consumption (never, rarely, times per week or per month). In the present study, total nut intake was converted to g/d. Regarding the nut intake of the selected participants, 72 patients never consumed nuts (0 g/d) (non consumers (NC)), and 47 patients were regular consumers (RC), i.e. participants with an intake of ≥2.9 g/d of nuts in the preceding year (nut serving size, 28 g). The groups NC and RC were frequency matched group by sex, age and smoking status (**Supporting Information Figure 1**). In the present study, sporadic consumers (between >0g/d and < 2.9 g/d of nut intake) were excluded to reduce "biological noise".

URINARY NUT METABOLOME ASSESSMENT

At baseline, a single 24 h urine sample was collected from each participant. Urine samples were divided into aliquots, coded and stored at -80 ºC until analysis. Sample preparation was based on previously published methodology and was applied for the metabolomic analysis [13, 15]. Briefly, the samples were centrifuged for 5 min at 12000 g. A 50 µL aliquot of the supernatant was mixed with an equal volume of Milli-Q water, and transferred into a 96-well plate for metabolomics analyses.

Metabolomic analyses were conducted using an HPLC-Q-ToF-MS [Agilent 1200 Series Rapid Resolution HPLC system coupled to a hybrid quadrupole TOF (Q-ToF) QSTAR Elite mass spectrometer (Applied Biosystems/MDS Sciex, Framingham, MA, USA)] in

negative ion mode due to the chemical characteristics of the metabolites (observed before in our previous articles [13, 15]) and following our previously published protocol [13]. 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 μL 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).

Data were extracted and aligned using MarkerView TM 1.2.1 (Applied Biosystems, MDS Sciex, Toronto, Ontario, Canada). The parameters used for the processing of raw data are listed in (**Supporting Information Table 1**. Before multivariate statistical analysis, mass feature data sets were log-transformed and Pareto-scaled using SIMCA-P +13.0 software (Umetrics, Umeå, Sweden). The mass features with coefficient of variation (CV) in urine samples greater than CV in pool of urine samples were removed to minimize the analytical variation. The mass features that were missing in at least 25% of samples from both groups were considered to be noise and they were excluded in the future analyses.

The differences in the urine metabolome between the groups of study (NC versus RC) were explored using partial least-squares discriminant analysis with orthogonal signal correction (OSC-PLS-DA) (Supporting Information Figure 3 [16]. The quality of the models was evaluated through 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). The validation of the models was evaluated with the permutation test (n=200) (20). As a final quality test, the whole data set was randomly split into four equal-size subsamples (25% of the sample each), three of which were used as the training set while the remaining one was used as the validation set. This process was repeated four times, each subsample being used as the validation set at least

once, and the correctly classified participants in each validation set (%) were calculated (**Supporting Information Tables 2**). Those mass features with the highest variable importance projection (VIP) values in the RC group (cut-off ≥ 2) were selected as the most relevant to explain the differences in urinary metabolomics profiles associated with nut intake. After that, a multistep procedure was used to annotate putative urinary markers of nut intake from the selected mass features [15]. Putatively annotated compounds were carried out by matching mass features with mass spectral databases (Human Metabolome Database [17], Metlin [18], MetFrag [19]) with a mass error tolerance of ±10 mDa (assigning a level 2 of the evidence in the identification in accordance with Metabolomics Standards Initiative criteria [20]).

COGNITIVE FUNCTION ASSESSMENT

Cognitive function was measured using the Mini-Mental State Examination (MMSE), which was administered at baseline and at 3-year follow-up. The MMSE is a validated method for assessing global cognitive function, and is widely used in both clinical practice and research [21, 22]. It evaluates five areas of cognitive functioning: orientation, registration, attention and calculation, recall, and language abilities. Overall scores range from 0 to 30, with high scores indicating better cognitive functioning (continuous variable).

To calculate change in cognitive function, MMSE scores at baseline were subtracted from MMSE scores at 3-year follow-up. Thus, the subjects were divided into those with cognitive decline, defined as a decrease of two points or more from baseline assessment to 3-year follow-up [21, 23] and those without it (dichotomous variable).

OTHER BASELINE COVARIATE ASSESSMENT

Trained geriatricians conducted a comprehensive assessment of health, functional status and anthropometric measures using standardized protocols [12]. Dietary intake of total

energy (kcal/d) and alcohol (g/d) in the previous year were estimated using the FFQ [14] and an Italian food composition table [24]. Physical activity in the previous year was self-reported and was classified as [25]: 1) sedentary (completely inactive or light-intensity physical activity, e.g. walking), 2) light (light-intensity physical activity for 2 to 4 h/wk), and 3) moderate to intense (light-intensity physical activity of at least > 4 h/wk or moderate-intensity physical activity of at least 1–2 h/wk). Smoking status (current, former or never-smoker), age, sex, BMI and education (years of schooling) were reported or calculated. Functional status was assessed using Katz's Activities of Daily Living (ADL) [26], and the Lawton and Brody Instrumental Activities of Daily Living (IADL) [27] scales. Depressive symptoms were evaluated using the Center for Epidemiologic Studies Depression Scale (CES-D). A CES-D score of ≥16 was defined as a depressed mood [28]. Diseases were ascertained by combining information from self-reported physician diagnoses, pharmacological treatments, medical history, clinical examinations and blood tests. Diseases considered in this analysis were renal impairment, diabetes, cardiovascular diseases (myocardial infarction, angina pectoris and peripheral arterial disease), hypertension and stroke [29]. Inflammatory markers were measured in serum samples. Interleukin (IL)-6 and IL-1 receptor antagonist (IL-1ra) were measured by high-sensitivity enzyme-linked immunoabsorbent assays (ELISAs) using commercial kits (BIOSOURCE International Inc., Camarillo, CA). The high-sensitivity C-reactive protein (hsCRP) was measured by an ELISA colorimetric competitive immunoassay that used purified protein and polyclonal anti-CRP antibodies. TNF-α was measured using multiplex technology (Human Serum Adipokine Panel B LINCOplex kit; Linco Research, Inc., St. Charles, MO) [12].

STATISTICAL ANALYSIS

Descriptive analyses were performed to summarize information about the baseline characteristics of the study population. Differences between nut exposure groups, as well as differences between groups of cognitive function and differences between included and excluded participants groups, were tested by using a Student's t-test, Mann-Whitney test or Chi-square test.

Nut exposure was assessed using either dietary or urinary markers. To explore the relationships between dietary and urinary markers of nut intake, Spearman's rank correlation coefficients were used.

Multimetabolite prediction biomarker model of nut exposure

To design multimetabolite biomarker panels associated with habitual dietary nut exposure, Tobit models [30], a class of censored regression model designed to mitigate the problem of zero-inflated data, were conducted to estimate β coefficients and their SEE with the identified urinary markers. The continuous variable on dietary marker of nut intake was used as dependent variable, where the threshold was a value of 0 g/d. Two full and two reduced multimetabolite biomarker panels related to nut intake were proposed using identified urinary metabolites. The combination of individual biomarkers' performance was evaluated using ROC curves. The likelihood ratio test was used for comparing the goodness of fit between the models.

Associations between nut exposure and cognitive decline

Associations between nut exposure, dietary and urinary markers (individuals and panels) and cognitive decline were analysed using two different but complementary statistical approaches: 1) associations with a change in cognitive function (continuous variable) over 3 years of follow-up, which were evaluated by estimating the standardized β coefficients and their 95% CI in linear regression models; and 2) associations with a decline in cognitive function (dichotomous variable) over 3 years of

follow-up, which were analysed by estimating the OR and its 95% CI in logistic regression models. Covariates in these statistical models were identified a priori as known risk factors or potential confounders. Four separate statistical models were performed: Model 1, adjusted for the baseline cognitive score (in order to correct for the "regression toward the mean"), for sex and age; Model 2, additionally adjusted for BMI, energy intake (except for urinary markers), alcohol consumption, education, physical activity and smoking status; and Model 3, additionally adjusted for depressive symptoms, stroke, cardiovascular diseases, hypertension and diabetes. Interactions with sex, age, BMI, education, smoking status and physical activity were evaluated between dietary nut marker and change in cognitive function by including product terms in the fully adjusted model. Moreover, the global performance of the associations between dietary and urinary markers of nut intake, adjusted for covariates (Model 3), and decline in cognitive function was evaluated using ROC curves and estimating the AUC (95% CI).

All *P* values presented are two-tailed and were considered to be statistically significant when $P<0.05$. Data from metabolomic and epidemiologic analyses were analysed using R software version 3.2.4 (http://www.r-project.org) and the SPSS package program version 21.0 (SPSS, Chicago, IL).

3. Results

Descriptive study and dietary markers of nut exposure

The baseline characteristics of the study population according to groups of dietary nut intake are presented in **Table 1**. There were 72 NC (0 g/d) and 47 RC (≥2.9 g/d). This study included 57 women and 62 men, with a mean age of 73 years. Participants in the RC group had a lower prevalence of disability in ADL and had a smaller decline

(change) in cognitive function than those in the NC group. Of the 119 participants, 38 had cognitive decline (**Supporting Information Table 3**).

Urinary metabolomic markers assessment

The results of the data acquisition quality and the quality of the models of metabolic profiling differentiation in the study are included in the **Supporting Information Figure 2 and 3)**.

A total of 18 urinary metabolites were identified or tentatively identified as discriminatory urinary metabolites related to dietary nut exposure including: 1) markers of the polyphenol microbial metabolism (urolithin A (glucuronide, sulphate and sluphoglucuronide), urolithin B (glucuronide), hydroxyhippuric acid, hydroxyphenylacetic acid); 2) a marker of the polyphenol intestinal metabolism (resveratrol-sulphate); 3) markers of the fatty acid metabolism (dodecanedioic and dimethylglutaric acids); 4) markers of the tryptophan metabolism (indole-3-acetic acid glucuronide and indoxyl sulphate / indoxylsulphuric acid); 5) a marker of the benzoxazinoid biosynthesis (dihydroxy-benzoxazinone) and 6) four unidentified markers (**Table 2**). According to the FFQ, there were statistically significant differences in wine consumption ($P = 0.011$) between the groups of dietary nut intake. Although among nuts, peanuts can also contain small amounts of resveratrol; it was assumed that resveratrol-sulphate is a biomarker of wine more than nut consumption [31]. Therefore, resveratrol-suphate metabolite was excluded from the subsequent analyses

In order to improve the discrimination between the two groups (NC and RC), two full multimetabolite biomarker panels of nut intake were evaluated using Tobit models to estimate β coefficients and their SEE: Model A) all 13 known identified urinary metabolites; and Model B) a selection of 7 identified urinary metabolite biomarkers (urolithin A and its three conjugates, urolithin B and its glucuronide, and dodecanedioic

acid), which were previously associated with nut exposure as well (11, 12, 34). Two reduced biomarker panels were also evaluated: Model C) a reduced panel with the 4 metabolite urinary biomarkers from Model A with the highest β coefficient (urolithin A glucuronide, hydroxyhippuric acid, hydroxyphenylacetic acid and dimethylglutaric acid); and Model D) a reduced panel with 2 metabolite biomarkers from Model B (urolithin A and its glucuronide) (**Table 3**). The reduced models were not statistically different from the full models in a likelihood ratio test (Models A and C: P =0.30; Models B and D: P = 0.35). Furthermore, the panels for Models A, B, C and D displayed 80.9, 74.5, 83.0 and 70.2 % sensitivity and 87.5, 69.4, 84.7 and 70.8 % specificity, with an AUC of 90.7, 76.7, 93.3 and 78.2 % (all P<0.001), respectively (**Supporting Information Figure 4**).

Association between habitual nut exposure and cognitive function

The associations between dietary and urinary (individual and combined) markers of nut intake and the change or the decline in the MMSE score at 3-year follow-up are reported in **Table 4**. In the fully adjusted linear regression models (Model 3), participants who reported a higher amount of nut intake in the FFQ (1 SD of difference) had better cognitive function (β: 0.25; 95% CI: 0.04, 0.46; P = 0.018). No statistically significant interactions were found between dietary nut intake and sex (P = 0.70), age (P = 0.66), BMI (P = 0.67), education (P = 0.47), smoking status (P = 0.40) and physical activity (P = 0.74) in relation to change in cognitive function (data not tabulated). Moreover, a statistically significant inverse association between nut intake and cognitive decline risk (OR: 0.78; 95% CI: 0.61, 0.99; P = 0.043) was observed. Regarding the urinary markers, participants who excreted a higher amount of urolithin A and its three conjugates (urolithin A glucuronide, urolithin A sulphate and urolithin A sulphoglucuronide), urolithin B and its glucuronide, dodecanedioic acid,

dimethylglutaric acid and dihydroxy--benzoxazinonealso had better cognitive function. Similar association between hydroxyphenylacetic acid and change in MMSE score was also observed, although the result was not statistically significant in the fully adjusted model. In addition, the four multimetabolite urinary biomarker panels of nut intake were inversely associated with the change in cognition. In the fully adjusted logistic regression models (Model 3), urolithin A, urolithin A sulphate, urolithin B, urolithin B glucuronide, hydroxyphenylacetic acid, dimethylglutaric acid and dihydroxy-benzoxazinone levels were inversely associated with cognitive decline. No statistically significant associations between urolithin A glucuronide, urolithin A sulphoglucuronide and dodecanedioic acid levels and cognitive decline were observed. In the fully adjusted model, multimetabolite urinary biomarker panels of nut intake (Models A, B and C), except for Model D, were also associated with cognitive decline (**Table 4**).

The ROC curves and the corresponding AUC (95% CI), as well as the calculation of the sensitivity and specificity of dietary and urinary (individual and combined) markers of nut intake for the prediction of cognitive decline adjusted for all the potential factors, are shown in **Figure 1** and (**Supporting Information Table 4)**. The AUC for the dietary marker and multimetabolite urinary biomarker models (Models A, B, C and D) was 73.2, 74.8, 72.8, 73.1 and 71.3, respectively, and all were statistically significant ($P < 0.001$). All sensitivity and specificity values from these models were higher than 60 and 70 %, respectively (**Supporting Information Table 4)**.

4. Discussion

Our prospective study in non-demented older subjects shows that high intake of nuts was associated with better cognitive function and a lower risk of cognitive decline over a 3-year follow-up. To our knowledge, this is the first study using both FFQ and

untargeted metabolomics data to investigate the association between nut intake and cognitive decline risk. In addition, our results support that the use of multiple nut biomarkers in epidemiological studies may provide a more accurate assessment of the nut exposure than the use of individual biomarkers and therefore a proper estimation of the associations with health outcomes.

We found an association between high intake of nuts and lower cognitive decline risk whether the nuts exposure is measured based on the traditional FFQ or using a multimetabolite biomarker model. In line with previous prospective studies, nuts consumption was associated with a better cognitive function [32] and with a lower global cognitive decline [33]. Evidence focused on nut exposure alone is limited, but several studies have been conducted on dietary patterns including nuts as a key component. The Mediterranean diet supplemented with nuts was associated with improved cognitive function [34]. In a previous InCHIANTI study, a polyphenol-rich diet intake was related to a lower cognitive decline [35]. The mechanism by which nut intake protects cognitive function during aging is not clearly defined. Oxidative stress, inflammation and reduced cerebral blood flow have been considered to be important mechanisms leading to cognitive decline in older subjects [36]. Thus, nuts, alone or as part of healthy dietary patterns, may exert beneficial effects against the development of cognitive decline due to their high concentrations of antioxidants [37], including polyphenols (e.g. proanthocyanidins in almonds and hazelnuts; ellagitannins in walnuts and hazelnuts), MUFA (e.g. oleic acid in almonds and hazelnuts) [38], PUFA (e.g. α-linolenic and linoleic acid in walnuts) and vitamins [10].

Taking into consideration the whole composition of nuts, a multibiomarker approach to assess overall consumption may be more accurate for predicting nut exposure than the use of a single one [11], as previously described by our group [13, 15].

In the present study, urinary multimetabolite biomarker models of nut exposure (mainly Model C composed by Urolithin A glucuronide, hydroxyhippuric acid, hydroxyphenylacetic acid and dimethylglutaric acid) presented a greater predictive ability as a biomarker of nut intake, than individual markers. The discriminative capacity of Model C, as well as of urolithin A glucuronide, to classify subjects according to cognitive decline was observed. These may be valuable dietary biomarkers of cognitive decline, because of the potential role of polyphenols and polyunsaturated fatty acids in reducing oxidative stress and inflammation [10]. However, no statistically significant differences across nut groups in relation to inflammatory markers were observed (data not tabulated).

Urolithin A was the most discriminant phenolic marker also observed elsewhere [15, 39, 40]. Urolithins are gut microbiota products from ellagic acid and ellagitannins. In the lower gastrointestinal tract, these compounds are converted into urolithins, which are absorbed and metabolized to finally circulate in blood reaching different tissues prior to excretion [41]. Hence, these results represent an important step ahead in the validation of these compounds as biomarkers of nut exposure. Recently, three urolithin phenotypes were observed [42]. A higher percentage of "phenotype B", which produced isourolithin A and/or urolithin B in addition to urolithin A, was observed in those participants with chronic disease associated with microbial imbalance. In this study, the prevalence of "Phenotype A" (only urolithin A conjugates excreted), "Phenotype B" and "Phenotype 0" (no detected urolithins) with cognitive decline was 20, 20 and 60 %, respectively ($P = 0.78$). This may be due to the low sample size ($n = 47$, loss due to phenotypic variation) (data not tabulated). Therefore, further studies are needed to evaluate whether this phenotypic variation could be a biomarker associated with cognitive decline.

The presence of other microbial-derived metabolites including flavan-3-ols (2-hydroxyphenylacetic acid) and procyanidins (hydroxyhippuric acid), from walnuts [13, 39, 43, 44] and almonds [45], respectively and markers of the tryptophan metabolism such as indole-3-acetic-acid-*O*-glucuronide and indoxyl sulphate/indoxylsulphuric acid, mainly from the consume of walnuts [46] highlight again an interplay between nut intake, gut microbiota and cognitive decline. Currently, the brain-gut-microbiome connection is a hot research topic. Nuts, which are rich in fibre, omega-3 fatty acids and polyphenols, increase healthy gut microbiota and may improve cognition [47–49]. For example, the excretion of urinary indoles reflects a variation in gut microbiota composition in relation to their role in inhibition or promotion of the growth of specific bacterial species, also observed in a number of diseases states [46].

Generally, evidence concerning nut exposure and cognition relies on the measurement of nut exposure using mainly self-reported dietary questionnaires. Consequently, studies using biomarkers of nut exposure are needed to confirm these potential protective effects. Therefore, the use of a metabolomic approach to identify and validate proper and predictive nutritional biomarkers is now highly promising [11].

The main strengths of this study were its longitudinal design and the assessment of habitual nut exposure with the use of a nut-derived metabolites panel as a nutritional biomarker. The combination of different metabolites as a nutritional biomarker provided a more accurate estimation than that provided by only a single biomarker [15]. Another strength was the use of a validated method assessment test to evaluate cognitive decline [21, 22]. Finally, our models were adjusted for the most important confounding variables related to nut exposure and cognitive decline; however, possible residual confounding cannot be excluded.

Nevertheless, this study had some limitations. First, it is important to bear in mind that no type specification of nut consumption was discerned in the administered FFQ, although the percentage consumption of individual tree nuts and peanuts consumed as a whole in Italy (EPIC study) was approximately 60 (where walnuts are more consumed than almonds and hazelnuts) and 32 %, respectively [50]. In addition, the mean ± SD intake of nuts in their study (n = 3961; 1.7 ± 0.2 g/d) was similar to that in our study (n = 119; 1.9 ± 3.2 g/d). However, the present study sample might not be representative of nut consumption in general Italian population, because InCHIANTI was performed in community-dwelling older subjects living in two sites in Tuscany (Italy). Second, the present study population was aged ≥ 65 and therefore may be less accurate in recalling food intake, although demented participants were excluded. Finally, because biomarker assessment was performed only once at baseline, it may not necessarily reflect the participants' long-term nut consumption. Although this is to be expected with aging [51], there was a decrease in the nut intake during the follow-up, which was 1.9 and 0.9 g/d at baseline and at the 3-year follow-up visit, respectively (data not tabulated). Their r was 0.46 (P < 0.001).

In conclusion, this study showed that a higher habitual exposure to nuts was associated with a lower risk of cognitive decline in a cohort of older individuals. Moreover, this study opens up a large area of research with more reliable and accurate tools for identifying and validating new biomarkers of nut exposure and evaluating their association with cognitive decline.

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Author contributions

LF, SB, AC, and CA-L designed the research; MR conducted the research; ST and MP-R performed sample analysis; MR, MP-R, AM performed statistical analysis; MR MP-R, RZ-R wrote the paper; ST, SB, LF, AC and CA-L provided critical revision, and CA-L had primary responsibility for the final content. All authors read and approved the final manuscript.

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Conflict of Interest

The authors have declared no conflict of interest.

Figure 1. ROC curves of the associations between nut exposure and cognitive decline.

Table 1. Baseline characteristics of the study population according to groups of habitual dietary nut intake[1]

	Total	**Non-consumers**	**Regular Consumers**	***P***
Participants, n (%)	119	72 (60.5)	47 (39.5)	–
Nut, g/d	1.9 ± 3.2[2]	0.0 ± 0.0	4.9 ± 3.3	<0.001
Energy, kcal/d	2014 ± 589	1804 ± 541	2337 ± 511	<0.001
Alcohol, g/d	17.3 ± 22.9	14.0 ± 18.7	22.3 ± 27.7	0.054
Demographics				
Sex, F, n (%)	57 (47.9)	36 (50.0)	21 (44.7)	0.57
Age, y	73.0 ± 5.9	73.5 ± 6.2	72.2 ± 5.2	0.23
BMI, kg/m²	27.5 ± 3.8	27.1 ± 3.7	27.9 ± 3.8	0.27
Physical Activity, n (%)				0.53
Sedentary	18 (15.1)	9 (12.5)	9 (19.1)	
Light	55 (46.2)	33 (45.8)	22 (46.8)	
Moderate to high	46 (38.7)	30 (41.7)	16 (34.0)	

Smoking status, n (%)				0.18
Never	51 (42.9)	26 (36.1)	25 (53.2)	
Current	35 (29.4)	24 (33.3)	11 (23.4)	
Former	33 (27.7)	22 (30.6)	11 (23.4)	
Education (years of school)	6.0 ± 3.5	5.9 ± 3.7	6.1 ± 4.2	0.84
Disability in ≥1 ADL, n (%)	6 (5.0)	6 (8.3)	0 (0.0)	0.042
Disability in ≥1 IADL, n (%)	14 (11.8)	8 (11.1)	6 (12.8)	0.78
Inflammatory markers				
IL-6, pg/mL	2.8 (1.9–3.9)[3]	2.4 (1.8–3.8)	3.0 (2.2–4.9)	0.11
TNF-α, pg/mL	4.4 (2.9–5.6)	4.2 (2.8–5.4)	4.8 (3.6–5.9)	0.30
hsCRP, μg/mL	2.7 (1.5–4.8)	2.5 (1.4–5.0)	3.0 (1.5–4.8)	0.36
IL-1ra, pg/mL	121.3 (93.4–187.1)	120.0 (84.7–193.9)	125.8 (93.8–178.5)	0.99
Diseases and conditions				
Renal impairment, n (%)	67 (57.8)	43 (61.4)	24 (52.2)	0.32
Diabetes, n (%)	12 (10.1)	9 (12.5)	3 (6.4)	0.28

Cardiovascular disease, n (%)	26 (21.8)	12 (16.7)	14 (29.8)	0.09
Hypertension, n (%)	62 (52.1)	38 (52.8)	24 (51.1)	0.86
Stroke, n (%)	11 (9.2)	7 (9.7)	4 (8.5)	0.82
Depressive symptoms, CES-D score ≥16, n (%)	33 (27.7)	19 (26.4)	14 (29.8)	0.69
Cognitive function				
Subjects with cognitive decline, n (%)	38 (31.9)	28 (38.0)	10 (21.0)	0.04
Baseline global cognitive function, MMSE score	25.7 ± 2.5	24.6 ± 4.4	25.7 ± 2.7	0.87
Global cognitive function change	-0.5 ± 3.4	-1.1 ± 3.7	0.4 ± 2.6	0.012

[1]Descriptive analyses were compared between groups of nut exposure with the use of a Student's t-test, Mann-Whitney test or Chi-square test as appropriate. No missing participants. ADL, Activities of Daily Living; CES-D, Center for Epidemiologic Studies Depression scale; hsCRP, high-sensitivity C-Reactive Protein; IADL, Instrumental Activities of Daily Living; IL-1ra, Interleukin-1 receptor antagonist; IL-6, Interleukin-6; MMSE, Mini-Mental State Examination; TNF-α, Tumors Necrosis Factor-α.

[2]Mean ± SD (all such values).

[3]Median (IQR) (all such values).

Table 2. Urinary metabolites tentatively identified as potential biomarkers of nut intake[1]

N. Cluster	RT (min)	Detected mass (m/z)	Theoretical mass (m/z)	Error (mDa)	Assignation	Potential biomarker	VIP
Markers of the polyphenol microbial metabolism							
1	6.45	227.0327	227.0350	2.3	$[M - H]^-$	Urolithin A	3.75
		228.0365	228.0384	1.9	$^{13}C[M - H]^-$		2.56
2	5.38	403.0626	403.0671	4.5	$[M - H]^-$	Urolithin A glucuronide	4.78
		404.0663	404.0705	4.1	$^{13}C[M - H]^-$		4.38
		227.0329	227.0350	2.1	$[M - H - glucuronide]^-$		3.43
3	6.64	306.9884	306.9918	3.4	$[M - H]^-$	Urolithin A sulphate	3.96
4	5.23	483.0187	483.0239	5.2	$[M - H]^-$	Urolithin A sulphoglucuronide	3.01
5	6.80	211.0387	211.0401	1.4	$[M - H]^-$	Urolithin B	2.00
6	6.30	387.0650	387.0721	7.1	$[M - H]^-$	Urolithin B glucuronide	2.44
	-	388.0708	388.0755	4.7	$^{13}C[M - H]^-$		2.23
-	-	211.0386	211.0401	1,5	$[M - H - glucuronide]^-$	-	-
7	5.00	151.0579	151.0594	1.5	$^{13}C[M - H - COO]^-$	Hydroxyhippuric acid	2.66
-	-	150.0529	150.0560	3,1	[M – H – COO]-	-	-
-	4.98	194.0367	194.0459	9,2	[M – H]-	-	-
8	4.50	151.0383	151.0401	1.8	$[M - H]^-$	2-Hydroxyphenylacetic acid	3.59
Markers of the polyphenol intestinal metabolism							
9	6.95	307.0265	307.0282	1.7	$[M - H]^-$	Resveratrol-sulphate	2.99
-	-	308.0285	308.0316	3.1	$^{13}C[M - H]$-	-	-

		227.0702	227.0714	1.2	[M – H – sulphate]$^{-}$		2.72
-	-	228.0732	228.0748	1.6	^{13}C[M – H – sulphate]$^{-}$	-	-
Markers of the fatty acid metabolism							
10	6.77	229.1429	229.1445	1.6	[M – H]$^{-}$	Dodecanedioic acid	2.15
		230.1468	230.1479	1.1	^{13}C[M – H]$^{-}$		2.06
11	4.07	160.0700	160.0697	0.3	^{13}C[M – H]$^{-}$	Dimethylglutaric acid	2.70
-	-	159.0664	159.0663	0.1	[M – H]-	-	-
		115.0769	115.0764	0.5	[M – H – COO]$^{-}$		2.09
-	-	97.0657	97.0659	0.2	[M – H – COO – H2O]-	-	-
Markers of the tryptophan metabolism							
12	5.28	175.0265	175.0594	0.3	^{13}C[M – H – glucuronide]$^{-}$	Indole-3-acetic acid glucuronide	2.08
-	-	174.0569	174.0560	0.9	[M – H – glucuronide]-	-	-
-	-	351.0911	351.0915	0.4	^{13}C[M – H]-	-	-
-	-	350.0878	350.0881	0.3	[M – H]-	-	-
13	4.55	212.0018	212.0023	0.5	[M – H]$^{-}$	Indoxyl sulphate / Indoxylsulphuric acid	2.58
		213.0048	213.0057	0.9	^{13}C[M – H]$^{-}$		2.05
		132.0448	132.0455	0.7	[M – H – sulphate]$^{-}$		2.13
-	-	425.0117	425.0119	0.2	[2M– H]$^{-}$	-	-
Markers of the benzoxazinoid biosynthesis							
14	4.93	180.0373	180.0302	7.1	[M – H]$^{-}$	Dihydroxy-benzoxazinone	3.14
Unidentified markers							
15	6.70	309.0409			[M – H]$^{-}$	Unknown A (sulphate derivative)	3.19

		310.0437			$^{13}C[M-H]^-$		2.27
		311.0381			$2^{13}C[M-H]^-$		2.48
16	6.15	359.1329			$[M-H]^-$	Unknown B (glucuronide derivative)	2.04
-	-	183.1024	-	-	[M – H – glucuronide]-	-	-
17	6.54	397.1102			$[M-H]^-$	Unknown C	2.66
-	-	398.1141	398,1136	0,5	$^{13}C[M-H]$-	-	-
18	6.22	373.1150			$[M-H]^-$	Unknown D	2.25
		374.1126			$^{13}C[M-H]^-$		2.34

[1]One-class OSC-PLS-DA model (NC versus RC) was used. NC, non-nut consumers; RT, retention time; VIP, variable importance projection; RC, regular nut consumers. Most important MS/MS fragments that were used in the identification fo the metabolites with VIP <2 are also included in the table.

Table 3. Multimetabolite prediction biomarker models associated with habitual nut intake[a]

Model	Dietary nut exposure urinary biomarker	β ± SEE	*P*	Model	Dietary nut exposure biomarker	β ± SEE	*P*
A	Urolithin A	-0.033 ± 1.196	0.98	**C**	Urolithin A glucuronide	2.905 ± 0.676	<0.001
	Urolithin A glucuronide	2.193 ± 1.032	0.034		Hydroxyhippuric acid	3.436 ± 0.894	<0.001
	Urolithin A sulphate	0.088 ± 1.332	0.95		Hydroxyphenylacetic acid	3.263 ± 0.763	<0.001
	Urolithin A sulphoglucuronide	0.011 ± 1.242	0.99		Dimethylglutaric acid	3.452 ± 1.124	<0.001
	Urolithin B	0.240 ± 1.392	0.86				
	Urolithin B glucuronide	0.670 ± 1.212	0.58				
	Hydroxyhippuric acid	3.453± 0.971	<0.001				
	Hydroxyphenylacetic acid	2.887 ± 0.795	<0.001				
	Dodecanedioic acid	1.102 ± 0.850	0.19				
	Dimethylglutaric acid	2.339 ± 1.220	0.055				
	Indole-3-acetic-acid-O-glucuronide	1.834± 0.965	0.057				
	Indoxyl sulphate/Indoxylsulphuric	0.688 ± 0.564	0.22				

	acid						
	Dihydroxy-benzoxazinone	-0.186 ± 0.881	0.83				
B	Urolithin A	1.380 ± 1.319	0.29	**D**	Urolithin A	1.899 ± 0.914	0.038
	Urolithin A glucuronide	3.023 ± 1.191	0.011		Urolithin A glucuronide	2.578 ± 0.921	0.005
	Urolithin A sulphate	1.811 ± 1.381	0.19				
	Urolithin A sulphoglucuronide	-2.163 ± 1.423	0.13				
	Urolithin B	-0.917 ± 1.570	0.56				
	Urolithin B glucuronide	0.791 ± 1.368	0.56				
	Dodecanedioic acid	1.245 ± 0.895	0.16				

[a]Tobit models were used.

Table 4. Associations between nut exposure and cognitive decline[a]

Nut exposure	Change in cognitive function						Decline in cognitive function					
	Model 1		Model 2		Model 3		Model 1		Model 2		Model 3	
	β (95% CI)	*P*-value	β (95% CI)	*P*-value	β (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
Dietary marker												
Nut intake	0.19 (0.00, 0.38)	0.046	0.25 (0.05, 0.46)	0.014	0.25 (0.04, 0.46)	0.018	0.87 (0.73, 1.04)	0.12	0.79 (0.63, 1.00)	0.05	0.78 (0.61, 0.99)	0.043
Urinary markers												
Urolithin A	0.86 (0.10, 1.61)	0.026	1.00 (0.24, 1.76)	0.010	0.85 (0.04, 1.66)	0.040	0.41 (0.19, 0.90)	0.027	0.37 (0.16, 0.82)	0.015	0.37 (0.15, 0.88)	0.024
Urolithin A glucuronide	0.64 (-0.04,	0.06	0.90 (0.20,	0.013	0.86 (0.15, 1.56)	0.018	0.74 (0.45, 1.20)	0.22	0.68 (0.40,	0.16	0.69 (0.40,	0.18

	1.33)		1.60)						1.16)		1.19)	
Urolithin A sulphate	0.96 (0.17, 1.75)	0.017	1.21 (0.40, 2.01)	0.004	1.14 (0.32, 1.95)	0.007	0.52 (0.27, 1.01)	0.05	0.41 (0.19, 0.86)	0.019	0.43 (0.20, 0.91)	0.028
Urolithin A sulphoglucuronide	0.64 (-0.12, 1.41)	0.10	0.97 (0.18, 1.76)	0.017	0.86 (0.05, 1.68)	0.039	0.69 (0.38, 1.25)	0.23	0.54 (0.27, 1.08)	0.08	0.57 (0.28, 1.16)	0.12
Urolithin B	1.23 (0.43, 2.04)	0.003	1.33 (0.47, 2.19)	0.003	1.28 (0.37, 2.20)	0.007	0.48 (0.23, 1.01)	0.05	0.38 (0.16, 0.89)	0.027	0.38 (0.15, 0.92)	0.032
Urolithin B glucuronide	0.92 (0.24, 1.61)	0.009	0.94 (0.20, 1.68)	0.014	0.88 (0.11, 1.65)	0.026	0.56 (0.32, 0.98)	0.041	0.50 (0.26, 0.95)	0.036	0.49 (0.25, 0.96)	0.037
Hydroxyhippuric acid	-0.24 (-1.21, 0.73)	0.62	-0.13 (-1.15, 0.90)	0.81	0.11 (-0.98, 1.20)	0.84	0.98 (0.52, 1.87)	0.96	0.85 (0.42, 1.72)	0.65	0.66 (0.30, 1.45)	0.30

Hydroxyphenylacetic acid	0.74 (-0.05, 1.53)	0.06	0.65 (-0.14, 1.45)	0.11	0.67 (-0.14, 1.49)	0.10	0.46 (0.26, 0.83)	0.010	0.47 (0.25, 0.86)	0.015	0.45 (0.24, 0.87)	0.017
Dodecanedioic acid	0.83 (-0.00, 1.66)	0.05	1.17 (0.30, 2.04)	0.009	1.00 (0.08, 1.93)	0.033	0.69 (0.38, 1.23)	0.21	0.48 (0.25, 0.95)	0.035	0.51 (0.25, 1.02)	0.06
Dimethylglutaric acid	1.50 (0.34, 2.66)	0.011	1.75 (0.57, 2.94)	0.004	1.77 (0.56, 2.98)	0.005	0.38 (0.16, 0.94)	0.036	0.29 (0.11, 0.78)	0.014	0.29 (0.10, 0.79)	0.016
Indole-3-acetic acid-*O*-glucuronide	-0.39 (-1.30, 0.53)	0.40	-0.55 (-1.47, 0.37)	0.24	-0.60 (-1.52, 0.32)	0.20	1.43 (0.76, 2.67)	0.27	1.52 (0.79, 2.92)	0.21	1.60 (0.81, 3.15)	0.17
Indoxyl sulphate	0.19 (-0.49, 0.87)	0.59	0.22 (-0.50, 0.94)	0.55	0.26 (-0.46, 0.98)	0.48	0.98 (0.62, 1.54)	0.92	0.88 (0.52, 1.47)	0.62	0.85 (0.50, 1.45)	0.56
Dihydroxy--	0.61	0.20	0.91	0.06	1.12	0.027	0.46	0.033	0.39	0.017	0.30	0.009

benzoxazinone	(-0.32, 1.54)		(-0.05, 1.87)		(0.13, 2.10)		(0.23, 0.94)		(0.18, 0.84)		(0.12, 0.74)	
Multimetabolite urinary biomarker panels[b]												
Model A	0.004 (0.001, 0.007)	0.004	0.006 (0.003, 0.008)	<0.001	0.005 (0.002, 0.007)	0.002	0.997 (0.995, 0.999)	0.010	0.996 (0.994, 0.999)	0.002	0.996 (0.994, 0.999)	0.003
Model C	0.004 (-0.001, 0.009)	0.11	0.006 (0.001, 0.011)	0.029	0.006 (0.001, 0.011)	0.029	0.997 (0.993, 1.00)	0.056	0.996 (0.992, 1.00)	0.028	0.995 (0.991, 0.999)	0.016
Model B	0.005 (0.002, 0.009)	0.003	0.007 (0.003, 0.011)	<0.001	0.006 (0.002, 0.010)	0.003	0.997 (0.995, 1.00)	0.026	0.996 (0.993, 0.999)	0.008	0.996 (0.993, 0.999)	0.013
Model D	0.011 (0.001, 0.021)	0.028	0.015 (0.005, 0.025)	0.004	0.012 (0.002, 0.022)	0.022	0.994 (0.987, 1.00)	0.07	0.992 (0.985, 1.00)	0.047	0.993 (0.985, 1.00)	0.07

[a]Linear and logistic regression models were used, and the following three separate models are presented: model 1, which was adjusted for baseline score of cognitive function, sex and age; model 2, which was further adjusted for BMI, energy intake (only diet), alcohol consumption, education, physical activity and smoking status; and model 3, which was further adjusted for depressive symptoms, stroke, cardiovascular disease, hypertension and diabetes.

[b] Multimetabolite urinary biomarkers models evaluated using Tobit models as follows: Model A) all 13 known identified urinary metabolite; Model B) a selection of 7 identified urinary metabolite biomarkers (urolithin A and its three conjugates, urolithin B and its glucuronide, and dodecanedioic acid; Model C) a reduced panel with 4 metabolite biomarkers from Model A (urolithin A glucuronide, hydroxyhippuric acid, hydroxyphenylacetic acid and dimethylglutaric acid); and Model D) a reduced panel with 2 metabolite biomarkers from Model B (urolithin A and its glucuronide).