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Original Article

Plasmatic Hippuric Acid as a Hallmark of Frailty in an Italian Cohort: The Mediation Effect of Fruit–Vegetable Intake

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Abstract

Frailty syndrome is an age-related condition involving a loss of resilience, susceptibility to adverse health outcomes, and poor quality of life. This study was conducted in the framework of InveCe.Ab, an ongoing longitudinal population-based study. Plasma from 130 older individuals (older adults aged 76–78 years) was analyzed and validated (on 303 participants) using mass spectrometry-based metabolomics approaches. Equivalence tests showed that metabolites from the central cellular metabolic pathways were equivalent in frail and fit participants. Hippuric acid was the only cometabolite that distinguished fit from frail older adults. Logistic regression analysis indicated that high hippuric acid levels are significantly associated with a reduction of the risk of frailty after 4 years. Mediation analysis using a Frailty Index, hippuric acid, and fruit–vegetable intake supported the role of fruit–vegetable consumption in the hippuric acid relationship with the Frailty Index. These data point to low plasma hippuric acid as a plausible hallmark of frailty status, associated with lower fruit–vegetable intakes.

Keywords: Frailty, Fruit–vegetable intakes, Hippuric acid, Mass spectrometry, Metabolism

Life expectancy is gradually rising worldwide, leading to an increase in older populations (1). Thus, the burden of aging-related morbidities is growing, and the importance of health quality has been underlined (2). One of the most problematic expressions in aged populations is frailty, defined as a geriatric syndrome marked by weakness, loss of physiologic reserve, and resistance to stressors, causing vulnerability to adverse outcomes (3). Susceptibility to stressors is influenced by biological, behavioral, environmental, and social risk factors, the main consequence being an increase in the risk of various adverse health outcomes, including disability, morbidity, falls, hospitalization, institutionalization, and death (4).

As a clinically relevant syndrome, frailty may be assessed using several tools, 2 being the most widely adopted: the "Frailty Phenotype," which takes into consideration 5 criteria: weight loss, exhaustion, weakness, walking slowness, and reduced physical activity (5) and the "Frailty Index (FI)," which combines a number of progressive impairments in a composite score (6). Although these methods perform acceptably to determine the frailty status, they have some limitations, including the subjectivity of individual answers, utilization costs, and lack of linkage with the biological mechanisms of frailty (7). The discovery of objective biomarkers, to identify populations at risk of frailty, is important for an appropriate clinical management.

Several biological factors appear to contribute to the clinical presentation of frailty, the main ones being the dysregulation of inflammatory processes (8,9), genomic instability (10), epigenetic changes (11), oxidative stress (12), and mitochondrial dysfunction (13). The broad spectrum of phenotypes in the frailty score is a critical

issue in the determination of frailty biomarkers. Metabolomics, with its ability to profile a biological system through the simultaneous measurement of hundreds of metabolites, can be used as a phenotyping tool.

Few studies have looked at the plasma metabolome in frailty (14–16), but none of them reached common conclusions, probably because of differences in study design, sample size, population, and analytical tools. To identify metabolic determinants of frailty, we employed targeted and untargeted metabolomics approaches to profile a representative population from an ongoing longitudinal population study. The aim was to comprehensively characterize plasma metabolites in relation to the frailty phenotype, possibly taking dietary habits into consideration, so as to offer strategies for better identification and management of frailty.

Material and Methods

Participant Population

This research was conducted in the framework of InveCe.Ab (Invecchiamento Cerebrale in Abbiategrasso), an ongoing longitudinal population-based study (ClinicalTrials.gov, NCT01345110). The study design and methods have been detailed elsewhere (17). All participants gave their informed consent, at each wave, as approved by the University of Pavia ethics committee. The study procedures were in accordance with the principles of the Declaration of Helsinki of 1964 and amendments.

Briefly, all the InvecCe.Ab participants underwent a multidimensional evaluation to assess social, clinical, and neuropsychological aspects. They were also asked to provide a blood sample for biochemical and biological analyses and DNA extraction. Sociodemographic variables were sex, age (calculated at each medical examination), and education (number of school years completed). The information was acquired with a questionnaire administered by trained interviewers. A baseline assessment of the InveCe.Ab study was carried out in 2010, enrolling 1 321 individuals, and continued with follow-up after 2, 4, and 8 years.

The present study relies on 4-year (2014, 1 010 participants) and 8-year (2018, 762 participants) examinations, from which we extracted 3 data sets (Figure 1).

The first (test set) was retrieved from the 2014 wave: 130 participants (65 frails, FI ≥0.25; 65 fits, FI ≤0.08) were analyzed for metabolomics profiling to highlight the metabolic elements that had a significant association with the FI. The second data set (validation set) was generated by 2014 population expansion: 265 participants (81 frails, FI ≥0.25; 60 prefrails, FI 0.08–0.25; 124 fits, FI ≤0.08) were retrieved from the same wave and used to validate the plasma metabolic determinant discovered in the test set and the association with the FI and fruit and vegetable consumption (mediation analysis). The third data set (longitudinal set) was selected among the 2014 nonfrail (110 fits and 59 prefrails) participants who underwent the 2018 clinical assessment (longitudinal evaluation). This third set was used to verify whether the metabolic determinants found in 2014 could be a predictor of frailty 4 years later.

Frailty Index

Frailty was assessed using the FI, which accounts for the individual's functional status, the presence of diseases, and physical and cognitive deficits (18,19). In our protocol, FI was based on a list of 32 health variables or deficits (Supplementary Table 1), recoded to 0 = absence of a deficit and 1 = expression of the deficit. These individual deficit scores were combined in an index, where 0 = no deficit and 1 = all 32 deficits. Three classes of participants were defined: fit corresponds to FI ≤0.08, prefrail to FI between 0.08 and 0.25, and frail corresponds to FI ≥0.25 (20).

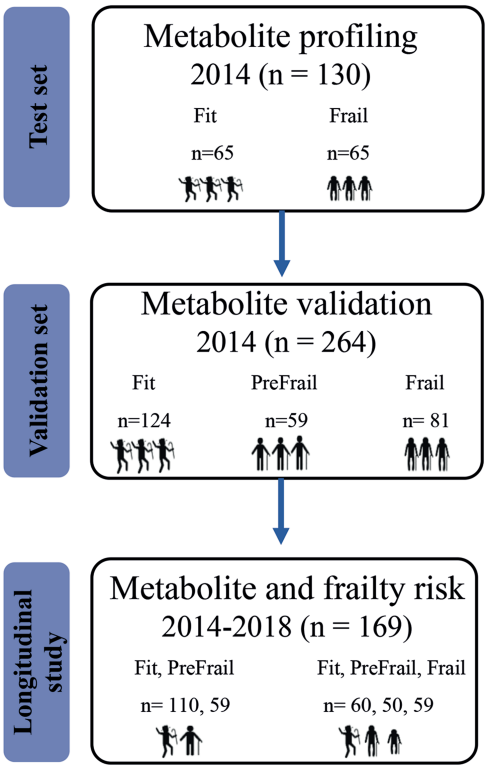

Figure 1. Schematic representation of the InveCe.Ab data sets.

Dietary Variables

Dietary variables related to the intake of fresh fruit and vegetable (Fruit–Veg) were obtained from simple questions on the current frequency of consumption ("Thinking about the last month, how often did you eat fresh fruit? How often did you eat raw vegetables?"). The variables indicating the consumption of fruit and vegetables could go from 0 to 3, corresponding to 0, 1, 2, or more than 3 times a week. An ad hoc score defined as the "fruit–vegetable consumption" score (Fruit–Veg) was constructed simply by summing the 2 scores, thus generating a synthetic variable between 0 and 6. Coffee/tea consumption was only assessed with one question on daily intake ("Do you habitually drink coffee/tea?" yes = 1; no = 0) and one question on the number of cups drunk per day ("How many cups of coffee/tea do you habitually drink per day?").

Targeted Plasma Metabolomics Analysis

A targeted quantitative approach using combined direct flow injection and liquid chromatography tandem mass spectrometry (AbsoluteIDQ 180 kit; Biocrates, Innsbruck, Austria; 5500 TripleQuad Sciex) was used on EDTA plasma samples stored at −80°C, as previously published (21). A metabolite was excluded from data analysis if 50% of all the sample concentrations for the metabolite were above the limit of detection. In total, 135 of the 186 metabolites were selected for statistical analysis (Supplementary Table 2). Twenty relevant metabolite sums and ratios reflecting enzymatic activities were calculated using metabolite concentrations to

understand enzyme activities and regulations better (Supplementary Table 3). The statistical significance of single metabolite concentrations or the sums and ratios were computed by univariate pairwise comparison Mann–Whitney–Wilcoxon test (JMP pro12, SAS).

Untargeted Metabolomics Approach (Flow Injection Analysis-Orbitrap-MS/MS)

For untargeted plasma metabolomics profiling, we used Flow Injection Analysis with an Agilent 1200 Series coupled to an LTQ-Orbitrap XL Mass Spectrometer (Thermo Fisher Scientific Waltham, MA), as previously reported (22). Briefly, metabolites were extracted by adding cold methanol (4:1, MeOH: plasma) to the plasma sample (20 µL). Samples were incubated at −80°C for 20 minutes then centrifuged for 15 minutes at 14 000 × *g*. The supernatant was collected, dried under nitrogen, and suspended in 25 µL of 0.1% formic acid. Replicate injections of the quality control sample, representative of the entire population, were run intermittently throughout the analysis.

A portion of the extract (8 µL) was analyzed by LTQ-Orbitrap XL Mass Spectrometer (Thermo Fisher Scientific) equipped with an electrospray source operated in negative and positive modes. Each run was carried out by injecting 8 µL of sample extract at a flow rate of 50 µL/min of mobile phase consisting of isopropanol/water (60:40, v/v) buffered with 5 mM ammonium at pH 9 for negative mode and methanol/water (60:40, v/v) with 0.1% formic acid at pH 3 for positive mode. Reference masses for internal calibration were used in continuous infusion during the analysis (*m/z* 210.1285 for positive and *m/z* 212.0750 for negative ionization). Mass spectra were recorded from *m/z* 50 to 1 000 with 60 000 resolutions. Source temperature was set to 240°C with 25 L/min drying gas and a nebulizer pressure of 35 psig. MS/MS fragmentation pattern of the significant features was collected and used to confirm metabolite identity. Before each sample, a blank sample (isopropanol/water [60:40, v/v] negative, methanol/water [60:40, v/v] with 0.1% formic acid positive) was run to minimize the carry-over effect. This method allows a rapid metabolic profiling of polar and nonpolar compounds. Lipids were considered only by classes due to the intrinsic method limitation in the discrimination of isobaric forms.

All data processing and analysis were done with Matlab R2016a (The Mathworks, Natick, MA) using an *in-house developed script* following the workflow proposed by Fuhrer et al. (23). The output *m/z* list was analyzed statistically (univariate pairwise comparison Mann–Whitney–Wilcoxon test, JMP pro12, SAS) to select statistically significant differences between frail and fit participants. Significantly altered features were identified by database searches (HMBD, http://www.hmdb.ca) in positive and negative ionization considering protonated/deprotonated ions. In Supplementary Table 4, bold type refers to metabolites with an accurate mass match less than 6 ppm and MS/MS fragmentation pattern similarity more than 99% relative to reference compounds.

The 2 one-sided tests procedure (JMP pro12, SAS) was used to test the metabolic equivalence between frail and fit participants. The upper and lower equivalence bound for each metabolic feature was specified using the standard deviation of the metabolite intensity in the quality control test (8 injections of pooled plasma samples) so metabolites whose intensity fell in this range were deemed equivalent. When 2 one-sided tests were significant ($p < .05$), the metabolite was considered equivalent.

Hippuric Acid Quantification

Hippuric acid levels were determined using an LC-8060 mass spectrometer (Shimadzu). The mass spectrometer was equipped with an ESI source operating in negative ion and selected reaction monitoring mode. The transitions identified during optimization of the method were *m/z* 178.5-134.15 (quantification transition) and *m/z* 178.5-77 (qualification transition) for hippuric acid; *m/z* 183-139.1 (quantification transition) and *m/z* 183-82.05 (qualification transition) for hippuric acid-D5 as internal standard (IS). The ion source settings were as follows: nebulized gas flow 3 L/min, heating gas flow 10 L/min, source temperature 240°C, and drying gas 10 L/min. Hippuric acid and hippuric acid-D5 were separated on a Gemini C18 column (50 × 2 mm; 5 µm particle size), using an 8-minute gradient from 0% to 98% B (acetonitrile), A (10 mM NH_4HCO_2 pH 3.5) at 200 µL/min.

Sample preparation

Twenty microliters of plasma were diluted with 60 µL of cold methanol and 85 ng of IS. Samples were vortexed, incubated for 20 minutes at −80°C, and centrifuged at 14 000 × *g* for 15 minutes; 5 µL aliquots of the supernatants were injected directly into the LC-MS system.

Calibration curves

All calibration curves included 1 zero point and 12 calibration points at 0.08, 0.34, 0.63, 0.85, 3.4, 6.3, 8.5, 12.7, 14.8, 17, 21.25, and 42.5 µg/mL of hippuric acid. The IS concentration was 17 ng/µL. Responses, expressed as the peak area ratio of the analysts, were plotted against the hippuric acid-D5 concentration, and the data were fitted with a linear regression curve. The quality of calibration curves was assessed from the determination coefficient (r^2) and by comparing the back-calculated concentrations of calibrators with the corresponding nominal values. Accuracy was determined by expressing the calculated concentration as a percentage of the nominal concentration. Accuracy had to be within 15% of the nominal value for each concentration; the precision, expressed by the CV (%), had not to exceed 15% for all concentrations (Supplementary Table 5).

Carry-over

The carry-over of the analytical instrumentation was checked by injecting hippuric acid at the highest concentration, followed by repeated injections of blank samples. Carry-over was considered absent because there was no detectable hippuric acid signal in the blank samples.

Recovery

Recovery was determined by comparing the peak area of hippuric acid-D5 spiked to plasma before extraction and the peak area of the analyte spiked to the same samples after extraction. The calculated recovery was 91 ± 0.002%.

Statistical Analysis

Descriptive data were presented as median and interquartile ranges; statistical significance of differences between 3 groups was assessed with the Kruskal–Wallis test, while between 2 groups the Mann–Whitney *U* test was used. The data underwent assumptions testing appropriate for linear regression and met the criteria of linearity, independence, and homoscedasticity. However, hippuric acid, FI, and Fruit–Veg violated the assumption of normality. In particular, the hippuric acid value in a Q–Q plot analysis showed a shape that could be improved by natural log transformation. For the other variables due to the large sample size and the robustness of regression models against violations of normality, we proceeded with this choice of method (24–26). The same has been applied to mediation analysis (27).

The association between H-Nlog, FI, and Fruit–Veg was evaluated with a linear regression model. The role of Fruit–Veg consumption as a potential mediator of the association between the plasma level of H-Nlog and the FI was evaluated following the Baron and Kenny method (28). Then we did a Bootstrap analysis using 5 000 bootstrapped samples and 95% bias-corrected confidence intervals, with the PROCESS macro version 3.5 developed by Hayes and Rockwood (27) to assess the significance of the mediation model. The size effect was calculated with the completely standardized effect of hippuric acid and the FI (29).

To check gender effects in the Fruit–Veg mediation between H-Nlog and FI, we introduced gender in a mediation model (Model 1; PROCESS, SPSS Macro).

To test the effect of H-Nlog on the incidence of frailty, frail participants of the 2014 assessment were excluded. Then we built a binary logistic regression model introducing 4-year frailty incidence as the dependent variable, with hippuric acid level, age, education, and gender as covariates. Statistical tests were computed with SPSS version 20.0 (SPSS, Chicago, IL), setting the significance threshold at $p < .05$.

Results

The results are presented in 3 sequential sections. First, the circulating metabolic determinants of frailty were isolated, highlighting the role of plasmatic hippuric acid. Second, the association of hippuric acid with frailty was confirmed in a larger sample, with a significant mediating role of Fruit–Veg intake. Third, plasma hippuric acid influenced the incidence of frailty 4 years later (Figure 1).

Metabolic Determinants in Frailty

We retrieved 130 plasma samples from the InveCe.Ab 2014 survey cohort and selected a balanced sample of fit and frail participants, whose characteristics are reported in Table 1. The participants were comparable for age, sex, and education. The FI was significantly higher in the frail groups than in the fit ones. Targeted metabolomics analysis on the plasma samples identified significant differences in the concentrations of 43 metabolites (mainly lipid species) and in 4 metabolite sums and ratios, although the difference between frail and fit participants was modest (Supplementary Figure 2A and Supplementary Table 3, 6).

To seek robust metabolic perturbations associated with frailty, we profiled the same plasma samples with rapid untargeted metabolomics analysis by flow injection–high-resolution mass spectrometry. Statistical analysis (Wilcoxon–Mann–Whitney, $p < .05$) identified 22 metabolites whose change in abundance differed significantly between frail and fit populations. As in the targeted approach, the deregulated metabolites were mainly lipid species, with only small differences between groups. The exception was hippuric acid, which was approximately 2 times lower (FC-1.8) in the frail group (Supplementary Figure 2B and Supplementary Table 4).

The lack of substantial metabolic differences between fits and frails using classical hypothesis testing prompted us to use equivalence testing, which confirmed the metabolic equivalence between the 2 populations. Among the 347 identified metabolic features, half of them (171/347) were equivalent in frails and fits. The equivalence of metabolic species (m/z) corresponded to lysophosphatidylcholines, lysophosphatidic acids, phosphatidylcholines, phosphatidic acids, mono-methyl-phosphatidylethanolamines, and sphingomyelins. Together with lipid classes, we also found an equivalent abundance of several amino acids, central cellular metabolic intermediates (lactate, pyruvate, citrate, erythrose 4-phosphate), and nucleic acids and derivatives (Supplementary Table 7).

Plasmatic Hippuric Acid Validation and Association With Frailty

Because hippuric acid was the only metabolite showing a robust plasmatic perturbation in the frailty group by untargeted metabolomics analysis, we developed an LC-MRM method for its absolute quantification in human plasma to validate the untargeted result. We confirmed the importance of hippuric acid levels in frailty by analyzing 264 participants (81 frails, 59 prefrails, 124 fits) retrieved from the InveCe.Ab 2014 follow-up. The characteristics of the participants are listed by FI in Table 2. Frail participants had low median plasma hippuric acid and Fruit–Veg consumption and a high FI. The mean concentrations of hippuric acid were 3.67, 3.04, and 2.71 µg/mL in the plasma of fit, prefrail, and frail, respectively. A significant drop of hippuric acid was detected in frails relative to fits (Figure 2A), but no significant difference was observed between the fit and prefrail groups. Frail females drove the significant decrement of plasma hippuric acid, while frail males had widely dispersed plasmatic hippuric acid levels (Figure 2B).

We tested the association between hippuric acid and frailty and found a significant inverse relationship with the FI (standardized beta: –0.156; $t = -2.714$; $p = .007$).

Hippuric Acid Relationship With Frailty and the Mediating Effect of Fruit–Vegetable Consumption

Hippuric acid is the glycine conjugate with benzoic acid and has long been associated with the microbial degradation of certain dietary components such as fruits, vegetables, whole grains, tea, and coffee (30,31). In view of the association between dietary patterns and risk of frailty (32–34), we analyzed Fruit–Veg consumption in our population. Like in other populations (35), vegetable intake was significantly lower in frails than fits (Table 2). It is known that coffee/tea intake can affect the plasma concentration of hippuric acid (36), so we considered the frequency of this consumption in relation to both hippuric acid and frailty. Coffee–tea intake (number of cups/day) was largely present, with only 8.8% who did not drink daily coffee or tea, and with a median of 2 cups a day (Table 2). The coffee–tea consumption showed a positive relationship with hippuric acid (standardized beta: 0.141; $t = 2.450$; $p = .015$), but no relationship with the FI (standardized beta: –0.001; $t = -0.023$; $p = 982$) so it could not be considered in the subsequent mediation analysis.

Considering the relation between dietary patterns, hippuric acid, and frailty, we then verified whether the link between hippuric acid and FI is mediated by Fruit–Veg consumption by using the 3-step classical approach of Baron and Kenny (28). Step 1 showed that FI was significantly inversely related to hippuric acid levels transformed as normal logarithm (H-Nlog) in a linear regression model (standardized beta: –0.157; $t = -2.571$; $p = .011$). Step 2 confirmed that Fruit–Veg was directly related to the H-Nlog (standardized beta: 0.123; $t = 2.023$; $p = .044$). Finally, in Step 3 FI was regressed on both Fruit–Veg and H-Nlog, showing that the association between H-Nlog and the FI was less than at Step 1 (standardized beta: –0.113; $t = -1.888$; $p = .060$; Table 3). These results support the hypothesis of a mediating role of Fruit–Veg consumption in hippuric acid levels. Bootstrap analysis of the mediation with FI as an outcome, H-Nlog as an independent variable, and Fruit–Veg as a mediator variable confirmed the significant indirect effect (the effect of

Table 1. Subset Population From the InveCe.Ab 2014 Survey Cohort Used for the First Step of Metabolomics Analysis

	Total (130)	Male (66)	Female (64)	p	Fit (65)	Frail (65)	*p*
Age (years)	77 (76–78)	77 (76–78)	76 (76–78)	.270	77 (76–78)	77 (76–78)	.797
Education (years)	5 (5–8)	7 (5–9)	5 (5–8)	.147	6 (5–9)	5 (5–8)	.075
Frailty Index (0–1)	0.16287 (0.04545–0.32942)	0.16288 (0.04166–0.32942)	0.16667 (0.06061–0.32954)	.541	0.04545 (0.01515–0.6061)	0.32812 (0.27273–0.42843)	<.001

Note: Descriptive characteristics of the participants, compared by sex and frailty status (Frailty Index), were reported as median (interquartile range), showing the significance (*p*) of a nonparametric test for 2 independent groups (Wilcoxon–Mann–Whitney).

Table 2. Subset Population From the InveCe.Ab 2014 Survey CohortTaken for the Second Step (metabolic validation analysis of hippuric acid in frailty)

	Total (264)	Fit (124)	Prefrail (59)	Frail (81)	*p*
Age (years)	77 (76–78)	76.5 (75.25–78)	77 (76–78)	77 (76–78)	.755
Gender (female %)	49.6	48.4	49.2	51.9	.886
Education (years)	5 (5–8)	5 (5–8)	5 (5–8)	5 (5–8)	.326
Hippuric acid (μg/mL di plasma)	2.31250 (1.20025 –3.93500)	2.60950 (1.36700 –5.01975)	2.58900 (1.12700–4.33200)	1.81100 (1.00950–3.20850)	.007
Frailty Index (0–1)	0.12121 (0.06060–0.27095)	0.06060 (0.03030–0.07294)	0.18182 (0.15152–0.21875)	0.32812 (0.27273–0.42843)	<.001
Fruit–vegetable* consumption (0–6)	6 (5, 6)	6 (5, 6)	5 (5, 6)	5 (4–6)	<.001
Coffee/tea consumption (cups/day)	2 (1–3)	2 (1–3)	2 (1–3)	2 (1, 2)	.351

Note: LC-MRM = liquid chromatography with multiple reaction monitoring. Descriptive characteristics of the participants with frailty status (Frailty Index) are reported as median (interquartile range), with significance (*p*) of a nonparametric test for *K* independent groups (Kruskal–Wallis) and chi-square for percentage.

*262 participants (female 129, male 133).

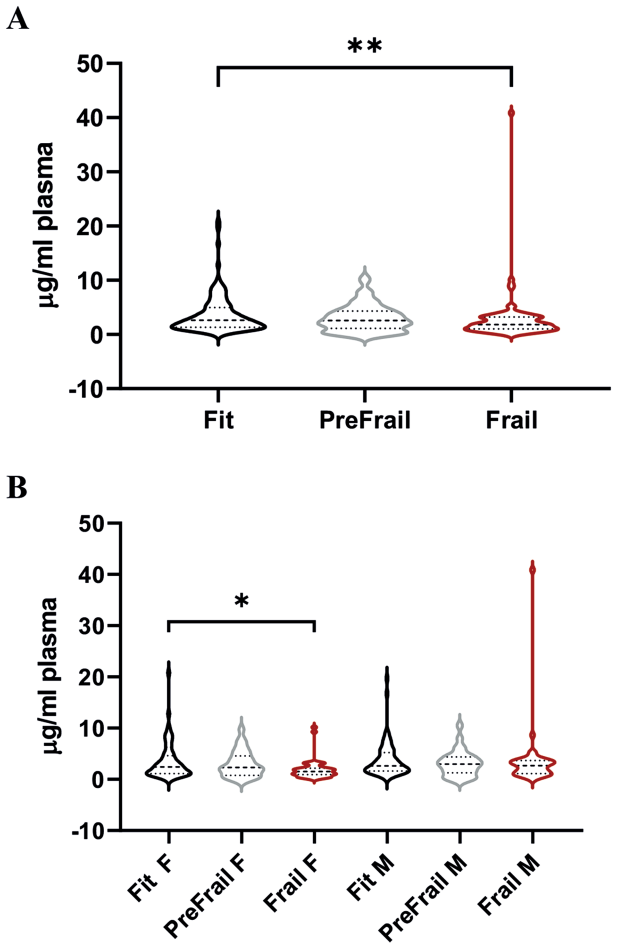

Figure 2. Violin plot graphs reporting the plasma concentrations (µg/mL) of hippuric acid in fit, prefrail, and frail participants (**A**), also considering gender (**B**). Asterisks mark significant differences, $*p < .05$, $**p < .01$; Kruskal–Wallis test. M = male; F = female.

the Fruit–Veg intake as a mediator variable between hippuric acid and FI): −0.0030 (95% CI: −0.0083 to −0.001). The introduction of gender as a covariate did not change the significance of the indirect effect of Fruit–Veg consumption on the FI, giving no interaction effect (interaction gender × Fruit–Veg: F: 1.207; $p = .27$). This was confirmed by a moderation analysis, with gender as a moderator between hippuric acid and FI (F: 1.792; $p = .18$). To assess the size of the effect regardless of the unit of measurement of the variables, a completely standardized effect of hippuric acid on FI was calculated in the bootstrap mediation model (effect −0.0297; 95% CI −0.0711 to −0.0031), showing a significant but small effect.

Plasma Hippuric Acid Levels Effects on the Incidence of Frailty

To test the predictive power of plasma hippuric acid for the risk of frailty, we examined participants who were nonfrail (fits and prefrails) in the 2014 assessment and reevaluated them for frailty in 2018 ($n = 174$). After 4 years there were 59 incident cases of frailty (33.9%), with no difference for sex, age, and years of education. The 2014-H-Nlog was significantly lower in the incident cases (nonfrail: 0.9085 + 1.06603; frail: 0.2614 + 2.1931; $t = 2.14$; $p = .036$) and for Fruit–Veg (nonfrail: 5.3559 + 1.05834; frail: 4.8526 + 1.50155; $t = 2.86$; $p = .005$). Logistic regression analyses showed that higher 2014-H-Nlog significantly reduced the risk of frailty after 4 years, also after introducing in the model Fruit–Veg consumption, age, gender, and education (beta: −0.237; OR: 0.789; 95% CI: 0.638–0.975). To exclude that plasmatic hippuric acid might change during aging, we investigated the hippuric acid patterns in fits and frails over 4 years (2014–2018). Longitudinal evaluation in both groups showed no significant changes in hippuric acid over time, also considering gender stratification (Supplementary Figure 2A and B). Fruit–Veg consumption alone was an independent predictor of subsequent frailty, showing a risk reduction with increased Fruit–Veg consumption (beta: −0.281; 95% CI: 0.597–0.955; Supplementary Table 8).

Discussion

We investigated the relationship between changes in circulating metabolites and frailty status to find objective biomarkers to identify people at risk of frailty. Primary metabolites could not serve as frailty biomarkers and only the secondary cometabolite hippuric acid showed substantial differences between fit and frail older adults. When we investigated links with the dietary variables, we confirmed that plasmatic hippuric acid was related to the Fruit–Veg consumption and could potentially serve as a biomarker of frailty. We observed metabolic fluctuations in agreement with those already reported in different frailty metabolic studies (14–16). We detected the modulation of lipid metabolism (15) and the deregulation of amino acids such as tryptophan and arginine as the main metabolic alterations in frail older adults (15,37).

Even though our metabolic fingerprint agrees with reported biochemical alterations, the small differences between frail and fit participants argue for the absence of robust metabolic markers of frailty. Furthermore, the equivalence tests on a wide range of identified metabolic species support the lack of major differences in primary metabolites in the frail population.

In contrast, we observed a significantly lower level of the secondary metabolite, hippuric acid, in the plasma of frail participants, particularly in frail females. Hippuric acid is a cometabolite derived from the degradation of plant (poly)phenols and aromatic amino acids by a range of gut microbes; the resulting benzoic acid is then subjected to glycine conjugation in the liver and kidney and finally excreted in urine (38). Low hippuric acid levels in blood and urine have been observed in several metabolomics studies in older participants with age-related diseases such as sarcopenia, hypomobility, and cognitive impairment (16,39,40). However, none of these studies

Table 3. Three-Step Analysis Applying Linear Regression Models Between Y and X (Action 1), M and X (Action 2), M + X Versus Y (Action 3)

	B	StB	t	*p*
Step 1: H-Nlog (**X**)–FI (**Y**)	−1.459	−0.157	−2.571	.011
Step 2: Fruit–Veg (**M**)–H-Nlog (**X**)	0.123	0.124	2.023	.044
Step 3: FI (**Y**) vs Fruit–Veg (**M**)–H-Nlog (**X**)				
Fruit–Veg	−0.146	−0.235	−3.922	<.001
H-Nlog	−0.070	−0.113	−1.888	.060

Notes: H-Nlog = log-normal concentration of hippuric acid; FI = Frailty Index; Fruit–Veg = fruit and vegetable intake. The table presents beta (*B*), standardized beta (StB), Student's *t*, and probability (*p*) with significance <.05.

examined the association between hippuric acid and the aged-related diseases or dietary patterns or changes in gastrointestinal microbiota diversity.

Our study illustrated the intrinsic relationships among plasma hippuric acid, frailty, and the intakes of plant (poly)-phenols. The inverse association between hippuric acid and the FI and between Fruit–Veg intake and the FI suggests that hippuric acid may act as a barometer of the decline of healthy dietary patterns in frailty. The association between Fruit–Veg consumption and frailty syndrome has already been described and even considering the heterogeneity between studies 2 meta-analyses indicated that a diet high in fruit, vegetables, and whole grains may be associated with a lower risk of frailty (41,42). The parallel independent ability of plasma hippuric acid and the Fruit–Veg intake to influence the risk of frailty syndrome suggests that single plasmatic determinations might be used to detect the developing frailty syndrome associated with an unhealthy diet early. The objective determination of plasma hippuric acid as a hallmark of frailty syndromes related to dietary habits may then overcome the subjective limitation of clinical assessment of frailty and the intrinsic bias of food question responses.

Regarding coffee and tea consumption, we confirmed the positive correlation with hippuric acid as previously described (36), but we did not find a relationship with frailty. This is only partly unexpected, as their protective effects, such as that on survival, though observed in adults have not been demonstrated for the older population (43). On the other hand, another positive effect of caffeine, such as on cognitive functions, does not translate into an unequivocal benefit from habitual intake in older people (44,45), probably because of the well-known tolerance effect that reduces the systemic impact of caffeine (46). Ultimately, no studies have analyzed the independent influence of coffee/tea consumption on frailty, but only "patterns" in which tea and coffee are components that contribute to the pattern score (47–49). Therefore, our results on coffee/tea consumption need to be interpreted within older people where, however, further research data are needed.

Strengths and Limitations of the Study

The strengths of our study are the longitudinal population-based design of the InveCe.Ab and the absolute plasma concentrations of hippuric acid used for statistical determinations.

These findings must be interpreted considering several limitations. First, we could not evaluate the contribution of microbial rearrangement to the low hippuric acid levels in frail participants, because we had no microbiome information. Therefore, we cannot quantify the contribution of diet to gut microbiota biodiversity. Second, the dietary habits of the InveCe.Ab senior participants were not obtained with a validated food-frequency questionnaire, but were assessed using simple questions about the weekly frequency of food consumption. This simplified approach provides limited information that makes impossible defining quantitative levels of fruit and vegetable intake, but on the other hand, it may overcome the difficulties and bias in assessing food portion sizes and the composition tables typical of food questionnaires (49,50). In this way, we obtained data about the eating habits of the participants that, although indicative, were suitable for our purposes. The debate on the best method to assess food intake remains open, because none of the proposed methods are free from disadvantages (51), especially for the older people (52). Finally, our data were gathered from an older adult cohort from a small northern Italian town: This might limit the generalizability of our findings to people in other countries with different distribution of years of age.

Conclusions

The present study links, for the first time to the best of our knowledge, significant plasma differences in the secondary metabolite hippuric acid with frailty syndrome, while circulating primary metabolites showed substantial equivalence between fit and frail older adults.

Our data point toward low plasmatic hippuric acid as a plausible hallmark of frailty status associated with a decline in Fruit–Veg intake. We further suggest that high hippuric acid may be associated with a lower risk of frailty as indicated by data comparison within a 4-year time window. The clinical/predictive potential of hippuric acid determination and its use as an objective frailty biomarker reflecting consumption of polyphenol-rich foods should now be validated in other populations where gut microbiome diversity may be considered too.

Supplementary Material

Supplementary data are available at *The Journals of Gerontology, Series A: Biological Sciences and Medical Sciences* online.

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Conflict of Interest

None declared.

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Author Contributions

Conceptualization: L.B., A.D., G.F., R.P., and A.G; clinical samples and data were provided by A.D. and A.G.; metabolomics analyses and data postprocessing were done by L.B., G.S., and G.d.S.; statistical analyses were done by L.B., M.C.M., and A.G.; L.B., R.P., and A.G. interpreted the results and wrote the original manuscript; A.D., C.B., O.P., C.C., M.C.M., and G.F. supervised the manuscript; project administration: C.C., A.G., and C.B.; funding acquisition: C.C. All the authors have read and agreed to this published version of the manuscript.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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