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Health associations of liver enzymes and inflammatory scores with urinary citrus flavonoid metabolites†

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Background: Dietary flavonoid intake is associated with a reduced risk of some cardiometabolic disorders, attributed in part to their claimed anti-inflammatory activity. Our aim was to investigate the potential association between specific urine flavonoid metabolites, liver enzymes, and inflammatory status in individuals with metabolic syndrome (MetS). *Methods*: In this cross-sectional study, clinical and dietary data from 267 participants, aged 55 to 75 years, participating in the PREDIMED Plus study (PREvención con DIeta MEDiterránea) were analyzed. At the baseline, spot urine samples were collected and seven urinary flavonoid metabolites were quantified using ultra-performance liquid chromatography coupled to triple quadrupole mass spectrometry (UPLC-Q-q-Q MS). Liver enzymes, inflammatory scores, and urinary flavonoid concentrations were inverse normally transformed. *Results*: Adjusted linear regression models showed an inverse association between urinary citrus flavanone concentrations and gamma-glutamyl transferase (GGT) (all p-values <0.05). Naringenin 7′-GlcUA was significantly associated with a lower aggregate index of systemic inflammation (AISI) ($B_{per\ 1SD}$ = −0.14; 95% CI: −0.27 to −0.02; p-value = 0.025) and systemic inflammation index (SII) ($B_{per\ 1SD}$ = −0.14; 95% CI: −0.27 to −0.02; p-value = 0.028).

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To investigate the relationship between flavanone subclasses and GGT levels, we fitted a score of citrus-flavanones, and subjects were stratified into quartiles. The highest values of the citrus-flavanone score (per 1-SD increase) were associated with lower GGT levels ($B_{per\ 1SD}$ = −0.41; 95% CI: −0.74 to −0.07), exhibiting a linear trend across quartiles (p-trend = 0.015). *Conclusion*: This cross-sectional study showed that higher urinary excretion of citrus-flavanone metabolites was associated with lower GGT levels in subjects diagnosed with MetS and obesity.

Introduction

Metabolic syndrome (MetS) is a health condition, which includes a cluster of metabolic risk factors for developing cardiovascular disease (CVD), type 2 diabetes (T2D), and non-alcoholic fatty liver disease (NAFLD).[1–3] NAFLD encompasses a wide range of diseases, ranging from simple steatosis to cirrhosis, leading to hepatic carcinoma in ending stages, in the absence of excessive alcohol intake.[1] Growing pieces of evidence suggest that NAFLD is the hepatic representation of MetS and it would be one of the main contributors to its pathogenesis.[3,4] MetS is closely related to a low-grade inflammatory status leading to oxidative stress, which can promote the activation of multiple inflammatory pathways, increasing the risk of liver dysfunction and CVD.[5,6] These hepatic and extrahepatic dysfunctions are marked by the increase in liver enzymes and hematologic disorders linked to MetS.[7–12]

In recent years, there has been considerable interest in healthy dietary patterns characterized by a higher intake of plant-based foods and their benefits on health outcomes.[13] In this sense, some studies have suggested that the Mediterranean diet (MedDiet), rich in fruits, vegetables, olive oil, and whole-grain cereals, could prevent and manage most diet-related noncommunicable diseases.[14,15] In human and *in vitro* studies, plant-derived (poly)phenolic compounds showed anti-inflammatory properties and the ability to inhibit oxidative stress.[16–20] However, the results from epidemiological studies are inconsistent and scarce;[16,21] probably due to the fact that evaluating accurately nutrient intake such as (poly) phenols from food frequency questionnaires (FFQ) is complex.[22] In this context, the use of dietary biomarkers for assessing directly the dietary intake/status could avoid the bias of the dietary self-reported data.[22,23] Experimental and human studies have evidenced that flavonoids might exert hepatoprotective properties,[24] cardiovascular benefits,[18,25,26] anti-metastatic activity, and improvement in the metabolic profile.[19] Flavonoids are widely present in many plant-based foods, such as berry fruits, red wine, citrus fruits, grapes, apples, tea, soybeans, and others.[27] These (poly)phenolic compounds consist of a diphenyl propane carbon skeleton, containing two benzene rings linked by a linear three-carbon chain (C6–C3–C6).[28] They can be categorized according to the degree of oxidation and saturation in the heterocyclic C-ring into flavones, flavanones, flavonols, flavan-3-ols, anthocyanins, and isoflavones.[28] Flavanones represent a major part of the total flavonoid intake in European countries, hesperetin and naringenin being the most abundant ones. They are mainly excreted in urine as phase II conjugates that are recognized as biomarkers of citrus-derived food consumption.[23]

Citrus fruits contain phenolic acids and more than 60 types of flavonoids, but they are also rich in vitamin C, folic acids, potassium, dietary fiber, and carotenoids.[29] Several animal studies suggested that citrus-flavanones have anti-inflammatory systemic effects and they protect against liver damage and vascular endothelial dysfunction.[19,30–32] Nevertheless, few human studies have evaluated the impact of flavonoids on health in individuals with MetS. We hypothesized that higher urinary excretion of these specific urinary flavonoid metabolites is associated with liver health and inflammatory status. Therefore, our primary objective was to investigate the interplay between selected urinary flavonoid metabolites, liver enzymes and inflammatory scores in subjects with MetS. The secondary aim was to evaluate the association between (poly) phenol-rich food intake and urinary flavonoid metabolite concentrations.

Materials and methods

Study population

For the present study, baseline data from the Navarra-Nutrition Centre were analysed in the framework of the PREDIMED-Plus study (**https://www.isrctn.com/ISRCTN89898870**). The study design of this clinical trial has been described elsewhere.[33] In brief, PREDIMED-Plus is a parallel-group multi-centre, controlled, randomized trial conducted in Spain (**https://www.predimedplus.com/**).[33] The PREDIMED-Plus study was designed to evaluate a weight-loss intervention based on an energy-reduced MedDiet, physical activity promotion, and behavioural support, in comparison to a non-restrictive Mediterranean diet pattern plus usual care intervention, for CVD events as a primary event.[33] Eligible participants were men (55–75 years old) and women (60–75 years old) with a body mass index (BMI) $\geq$ 27 kg m^{-2} to <40 kg m^{-2} who were diagnosed with MetS at enrolment according to the guidelines from the International Diabetes Federation/National Heart, Lung and Blood Institute/American Heart Association. For the study, participants were excluded if they had several medical conditions (*i.e.* history of previous CVD, including angina, cancer or history of malignancy, cirrhosis or liver dysfunction; alcohol or drug abuse; individuals with acute infection or inflammation; treatment with immunosuppressive, cytotoxic agents, and systemic corticosteroids).[33] The study protocol was approved by the Research Ethics Committee for clinical investigations at the University of Navarra (053/2013), according to the ethical

standards of the Declaration of Helsinki. All participants provided written informed consent. Two hundred sixty six participants with available urine samples were included in this cross-sectional study.

Dietary assessment

Dietary data during the last year were collected using a validated semiquantitative food frequency questionnaire (FFQ), including 143 items commonly consumed in Spain.[34] Frequencies of consumption of the food items were registered at 9 levels: never or seldom, 1–3 times per month, once per week, 2–4 times per week, 5–6 times per week, once per day, 2–3 times per day, 4–6 times per day, and >6 times per day. Reported frequencies of food intake were converted into frequencies per day and multiplied by the weight of the typical portion size indicated to obtain the intake information in g d^{-1}.[34] To evaluate dietary consumption and urinary flavonoid metabolites, we focused on flavonoid-rich foods such as vegetables, fruits, whole grains, and coffee. This FFQ was performed by trained dietitians in a face-to-face interview.[33] Adherence to the energy-reduced MedDiet was assessed using a 17-item questionnaire,[35] a version of the 14-point Mediterranean Diet Adherence Screener (MEDAS) score validated in the PREDIMED study.[36] The 17-item questionnaire has a more restrictive threshold for some caloric-dense foods and additional items aimed to reduce the caloric intake.[35]

Assessment of liver function and inflammatory status

After an overnight fast, the first spot urine and blood samples were collected. Aliquots of serum, plasma, and urine were collected, coded and stored at −80 °C.[33] Liver function was evaluated by hepatic enzymes, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), and GGT. All enzymes were measured using standardized and validated analytical procedures in accredited laboratories for clinical analysis. Blood cell counts were used to calculate inflammatory indexes, such as the aggregate index of systemic inflammation (AISI: neutrophils × platelets × monocytes/lymphocytes) and the systemic inflammatory index (SII: neutrophils × platelets/lymphocytes).[37]

Covariable assessment

At the baseline, sociodemographic, clinical (personal medical history and use of medications), physical activity, lifestyle (alcohol intake and smoking status), and anthropometric data were collected by personnel of the study according to the study protocol.[33] On each visit, the waist circumference (cm), body weight (kg) and height (cm) were measured using standardized procedures and calibrated equipment. BMI was calculated as weight (kg) divided by the square of the height (m). Blood pressure was measured three times using a validated semi-automatic oscillometer (OmronHEM-705C, the Netherlands) after 5 minutes of rest while the participants were in a seated position. Physical activity (Metabolic Equivalent of Task (MET)-minute per week) was evaluated using the short Registre Gironi del Core validated questionnaire.[38,39] T2D was ascertained by the previous diagnosis or if they met one of the following criteria: (1) glycated haemoglobin (HbA1c) ≥6.5%, (2) use of antidiabetic medication or (3) fasting glucose ≥126 mg dl^{-1} considering the American Diabetes Association guidelines.[40] Biochemical analyses, including glucose, insulin, triglycerides, and total cholesterol levels, were conducted by standard enzymatic assays, allowing for laboratory protocols.[33]

Urine flavonoid metabolite assessment

The target flavonoid metabolites in spot urine were first extracted by micro-Solid-Phase Extraction (μ-SPE) and then measured by ultra-performance liquid chromatography and triple-quadrupole mass spectrometry (UPLC-Q-q-Q MS) with a validated method with minor modifications.[41] Firstly, the spot urine was diluted (v : v 1 : 10) with HPLC water (Sigma-Aldrich, Steinheim, Germany). The diluted urine was acidified with 4% phosphoric acid (v : v 1 : 1) and 600 μl of the mixture was loaded to the μ-SPE plate (Oasis 96-well reversed-phase HLB sorbent plate, Waters, Eschborn, Germany). The isotope internal standards (±)-catechin-2,3,4-$^{13}C_3$ (0.54 mg ml^{-1}, Sigma-Aldrich, Steinheim, Germany) and ferulic acid-1,2,3-$^{13}C_3$ (0.99 mg ml^{-1}, Sigma-Aldrich, Steinheim, Germany) were added to the samples before SPE for the calculation of the recovery rate. The loaded samples were washed with HPLC water (200 μl), 0.2% acetic acid (200 μl) and then eluted with 60 μl methanol.

The flavonoid metabolites were identified and quantified with authentic standards. 3′-Methoxy-(−)-epicatechin (3′-ME-EC) was obtained from GERBU Biotechnik (Heidelberg, Germany). Naringenin-4′-glucuronide (naringenin 4′-GlcUA), naringenin-7′-glucuronide (naringenin 7′-GlcUA) and hesperetin-3′-glucuronide (hesperetin 3′-GlcUA) were obtained from Toronto Research Chemicals (Toronto, ON, Canada). Kaempferol-3-glucuronide (kaempferol-3-GlcUA) was obtained from Extrasynthese (Genay, France). Myricetin and isorhamnetin were obtained from Sigma-Aldrich (Steinheim, Germany). The analysis was performed on a Shimadzu LCMS 8060 (Shimadzu, Kyoto, Japan). Briefly, 5 μl of eluted samples was injected through a Raptor Biphenyl column 2.1 × 50 mm, 1.8 μm (Restek, Bellefonte, USA) coupled with a guard cartridge 5 × 2.1 mm, 2.7 μm (Restek, Bellefonte, USA). The UPLC gradient included a 14-minute run joined with a 2-minute equilibration and the mobile phases were water and acetonitrile (Sigma-Aldrich, Steinheim, Germany) both acidified with 0.1% formic acid. The run was performed at 0.5 ml min^{-1} flow rate at 30 °C. The details of the analysis parameters were previously described.[41] The targeted compounds were identified and quantified with 1 to 3 transitions in multiple reaction monitoring (MRM) mode in negative ion mode. Data acquisition and analysis were performed on LabSolutions software (SHIMADZU, Kyoto, Japan). The concentration of flavonoid metabolites was adjusted for urine creatinine levels[42] before data analysis.

Statistical analysis

Descriptive variables are expressed as mean (SD). Categorical variables are shown as number (*n*) and percentage (%).

Baseline comparison between men and women was applied using the Pearson chi-square test (χ^2) for categorical variables or Student's *t*-test. The normality of the continuous markers was evaluated by the Shapiro–Wilk test. Liver enzymes, inflammatory scores, and the concentrations of urinary flavonoid metabolites were normalized and scaled to multiples of 1 SD with the rank-based inverse normal transformation, according to Blom's method.[43] Spearman's correlation analyses assessed the strength of relationships between urinary flavonoid metabolites, flavonoid-rich foods, liver enzymes, and inflammatory scores.

Linear regression models were applied to explore the association between transformed urinary flavonoid metabolites, liver enzymes, and inflammation scores (as outcomes). Results are presented as unstandardized *B*-coefficients and a 95% confidence interval (CI). All linear regression models were adjusted for several potential confounders, including sex, age (continuous, years), smoking status (never, former, current), marital status (single, married, widow, divorced or separated), educational level (primary education, secondary education, or academic/graduate), physical activity (continuous, MET-min per week), sleeping hours (continuous, h per day), energy intake (continuous, kcal d^{-1}), intake of vitamins (yes/no) and alcohol consumption (continuous, g d^{-1}). To account for multiple testing, we corrected *p*-values of the linear regression adjusted associations between a 1-SD increase in urine (poly)phenol metabolite levels and markers, using the Simes false discovery rate (FDR) procedure. An FDR-*p*-value <0.05 was considered to be statistically significant.

To evaluate the potential association between GGT and flavanones, we calculated the flavanone score by combining all the urine citrus-flavanone metabolites (naringenin 4′-GlcUA, naringenin 7′-GlcUA and hesperetin 3′-GlcUA) weighted with their respective individual coefficients from the fitted linear regression model. The flavanone score was calculated by summing all the weighted citrus-flavanones. This score was stratified into quartiles in the linear regression model adjusted for the same confounders as aforementioned. To assess the linear *p*-trend across quartiles, we calculated the median metabolite concentration within each quartile, which was included in the linear regression model as a continuous variable. All statistical tests were 2-sided, and *p*-values of less than 0.05 were considered statistically significant. All statistical analyses were conducted with Stata version 16.0 (StataCorp LP, College Station, TX, USA).

Results

Study population characteristics and flavonoid-based urinary metabolites

The baseline characteristics of study participants are shown in Table 1. Two hundred sixty-six participants were evaluated (153 men, 64.5 ± 5.4 years and 114 women, 67.5 ± 3.9 years). Women were older (men = 64.6 ± 5.4 years; women = 67.5 ± 3.9 years, $p < 0.001$) and had higher BMI (men = 31.8 ± 3.1 kg m^{-2}; women = 32.8 ± 3.7 kg m^{-2}, $p = 0.021$) than men. On the other hand, men were more active and more probably smokers, showing higher energy ($p = 0.004$), coffee ($p = 0.040$), red wine ($p < 0.001$) and alcohol intake ($p < 0.001$) compared to women. In contrast, women showed higher intake of vegetables (men = 318.2 ± 123.0 g d^{-1}; women = 354.1 ± 124.0 g d^{-1}, $p = 0.020$) than men. No differences between men and women were found in the intake of total fruit, whole wheat bread, citrus fruit, and total nuts. The mean intake of other dietary components is presented in the electronic supplemetary information (ESI) (Table 1S†). Adherence to MedDiet did not show differences according to sex. Regarding clinical parameters, men had higher levels of ALT ($p = 0.004$) and AST ($p = 0.043$) than women (Table 1). On the other hand, women had higher insulin ($p = 0.025$) and total cholesterol levels ($p < 0.001$) compared with men.

Urinary flavonoid excretions are presented in Table 2. Stratification by sex was also conducted due to the significant differences seen in dietary intake between men and women. In general, women presented significantly higher excretion of flavonoid metabolites compared to men. Nevertheless, there was no difference in the urinary excretion of kaempferol-3-GlcUA (men = 0.1 ± 0.2; women = 0.2 ± 0.3 nmol g^{-1} creatinine; $p = 0.051$) when comparing men and women. Regarding the mean excretion of flavonoids in the total sample, the highest excretion was for 3′-ME-EC (1.2 ± 1.1 nmol g^{-1} creatinine), followed by flavanone metabolites, 3′-GlcUA (19.3 ± 38.2 nmol g^{-1} creatinine), naringenin 4′-GlcUA (18.0 ± 38.3 nmol g^{-1} creatinine) and naringenin 7′-GlcUA (11.0 ± 25.9 nmol g^{-1} creatinine). Meanwhile, flavonols showed the lowest urinary excretion: the mean urinary excretion for myricetin was (5.2 ± 9.2 nmol g^{-1} creatinine), isorhamnetin (0.5 ± 0.4 nmol g^{-1} creatinine), and kaempferol-3-GlcUA (0.1 ± 0.2 nmol g^{-1} creatinine).

Relationship between dietary intake, liver enzymes and inflammatory indexes

The correlations between the dietary intake of selected flavonoid-rich foods estimated from FFQ, liver enzymes and inflammatory scores are presented in Table 3. Inverse associations among liver enzymes, fruit, and vegetable intake (rho values of −0.134 to −0.178) were found. For example, we observed that GGT levels had significant inverse correlations with the intake of total fruit (rho = −0.166), fruits and vegetables (rho = −0.178), and the groups composed of fruits, vegetables and cereals (rho = −0.161). Concerning inflammatory scores, the AISI showed an inverse correlation with citrus fruit intake (rho = −0.138). In addition, a positive association between onion intake and SII was found (rho = 0.202).

Correlations between dietary intake and urinary flavonoid excretion

Considering the potential correlations between dietary consumption and outcomes (liver enzymes and inflammatory scores), we evaluated the association between dietary intake, selected urinary flavonoid excretion, and flavonoid-rich food from main vegetables, fruit sub-groups, and whole grain cereals in our sample. Table 4 shows the correlations between

Table 1 Baseline characteristics of participants concerning sociodemographic, clinical and lifestyle features categorized by sex

	All (n = 267)	Men (n = 153)	Women (n = 114)	p-Value[a]
Age (years)	65.8 (5.1)	64.6 (5.4)	67.5 (3.9)	<0.001
BMI (kg m^{-2})	32.2 (3.4)	31.8 (3.1)	32.8 (3.7)	0.021
Hypertension (%)	252 (94.4)	144 (94.1)	108 (94.7)	0.828
Type 2 diabetes (%)	100 (37.5)	61 (39.9)	39 (34.2)	0.345
Glucose (mg dL^{-1})	119.3 (32.7)	120.9 (35.0)	117.3 (29.4)	0.385
Insulin (mIU L^{-1})	14.0 (9.0)	13.0 (7.1)	15.5 (10.9)	0.025
HOMA-IR	4.2 (3.2)	3.9 (2.4)	4.6 (4.2)	0.065
Total cholesterol (mg dL^{-1})	200.3 (36.5)	192.2 (34.2)	211.3 (36.7)	<0.001
Triglycerides (mg dL^{-1})	148.0 (61.9)	151.1 (69.0)	144.0 (50.9)	0.355
ALT (U L^{-1})	28.0 (20.6)	31.2 (24.5)	23.8 (12.3)	0.004
AST (U L^{-1})	23.7 (13.4)	25.2 (16.3)	21.8 (7.3)	0.043
GGT (U L^{-1})	42.0 (41.0)	44.9 (41.5)	38.3 (40.2)	0.193
AISI index	270.8 (385.5)	249.8 (270.3)	299.1 (500.6)	0.306
SII index	381.0 (182.3)	374.8 (180.7)	389.3 (184.9)	0.523
Alcohol intake (g d^{-1})	12.8 (17.6)	19.7 (19.8)	3.6 (7.2)	<0.001
Leisure time physical activity (MET min per week)	3104 (2753)	3690 (3101)	2316 (1953)	<0.001
Total energy intake (g d^{-1})	2609 (538)	2689 (535)	2500 (526)	0.004
Adherence to MedDiet[b]	8.9 (2.5)	8.8 (2.4)	9.0 (2.5)	0.448
Total fruits (g d^{-1})	425.5 (221.6)	407.3 (221.6)	450.0 (220.4)	0.120
Total vegetables (g d^{-1})	333.6 (124.5)	318.2 (123.0)	354.1 (124.0)	0.020
Citrus fruit (g d^{-1})	151.4 (125.5)	144.1 (127.2)	161.2 (123.0)	0.269
Whole wheat bread (g d^{-1})	55.2 (92.2)	50.6 (97.3)	61.4 (85.0)	0.345
Total nuts (g d^{-1})	15.0 (18.1)	15.6 (18.1)	14.3 (18.2)	0.549
Coffee intake (g d^{-1})	37.2 (51.4)	42.8 (52.0)	29.7 (49.7)	0.040
Total red wine (g d^{-1})	61.2 (105.7)	91.3 (120.8)	20.7 (61.8)	<0.001
Medications, n (%)				
Cholesterol-lowering treatment (%)	117 (43.8)	67 (43.8)	50 (43.9)	0.991
Any anti-diabetic treatment (%)	69 (25.8)	43 (28.1)	26 (22.8)	0.328
Smoking habits (%)				<0.001
Never smoker	105 (39.3)	29 (19.0)	77(66.7)	
Former smoker	125 (46.8)	97 (63.4)	28 (24.6)	
Current smoker	37 (13.9)	27 (17.6)	10 (8.8)	

Data were calculated by chi-square or Student's t-test as appropriate. Results are expressed as n (%) or mean (standard deviation). [a] p for differences between sexes. [b] Mediterranean dietary score from 0 to 17 points. Abbreviations: BMI, body mass index; HOMA-IR, homeostatic model assessment for insulin resistance; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; AISI, aggregate index of systemic inflammation; SII, systemic inflammation index; MET, metabolic equivalent; MedDiet, Mediterranean diet. AISI index = ((neutrophils × monocytes × platelets)/lymphocytes); SII index = ((neutrophils × platelets)/lymphocytes).

Table 2 Concentrations of spot urinary flavonoid metabolites categorized by sex

Urinary metabolites nmol g^{-1} creatinine)	All (n = 267)	Men (n = 153)	Women (n = 114)	p-Value[a]
Flavanol				
3′-ME-EC	1.2 (1.1)	1.0 (0.6)	1.5 (1.5)	<0.001
Flavanones				
Naringenin 4′-GlcUA	18.0 (38.3)	13.5 (34.5)	24.0 (42.2)	0.027
Naringenin 7′-GlcUA	11.0 (25.9)	7.9 (22.8)	15.2 (29.2)	0.022
Hesperetin 3′-GlcUA	19.3 (38.2)	13.7 (25.1)	26.7 (49.8)	0.006
Flavonols				
Kaempferol-3-GlcUA	0.1 (0.2)	0.1 (0.2)	0.2 (0.3)	0.051
Myricetin	5.2 (9.2)	3.7 (3.6)	7.3 (13.2)	0.001
Isorhamnetin	0.5 (0.4)	0.4 (0.3)	0.7 (0.5)	<0.001

Data were calculated by Student's t-test. Results are expressed as mean (standard deviation). [a] p for differences between sexes. Abbreviations: 3′-ME-EC, 3′-methoxy-(−)-epicatechin.

urinary flavonoid excretion and the dietary intake of key plant-based food groups. Of note is that urinary excretion of 3′-ME-EC exhibited significant correlations with apples (rho = 0.157), chard (rho = 0.143), total fruits (rho = 0.165), and vegetables (rho = 0.200). Regarding urine flavanone excretion, naringenin 4′-GlcUA was moderately correlated with citrus, olive, walnuts, and nuts (rho = 0.202 to 0.286). Similarly, naringenin 7′-GlcUA was positively correlated with olives, walnuts, nuts, total fruit, other vegetables, and whole wheat bread (rho = 0.137 to 0.181). A strong positive correlation was also observed between citrus fruit intake and naringenin 7′-GlcUA (rho = 0.348). Moreover, a moderate to strong correlation was identified between hesperetin 3′-GlcUA, total fruit (rho = 0.298) and citrus fruit (rho = 0.447). Weaker correlations were observed between urinary excretion of flavonols such as kaempferol-3-GlcUA and apple (rho = 0.148), walnut (rho = 0.191), and nut (rho = 0.179) consumption. Myricetin was correlated with apple, total fruit, and vegetable intake (rho = 0.126 to 0.139). On the other hand, isorhamnetin excretion was correlated with citrus fruit (rho = 0.129), chard (rho = 0.175), and total vegetable (rho = 0.138) intake.

Associations between urinary flavonoid metabolites, liver enzymes, and inflammatory scores

Table 5 presents the association between urinary flavonoid metabolites and liver enzymes. The adjusted models showed

Table 3 Spearman's correlations (rho values) between transformed liver enzymes, inflammatory markers and dietary food intake from FFQ

Markers	Watermelon	Citrus	Onion	Total fruit	Total vegetables	Fruits and vegetables	Fruit, vegetables and cereals
ALT	−0.160**	−0.098	−0.144*	−0.152*	−0.018	−0.123*	−0.114
AST	−0.101	−0.050	−0.134*	−0.086	0.023	−0.057	−0.048
GGT	−0.110	−0.112	−0.110	−0.166**	−0.111	−0.178**	−0.161**
AISI index	−0.017	−0.138*	0.138*	−0.088	0.070	−0.018	0.011
SII index	0.023	−0.057	0.202**	0.024	0.105	0.086	0.117

Data were expressed as Spearman's rho values. Liver and inflammatory markers were inverse normally transformed. Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; AISI, aggregate index of systemic inflammation index; SII, systemic inflammation index. $p < 0.05$*; $p < 0.01$**.

Table 4 Spearman's correlations (rho values) between dietary intake of fruits, vegetables and cereals from FFQ data with transformed urinary flavonoid metabolites

	Flavanol	Flavanones			Flavonols		
Foods	3′-ME-EC	Naringenin 4′-GlcUA	Naringenin 7′-GlcUA	Hesperetin 3′-GlcUA	Kaempferol-3-GlcUA	Myricetin	Isorhamnetin
Fruits							
Citrus	0.119	0.286***	0.348***	0.447***	0.114	0.093	0.129*
Apple	0.157*	0.066	0.069	0.081	0.148*	0.126*	0.092
Watermelon	0.104	−0.005	0.009	0.091	−0.054	0.059	−0.001
Olive	−0.014	0.204***	0.181**	0.154*	0.088	−0.005	−0.086
Walnuts	0.022	0.202***	0.154*	0.174**	0.191**	−0.022	0.037
Total nuts	0.015	0.222***	0.169**	0.184**	0.179**	−0.001	0.009
Total fruits	0.165**	0.137*	0.181**	0.298***	0.039	0.139*	0.094
Vegetables							
Chard	0.143*	0.083	0.051	0.157*	0.061	0.048	0.175**
Onion	0.115	0.012	0.013	0.050	−0.012	0.054	−0.031
Other vegetables	0.004	0.094	0.137*	0.157*	0.076	0.078	0.148*
Total vegetables	0.200**	0.011	0.024	0.087	0.014	0.138*	0.138*
Cereals							
Whole wheat bread	0.116	0.130*	0.149*	0.116	0.056	0.010	0.037
Whole grains	0.092	−0.046	−0.030	−0.007	0.014	0.045	0.068

Data were expressed as Spearman's rho values. $P < 0.05$*; $p < 0.01$**; $p < 0.001$***. Abbreviations: 3′-ME-EC, 3′-methoxy-(−)-epicatechin.

that specific citrus-flavanones: naringenin 4′-GlcUA (B_{per} 1SD = −0.15; 95%CI: −0.26 to −0.03; p-value = 0.014), naringenin 7′-GlcUA (B_{per} 1SD = −0.12; 95%CI: −0.24 to −0.01; p-value = 0.038), and hesperetin 3′-GlcUA (B_{per} 1SD = −0.12; 95%CI: −0.24 to −0.002; p-value = 0.047) were inversely associated with GGT levels. Nevertheless, there were no significant associations between other urinary flavonoids (3′-ME-EC, kaempferol-3-GlcUA, myricetin, and isorhamnetin) and hepatic enzymes. Related to inflammatory scores, as shown in Table 6, only a significant relationship was found between urinary excretion of naringenin 7′-GlcUA and AISI (B_{per} 1SD = −0.14; 95%CI: −0.27 to −0.02; p-value = 0.025) and SII (B_{per} 1SD = −0.14; 95%CI: −0.27 to −0.02; p-value = 0.028). However, p-values after the FDR adjustment did not reach statistical significance ($p < 0.05$) in any model. Because of the significant relationship between citrus-flavanone urinary metabolites and GGT levels, we explored the association between GGT levels and the weighted score merging citrus-flavanone metabolites (naringenin 4′-GlcUA, naringenin 7′-GlcUA, and hesperetin 3′-GlcUA) stratified into quartiles in Fig. 1. The linear regression model adjusted for the same confounders indicated that individuals with higher values of the citrus-flavanone score were associated with lower GGT levels (B_{per} 1SD increase: −0.41; 95%CI: −0.74 to −0.07), showing a linear trend across quartiles (p-trend = 0.015). Also, we observed that the mean of citrus-flavanone urinary excretion increased across quartiles of the score. For $Q1$ = 1.64 (nmol g^{-1} creatinine), $Q2$ = 3.98 (nmol g^{-1} creatinine), $Q3$ = 22.76 (nmol g^{-1} creatinine), and $Q4$ = 166.48 (nmol g^{-1} creatinine).

Discussion

In this cross-sectional study conducted in a subsample of the PREDIMED-Plus trial, we found that specific citrus flavanone urinary metabolites (naringenin 4′-GlcUA, naringenin 7′-GlcUA, and hesperetin 3′-GlcUA) are associated with lower GGT levels in individuals diagnosed with MetS. Individually, we observed an inverse association between naringenin 7′-GlcUA and inflammatory scores (AISI and SII), but those

Table 5 Beta-coefficients (95% CIs) for specific transformed urine flavonoid metabolites (independent variable) and transformed liver markers (dependent variable) in individuals diagnosed with MetS by using a linear regression model

	ALT				AST				GGT			
Transformed urinary metabolites	*B* coefficient	95% CI	*p*-Value	FDR-adjusted *p*-value	*B* coefficient	95% CI	*p*-Value	FDR-adjusted *p*-value	*B* coefficient	95% CI	*p*-Value	FDR-adjusted *p*-value
Flavanol												
3′-ME-EC	−0.01	(−0.14, 0.11)	0.819	0.967	0.003	(−0.13, 0.13)	0.969	0.969	−0.02	(−0.14, 0.11)	0.785	0.967
Flavanones												
Naringenin 4′-GlcUA	−0.03	(−0.15, 0.09)	0.662	0.927	−0.01	(−0.13, 0.12)	0.914	0.936	−0.15	(−0.26, −0.03)	**0.014**	0.327
Naringenin 7′-GlcUA	−0.04	(−0.16, 0.08)	0.549	0.915	0.02	(−0.10, 0.14)	0.746	0.922	−0.12	(−0.24, −0.01)	**0.038**	0.329
Hesperetin 3′-GlcUA	−0.08	(−0.20, 0.04)	0.184	0.627	−0.03	(−0.16, 0.09)	0.593	0.899	−0.12	(−0.24, −0.002)	**0.047**	0.329
Flavonols												
Kaempferol-3-GlcUA	−0.07	(−0.18, 0.05)	0.270	0.727	−0.05	(−0.17, 0.07)	0.413	0.899	−0.04	(−0.15, 0.08)	0.522	0.914
Myricetin	0.005	(−0.12, 0.13)	0.939	0.967	−0.04	(−0.16, 0.08)	0.517	0.899	−0.01	(−0.13, 0.11)	0.890	0.967
Isorhamnetin	−0.01	(−0.14, 0.12)	0.908	0.967	0.01	(−0.12, 0.14)	0.880	0.936	0.03	(−0.09, 0.16)	0.629	0.927

Models were adjusted for sex, age, smoking status (never, former, current), marital status (single, married, widow, divorced or separated), educational level (primary education, secondary education, or academic/graduate), physical activity (MET-min per week), sleeping hours (h per day), energy intake (kcal/d), intake of vitamins and alcohol consumption (g/d). FDR (false discovery rate) controlling adjustments were conducted by applying the method of Simes. Liver markers were inverse normally transformed. Abbreviations: CI, confidence interval; 3′-ME-EC, 3′-methoxy-(−)-epicatechin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; FDR, false discovery rate.

associations were not significant after FDR correction. Despite this, biologically, the hypothesis that the metabolites derived from citrus fruit consumption could have beneficial effects on liver health and MetS components is justified.[24,30,31,44] For example, several animal studies indicated that naringenin could decrease the levels and expression of liver enzymes and pro-inflammatory cytokines, which can prevent hepatic damage produced by exposure to ethanol.[45,46] A recent study evaluated the effect of MedDiet intervention on plasma citrus flavonoids (glycoside and aglycone) and their associations with biomarkers of inflammation and oxidative stress in individuals diagnosed with type 2 diabetes.[47] After twelve weeks of intervention, the participants showed increased plasma levels of citrus-derived metabolites and reduced levels of the pro-inflammatory cytokine interleukin-6 in the MedDiet group compared to the control group.[47] It should be kept in mind that oxidative stress and inflammation are the key drivers for liver diseases, leading to metabolic alterations in lipid and hepatic/extra-hepatic glucose homeostasis.[5,6,48]

In recent years, flavonoids have attracted interest in scientific research focused on CVD.[49] In a prospective cohort study among 69 622 women from the Nurses' Health Study, citrus fruits/juices were the main dietary flavanone, but also the highest intake of flavanones was associated with a reduced relative risk of ischemic stroke compared to the lowest consumption.[21] Consistent with this result, a meta-analysis of prospective cohort studies evidenced that the intake of flavanones was inversely associated with the risk of CVD.[49] It has to be mentioned that our population presented MetS and a high risk of CVD. This connection is critical considering that cardiometabolic disorders are linked to impaired hepatic and glucose metabolism in subjects diagnosed with MetS.[50,51]

Thus, these findings suggest that citrus-flavanone metabolites appear to have prominent results on cardiometabolic risk factors in humans and could be important to reduce the liver disease burden in subjects diagnosed with MetS. The beneficial effects of citrus flavanones (*e.g.*, radical scavenging, anti-inflammatory activity, and microbiota modulation properties) on liver diseases and associated complications may be due to the biochemical structures (the number and specific position of the hydroxyl groups in the A and B rings) and the presence of the sugar moiety.[52]

A higher intake of plant-based flavonoid-rich foods is a feature of the Mediterranean dietary pattern.[53–55] Interestingly, we observed that urinary excretion of some flavanone metabolites such as naringenin 4′-GlcUA, naringenin 7′-GlcUA, and hesperetin 3′-GlcUA was positively correlated with citrus fruit consumption. It is recognized that citrus fruits are the main dietary source of flavanones and their concentrations are higher in fruit tissue compared to juice.[56] Proline, betaine and flavanone glucuronides, such as hesperetin and naringenin, have been proposed as biomarkers of citrus intake.[57] Our findings are in line with the studies on the evaluation of specific metabolites that reflect the consumption of citrus fruit. Tomás-Navarro *et al.* found that the main flavanones detected in urine were phase II conjugated derivatives of naringenin

Table 6 Beta-coefficients (95% CIs) for specific transformed urine flavonoid metabolites and inflammatory markers (outcome) in individuals diagnosed with MetS by using a linear regression model

	AISI index				SII index			
Transformed urinary metabolites	*B* coefficient	95% CI	*p*-Value	FDR-adjusted *p*-value	*B* coefficient	95% CI	*p*-Value	FDR-adjusted *p*-value
Flavanol								
3′-ME-EC	−0.06	(−0.19, 0.07)	0.381	0.852	−0.05	(−0.19, 0.08)	0.414	0.852
Flavanones								
Naringenin 4′-GlcUA	−0.12	(−0.24, 0.01)	0.073	0.359	−0.08	(−0.20, 0.05)	0.241	0.703
Naringenin 7′-GlcUA	−0.14	(−0.27, −0.02)	**0.025**	0.327	−0.14	(−0.27, −0.02)	**0.028**	0.327
Hesperetin 3′-GlcUA	−0.11	(−0.24, 0.01)	0.082	0.359	−0.10	(−0.23, 0.03)	0.121	0.471
Flavonols								
Kaempferol-3-GlcUA	−0.06	(−0.19, 0.06)	0.337	0.843	−0.03	(−0.15, 0.10)	0.655	0.927
Myricetin	−0.08	(−0.21, 0.04)	0.197	0.627	−0.05	(−0.18, 0.08)	0.453	0.881
Isorhamnetin	−0.02	(−0.15, 0.12)	0.775	0.967	−0.12	(−0.25, 0.01)	0.076	0.359

Models were adjusted for sex, age, smoking status (never, former, current), marital status (single, married, widow, divorced or separated), educational level (primary education, secondary education, or academic/graduate), physical activity (MET-min per week), sleeping hours (h per day), energy intake (kcal d^{-1}), intake of vitamins and alcohol consumption (g d^{-1}). Inflammatory markers were inverse normally transformed. Abbreviations: CI, confidence interval; 3′-ME-EC, 3′-methoxy-(−)-epicatechin; AISI, aggregate index of systemic inflammation; SII, systemic inflammation index; FDR, false discovery rate.

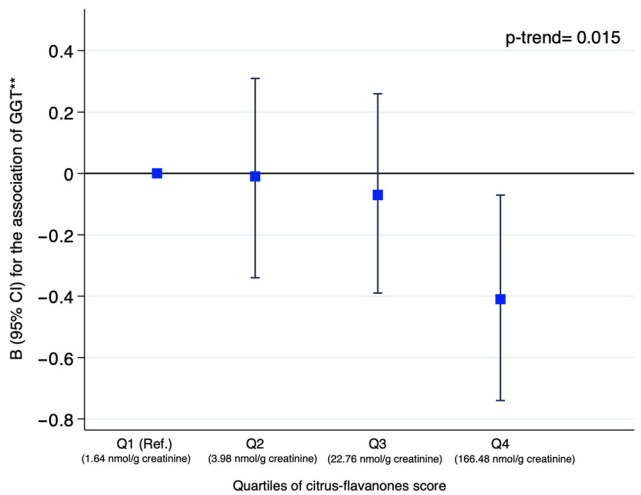

Fig. 1 The graph represented the association between GGT **(inverse normally transformed) and quartiles of the citrus-flavanone score (per 1-SD increase). Fig. 1 shows the *B* (95% CI) from the linear regression model. The model was adjusted for sex, age, smoking status, marital status, educational level, physical activity, sleeping hours, energy intake, intake of vitamins and alcohol consumption. The *p* for trend in the analysis was calculated for the association across quartiles. The mean of citrus-flavanone urinary excretion (nmol g^{-1} creatinine) for *Q*1 = 1.64; *Q*2 = 3.98; *Q*3 = 22.76; *Q*4 = 166.48 (these values are expressed in crude).

and hesperetin after ingesting 400 ml of citrus fruit (orange and mandarin).[23] Previous studies from the European Prospective Investigation into Cancer (EPIC)[53,58] and PREDIMED-Plus showed that flavonoids were the main contributors to total (poly)phenol intake in the Spanish population.[54,55] On average, the consumption of citrus fruit was 151 g per day (around 35% of the total mean fruit intake) in our population. These findings are relevant, considering that a serving of orange juice (250 ml) has 25–65 mg of flavanones (as aglycones), and the flesh of orange (200 g) has a range of 125–375 mg.[56] However, flavonoid composition of citrus fruit and juices is heterogeneous due to the use of different methods for quantifying and the different units of measurement.[56] Also, the flavonoid content of citrus fruit can depend on many factors, including the variety, the cultivar, the geographical origin, harvesting techniques or weather.[56] In our study, despite no differences in citrus intake among sexes, differences in urinary flavanone excretion were statistically significant. It is well documented that the bioavailability of (poly) phenols can be influenced by several factors.[59] The interindividual variation in the bioavailability of flavonoids depends upon the form in which they are ingested. On the other hand, the transformations suffered during gastrointestinal digestion and metabolism (phases I and II) depend on genetic polymorphisms, sex, age, and the diversity of the colonic microbiota.[59,60] The gut microbiota composition influences the flavanone bioavailability, but also a bidirectional interaction exists between citrus flavanones and gut microbiota.[52] It is suggested that (poly)phenols could modulate the gut barrier and improve intestinal microbiota homeostasis in metabolic disease.[52,61]

By identifying the selected flavanones (naringenin 4′-GlcUA, naringenin 7′-GlcUA, and hesperetin 3′-GlcUA) as biomarkers that reflect citrus fruit consumption, we evaluated the relationship between citrus flavanones and liver enzymes, combining these urinary citrus-derived flavanones in weighted scores. Our results showed that higher values of the flavanone score (Q4) were associated with lower GGT levels in a dose–response manner. In the same line, a study evaluated the relationship between flavonoid intake and the risk of NAFLD measured by the fatty liver index (FLI). In a cohort study including 17 685 participants from the National Health and Nutrition Examination Survey (2005–2010), the authors showed that the lowest intake of flavonoids was associated with higher FLI,

C-reactive protein (CRP) and liver enzyme levels. Meanwhile, higher flavonoid intake was associated with less likelihood of NAFLD [third tertile *vs.* first tertile; OR = 0.81 (95% CI: 0.78, 0.86)].[62] As is known, the beneficial effects of citrus flavanones depend on their bioavailability.[52] Citrus flavanone glycosides such as hesperidin and naringin are resistant to enzymatic breakdown in the stomach and small intestine, so they are mainly metabolized in the colon, releasing aglycone forms (hesperetin and naringenin) that are further metabolized into phase II metabolites with an intact structure and also catabolized into several ring-fission products.[52,63]

In our study, we only evaluated urinary structurally related flavanone phase II metabolites. Being gut microbial metabolites, the T_{max} of the flavanone conjugates is around 5–7 hours post-consumption, therefore longer than that for other structurally related flavonoid metabolites that are usually absorbed in the small intestine and have a T_{max} of 1–2 hours.[60,63] Thus, this could be a reason why these flavanone metabolites moderately correlated with citrus consumption in the FFQ data and may be good biomarkers of habitual citrus consumption.

Mechanisms underlying the potential hepatoprotective effects of naringenin and hesperetin in humans are still poorly understood. In *in vivo* and *in vitro* studies, naringenin and hesperetin metabolites could reduce membrane phospholipid damage, thus preventing lipid peroxidation.[24,64] These effects may be due to their higher lipophilicity, decreasing the membrane fluidity and reducing the interaction between free radicals and lipids.[24,64] Also, citrus flavanones showed a great capacity to decrease the free radical scavenging activity, increase the expression of peroxisome proliferator-activated receptor α genes, and mitigate the activation of inflammatory pathways, avoiding liver damage, which are crucial approaches for NAFLD prevention.[24,30,64,65] Despite the noteworthy results of experimental studies of citrus flavanone effects on the liver, randomized control trials (RCT) in humans that established safe and tolerable dosage ranges are lacking. A randomized cross-over trial evaluated the effect of 600 ml d^{-1} orange juice intake on twenty-nine men over 50 years with hypercholesterolemia for 4 weeks.[66] The participants presented improvements in antioxidant parameters and endothelial function.[66] Another RCT tested the intake of a (poly)phenolic extract from citrus fruits that contained around 20% naringenin in ninety-five participants with overweight. After twelve weeks of intervention, the participants showed significant improvements in body composition, inflammatory markers (CRP and fibrinogen), and oxidative status.[67]

Most of the data concerning flavonoids' benefits on health outcomes have been collected from prospective studies and have some limitations regarding the quantification of flavonoids using FFQ in most studies.[68] These discrepancies could be partially explained by the incomplete representation of flavonoid food sources on FFQs, and the misclassification of the dietary flavonoid content of foods due to the lack of good and reliable (poly)phenol composition databases, which cannot avoid potential bias.[68] In this context, the development of sensitive, robust and accurate analytical methods to quantify (poly)phenol intake in biofluids to improve currently available (poly)phenol composition databases is needed.[68]

The strength of the present study is the large sample size of patients with relevant data. Furthermore, we applied a validated analytical method to accurately identify and measure flavonoid metabolites with authentic standards. Nevertheless, our study has some limitations. First, this study has a cross-sectional design and our findings cannot establish causation. Second, this Mediterranean cohort was older in age and had MetS. Thus, our results cannot be generalized to other ethnic, younger and/or healthier individuals. However, it is plausible that plant-based foods have beneficial effects on health outcomes.[13] Third, the flavonoid metabolites evaluated in this study were limited and therefore influence our conclusions, but the flavanone metabolites used in the present study are well correlated with citrus fruit intake, and they have been suggested as biomarkers of citrus consumption.[23,55,57]

Conclusion

Our results suggest that higher urinary excretion of citrus-flavanone metabolites, such as naringenin 4′-GlcUA, naringenin 7′-GlcUA, and hesperetin 3′-GlcUA, might be associated with reduced levels of GGT. Further well-designed randomized trials are still needed to establish these associations and the amount of consumption of citrus foods that is required to protect against liver damage in individuals diagnosed with MetS.

Author contributions

Conceptualization, V.B.-V., I.A., M.A.Z., A.R.-M., and J.A.M; formal analysis, V.B.-V., C.R., Y.X., I.A., M.A.Z., A.R.-M., and J.A.M.; investigation, V.B.-V., Y.X., C.R., I.A., M.A.Z., M.A.M.-G., P.B.-C, F.V.-S, V.M.S, Z.V.-R, C.S.-O., M.D.-F., C.C., R.E., R.M.L.-R., M.F., G.B., N.B., J.S.-S, F.J.T., J.A.T., D.R., J.K., X.P., L.D., A.R.-M., J.A.M; writing—original draft, V.B.-V., Y.X., C.R., I.A., M.A.Z., A.R.-M., F.V.-S, V.M.S, and J.A.M.; writing—review and editing, V.B.-V., Y.X., C.R., I.A., M.A.Z., M.A.M.-G., P.B.-C, F.V.-S, V.M.S, Z.V.-R, C.S.-O., M.D.-F., C.C., R.E., R.M.L.-R., M.F., G.B., N.B., J.S.-S, F.J.T., J.A.T., D.R., J.K., X.P., L.D., A.R.-M., J.A.M.; supervision, C.R., I.A., M.A.Z., A.R.-M., J.A.M. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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