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Urinary metabolomics of phenolic compounds reveals biomarkers of type-2 diabetes within the PREDIMED trial

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ABSTRACT

Background: Phenolic compounds have been associated with protective effects against type-2 diabetes (T2D). We used a metabolomics approach to determine urinary phenolic metabolites associated with T2D and fasting plasma glucose.

Methods: This case-control study within the PREDIMED trial included 200 participants at high cardiovascular risk, 102 of whom were diagnosed with T2D. A panel of urinary phenolic compounds were analysed using a novel method based on liquid chromatography coupled to mass spectrometry. Multivariate statistics and adjusted logistic regressions were applied to determine the most discriminant compounds and their association with T2D. The relationship between the discriminant phenolic compounds and plasma glucose was assessed using multivariable linear regressions.

Results: A total of 41 phenolic compounds were modeled in the orthogonal projection to latent structures discriminant analysis, and after applying adjusted logistic regressions two were selected as discriminant: dihydrocaffeic acid (OR = 0.22 (CI 95 %: 0.09; 0.52) per 1-SD, p-value = 0.021) and genistein diglucuronide (OR = 0.72 (CI 95%: 0.59; 0.88) per 1-SD, p-value = 0.021). Both metabolites were associated with a lower risk of suffering from T2D, but only dihydrocaffeic acid was inversely associated with plasma glucose (β = −17.12 (95 % CI: −29.92; −4.32) mg/dL per 1-SD, p-value = 0.009).

Conclusions: A novel method using a metabolomics approach was developed to analyse a panel of urinary phenolic compounds for potential associations with T2D, and two metabolites, dihydrocaffeic acid and genistein diglucuronide, were found to be associated with a lower risk of this condition.

Abbreviations: , AMPK1, Adenosine monophosphate-activated protein kinase; , AUC, area under the curve; , CV, cross-validation; , FDR, false discovery rate; , FC, fold change; , LTQ-Orbitrap-HRMS, linear ion trap quadrupole-Orbitrap-high-resolution mass spectrometry; , MET, metabolic equivalent for task; , OPLS-DA, orthogonal projection to latent structures discriminant analysis; , PPARy, peroxisome proliferator-activated receptor y; , ROC, receiver operator characteristic; , SIRT1, sirtuin 1; , SPE, solid-phase extraction; , T2D, type-2 diabetes; , VIP, variable importance in projection.

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1. Introduction

Polyphenols are secondary metabolites with a wide diversity of chemical structures. The intake of polyphenols can be estimated through dietary questionnaires and food-composition tables [1]. Despite recent improvements, these methods have limitations due to the variability of the phenolic content in foods, which can be modified by processing, cooking, plant variety, or season of harvest [2–4]. In addition, the bioavailability of polyphenols varies depending on the food with which they are ingested [5]. Thus, the most reliable way to measure exposure to dietary polyphenols is by analysing biological samples, especially urine, as polyphenols and their metabolites are largely excreted in urine 24–48 h after ingestion [6,7]. Individual polyphenols measured through food frequency questionnaires and analysis of biological samples have been linked with a lower risk of type-2 diabetes (T2D) [8]. The Mediterranean Diet, which is rich in plant-based foods and characterized by a high phenolic intake, has also been associated with a lower T2D incidence [9,10]. In recent years, research on T2D prevention and control has grown in response to the increasing global prevalence of the disease and associated mortality rate, with 700 million people predicted to be affected by 2045 [11].

Metabolomics has emerged as a tool for the identification of disease biomarkers, allowing earlier detection and improved monitoring, which will enable the development of personalized treatment plans for patients [12]. For example, in 2022, Larkin et al. detected biomarkers of cancer in blood samples of patients with nonspecific symptoms [13]. However, metabolomics-based research still faces challenges, such as the standardization of methods across laboratories, finding a consensus on data interpretation, and the promotion of data-sharing [14].

Metabolomics has also been applied to identify biomarkers related to T2D and its risk factors. Most of this research has focused on different lipid classes, such as ceramides or sphingolipids, bile acids, and amino acids, especially branched-chain and aromatic amino acids [15,16]. However, the relationship of polyphenols and their metabolites with T2D has been minimally explored using metabolomics. The application of this approach could shed light on polyphenols involved in the pathways leading to T2D, as well as their effect on the molecular mechanisms underlying the development of the disease. Ultimately, a greater understanding of the pathogenesis of T2D will facilitate the implementation of new prevention strategies.

Therefore, the aim of the present work was to develop a metabolomics-based method to analyse a panel of urinary phenolic compounds for potential associations with T2D in participants of the PREDIMED (PREvención con DIeta MEDiterránea) trial with and without T2D at baseline. To this end, we employed high precision analytical techniques based on linear ion trap quadrupole-Orbitrap-high-resolution mass spectrometry (LTQ-Orbitrap-HRMS), which allowed us to identify a great variety of unknown phenolic compounds. The most discriminant compounds related to T2D were then identified with multivariate statistics, which enabled us to simultaneously assess a large number of phenolic compounds. Finally, we investigated the associations of the selected individual compounds with T2D and fasting plasma glucose levels using multivariable-adjusted regressions.

2. Materials and methods

2.1. Study design

The present work is a case-control sub-study using baseline data of the PREDIMED trial, a multicentre, parallel-group, randomized, controlled trial conducted in Spain from 2003 to 2010. The methods and design of this study have been described in detail elsewhere [17,18]. Its main aim was to assess the effect of a Mediterranean diet enriched with olive oil or nuts on the primary prevention of cardiovascular disease. It included 7447 participants aged 55–80 years at high cardiovascular risk who had T2D or at least three of the following major risk factors: current smoking, hypertension, dyslipidaemia, overweight/obesity or a family history of premature cardiovascular disease. To carry out the study, 200 participants from the PREDIMED-Hospital Clinic recruitment center (Barcelona) were randomly selected, 102 of whom were diagnosed with T2D. Participants who reported extreme total energy intakes (>3500 or <500 kcal/day in women or >4000 or <800 kcal/day in men) were excluded from the analysis.

The Institutional Review Board (IRB) of the Hospital Clinic (Barcelona, Spain) accredited by the US Department of Health and Human Services (DHHS) update for Federal-wide Assurance for the Protection of Human Subjects for International (Non-US) Institutions #00000738 approved the study protocol on July 16, 2002. All participants provided informed consent and signed a written consent form.

2.2. Covariate assessment

Trained dietitians completed a semi-quantitative 137-item food frequency questionnaire in interviews with participants, as well as a 14-item questionnaire to assess their adherence to the Mediterranean diet [19]. Participants were considered to suffer from hypercholesterolemia or hypertension if they had a previous diagnosis and/or were under cholesterol-lowering or antihypertensive medication, respectively. Trained personnel measured body weight, height, waist circumference, and blood pressure. Body mass index (BMI) was calculated as weight in kg divided by height in m^2. Physical activity (metabolic equivalent tasks per minutes per day, METs min/day) was assessed with a validated Spanish version of the Minnesota physical activity questionnaire [20]. Plasma glucose, total cholesterol, triglycerides, and HDL cholesterol were determined by standard enzymatic methods, and LDL cholesterol was calculated by the Friedewald equation [18].

2.3. Ascertainment of type-2 diabetes

For the present analysis, the main endpoint was the prevalence of T2D, which was defined as previous clinical diagnosis of T2D, or glycated hemoglobin (HbA1c) $\geq$ 6.5%, or use of antidiabetic medication at baseline, or fasting plasma glucose > 126 mg/dl in both the screening visit and baseline visit.

2.4. Phenolic metabolic profiling

Biological samples were collected after an overnight fast, coded, and stored at − 80 °C until analysis. Phenolic compounds were isolated using a method previously validated by our group with minor modifications [21]. Briefly, 50 μL urine samples were diluted 1:20 (v:v) with Milli-Q Water (Bedford, MA, USA), and 100 μL of the internal standard abscisic acid-d_6 (Santa Cruz Biotechnology, Santa Cruz, CA) was added. The sample dilution was acidified with 2 μL of formic acid (Panreac Química S.A., Barcelona, Spain) and centrifuged at 15,000 g at 4 °C for 4 min. The acidified urines underwent a solid-phase extraction (SPE) in Water Oasis HLB 96-well plates 30 μm (30 mg) (Water Oasis, Milford, MA, USA). First, the 96-well plate was activated with methanol (Sigma-Aldrich, St. Louis, MO, USA) and 1.5 M formic acid, and after loading the samples, a clean-up step was performed with 1.5 M formic acid and methanol (0.5 %). The phenolic compounds were then eluted with methanol acidified with 1.5 M formic acid, evaporated to dryness with nitrogen gas and reconstituted with 100 μL formic acid (0.05 %). After 20 min of vortex mixing, the samples were filtered through 0.22 μm polytetrafluoroethylene 96-well plate filters (Millipore, Massachusetts, USA).

The analysis was performed on an Accela chromatograph (Thermo Scientific, Hemel Hempstead, UK) coupled to an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Hemel Hempstead, UK) equipped with an H-ESI source working in negative mode as described elsewhere [21]. Chromatographic separation was performed on a Kinetex F5 100 Å Column (50 × 4.6 mm, 2.6 μm) from Phenomenex (Torrance, CA, USA). Mobile phases A and B were, respectively, 0.05 % formic acid in water

and 0.05 % formic acid in acetonitrile. The following linear gradient was used: held at 98%A for 1.7 min, decreased to 92%A for 3 min, decreased to 80%A for 1.3 min, decreased to 70%A for 1.3 min, decreased to 50 % for 0.1 min, decreased to 0 % for 1.3 min, then returned to initial conditions for 1.7 min and re-equilibrated for 3 min. The flow rate was set at 0.750 μL/min and the injection volume was 5 μL.

The collected UHPLC-HRMS data (. RAW file) were converted into. abf files using the Reifycs Abf Converter and then further processed using the software MS-DIAL (version 4.24) [22]. In this regard, automatic peak finding and LOWESS normalization were performed. The mass range 100–1500 *m/z* was searched for peaks with a minimum peak height of 10,000 cps. The MS tolerance for peak centroiding was set to 0.01 Da. Retention time information was excluded from the calculation of the total score. For identification, accurate mass tolerance was 0.01 Da. The identification step was based on mass accuracy and isotopic patterns, and the annotation was carried out by manually comparing the peaks with the theoretical phenolic compounds.

Then, for annotation confirmation, a pool of representative samples was injected in the UHPLC-Orbitrap-HRMS equipment and a data-dependent scan was carried out with the use of a parent ion list, using the ions tentatively annotated previously. The data were analysed using Xcalibur software v2.0.7 (Thermo Fisher Scientific, San Jose, CA, USA) and the fragments were manually checked.

2.5. Creatinine determination

Creatinine was measured by an adapted Jaffé alkaline picrate method for 96-well plates, as described by Medina-Remón et al. [23]. As phenolic compounds were expressed as peak areas, without quantitation, it was not possible to normalize their values by creatinine concentration. Therefore, we introduced creatinine in the adjustment models to account for differences in urinary excretion.

2.6. Statistical analyses

Supervised analysis of multivariate data was carried out using SIMCA software (Umetrics) and orthogonal projection to latent structures discriminant analysis (OPLS-DA). This model was selected over a partial least squares discriminant analysis, as the orthogonal projection allows a better separation between groups when the intra-group variability is high [24]. To investigate the presence of outliers, Hotelling's T2 was applied, using a 95% limit for suspicious outliers and 99 % for strong outliers. Goodness-of-prediction (Q2 Y) and goodness-of-fit (R2 Y) were used as validation parameters, adopting a Q2 Y prediction ability of $>$ 0.5 as the acceptability threshold. ANOVA applied to cross-validated residuals (CV-ANOVA) was used for the cross-validation of the model, with a p-value < 0.05 as a threshold. The p-value of the CV-ANOVA indicates the probability for an OPLS-DA model, with this F-value being the result only of chance [25]. Finally, a permutation test (200 permutations) was done to exclude overfitting. The variable importance in projection (VIP) was used to extrapolate the possible marker compounds, i.e., those with a VIP score > 1. For each phenolic metabolite with a VIP score > 1, the fold change comparing T2D versus T2D-free participants was calculated.

Logistic regression models were applied to assess the association of each phenolic compound (1-SD increment in transformed concentration of metabolites) with T2D, adjusting for covariates and confounders that could alter this relationship. Thus, we adjusted for age, sex, BMI, smoking habit, educational level, physical activity, total energy intake and hypercholesterolemia. Individual phenolic compounds and creatinine were natural logarithmically transformed to normalize their distributions, as were confounders that did not follow normal distribution (physical activity and total energy intake). The p-values of the logistic-adjusted associations were adjusted using the false discovery rate (FDR)-adjusted procedure to account for multiple testing [26]. Therefore, those metabolites with a VIP score > 1 and a statistically significant p-value (<0.05) were considered as discriminant for T2D status and were used as independent variables in the following regression models. Receiver operating characteristic (ROC) curve analysis was used to assess the accuracy of the discriminant phenolic compounds.

To further explore the relationship between the discriminant phenolic compounds and T2D, multivariable regression models were used to assess their association with plasma glucose levels (mg/dL). Three adjustment models of increasing complexity were used. Model 1 was minimally adjusted for age, sex, and creatinine. Model 2 was further adjusted for smoking habit, educational level, BMI, physical activity, total energy intake, and hypercholesterolemia. As antidiabetic drugs were only used by diabetic participants with higher levels of glucose, their inclusion in the model might be an overadjustment. We therefore also applied model 3, which was additionally adjusted for antidiabetic drug usage.

Logistic and multivariable adjusted regression models were generated using Stata 16.0. P-value < 0.05 was considered statistically significant.

3. Results

3.1. General characteristics

The descriptive characteristics of the 200 participants are listed in Table 1. Their mean + SD age was 66.1 + 5.3 years and approximately half were women (54.5 %). 102 participants were diagnosed with T2D and 98 were free of T2D. The two groups were well-balanced in terms of BMI, physical activity, and total energy intake. A higher percentage of participants with hypercholesterolemia was observed in the non-T2D group (93.9 %). 15 % of the total participants were current smokers, the percentage being similar in both groups. Regarding education, a higher percentage of participants with T2D had received a high or medium level of education (40.2 %) compared to those without T2D (26.5 %).

3.2. Phenolic compound identification

The HRMS-based metabolomics analysis permitted the tentative identification of 79 phenolic compounds according to their exact mass and isotopic ratio. Among the 41 marker compounds selected using the VIP method (VIP score > 1), 17 were identified by their exact mass, isotopic ratio, and fragmentation pattern. The results are presented in

Table 1
Baseline characteristics of all the participants according to T2D status.

Characteristics	All (n=200)	Participants without T2D (n=102)	Participants with T2D (n=98)	p-value
Women, n(%)	109 (54.5)	56 (57.1)	53 (52.0)	0.462
Age, years	66.1 + 5.3	65.7 + 5.02	66.5 + 5.6	0.326
BMI, kg/m2	29.5 + 3.4	29.3 + 3.3	29.7 + 3.5	0.363
Hypercholesterolemia	158 (79.0)	92 (93.9)	66 (64.7)	< 0.001
Current smokers	30 (15.0)	15 (15.31)	15 (14.71)	0.958
High or medium education	67 (33.5)	26 (26.5)	41 (40.2)	0.041
Physical activity, METS-min/day	291.0 + 266.9	286.2 + 263.4	295.6 + 271.4	0.805
Total energy intake, kcal/day	2394.1 + 500.8	2419.9 + 471.3	2369.2 + 528.7	0.475

T2D, type-2 diabetes; METS, metabolic equivalents.
Continuous variables are shown as means + SDs, and categorical variables are shown as percentages.
T-test or chi-square test as appropriate.

the Appendix (Table A1). Due to the low concentrations of the urine samples and the difficulty in obtaining MS fragments, some compounds were tentatively identified according to the mass accurate measurements and the isotopic pattern, with a level 4 identification [27].

3.3. Determination of discriminant compounds

The OPLS-DA was built to model the raw data in a supervised manner and extrapolate the VIP marker discriminant compounds. The OPLS-DA analysis resulted in a model with R2 Y = 0.43 and Q2 Y = 0.101. VIP values were obtained for each variable in the OPLS-DA model and the 41 selected compounds (VIP > 1.0) were consecutively identified by product ion scan analysis (MS^2) (Table A1). The multivariate supervised statistical method allowed the identification of the discriminant compounds and provided a good separation between participants with and without T2D in the score plot (Fig. 1).

Table 2 shows the 41 phenolic compounds with a VIP score > 1 grouped according to their polyphenol class. The average fold change (FC) was calculated to compare the samples of participants with and without T2D. Among all the compounds, 27 had a negative logFC, indicating their levels were higher in participants free of T2D.

In the logistic-adjusted regression analysis, two phenolic compounds were associated with T2D after the FDR correction: dihydrocaffeic acid and genistein diglucuronide. Dihydrocaffeic acid is a colonic metabolite of caffeic and ferulic acid, although it can also be found in olives [28,29]. The aglycone of genistein diglucuronide can be found in vivo after the intake of soy, and in smaller proportions after consumption of nuts, vegetables, and fruits [30]. Both dihydrocaffeic acid and genistein diglucuronide showed an inverse association with T2D (OR = 0.22 (CI 95 %: 0.09; 0.52) per 1-SD, *p*-value = 0.021 and OR = 0.72 (CI 95 %: 0.59; 0.88) per 1-SD, *p*-value = 0.021, respectively), although the logFC of dihydrocaffeic acid was lower compared to genistein diglucuronide (−0.27 and −1.92, respectively). Dihydrocaffeic acid and genistein diglucuronide were both identified by product ion scan analysis (MS^2), comparing the fragments to those in the literature [31,32].

Fig. 2 illustrates the ROC curve of urinary dihydrocaffeic acid and genistein diglucuronide in relation to T2D adjusted by the potential confounders. The area under the curve was 0.779 and 0.783 for dihydrocaffeic acid and genistein diglucuronide, respectively, indicating that both compounds were discriminant for T2D. Dihydrocaffeic acid predicted a non-diabetic status with 70.97 % of sensitivity and 65.66 % of specificity, whereas for genistein diglucuronide, the sensitivity was 75.61 % and specificity 65.22 %.

3.4. Discriminant compounds and fasting plasma glucose

The associations between plasma glucose and the discriminant phenolic compounds according to the three adjustment models are presented in Table 3. Dihydrocaffeic acid showed a significant inverse association with plasma glucose in model 2 (β = −17.12 (95 % CI: −29.92; −4.32) mg/dL per 1-SD, *p*-value = 0.009), but in model 3, which included the use of antidiabetic drugs, the relationship was no longer significant and the β coefficient decreased. However, a strong positive correlation was found between antidiabetic drug usage and plasma glucose (data not shown), so its inclusion as a confounder could be an overadjustment. A comparison of the associations of dihydrocaffeic acid with plasma glucose using adjustment models 2 and 3 is presented in Fig. 3. Genistein diglucuronide did not show any significant association in any adjustment model.

4. Discussion

In the present case-control substudy of the PREDIMED trial, we identified urinary phenolic compounds associated with the risk of T2D in participants at high CVD risk using a metabolomics approach based on UHPLC-Orbitrap-HRMS. The metabolites dihydrocaffeic acid and genistein diglucuronide were associated with lower T2D risk, and dihydrocaffeic acid was also inversely associated with plasma glucose levels.

There is extensive evidence from epidemiological studies that dietary polyphenols reduce T2D risk [9]. This relationship could be explained by the anti-inflammatory properties of phenolic compounds, as cellular inflammation plays a key role in the development of T2D. Polyphenols can modulate the transcription of genes involved in inflammatory pathways, such as PPARγ (peroxisome proliferator-activated receptor γ), SIRT1 (Sirtuin 1), or AMPK1 (adenosine monophosphate-activated protein kinase) [33,34]. In addition, they can reduce glucose absorption by inhibiting the sodium-dependent glucose transporter 1 (SGLT1) and increase its uptake in tissues through activation of glucose transporter 4 (GLUT4) [35]. Through prebiotic effects, polyphenols can stimulate the growth of microbial species in the gut with a beneficial impact on metabolic diseases [36].

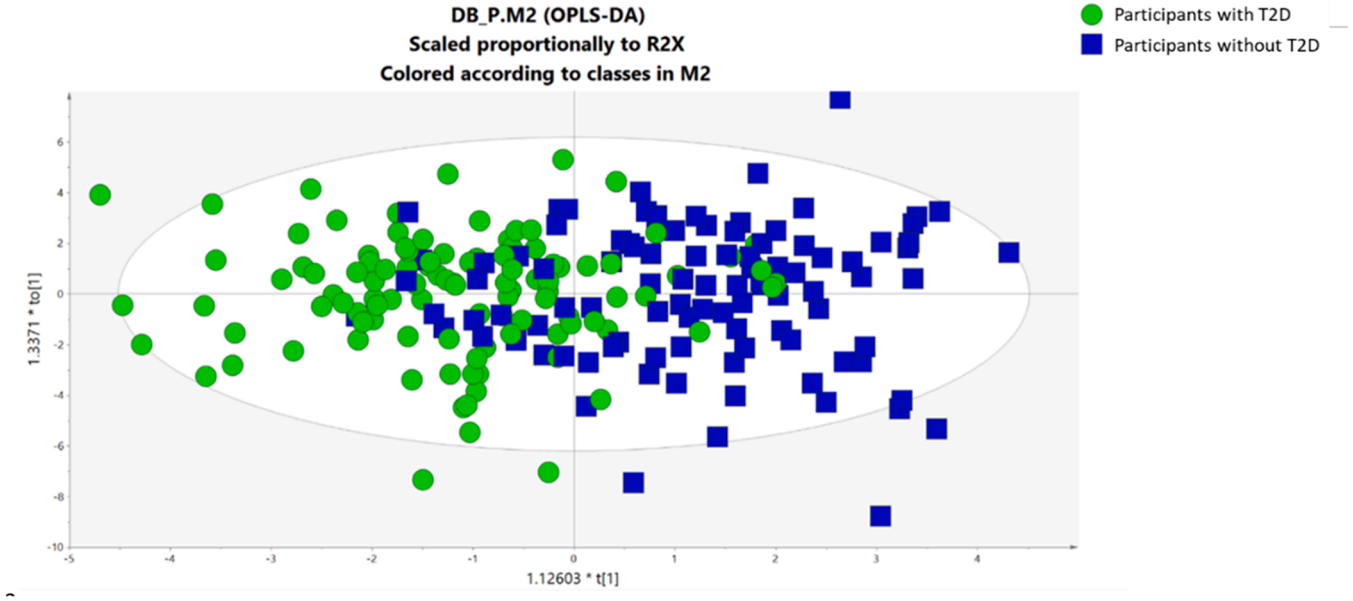

Fig. 1. OPLS-DA score plot built considering the urinary phenolic profile of the participants with and without T2D.

Table 2
Average logFC of the marker compounds (VIP > 1) of T2D and their polyphenol class. The OR (CI 95%) and *p*-value obtained for each compound in the logistic regression analysis are also shown.

Polyphenol class	Tentative metabolite identification	LogFC	OR (CI 95%) per 1-SD	*p*-value
Flavonoids	Epicatechin diglucuronide	-4.3481	0.74 (0.46; 1.17)	0.244
	Hesperetin	-2.2667	0.89 (0.76; 1.04)	0.214
	Daidzein sulfate	-2.1385	0.74 (0.60; 0.92)	0.068
	Genistein diglucuronide	-1.9158	0.72 (0.59; 0.88)	0.021
	Hesperetin diglucuronide	-1.6719	0.95 (0.84; 1.07)	0.419
	Genistein	-1.2496	0.73 (0.52; 1.03)	0.148
	Daidzein glucuronide	-1.2200	0.87 (0.71; 1.07)	0.244
	Hesperetin glucuronide	-1.0218	0.89 (0.77; 1.04)	0.214
	Daidzein	-0.4381	0.85 (0.73; 1.00)	0.128
	Naringenin diglucuronide	-0.3238	0.78 (0.62; 0.99)	0.117
	Naringenin disulfate	-0.0045	0.91 (0.68; 1.23)	0.595
	Epicatechin glucuronide	0.0821	0.94 (0.75; 1.18)	0.628
	Equol sulfate	1.1369	0.94 (0.65; 1.35)	0.740
	Hesperetin sulfate	1.7829	0.89 (0.75; 1.06)	0.244
	Equol glucuronide	2.6864	1.31 (1.07; 1.61)	0.068
Lignans	Enterolactone glucuronide	-0.5135	0.79 (0.62; 1.03)	0.148
Phenolic acids	Gallic acid diglucuronide	-4.4063	0.88 (0.72; 1.07)	0.244
	Benzoic acid diglucuronide	-2.7038	1.01 (0.78; 1.30)	0.961
	Hydroxybenzoic acid diglucuronide	-2.5572	0.71 (0.55; 0.92)	0.068
	3-Hydroxyphenylacetic acid	-1.6165	0.71 (0.56; 0.91)	0.068
	Caffeic acid diglucuronide	-0.8731	0.77 (0.61; 0.99)	0.117
	Vanillic acid disulfate	-0.7186	0.82 (0.60; 1.11)	0.244
	Vanillic acid sulfate	-0.5185	0.72 (0.48; 1.08)	0.186
	Benzoic acid glucuronide	-0.4855	0.75 (0.54; 1.03)	0.148
	Protocatechuic glucuronide	-0.3934	0.74 (0.48; 1.14)	0.244
	Protocatechuic acid	-0.3314	0.74 (0.47; 1.18)	0.244
	Dihydrocaffeic acid	-0.2748	0.22 (0.09; 0.52)	0.021
	Hippuric acid glucuronide	-0.0289	0.67 (0.47; 0.93)	0.082
	Hydroxyphenylpropionic acid sulfate	0.1788	1.19 (0.94; 1.51)	0.214
	Hydroxyphenylpropionic acid glucuronide	0.2362	1.14 (0.89; 1.46)	0.337
	Chlorogenic acid sulfate	0.2885	1.15 (0.98; 1.35)	0.148
	3-Hydroxyphenylacetic acid disulfate	0.3268	1.19 (0.91; 1.56)	0.244
	Caffeic acid sulfate	0.3724	1.31 (0.99; 1.72	0.145
	Ferulic acid sulfate	0.4121	1.41 (1.06; 1.87)	0.077
	Coumaric acid glucuronide	0.4505	1.32 (1.01; 1.73)	0.123
	Chlorogenic acid glucuronide	0.7417	1.22 (1.01; 1.47)	0.117
	Chlorogenic acid	1.0310	1.49 (1.08; 2.05)	0.077
Stilbenes	Dihydroresveratrol disulfate	0.2885	1.15 (0.98; 1.35)	0.148
Other	Urolithin A glucuronide	-0.3752	0.86 (0.77; 0.97)	0.077
	Urolithin C	-0.3273	0.80 (0.65; 0.99)	0.117
	Urolithin B diglucuronide	-0.3259	0.79 (0.61; 1.03)	0.148

FC, fold change; orthogonal projection to latent structures discriminant analysis, OPLS-DA; variable importance in projection, VIP; type-2 diabetes, T2D; OR, odds ratio; CI, confidence interval.
Log-transformation was applied to raw values of phenolic compounds.
Logistic regressions were adjusted for sex, age, smoking habit, educational level, BMI, physical activity, total energy intake, and hypercholesterolemia.
P-value was FDR-adjusted.

Most of the studies that report a negative association between polyphenols and T2D in humans are based on estimations of phenolic intake through dietary recall and food-composition tables rather than analysis of biological samples[37,38]. However, these methodologies are subject to errors due to systematic bias and subjectivity, and they do not consider other aspects such as bioavailability and the formation of new compounds through endogenous metabolism [7]. Thus, the resulting data may not reflect real exposure to biologically active compounds. In the present study, we employed liquid chromatography and HRMS, which provide highly accurate mass determinations and fragmentation patterns from multi-stage mass fragmentation and allow the structural elucidation of known and unknown compounds [39,40]. Therefore, we were able to identify a great variety of phenolic compounds in urine samples and to measure exposure to bioactive compounds objectively and accurately.

In our study, dihydrocaffeic acid was associated with a lower risk of T2D. This compound is thought to be a metabolite originated by the gut microbiota in the colon from the cinnamic acids caffeic, ferulic and chlorogenic acids [28,41,42]. These phenolic compounds can be found in different foods and beverages that form an essential part of the Mediterranean diet, such as coffee, vegetables, and fruits [29]. Previous research has reported that the beneficial effects of dihydrocaffeic acid are related to oxidative and the insulin/IGF-1 pathway [43]. However, to our knowledge, no studies until now have analysed the association of dihydrocaffeic acid with T2D or plasma glucose levels in humans.

Dihydrocaffeic acid precursors have shown that they can provide benefits against T2D through different molecular pathways, as described in Fig. 4. A study performed in rats reported that caffeic acid has a protective effect against hyperglycemia and insulin resistance, with several possible mechanisms involved. It has been suggested that caffeic acid modulates the purinergic and cholinergic pathways, thus reducing oxidative stress and inflammation [44]. It is also thought to decrease the production of proinflammatory factors, such as cytokines or leptin [45,46]. Furthermore, Un et al. found that glucokinase was down-regulated by caffeic acid, leading to an attenuation of hepatic glucose output [47]. Regarding ferulic acid, it has been shown that it reduces ß-cell dysfunction by increasing the activity of antioxidant enzymes and modulating others that are key to glucose production, as glucose-6-phosphatase and phosphoenolpyruvate carboxykinase [48]. There are numerous studies that demonstrate that chlorogenic acid can reduce blood glucose in humans. In a clinical trial, it was shown that it can enhance insulin sensibility and ameliorate insulin resistance [49]. Chlorogenic acid, a precursor of dihydrocaffeic acid through its cleavage

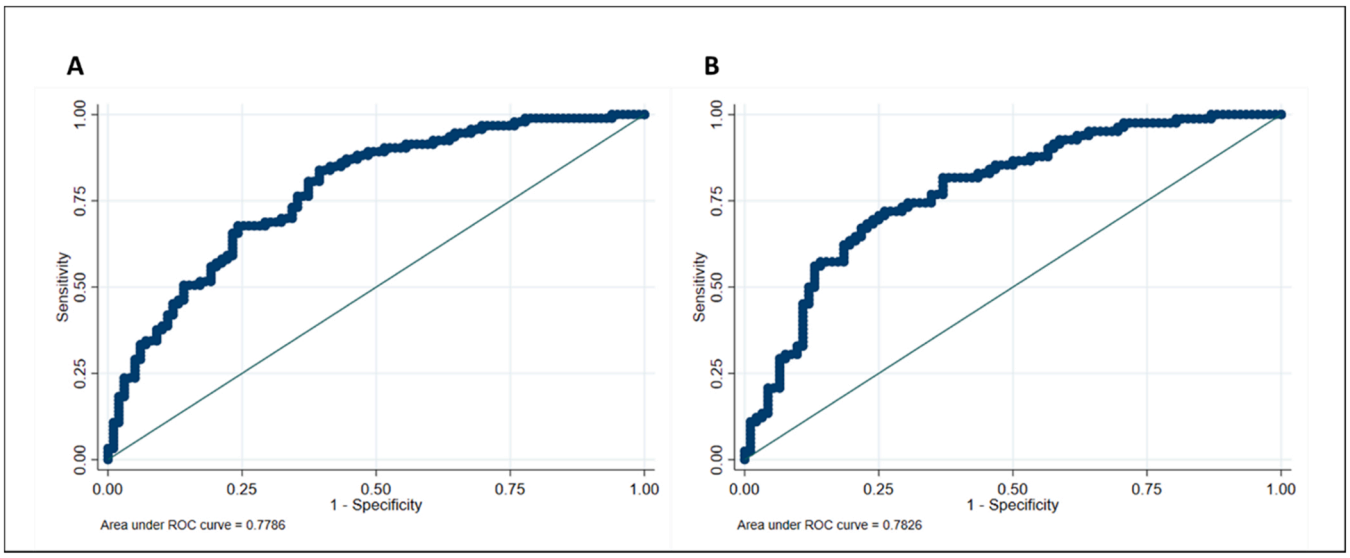

Fig. 2. Receiver operator characteristic curves for the predictive value of dihydrocaffeic acid (A) and genistein diglucuronide (B) regarding absence of type-2 diabetes.

Table 3
Multivariable linear regression between the discriminant phenolic compounds and plasma glucose.

		Dihydrocaffeic acid		Genistein diglucuronide	
		β (95% CI) per 1-SD	*p*-value	β (95% CI) per 1-SD	*p*-value
Plasma glucose (mg/dL)	*Model 1*	-12.87 (−26.61; 0.87)	0.066	-0.90 (−4.38; 2.57)	0.608
	Model 2	-17.12 (−29.92; −4.32)	0.009	-2.10 (−5.42; 1.23)	0.216
	Model 3	-10.15 (−22.46; 2.17)	0.106	0.42 (−3.48; 2.63)	0.785

ß, difference between groups; CI, confidence interval.
Log-transformation was applied to raw values of phenolic compounds.
Model 1: age and sex.
Model 2: age, sex, smoking habit, educational level, BMI, physical activity, total energy intake, and hypercholesterolemia.
Model 3: age, sex, smoking habit, educational level, BMI, physical activity, total energy intake, hypercholesterolemia, and use of antidiabetic drugs.

into caffeic and quinic acids, can modulate the activity of enzymes involved in glucose metabolism, such as α-amylase, or glucose-6-phosphatase [50,51]. It also reduces the decrease in the expression of IRS-1 and GLUT-4 typically observed after high glucose exposure [52]. In addition, chlorogenic acid can reduce the production of reactive oxygen species and protect against oxidative stress [53].

In the present study, we found that dihydrocaffeic acid was associated with lower concentrations of plasma glucose, suggesting that it exerts the same beneficial effects on glucose metabolism as its precursors, probably due to chemical structural similarity. Interestingly, when the use of antidiabetics drugs was included in the analysis this association was no longer significant. Nevertheless, this could be due to an overadjustment, as the participants using antidiabetic drugs were diabetics with high levels of plasma glucose. Overall, these findings suggest that this microbial caffeic acid metabolite has beneficial biological activity against T2D.

We found that higher levels of genistein diglucuronide were associated with a lower risk of suffering from T2D. Genistein diglucuronide is the major phase-II metabolite first to appear in plasma after genistein consumption, followed by single-conjugated metabolites [54]. The

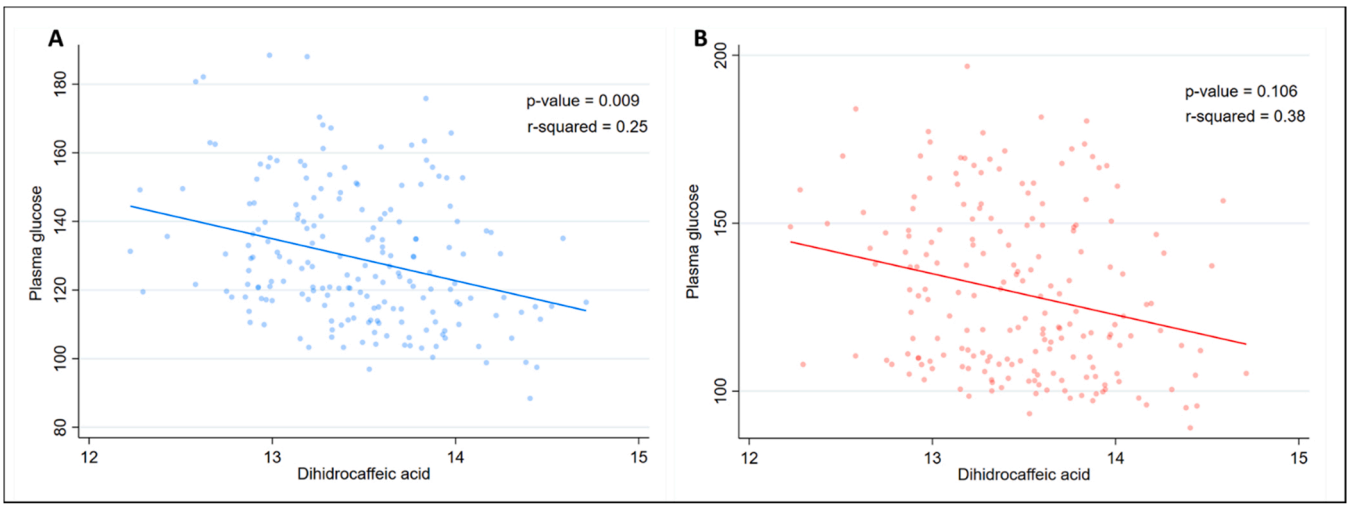

Fig. 3. Comparison of the multivariable linear regression between dihydrocaffeic acid and plasma glucose adjusted for (A): age, sex, smoking habit, educational level, BMI, physical activity, total energy intake, and hypercholesterolemia; and (B) further adjusted for the use of antidiabetic drugs.

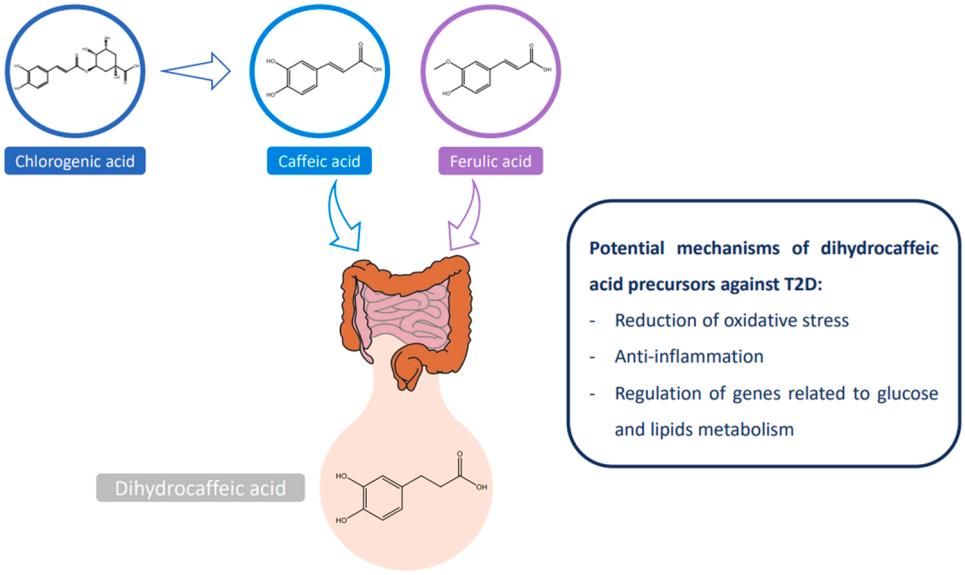

Fig. 4. Summary of dihydrocaffeic acid precursors (chlorogenic, caffeic, and ferulic acid) and their potential mechanisms against T2D. T2D, type 2 diabetes.

relationship between genistein aglycone and T2D has been widely investigated, and several studies have associated this isoflavone with a reduced risk of developing the disease, lower glucose levels, and improved insulin sensitivity [55,56]. Most of these studies were clinical trials that assessed the effects of daily genistein supplementation [57, 58]. Genistein is a phytoestrogen naturally found in soy in high concentrations, with small amounts present in other products consumed in the Mediterranean diet, such as nuts, vegetables, and fruits, [30]. Several mechanisms have been proposed to explain the protective effect of genistein against T2D. A study performed in mice suggested that it improves insulin release by inducing pancreatic β cells proliferation [59]. Mezei et al. observed a reduction of triglycerides and cholesterol through the activation of PPARγ, which is involved in glucose and lipid metabolism [60]. Therefore, genistein diglucuronide could exert a protective effect against T2D through similar mechanisms, as genistein may be released from the diglucuronide during its transport through blood or upon reaching an organ [54].

The present study has both strengths and limitations. Limitations include the relatively small sample size and the high cardiovascular risk status of the participants, which restricts the extrapolation of the results to other populations. In addition, the nature of the study precludes determination of causality. On the other hand, the main strength of our study is the use of metabolomics based on HRMS to evaluate a wide variety of phenolic compounds in biological samples. In addition, this study involved a free-living population, and the results reflect real-life conditions. Finally, the methodology here developed could be applied in other human studies to find new metabolite biomarkers of foods or disease.

5. Conclusions

A novel method using a metabolomics approach was developed to determine the association of urinary phenolic compounds with T2D revealed that two metabolites, dihydrocaffeic acid and genistein diglucuronide, were associated with a lower risk of T2D in a Mediterranean population at high cardiovascular risk. Further research is needed to explore the effects of these metabolites on the pathogenesis of T2D and their usefulness as tentative biomarker for T2D prediction.

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CRediT authorship contribution statement

Conceptualization, I.D.L. and R.M.L.M.; Formal analysis, I.D.L., J.L.C., A.V.Q., O.J.; Writing – original draft, I.D.L.; Writing – review and editing, J.L.C., A.V.Q., O.J., M.A.M.G., F.B.H., M.F., E.R., R.E., R.M.L.R.

Declaration of Competing Interest

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Data availability

Data will be made available on request.

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Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki and was approved by the Institutional Review Board of the 11 participating centers. The study was registered with the International Standard Randomized Controlled Trial Number (ISRCTN) 35739639.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.114703.

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