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# Plasma Metabolite Profiles Associated with the Amount and Source of Meat and Fish Consumption and the Risk of Type 2 Diabetes

*Jesús García-Gavilán, Stephanie K. Nishi, Indira Paz-Graniel, Marta Guasch-Ferré, Cristina Razquin, Clary B. Clish, Estefanía Toledo, Miguel Ruiz-Canela, Dolores Corella, Amy Deik, Jean-Philippe Drouin-Chartier, Clemens Wittenbecher, Nancy Babio, Ramon Estruch, Emilio Ros, Montserrat Fitó, Fernando Arós, Miquel Fiol, Lluís Serra-Majem, Liming Liang, Miguel A. Martínez-González, Frank B. Hu, and Jordi Salas-Salvadó**

**Scope: Consumption of meat has been associated with a higher risk of type 2 diabetes (T2D), but if plasma metabolite profiles associated with these foods reflect this relationship is unknown. The objective is to identify a metabolite signature of consumption of total meat (TM), red meat (RM), processed red meat (PRM), and fish and examine if they are associated with T2D risk.**

**Methods and results: The discovery population includes 1833 participants from the PREDIMED trial. The internal validation sample includes 1522 participants with available 1-year follow-up metabolomic data. Associations between metabolites and TM, RM, PRM, and fish are evaluated with elastic net regression. Associations between the profiles and incident T2D are estimated using Cox regressions. The profiles included 72 metabolites for TM, 69 for RM, 74 for PRM, and 66 for fish. After adjusting for T2D risk factors, only profiles of TM (Hazard Ratio (HR): 1.25, 95% CI: 1.06-1.49), RM (HR: 1.27, 95% CI: 1.07-1.52), and PRM (HR: 1.27, 95% CI: 1.07-1.51) are associated with T2D.**

**Conclusions: The consumption of TM, its subtypes, and fish is associated with different metabolites, some of which have been previously associated with T2D. Scores based on the identified metabolites for TM, RM, and PRM show a significant association with T2D risk.**

## 1. Introduction

The importance of nutrition in preventing noncommunicable diseases is well established. The current evidence suggests that the consumption of several animal-origin foods such as red meat (RM) and processed red meat (PRM) is associated with an increased risk of these conditions, especially cardiometabolic diseases.[1,2] RM and its derivatives are the main protein sources in the Western diet. Several cross-sectional and longitudinal studies have shown positive relationships between the consumption of RM and PRM with adverse health outcomes such as hypertension,[3] abdominal obesity,[4,5] metabolic syndrome,[4,6] type-2 diabetes (T2D),[7,8] and cardiovascular diseases (CVD).[9,10] Furthermore, the International Agency for Research on Cancer (IARC) has classified RM as a group 2A possible carcinogen and PRM as a group 1 carcinogen.[11]

J. García-Gavilán, S. K. Nishi, I. Paz-Graniel, N. Babio, J. Salas-Salvadó
Departament de Bioquímica i Biotecnologia
Unitat de Nutrició Humana, Hospital Universitari San Joan de Reus
Universitat Rovira i Virgili
Reus 43202, Spain
E-mail: jordi.salas@urv.cat

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J. García-Gavilán, S. K. Nishi, I. Paz-Graniel, N. Babio, J. Salas-Salvadó
Institut d'Investigació Sanitària Pere Virgili (IISPV)
Reus 43204, Spain

J. García-Gavilán, S. K. Nishi, I. Paz-Graniel, C. Razquin, E. Toledo, M. Ruiz-Canela, D. Corella, N. Babio, R. Estruch, E. Ros, M. Fitó, F. Arós, M. Fiol, L. Serra-Majem, M. A. Martínez-González, J. Salas-Salvadó
Consorcio CIBER, Fisiopatología de la Obesidad y Nutrición (CIBERObn)
Instituto de Salud Carlos III (ISCIII)
Madrid 28029, Spain

S. K. Nishi
Toronto 3D (Diet, Digestive Tract and Disease) Knowledge Synthesis and Clinical Trials Unit
Toronto, ON M5C 2T2, Canada

Both RM and PRM contain a notable amount of saturated fatty acids (SFA) which have been associated with alterations in lipid metabolism, insulin sensitivity, and adipose tissue inflammation, increasing the risk of developing CVD and T2D.[12] Additionally, the cooking processes of RM and PRM at high temperatures can form carcinogenic compounds such as heterocyclic amines.[13] On the other hand, fish, which is also a source of animal protein containing minerals and vitamins, or, in the case of fatty fish, omega 3 fatty acids, has not been consistently associated with such health risks.[3,5,14,15]

Until now, classical nutritional epidemiology has related lifestyle exposures to the development of chronic pathologies, but without deeping into the biological mechanisms implicated in these relationships. This type of epidemiologic approach is the so-called "black box approach" or "black box epidemiology."[16] The transition from a "black box approach" to a "systems approach" may be facilitated by identifying potential biological mechanisms underlying the established associations between diet and disease. Nutritional metabolomics allows us to investigate the relationship between diet and the risk of developing pathologies.[17]

Previous studies have associated meat consumption with an increased risk of T2D[7,8] and some metabolites related to the consumption of meat and fish have been also associated with some cardiometabolic pathways.[18] These findings can indicate a possible mediator effect between these foods and the development of pathologies such as T2D. However, no previous studies have investigated if plasma metabolite profiles correlated with the intake of total meat (TM), RM, PRM, and fish, are also associated with the development of T2D.

In the present study, we aim to identify the plasma metabolite profiles associated with the consumption of TM, RM, PRM, and fish using data from the PREvención con DIeta MEDiterránea (PREDIMED) study, and then examine whether these metabolite profiles are associated with an increased risk of T2D development independently of recognized conventional risk factors. We hypothesize that plasma metabolite profiles differ between participants based on their consumption of total meat, subtypes of meat (RM, PRM), and fish.

## 2. Experimental Section

### 2.1. Study Population

#### *2.1.1. Discovery Population*

The current study was carried out in the context of the PREDIMED study, a multicenter randomized controlled trial conducted in Spain from 2003 to 2010 where the main objective was to examine the effect of the Mediterranean diet (MedDiet) on the primary prevention of CVD in a population at high cardiovascular risk. A complete description of the PREDIMED study protocol

S. K. Nishi
Clinical Nutrition and Risk Factor Modification Centre
St. Michael's Hospital, Unity Health Toronto
Toronto, ON M5C 2T2, Canada

M. Guasch-Ferré, C. Wittenbecher, M. A. Martínez-González, F. B. Hu
Department of Nutrition
Harvard TH Chan School of Public Health
Boston, MA 02115, USA

M. Guasch-Ferré, F. B. Hu
Channing Division for Network Medicine, Department of Medicine
Brigham and Women's Hospital and Harvard Medical School
Boston, MA 02115, USA

C. Razquin, E. Toledo, M. Ruiz-Canela, M. A. Martínez-González
Department of Preventive Medicine and Public Health, Navarra Health Research Institute (IDISNA)
University of Navarra
Pamplona 31008, Spain

C. B. Clish, A. Deik
The Broad Institute of Harvard and MIT
Boston, MA 02142, USA

D. Corella
Department of Preventive Medicine
University of Valencia
Valencia 46020, Spain

J.-P. Drouin-Chartier
Centre Nutrition, Santé et Société, Institut sur la Nutrition et les Aliments Fonctionnels
Faculté de Pharmacie, Université Laval
Québec G1V 0A6, Canada

C. Wittenbecher
Department of Molecular Epidemiology
German Institute of Human Nutrition Potsdam-Rehbruecke
14558 Nuthetal, Germany

C. Wittenbecher
German Center for Diabetes Research
85764 Neuherberg, Germany

R. Estruch
Department of Internal Medicine, Institut d'Investigacions Biomèdiques August Pi Sunyer
Hospital Clinic
University of Barcelona
Barcelona 08036, Spain

E. Ros
Lipid Clinic, Department of Endocrinology and Nutrition, Agust Pi i Sunyer Biomedical Research Institute (IDIBAPS)
Hospital Clinic, University of Barcelona
Barcelona 08036, Spain

M. Fitó
Cardiovascular and Nutrition Research Group
Institut de Recerca Hospital del Mar
Barcelona 08003, Spain

F. Arós
Department of Cardiology
University Hospital of Alava
Vitoria 01009, Spain

M. Fiol
Health Research Institute of the Balearic Islands (Idisba)
University of Balearic Islands and Hospital Son Espases
Palma de Mallorca 07122, Spain

L. Serra-Majem
Research Institute of Biomedical and Health Sciences IUIBS
University of Las Palmas de Gran Canaria
Las Palmas 35001, Spain

L. Liang, F. B. Hu
Department of Epidemiology
Harvard T. H. Chan School of Public Health
Boston, MA 02115, USA

L. Liang
Department of Statistics
Harvard T. H. Chan School of Public Health
Boston, MA 02115, USA

could be found on the study website (http://www.predimed.es/) and in previous publications.[19,20] All study participants provided written informed consent, and the Institutional Review Boards of each of the respective study centers approved the protocol. The PREDIMED trial was registered at ISRCTN (http://www.isrctn.com/, ISRCTN35739639).

The present analysis involved participants from two nested case-cohort studies designed for metabolite profiling within the PREDIMED study (the first study for CVD, as the primary event of the trial, and the second study for T2D, as a secondary event) (Figure S1, Supporting Information). Participants from the first study, the PREDIMED-CVD study, consisted of 229 incident CVD cases and 788 subcohort participants free of CVD at baseline (overlapping $n = 37$),[21,22] while the participants from the second study, the PREDIMED-T2D study, consisted of 251 incident T2D cases, and 641 subcohort participants without T2D at baseline (overlapping $n = 53$).[23,24] All participants with available baseline metabolomics information from the two studies and who had completed validated semi-quantitative 137-item food frequency questionnaires (FFQs) were selected ($n = 1882$). However, participants were excluded from the analysis when they had missing values in FFQs at baseline ($n = 11$), a daily energy intake <500 and >3500 kcal day$^{-1}$ for women or <800 and >4000 kcal day$^{-1}$ for men ($n = 34$), or missing values in ≥20% metabolites ($n = 4$). The final sample included a total of 1833 participants at baseline that 633 had been allocated to the MedDiet supplemented with extra virgin olive oil group, 629 to the MedDiet supplemented with the nuts group, and 571 to the control diet group.

#### 2.1.2. Validation Population

To validate the metabolite profile, an internal validation analysis was conducted using data from the same PREDIMED study participants who had data on diet and metabolomics at 1-year of follow-up ($n = 1522$).

#### 2.1.3. Dietary Assessment

A 137-item semi-quantitative validated FFQ was given to all participants at baseline and yearly thereafter by trained dietitians in face-to-face interviews.[25] Energy and nutrient intake were estimated according to Spanish food composition tables.[26,27] Information on self-reported TM, RM, PRM, and fish were derived from the FFQ. TM consumption was the sum of RM, white meat, and PRM consumptions. RM consumption was the sum of the pork, veal, beef, and lamb consumptions. PRM consumption was the sum of offal, ham, sausages, pâté, hamburgers, and bacon. Fish consumption was considered the sum of white fish, blue fish, and seafood.

#### 2.1.4. Other Measurements and Covariates

Data related to lifestyle habits, medical conditions, and medications were collected at baseline and annual visits by trained staff. Weight, height, waist circumference, and blood pressure (by triplicate) were measured according to the study protocol. Physical activity was assessed with the validated Spanish version of the Minnesota Leisure-Time Physical Activity questionnaire at baseline and annually.[28]

#### 2.1.5. Metabolite Profiling

For the metabolomics analysis, baseline and 1-year overnight (>8 h) fasting plasma EDTA samples were collected, processed, and stored in –80 °C freezers at each recruiting center. Cases and subcohort participant samples were then randomly sent and analyzed in pairs (baseline and 1-year visit) for metabolomics assays. Quantitative metabolic profiling of the plasma samples was performed at the Broad Institute of Harvard University and the Massachusetts Institute of Technology using a combination of different instruments and methods previously described[29] that include high-throughput liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Nexera X2 U-HPLC coupled to a Q-Exactive mass spectrometer for AAs and profile polar metabolites and Nexera X2 U-HPLC coupled to an Exactive Plus orbitrap MS for lipids). After quality filtering and standardization, 399 named metabolites were qualified for primary analyses. Due to a high number of missing values (i.e., >20%) 11 metabolites and additional three metabolites, considered as internal standards (1,2-didodecanoyl-sn-glycero-3-phosphocholine, valine-d8, and phenylalanine-d8), were removed. In total, 385 metabolites were used in the final analysis.

Details of the LC-MS/MS methodologies utilized have been previously published.[30–32] Amino acids (AAs) and other polar metabolites were profiled with a Nexera X2 U-HPLC (Shimadzu Corp., Marlborough, MA, USA) coupled to a Q-Exactive mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA). Metabolites were extracted from 10 µL plasma using 90 µL of acetonitrile/methanol/formic acid (74.9:24.9:0.2 vol:vol:vol) containing stable isotope-labeled internal standards (valine-d8 [Sigma-Aldrich] and phenylalanine-d8 [Cambridge Isotope Laboratories]). The samples were centrifuged at 9000 × *g* for 10 min at 4 °C, and the supernatants were injected directly onto a 150 × 2-mm, 3-µm Atlantis HILIC column (Waters). The column was eluted isocratically at a flow rate of 250 µL min$^{-1}$ with 5% mobile phase A (10 mmol ammonium formate L$^{-1}$ and 0.1% formic acid in water) for 0.5 min followed by a linear gradient to 40% mobile phase B (acetonitrile with 0.1% formic acid) over 10 min. MS analyses were carried out using electrospray ionization in the positive-ion mode, and full-scan spectra were acquired over 70–800 $m/z$. For the AAs, mass spectrometry analyses were carried out using electrospray ionization in the positive-ion mode using full-scan analysis over 200–1100 $m/z$. Raw data were processed using Trace Finder version 3.1 and 3.3 (Thermo Fisher Scientific) and Progenesis QI (Nonlinear Dynamics). Polar metabolite identities were confirmed using authentic reference standards.

Fatty acids and other lipids were also profiled using a Nexera X2 U-HPLC (Shimadzu Corp., Marlborough, MA, USA) coupled to an Exactive Plus orbitrap MS (Thermo Fisher Scientific) and were extracted from 10 µL plasma using 190 µL of isopropanol containing 1,2-didodecanoyl-sn-glycerol-3-phosphocholine (Avanti Polar Lipids) as an internal standard. The lipid extraction (2 µL) was injected onto a 100 × 2.1-mm, 1.7-µm ACQUITY BEH C8 column (Waters). The column was eluted iso-

 

cratically with 80% mobile-phase A of (95:5:0.1 vol:vol:vol 10 mM ammonium acetate/methanol/formic acid) for 1 min followed by a linear gradient to 80% mobile-phase B (99.9:0.1 vol:vol methanol/formic acid) over 2 min, a linear gradient to 100% mobile-phase B over 7 min, and then 3 min at 100% mobile-phase B. Lipids were identified using the head group, total acyl carbon numbers, and total acyl double bond content. Pairs of pooled plasma reference samples were analyzed in intervals of 20 participant samples to enable assessment of data quality and to facilitate data standardization across the analytical queue and sample batches. One sample from each pair of the pooled references functioned as a passive quality control sample to assess the analytical measurement reproducibility of each metabolite, whereas the other pooled sample was used to standardize using a "nearest neighbor" approach. Standardized values were calculated using the ratio of the value in each sample over the nearest pooled plasma reference multiplied by the median value measured across the pooled references.

#### 2.1.6. Statistical Analysis

Participants' baseline characteristics were presented as median and interquartile range (IQR) when variables were quantitative or percentages (*n*) when variables were categorical. The identification and selection of metabolites associated with TM, RM, PRM, and fish consumption was done using plasma metabolomics data from the PREDIMED study at baseline as the training set (i.e., discovery population). As testing sets (i.e., validation population), 1-year data from the PREDIMED study were used.

Missing values were imputed for metabolites with less than 20% missing using the random forest imputation approach ("missForest" function from the "missForest" R package) as previous publications have recommended.[33–35] Before conducting the multivariate analysis, autoscaling of the metabolomic data was done, such that the data were centered and scaled with the SD as the scaling factor.[36] Gaussian linear regression models were used with the elastic net penalty ("glmnet" R package) to build the models for TM, PRM, RM, and fish consumption because of the high dimensionality and collinear nature of data. In the discovery population (PREDIMED baseline data), a 10-fold cross-validation (CV) approach was performed such that the sample was split into training and validation sets at 90% and 10% of the sample, respectively. Furthermore, a 10-fold CV was performed within the training sets to determine the optimal value of the tuning parameter ($\lambda$) to yield the minimum mean squared error (minMSE). The minMSE value was estimated using the argument s = "lambda.min" in the cv.glmnet function ("glmnet" R Package). The coefficients from each CV iteration were reported after evaluating the $\lambda$ selection. Additionally, the $\alpha$ parameter was evaluated from 0 (i.e., a Ridge regression) to 1 (i.e., a Lasso regression) in 0.1 increments to test the best parameters for these analyses. The best-predicting accuracy in the validation sets was obtained with $\alpha = 0.2$ for TM, 0.3 for RM, 0.4 for PRM, and 0.1 for fish models. Additionally, for each training validation data set pair, weighted models were constructed using the coefficients of the metabolites resulting from each elastic net regression in the training set. Pearson correlation coefficients were determined between TM, PRM, RM, and fish consumption and the metabolites profiles in both the discovery and internal validation populations. For reproducibility, regression coefficients were reported using 10 iterations of the 10-fold CV elastic net regression in the entire data set. These analyses were based on consistency among CV runs and, therefore, any *p*-value was derived.

Cox regressions with Barlow weights and robust variance estimator were run to evaluate the associations between the identified metabolites profiles of TM, RM, PRM, and fish consumption and T2D risk (245 events at baseline and 161 incident events at 1 year) within the T2D nested case-cohort study. Three multivariate models were assessed. For the baseline analysis, the first model, the basic model, was adjusted for age (years), sex, and propensity scores (see Supplementary Methods, Supporting Information),[20] and was stratified by intervention group and recruitment center. The second model was additionally adjusted by body mass index (kg $m^{-2}$), smoking status (current, former, or never), and alcohol consumption in g $day^{-1}$ (and adding a quadratic term), educational level (primary, secondary, or college), physical activity (METs $min^{-1}$ $day^{-1}$), family history of CHD (yes/no), total energy intake (kcal $day^{-1}$), and intakes of vegetables, fruits, cereals, nuts, olive oil, eggs, legumes, and dairy. The third model included all covariables from model 2 in addition to the consumption of TM, RM, PRM, or fish at baseline from which the metabolite set was derived, respectively. For the 1-year analyses, the metabolite signatures were associated with incident T2D adjusting for baseline data but excluding T2D cases during the first year (same models than baseline analyses).

Several sensitivity analyses were performed. First, binomial linear regression models were used with the elastic net penalty employing extreme tertiles (T1 vs T3) for each of the analyzed meat and fish group categorizations (TM, RM, PRM, and fish) instead of treating the exposures as continuous data. Second, Pearson correlations were performed between the intake of meat-food groups and fish with each metabolite profiles to test the specificity of each signature. Third, the sample was splitted by sex and ran the Cox regression models with Barlow weights and robust variance with the identified metabolite signatures of TM, RM, PRM, and fish as continuous at baseline and 1-year visit. Finally, the sample by intervention group was also splitted and ran again the Cox regression models using the identified metabolite signatures as continuous at baseline and 1-year visit. Finally, the metabolite profiles were performed again without those participants who developed CVD and validated them using Pearson correlations between the consumption of foods groups with each metabolite profiles.

All analyses were considered statistically significant when $p < 0.05$ and were performed using R version 3.6.1 statistical software (R Foundation for Statistical Computing).

## 3. Results

### 3.1. Characteristics of the Study Participants

**Table 1** shows the baseline and 1-year characteristics of the study participants according to extreme tertiles of TM intake and Figure S1, Supporting Information shows the flow-chart of participants. The median [IQR] of TM consumption at baseline was 130.6 [97.6, 164.9] g $day^{-1}$ and after 1 year was 119.6 [90.0, 152.0] g $day^{-1}$. Concerning RM, PRM, and total fish consumption, the

 

**Table 1.** Characteristics of study subjects and according to extreme tertiles [T1 vs T3] of total meat intake.

| | Baseline visit[a)] | | | 1 Year visit[a)] | | |
|---|---|---|---|---|---|---|
| | T1 ($n$ = 613) | T3 ($n$ = 608) | Total sample ($n$ = 1833) | T1 ($n$ = 511) | T3 ($n$ = 501) | Total sample ($n$ = 1522) |
| Age [years] | 69 [64, 73] | 66 [62, 71] | 67 [62, 72] | 68 [63, 74] | 66 [62, 71] | 67 [62, 72] |
| Sex, women, $n$[%] | 392 [64] | 286 [47] | 1058 [58] | 306 [60] | 253 [51] | 875[58] |
| Body mass index [kg m$^{-2}$] | 29.8 [27.5, 32.3] | 29.8 [27.5, 32.3] | 29.7 [27.4, 32.2] | 30.0 [27.5, 32.4] | 29.4 [27.3, 31.8] | 29.6 [27.3, 32.1] |
| Waist circumference [cm] | 100 [93, 106] | 101 [95, 108] | 100 [93, 107] | 101 [94, 107] | 101 [94, 107] | 100 [93, 106] |
| Type 2 diabetes, $n$ [%] | 186 [30] | 194 [32] | 535 [29] | 162 [32] | 154 [31] | 463 [30] |
| Hypercholesterolemia, $n$ [%] | 485 [79] | 450 [74] | 1411 [77] | 378 [74] | 371 [74] | 1152 [76] |
| Hypertension, $n$ [%] | 537 [88] | 529 [87] | 1603 [87] | 441 [86] | 434 [87] | 1318 [87] |
| Family history of CVD, $n$ [%] | 150 [24] | 138 [23] | 451 [25] | - | - | - |
| Current smoking, $n$ [%] | 80 [13] | 111 [18] | 287 [16] | 70 [14] | 93 [19] | 215 [14] |
| Total meat [g day$^{-1}$] | 83 [60, 98] | 184 [166, 211] | 131[98, 165] | 77 [59, 91] | 170 [152, 195] | 120 [90, 152] |
| Total fish [g day$^{-1}$] | 90 [61, 124] | 103 [75, 141] | 97 [65, 129] | 100 [70, 127] | 108 [77, 140] | 104 [76, 135] |
| Red meat [g day$^{-1}$] | 21 [10, 31] | 84 [53, 96] | 43 [21, 74] | 20 [10, 31] | 64 [41, 86] | 31 [20, 53] |
| White meat [g day$^{-1}$] | 21 [21, 64] | 74 [64, 86] | 64 [31, 74] | 31 [21, 64] | 74 [64, 86] | 64 [31, 74] |
| Processed meat [g day$^{-1}$] | 16 [9, 24] | 36 [25, 47] | 25 [15, 36] | 13 [5, 21] | 29 [20, 42] | 21 [12, 31] |
| Vegetables [g day$^{-1}$] | 303 [219, 387] | 321 [244, 434] | 311 [233, 404] | 315 [234, 402] | 339 [256, 434] | 332 [250, 415] |
| Fruit [g day$^{-1}$] | 328 [207, 470] | 332 [231, 469] | 328 [220, 474] | 334 [235, 491] | 377 [259, 514] | 360 [250, 503] |
| Legumes [g day$^{-1}$] | 17 [13, 26] | 17 [16, 25] | 17 [13, 25] | 21 [16, 26] | 21 [16, 26] | 21 [16, 26] |
| Cereals [g day$^{-1}$] | 200 [141, 279] | 237 [176, 304] | 216 [166, 291] | 106 [138, 269] | 115 [176, 302] | 109 [162, 290] |
| Dairy [g day$^{-1}$] | 332 [226, 565] | 310 [228, 532] | 325 [228, 550] | 331 [225, 553] | 323 [228, 528] | 321 [228, 537] |
| Olive oil [g day$^{-1}$] | 35 [25, 50] | 50 [25, 50] | 50 [25, 50] | 50 [25, 50] | 50 [25, 50] | 50 [25, 50] |
| Nuts [g day$^{-1}$] | 6 [0, 15] | 6 [2, 17] | 6 [0, 15] | 4 [2, 30] | 4 [4, 28] | 4 [2, 28] |
| Wine [g day$^{-1}$] | 0 [0, 79] | 14 [0, 100] | 7 [0, 100] | 0 [0, 79] | 14 [0, 100] | 7 [0, 100] |
| Alcohol [g day$^{-1}$] | 1 [0, 10] | 5 [1, 16] | 2 [0, 11] | 1 [0, 10] | 5 [0, 12] | 3 [0, 11] |
| Total energy [kcal day$^{-1}$] | 2075 [1749, 2430] | 2468 [2111, 2855] | 2228 [1907, 2618] | 2056 [1763, 2405] | 2423 [2091, 2846] | 2227 [1903, 2597] |

[a)] Values are medians [IQR] for continuous variables or number [%] for categorical variables. T, tertiles.

median consumption values were 42.9 [21.4, 74.3], 25.3 [14.8, 36.4], and 97.1 [65.4, 129.2] g day$^{-1}$ at baseline, and 31.4 [20.0, 52.9], 20.5 [11.9, 30.7], and 103.5 [76.3, 135.0] g day$^{-1}$ at 1-year, respectively. Participants with higher TM consumption at baseline were less likely to be women and more likely to smoke. Table S1, Supporting Information provides the baseline and 1-year characteristics of study participants according to tertiles of fish, RM, and PRM consumption and Table S2, Supporting Information shows the characteristics of study participants according to the PREDIMED-T2D case-control subcohort included in the Cox models. Participants showed similar characteristics regardless of the consumption of RM, PRM, and fish. In the PREDIMED-T2D subcohort, control participants were more likely to be female and to have hypercholesterolemia, but less likely to have hypertension and to smoke at baseline and 1-year.

### 3.2. Identification of Meat-and-Fish-Related Metabolites

**Table 2** summarizes the Pearson correlation coefficients, as well as the number of selected metabolites, between the metabolite profile models and the TM, its subtypes, and total fish consumption values in the PREDIMED baseline data (discovery population) and 1-year data (validation population). The Pearson correlations between metabolite profiles and food consumption at baseline were 0.41 (95% CI: 0.37, 0.45) for TM intake, 0.37 (95% CI: 0.33, 0.41) for RM intake, 0.39 (95% CI: 0.35, 0.43) for PRM intake, and 0.45 (95% CI: 0.42, 0.49) for fish intake. At 1-year, the Pearson correlations were 0.31 (95% CI: 0.26, 0.35) for TM intake, 0.32 (95% CI: 0.28, 0.37) for RM intake, 0.22 (95% CI: 0.17, 0.26) for PRM intake, and 0.33 (95% CI: 0.29, 0.38) for fish intake. Figure S2, Supporting Information displays Pearson correlations between the intake of meat-food groups and fish with each metabolite profiles to test the specificity of each signature. TM consumption was associated with 72 metabolites, RM with 69, PRM with 74, and fish consumption with 66. The respective means of the metabolites' regression coefficients are shown in Figure S3 and Table S3, Supporting Information. **Figure 1** shows Venn diagrams with overlapping metabolites identified the different food intake approaches. The metabolites with the strongest negative association with TM cosumption were lactate, 14:0 sphingomyelin (SM), and 40:6 phosphatidylcholine (PC), with RM cosumption lactate, 18:2 carnitine, and 10:2 carnitine, with PRM consumption glycine, 34:0 phosphatidylethanolamine (PE), and 40:10 PC, and with fish consumption 18:0 cholesterol ester (CE), dimethylguanidino valeric acid (DMGV), and 34:0 PE. Whereas the metabolites with the strongest positive association with TM cosumption were

 

**Table 2.** Pearson correlation coefficients between metabolite profiles and food intakes.

| Assessment | Baseline visit | | | | 1 year visit |
|---|---|---|---|---|---|
| | Pearson correlation (95% CI)[a)] | Total metabolites[b)] | Metabolites with positive coefficients | Metabolites with negative coefficients | Pearson correlation (95% CI)[a)] |
| Total meat | 0.41 (0.37, 0.45) | 72 | 28 | 44 | 0.31 (0.26, 0.35) |
| Red meat | 0.37 (0.33, 0.41) | 69 | 31 | 38 | 0.32 (0.28, 0.37) |
| Processed red meat | 0.39 (0.35, 0.43) | 74 | 28 | 46 | 0.22 (0.17, 0.26) |
| Total fish | 0.45 (0.42, 0.49) | 66 | 28 | 38 | 0.33 (0.29, 0.38) |

[a)] The Pearson's coefficients reflect the correlation between FFQ food intakes and predicted food intakes based on the previously food-specify metabolite profiles within the discovery cohort; [b)] Number of metabolites obtained 10 times in the 10-fold cross-validation procedure for the elastic net continuous regression, using the lambda.min option.

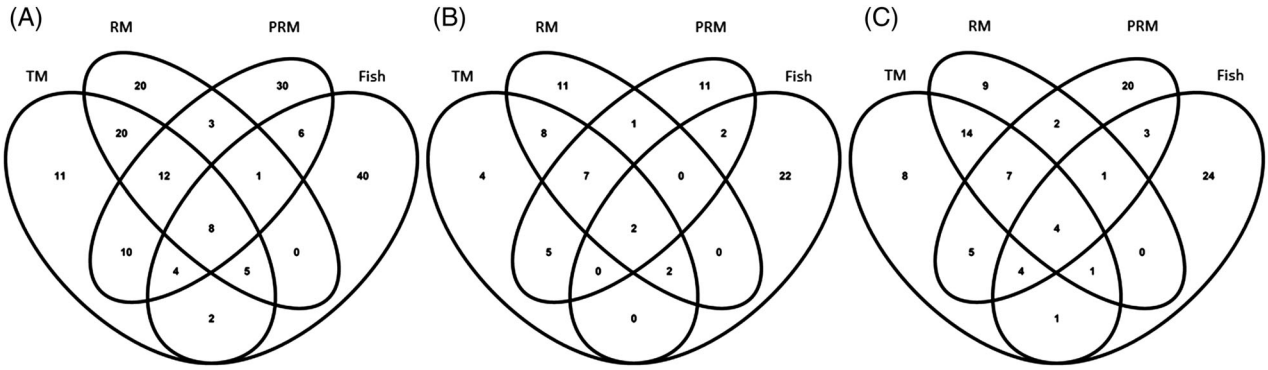


**Figure 1.** Venn diagrams showing the overlapping metabolites identified using the different food intake approaches by means of the elastic net continuous regression. A) Considering all selected metabolites; B) considering metabolites with positive coefficients; C) considering only metabolites with negative coefficients. PRM, processed meat; RM, red meat; TM, total meat.

isoleucine, creatine, and lactose, with RM consumption 38:4 PC plasmalogen, cotinine, and isoleucine; with PRM consumption leucine, uric acid, and 36:5 PC plasmalogen, and with fish consumption sorbitol, trimethyl benzene, and 22:6 CE. Regarding overlapping metabolites, there are two overlapping metabolites (C36:5 PC plasmalogen A and cotinine) in all food groups and only one metabolite (cortisone) between RM and PRM with positive coefficients. Likewise, there are four overlapping metabolites (glycine, C34:0 PE, C22:4 CE, and cyclohexylamine) associated with all food groups, one metabolite (N4-Acetylcytidine) between TM and fish, and only two metabolites (C22:1 SM and uridine) between RM and PRM with negative coefficients.

Sensitivity analysis using extreme tertiles of TM, RM, PRM, and fish intake in the elastic net logistic regression showed comparable results in terms of the metabolites selected (data not shown). Similarly, when we repeated the selection and validation of the metabolite profiles for each food excluding those participants with incident CVD as a sensitivity analysis, all selected metabolites were also in the previously performed metabolite profiles, showing similar associations (in magnitude and direction), and the Pearson correlations at baseline and also at 1-year visits (Tables S6 and S7, Supporting Information).

### 3.3. Association of Meat-and-Fish-Related Metabolites with T2D

At baseline, the HR and 95% CI for T2D per SD increment in the metabolite profile model of TM consumption was 1.25 (1.06, 1.49; *p*-value = 0.009), for RM it was 1.27 (1.07, 1.52; *p*-value = 0.007), for PRM it was 1.27 (1.07, 1.51; *p*-value = 0.007), and for fish it was 1.12 (0.98, 1.29; *p*-value = 0.107) after adjusting for lifestyle and dietary risk factors (**Table 3**, model 2). At 1-year (i.e., using the internal validation population), the HR and 95% CI for T2D per SD increment in the metabolite profile model of TM consumption was 0.87 (0.70, 1.08; *p*-value = 0.196), for RM this was 0.85 (0.68, 1.06; *p*-value = 0.155), for PRM it was 1.12 (0.90, 1.41; *p*-value = 0.309), and for fish it was 1.13 (0.94, 1.35; *p*-value = 0.198) also after adjusting for lifestyle and dietary risk factors (Table 3, model 2). The results were similar even when the models were additionally adjusted for their respective self-reported food group consumption (Table 3, model 3).

In the secondary analyses, when we divided the database of T2D participants by sex (Table S4, Supporting Information), in men, but not in women, similar results to the main analysis in the Cox models at baseline were observed (TM HR: 1.68, 95% IC: 1.24, 2.27; RM HR: 1.55, 95% CI: 1.19, 2.03; PRM HR: 1.40, 95% CI: 1.08, 1.84; Fish HR: 1.16, 95% CI: 0.93, 1.45). No significant associations have been observed at 1-year, except for the TM for men (HR: 0.66, 95% IC: 0.46, 0.96) and PRM for women (HR: 1.41, 95% IC: 1.01, 1.98). Similarly, when we divided the database of T2D participants by intervention group, participants in the MedDiet group showed a 40% and 45% increase in the risk of developing T2D for each SD increase in the TM-signature model and for the RM-signature model, while participants in the low-fat diet group only showed a 49% increase in the risk of developing T2D for each SD increase in the PRM-signature model. At 1-year, no associations between the food metabolic signatures

**Table 3.** HRs (95% CIs) for incident type 2 diabetes according to metabolite profiles of total meat and subtypes, and total fish intakes in the PREDIMED population.

| | Type 2 diabetes | | | |
|---|---|---|---|---|
| | Baseline visit[a)] | | 1 year visit[b)] | |
| | HR (95% CI) | *p* | HR (95% CI) | *p* |
| Cases/total participants | 245/923 | | 161/704 | |
| Total meat | | | | |
| Model 1 | 1.21 (1.03, 1.42) | 0.019 | 0.92 (0.76, 1.11) | 0.381 |
| Model 2 | 1.25 (1.06, 1.49) | 0.009 | 0.87 (0.70, 1.08) | 0.196 |
| Model 3 | 1.27 (1.07, 1.51) | 0.007 | 0.84 (0.67, 1.05) | 0.129 |
| Red meat | | | | |
| Model 1 | 1.25 (1.06, 1.47) | 0.009 | 0.89 (0.73, 1.08) | 0.381 |
| Model 2 | 1.27 (1.07, 1.52) | 0.007 | 0.85 (0.68, 1.06) | 0.155 |
| Model 3 | 1.28 (1.07, 1.53) | 0.007 | 0.84 (0.66, 1.06) | 0.138 |
| Processed meat | | | | |
| Model 1 | 1.19 (1.01, 1.40) | 0.036 | 1.11 (0.90, 1.37) | 0.337 |
| Model 2 | 1.27 (1.07, 1.51) | 0.007 | 1.12 (0.90, 1.41) | 0.309 |
| Model 3 | 1.32 (1.10, 1.58) | 0.003 | 1.09 (0.87, 1.37) | 0.469 |
| Fish | | | | |
| Model 1 | 1.04 (0.92, 1.18) | 0.540 | 0.94 (0.81, 1.09) | 0.412 |
| Model 2 | 1.12 (0.98, 1.29) | 0.107 | 1.13 (0.94, 1.35) | 0.198 |
| Model 3 | 1.16 (1.00, 1.35) | 0.051 | 1.11 (0.93, 1.33) | 0.261 |

Model 1: adjusted for age (years), sex, and propensity scores; stratified by intervention group and recruitment center. Model 2: model 1 + BMI, smoking status (never, former, or current smoker), alcohol intake and squared alcohol intake (grams per day), education level (primary, secondary, academic) physical activity (metabolic-equivalent minutes per day), family history of CHD (yes/no), total energy intake (kcal per day) and intakes of vegetables, fruits, cereals, nuts, olive oil, eggs, legumes, and dairy. Model 3: model 2 + intake of total meat, fish, red meat, or processed meat, respectively, from which the metabolite set was derived. BMI, body mass index; CHD, coronary heart disease; CI, confidence interval;HR, hazard ratio; T2D, type 2 diabetes. [a)] Analysis of T2D risk was conducted in the 923 participants form the PREDIMED case-cohort database. Cox proportional hazard models, with Barlow weights were used to estimate HRs and their 95% CIs for T2D. Person-time of follow-up was calculated as the interval between the baseline data and the date of T2D event, death, or date of the last participant contact, whichever came first. HRs refer to 1-SD increase in correlated multi-metabolite score; [b)] Total meat and subtypes intakes, total fish intake, metabolic signatures, and covariates were assessed at the first year, and outcome was the incident T2D events occurred after the first-year visit through to the end of follow-up. The models were the same as in the baseline models. 704 participants for T2D were included in the analyses.

considered and T2D risk was observed. Only the PRM-signature was associated with a 77% increase in the risk of developing T2D in the low-fat diet group (Table S5, Supporting Information).

## 4. Discussion

To our knowledge, this is the first study to examine the association between metabolite signatures of meat and fish and the risk of T2D. We identified several sets of metabolites, associated with TM, RM, PRM, and fish which showed moderate significant Pearson correlation coefficients with the respective self-reported food intakes from participants of the PREDIMED study. Additionally, all identified meat-related metabolite signatures showed a significant positive association with T2D after adjusting for potential confounders at baseline.

A few observational studies and clinical trials have previously evaluated and related the TM or fish consumption with plasma or serum metabolites using different metabolomics platforms[37–44] and some of these metabolites were found to overlap in our multi-metabolite signatures, suggesting that on their own may not be adequate as unique biomarkers as they do not appear to have enough discriminating ability to distinguish between the consumption of these food groups. The identification of metabolite profiles that include more metabolites than those typically associated with dietary meat or fish intake may reflect metabolite variations associated with the whole diet, help to discern between the type of product consumed, and provide greater knowledge of new candidate metabolic pathways to be altered by consumption of these food groups.

In the EPIC-Oxford cohort, a profile of 118 metabolites was determined according to the participants diet.[45] Meat-eaters displayed higher levels of several acylcarnitines (C0, C14:1, C2, C4, and C5), arginine, creatinine, serotonin, several PC species, and SMs compared to vegeterians, vegans, or fish-eaters. In the present study, positive associations were also observed between creatinine and some PCs with TM and RM consumption, while SMs were negatively associated with TM, RM, and PRM. Another study conducted on a Chinese population found similar associations.[43] In a cross-sectional analysis conducted in 7012 Japanese adult women and men, a promising TM metabolomic biomarker with a profile of six AAs, peptides, and analogs that included hydroxyproline, 2-aminobutyrate, creatine, carnitine, 3-MH, and beta-alanine was identified.[46] Our multi-metabolite signature also included some of these AAs, but also other

metabolites, highlighting the presence of isoleucine, several short- and medium-chain carnitines, glutamate, proline, and glycine.

Our fish-related metabolite profiles showed a strong positive association with plasma sorbitol, a polyol metabolized in the liver which is able to modify adenosine levels in humans and increase peripheral uric acid concentrations.[47] Of note, meat consumption was also positively associated with uric acid. Higher levels of both sorbitol and uric acid have previously been associated with the pathogenesis of T2D,[48,49] which is consistent with our results where a higher prevalence of T2D was shown in those participants in the higher consumption tertile of these foods.

Eight overlapping metabolites were observed between our four food metabolite signatures (for TM, RM, PRM, and fish), 12 overlapping metabolites between the signatures of meat groups, and 20 metabolites overlapped between TM and RM (the highest number of overlapping metabolites between signatures, probably because RM consumption was higher in our population compared to PRM and fish). Only the associations for 5-acetylamino-6-amino-3-methyluracil (AAMU) and C38:4 PC plasminogen showed an inverse association between meat groups (positive associations) and fish (negative associations) in the group of eight overlapping metabolites. AAMU is an aromatic amide related to caffeine metabolism.[50] Caffeine was also selected with a positive coefficient in the three meat signatures, but not in the case of fish. Previous studies have demonstrated that smokers, whose sense of taste is usually modified by the smoking habit, present with a higher intake of meat and coffee, and a lower intake of fish,[51] which potentially could explain the results of our study. Our participants in the highest tertile of the meat groups had a higher consumption of coffee (data not shown) and higher prevalence of smokers compared to those in the lowest tertile, while no significant difference in the prevalence of smokers was observed in the case of fish consumption.

C38:4 PC plasmalogen is a metabolite classified as an oxidative stress biomarker[52] that has shown to be correlated with RM and PRM.[53,54] Low levels of plasmalogens under high oxidative stress conditions, as occurs in a population with several cardiovascular risk factors, can be indicative of their function as scavengers protecting other phospholipids, lipids, and lipoproteins from oxidative reactions[55] and may explain the inverse association we observed between plasmalogens and fish consumption, a recognized dietary source of omega-3 long-chain polyunsaturated fatty acids.[56] The associations for the other six metabolites (C22:4 CE, C34:0 PE, cyclohexylamine, glycine, C36:5 PC plasminogen, and cotinine) had the same direction in across the groups of TM, RM, PRM, and fish. In some cases, like glycine, the metabolite has been previously negatively associated with the consumption of foods from animal-origin,[45,57] but in other cases, such as C34:0 PE, lactose, and GABA, a positive association has been seen with dairy food,[58] contrary to our results.

Several epidemiological studies have demonstrated detrimental effects of RM and PRM consumption on cardiometabolic health.[4,5,7,9] In the present study, a total of 20 and 30 metabolites were associated with RM or PRM consumption, respectively. Beta-alanine and N-carbamoyl-beta-alanine were only negatively associated with RM. Beta-alanine is considered a biomarker of RM and TM consumption and N-carbamoyl-beta-alanine is derived from the former, which explains its inverse association. Numerous carnitines were associated with RM intake (C5, C7, and C26 were positively associated) and PRM intake (C18:1-OH, C20:4, and C4 were negatively associated). These molecules are cofactors of fatty acid metabolism that transport fatty acids into the mitochondria and stimulate beta-oxidation.[59] Some of these carnitines had already been previously associated with RM or PRM[45,60] and, curiously, in the present analyses total carnitine was only associated with fish. These observed differences may be related to the processing and the addition of additives in PRM. Even so, plasma carnitine levels can also be altered by some physiological conditions associated to age or sex, so we cannot rule out that their selection is a consequence of the characteristics of the population. Other specific metabolites included in the PRM signature were arginine and ADMA, while ornithine and SDMA were selected for the RM signature, both metabolites related to the arginine/nitric oxide pathway.[61] A possible explanation may lie in the content of dietary nitrites, common in these products. Nitrites can increase the bioavailability of arginine and redirect its flow from urea/ornithine production to nitric oxide/citrulline formation.[62]

A number of metabolites only overlapped between the RM and PRM signatures, but not with the TM signature (e.g., cortisone and uridine) or on the contrary, they were only selected in the TM signature (mainly different families of lipid species, but also metabolites such as succinate, thyroxine, or xanthosine). An explanation for these differences is currently unknown, and therefore future research is warranted to confirm the specific relationships and the potential role as a biomarker of these metabolites with the consumption of these foods.

T2D has previously been associated with the consumption of TM, RM, and PRM[7,8] and with some of the metabolites that are part of their metabolite profiles.[63] For example, amino acids such as isoleucine, leucine, or glutamate, that we associated with higher consumption of TM, RM, and PRM in our metabolite profiles, have been previously associated with insulin resistance and increased risk of T2D. In addition, some lipids that showed a positive association with the consumption of TM, RM or fish in our metabolite profiles (C34:2 PE, C38:3 PC, C38:4 DAG, C51:1 TAG) have been associated with a lower risk of T2D.[63] The consumption of these food groups might promote metabolic alterations through some pathways such as proteolysis, de novo lipogenesis, or fatty acid oxidation, increasing the risk of T2D. However, more specific studies are needed to evaluate these hypotheses. After adjusting for potential confounders, we observed that our TM, RM, and PRM metabolic signatures were associated with a 25%, 27%, and 27% higher risk of T2D, respectively, but no significant association were observed with fish metabolite profiles. Additionally, we did not find associations between these signatures with 1-year T2D incidence. These contradictory results may be due to reverse causality as a consequence of the dietary interventions of the trial, as we have observed in our secondary analyses.

Limitations and strengths of our study have to be considered. First, the use of FFQs to determine food consumption may be prone to measurement error and participant recall bias despite being administered by trained dietitians. However, the validity and reproducibility of the FFQ have been previously assessed and confirmed for use in the present population.[25] Second, we are unable to establish a cause–effect relationship between metabolic signatures and T2D because of the observational design of our

study. Furthermore, our analyses were conducted in a single elderly Mediterranean population with high cardiovascular risk, and although the sensitivity analyzes showed similar results to the principal analyses, we cannot rule out that part of these results are the consequence of some pathogenic factors related to CVD development that are associated, for example, with elevated BCAAs.[21] For this reason, our results cannot be generalized and should be replicated and validated in other populations. Third, our analyses were restricted to only 385 metabolites. For this reason, we cannot ensure that there are no other unknown relevant metabolites associated with meat or fish consumption outside of our data set that could be identified using other approaches. Finally, plasma metabolite profiles are reflective of metabolic homeostasis, which may be influenced by food intake, but also other physiological processes. The present study was not designed to distinguish between biomarkers of intake nor individual metabolic responses. Despite these limitations, this study has several strengths. Our analyses were carried out with a large sample, possible confounding was controlled for with several covariates, and the metabolite profile signatures were estimated with more than 350 metabolites. Additionally, our signatures were built with agnostic machine-learning processes using well-characterized metabolites and were cross-validated internally in the discovery population using baseline data and, later, were replicated using data from a validation population at 1 year.

In conclusion, four sets of metabolites were associated with the consumption of TM, RM, PRM, and fish, and the scores based on the identified metabolites were associated with higher risk of T2D in a Mediterranean population at high risk of cardiovascular disease. Although they include some metabolites previously associated with T2D, the metabolite profiles were not associated with an increased risk of T2D at 1-year. More specific studies are needed to develop more precise metabolite signatures that reflect the metabolic response to the consumption of these products per se and to understand the metabolic pathways implicated in T2D development.

## Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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## Conflict of Interest

J.S.-S. is a non-paid member of the Scientific Committee of the International Nut and Dried Fruit Foundation. He has received grants/research support from the American Pistachio Growers and International Nut and Dried Fruit Foundation through his institution. He has received honoraria from Nuts for Life, Danone and Eroski. He reports personal fees from Danone. He is a member of the executive committee of the Instituto Danone Spain. S.K.N. is a volunteer member of the not-for profit group Plant Based Canada. Any other co-authors have a conflict of interest that is relevant to the subject matter or materials included in this work.

## Author Contributions

J.G.G., M.G.F., M.R.-C., C.R., E.T., M.A.M.-G., J.S.S., F.B.H. designed the research; D.C., R.E., M.F., L.S.M., M.A.M.-G., J.S.S. coordinated the subject recruitment at the outpatient clinics and clinical data collection in Prevención con Dieta Mediterránea (PREDIMED); C.B.C. conducted the metabolomics data analysis; J.G.G. conducted the statistical analysis and drafted the manuscript; J.S.S. are the guarantors of this work, and, as guarantors, take responsibility for the integrity of the data and the accuracy of the data analysis; J.G.G., M.A.M.-G., J.S.S., F.B.H. had access to all the data in the study; J.G.G., S.N., I.P.G., N.B., M.F., J.-P.D.-C., E.T., C.W., M.A.M.-G., J.S.S., F.B.H. interpreted the data; and all authors: made critical revisions to the manuscript for key intellectual content and read and approved the final manuscript.

## Data Availability Statement

The dataset generated and/or analyzed during the current study are not publicly available due the lack of authorization from PREDIMED participants. Requestors wishing to access the PREDIMED trial data used in this study can make a request to the corresponding author and it will then be passed to members of the PREDIMED Steering Committee for deliberate.

## Keywords

fish, metabolomics, meat, PREDIMED, type 2 diabetes

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