Glycolysis/gluconeogenesis- and tricarboxylic acid cycle–related metabolites, Mediterranean diet, and type 2 diabetes

Marta Guasch-Ferré,[1,2,3] José L Santos,[4] Miguel A Martínez-González,[1,5,6] Clary B Clish,[7] Cristina Razquin,[5,6] Dong Wang,[1] Liming Liang,[8] Jun Li,[1] Courtney Dennis,[7] Dolores Corella,[6,9] Carlos Muñoz-Bravo,[10] Dora Romaguera,[6,11] Ramón Estruch,[6,12] José Manuel Santos-Lozano,[6,13] Olga Castañer,[6,14] Angel Alonso-Gómez,[6,15] Luis Serra-Majem,[6,16] Emilio Ros,[6,17] Sílvia Canudas,[2,6] Eva M Asensio,[9] Montserrat Fitó,[6,14] Kerry Pierce,[7] J Alfredo Martínez,[6,18] Jordi Salas-Salvadó,[2,6] Estefanía Toledo,[5,6] Frank B Hu,[1,3] and Miguel Ruiz-Canela[5,6]

[1]Department of Nutrition, Harvard TH Chan School of Public Health, Boston, MA, USA; [2]Human Nutrition Unit, Faculty of Medicine and Health Sciences, Pere Virgili Health Research Institute, Rovira i Virgili University, Reus, Spain; [3]Channing Division of Network Medicine, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA, USA; [4]Department of Nutrition, Diabetes and Metabolism, School of Medicine, Pontificia Universidad Católica de Chile, Santiago, Chile; [5]Department of Preventive Medicine and Public Health, IdiSNA (Health Research Institute of Navarra), University of Navarra, Pamplona, Spain; [6]The Spanish Biomedical Research Center in Physiopathology of Obesity and Nutrition, Health Institute Carlos III, Madrid, Spain; [7]Broad Institute of MIT and Harvard University, Cambridge, MA, USA; [8]Department of Biostatistics, Harvard TH Chan School of Public Health, Boston, MA, USA; [9]Department of Preventive Medicine, University of Valencia, Valencia, Spain; [10]Department of Public Health and Psychiatry, University of Málaga, Málaga, Spain; [11]Health Research Institute of the Balearic Islands (IdISBa), University Hospital Son Espases, Mallorca, Spain; [12]Department of Internal Medicine, Department of Endocrinology and Nutrition Biomedical Research Institute August Pi Sunyer (IDI-BAPS), Hospital Clinic, University of Barcelona, Barcelona, Spain; [13]Department of Family Medicine, Primary Care Division of Sevilla, San Pablo Health Center, Sevilla, Spain; [14]Cardiovascular and Nutrition Research Group, Hospital del Mar Research Institute (IMIM), Barcelona, Spain; [15]Bioaraba Health Research Institute; Osakidetza Baseque Health Service, Araba University Hospital; Unibersity of the Basque Country UPV/EHU; Vitoria-Gasteiz, Spain; [16]Research Institute of Biomedical and Health Sciences (IUIBS), University of Las Palmas de Gran Canaria and Service of Preventive Medicine, Complejo Hospitalario Universitario Insular Materno Infantil (CHUIMI), Canary Health Service, Las Palmas de Gran Canaria, Spain; [17]Lipid Clinic, Department of Endocrinology and Nutrition Biomedical Research Institute August Pi Sunyer (IDI-BAPS), Hospital Clinic, University of Barcelona, Barcelona, Spain; and [18]Department of Nutrition, Food Sciences, and Physiology, Center for Nutrition Research, University of Navarra, Pamplona, IMDEA Food, Madrid, Spain

ABSTRACT

Background: Glycolysis/gluconeogenesis and tricarboxylic acid (TCA) cycle metabolites have been associated with type 2 diabetes (T2D). However, the associations of these metabolites with T2D incidence and the potential effect of dietary interventions remain unclear.

Objectives: We aimed to evaluate the association of baseline and 1-y changes in glycolysis/gluconeogenesis and TCA cycle metabolites with insulin resistance and T2D incidence, and the potential modifying effect of Mediterranean diet (MedDiet) interventions.

Methods: We included 251 incident T2D cases and 638 noncases in a nested case-cohort study within the PREDIMED Study during median follow-up of 3.8 y. Participants were allocated to MedDiet + extra-virgin olive oil, MedDiet + nuts, or control diet. Plasma metabolites were measured using a targeted approach by LC–tandem MS. We tested the associations of baseline and 1-y changes in glycolysis/gluconeogenesis and TCA cycle metabolites with subsequent T2D risk using weighted Cox regression models and adjusting for potential confounders. We designed a weighted score combining all these metabolites and applying the leave-one-out cross-validation approach.

Results: Baseline circulating concentrations of hexose monophosphate, pyruvate, lactate, alanine, glycerol-3 phosphate, and isocitrate were significantly associated with higher T2D risk (17–44% higher risk for each 1-SD increment). The weighted score including all metabolites was associated with a 30% (95% CI: 1.12, 1.51) higher relative risk of T2D for each 1-SD increment. Baseline lactate and alanine were associated with baseline and 1-y changes of homeostasis model assessment of insulin resistance. One-year increases in most metabolites and in the weighted score were associated with higher relative risk of T2D after 1 y of follow-up. Lower risks were observed in the MedDiet groups than in the control group although no significant interactions were found after adjusting for multiple comparisons.

Conclusions: We identified a panel of glycolysis/gluconeogenesis-related metabolites that was significantly associated with T2D risk in a Mediterranean population at high cardiovascular disease risk. A MedDiet could counteract the detrimental effects of these metabolites. This trial was registered at controlled-trials.com as ISRCTN35739639. *Am J Clin Nutr* 2020;111:835–844.

Keywords: glycolysis metabolites, tricarboxylic acid cycle metabolites, metabolomics, type 2 diabetes, insulin resistance

Introduction

Metabolomics is a rapidly evolving discipline that offers a new avenue for identifying novel biomarkers before the onset of diabetes beyond classical risk factors (1). Metabolomic

studies have revealed that several blood sugars, sugar-related metabolites, components of the glycolysis/gluconeogenesis pathway, and tricarboxylic acid (TCA) cycle intermediates have been associated with insulin resistance, prediabetes, and diabetes in case-control, cross-sectional, and prospective studies (2–8). Interestingly, several metabolites belonging to the glycolysis/gluconeogenesis pathway and TCA cycle show relevant changes in plasma concentrations after oral glucose challenges (9, 10). Among them, lactate (the end product of anaerobic glycolysis) also showed differential changes in its circulating concentrations during the oral-glucose-tolerance test (OGTT) by insulin resistance status (9). Moreover, circulating lactate is a relevant predictor of subsequent T2D incidence in several epidemiologic studies (11–13). However, these studies only assessed plasma lactate at baseline and did not perform a broader assessment of other lactate-related metabolites involved in glucose homeostasis.

Although available literature has pointed to a link between some glycolysis/gluconeogenesis or TCA cycle plasma metabolites and prediabetes or T2D, to our knowledge no previous longitudinal study has assessed the association of these metabolites with future T2D incidence in initially nondiabetic subjects. Importantly, existing studies have not integrated longitudinal data with the potential effect of dietary interventions. This integration is needed to evaluate the associations of interest in a comprehensive manner and to provide support for public health actions. In this context, no large, long-term study has assessed whether dietary interventions can modify the relation between metabolomic profiles composed of gluconeogenesis-pathway metabolites and T2D risk. Therefore, the aim of the present study was to evaluate the association of baseline and 1-y changes in plasma glycolysis/gluconeogenesis-related metabolites and TCA cycle intermediates with insulin resistance and T2D risk; and to examine whether these associations might be mitigated by dietary interventions based on the Mediterranean diet (MedDiet) among participants at high cardiovascular disease (CVD) risk.

Methods

Study design and participants

The present study was a nested case-cohort study within the PREDIMED trial (ISRCTN35739639). Briefly, the PREDIMED trial (www.predimed.es) was conducted from 2003 through 2010 in Spain and aimed to evaluate the effects of the MedDiet for the primary prevention of CVD. At baseline, 7447 participants aged 55–80 y with high CVD risk, but initially free from diagnosed CVD, were allocated to 1 of 3 dietary interventions: *1*) MedDiet supplemented with extra-virgin olive oil (provided to participants for free); *2*) MedDiet supplemented with mixed tree nuts (provided to participants for free); or *3*) a control group that received advice to follow a low-fat diet (and participants received nonfood gifts). Detailed information about the PREDIMED trial has been published elsewhere (14, 15).

In the present case-cohort study, we have included all the available incident T2D cases diagnosed during a median follow-up of 3.8 y and a random subsample of 20% of participants free of T2D at baseline and who had available EDTA plasma samples (16). Among all participants free of diabetes at baseline ($n = 3541$), we selected for the present analysis 889 participants (**Supplemental Figure 1**), including 251 incident T2D cases with available plasma samples and a subcohort of 691 randomly selected participants (638 noncases and 53 overlapping cases). Among the total selected subset of 889 participants, 656 had available blood samples after 1 y of follow-up (499 noncases and 157 cases that occurred after 1 y of follow-up) and they were included in the 1-y change analyses. The protocol was approved by the research ethics committees at all study locations, and all participants provided written informed consent.

Ascertainment of T2D cases

The PREDIMED protocol included T2D as a prespecified secondary endpoint of the trial among participants initially free of diabetes. At baseline, prevalent T2D was identified by clinical

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Supplemental Tables 1 and 2 and Supplemental Figures 1 and 2 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/ajcn/.

The data sets generated and analyzed during the current study are not publicly available owing to national data regulations and for ethical reasons, including the possibility that sharing some information might compromise research participants' consent because our participants only gave their consent for the use of their data by the original team of investigators. However, investigators interested in analyzing the PREDIMED data set used for the present article may submit a brief proposal and statistical analysis plan to the corresponding author. Upon approval from the PREDIMED Steering Committee, data analysis will be made available to them using an onsite secure access data enclave.

MG-F and JLS equally contributed to this work.

Address correspondence to MR-C (e-mail: mcanela@unav.es).

Abbreviations used: CVD, cardiovascular disease; FDR, false discovery rate; MCT1, monocarboxylate transporter 1; MedDiet, Mediterranean diet; OGTT, oral-glucose-tolerance test; TCA, tricarboxylic acid; T2D, type 2 diabetes.

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diagnosis and/or use of antidiabetic medication. The diagnosis of incident T2D during follow-up has been described elsewhere (17) and followed the American Diabetes Association criteria (18), namely 2 confirmations of fasting plasma glucose ≥7.0 mmol/L or 2-h plasma glucose ≥11.1 mmol/L, after a standard 2-h 75-g OGTT. Blinded study physicians collected information on the outcomes. Blinded to the intervention assignment, the Clinical End-Point Ascertainment Committee adjudicated the T2D events according to standard criteria. Information on incident cases of T2D was collected from continuous contact with participants and primary health care physicians, annual follow-up visits, yearly ad hoc reviews of medical charts, and annual consultation of the National Death Index.

Covariate assessment

At baseline and at yearly follow-up visits, questionnaires assessing medical conditions, family history of disease, and risk factors were collected. Trained personnel measured participants' body weight, height, waist circumference, and blood pressure (in triplicate) according to the study protocol. BMI was calculated as kg/m^2. Physical activity was assessed using the validated Spanish version of the Minnesota Leisure-Time Physical Activity questionnaire (19). Participants were considered to have hypercholesterolemia or hypertension if they had previously been medically diagnosed, and/or they were being treated with cholesterol-lowering or antihypertensive agents, respectively.

Study samples and metabolomics profiling

All analyses used fasting (≥8 h) plasma EDTA samples collected at baseline and at year 1 of intervention. Samples were processed at each recruiting center no later than 2 h after collection and stored at −80°C. Pairs of samples (baseline and first-year visit) from cases and subcohort participants were randomly distributed before being shipped to the Broad Institute in Cambridge, MA, for metabolomics assays. Using a targeted approach, LC–tandem MS was used to quantitatively profile polar metabolites including organic acids, sugar phosphates, purines, pyrimidines, bile acids, and anionic (carboxylate-containing) metabolites. Internal standard peak areas were monitored for quality control and to ensure system performance throughout analyses. Pooled plasma reference samples were also inserted every 20 samples as an additional quality control. The raw data were processed using MultiQuant software (AB SCIEX) to integrate chromatographic peaks and the data were visually inspected to ensure the quality of signal integration. Details of the LC–tandem MS platform can be found elsewhere (20).

For this analysis we used plasma concentrations of metabolites involved in the pathways of glycolysis, gluconeogenesis, and the TCA cycle, namely fructose 6-phosphate, fructose 1,6-bis phosphate, 3-phosphoglycerate, phosphoenolpyruvate, pyruvate, lactate, alanine, glycerol-3-phosphate, citrate, aconitate, isocitrate, fumarate, malate, and succinate (for Human Metabolome Database numbers see **Supplemental Figure 2**; http://www.hmdb.ca/). These products were considered representative metabolites because the method could not chromatographically solve the isomers and therefore did not have unique multiple reaction monitoring transitions in MS. For this reason, in this article we have used the general names for these molecules, i.e., hexose monophosphate for fructose-6-phosphate, hexose diphosphate for fructose 1,6-bis phosphate, and fumarate/maleate for fumarate. We observed 2 missing values in the measurement of 3-phosphoglycerate, 4 missing values in phosphoenolpyruvate, 109 in pyruvate, and 319 in hexose diphosphate.

Participants' triglyceride, total cholesterol, LDL cholesterol, and HDL cholesterol were measured using fasting plasma samples at baseline. Serum glucose, triglyceride, total cholesterol, and HDL-cholesterol concentrations were measured using standard enzymatic methods and LDL-cholesterol concentrations were calculated with the Friedewald formula. Plasma glucose was measured using an enzymatic method to convert glucose to 6-phosphogluconate (ADVIA Chemistry Systems). The intra- and interassay CVs were 1.2% and 1.6%, respectively. Insulin concentrations were measured using an immunoenzymometric assay (ADVIA Chemistry Systems) with intra- and interassay CVs equal to 3.7 and 4.4, respectively. Insulin resistance was calculated by using the HOMA-IR index [HOMA-IR = fasting insulin (μU/mL) × fasting glucose (mmol/L)/22.5].

Statistics

Individual glycolysis/gluconeogenesis-related metabolite concentrations were normalized and scaled to multiples of 1 SD using the rank-based inverse normal transformation. Weighted proportional hazards Cox regression models using Barlow weights to account for the overrepresentation of cases, as recommended for case-cohort designs (21), were applied to estimate HRs and the 95% CIs of T2D, comparing participants in each quartile with the lowest quartile as well as per 1-SD increment in individual metabolites. Follow-up time was calculated from the date of enrollment to the date of diagnosis of T2D for cases, and to the date of the last visit or the end of the follow-up period for noncases. Models were adjusted for age, sex, intervention group, smoking, BMI, physical activity, hypertension, dyslipidemia, and baseline plasma glucose (centered on the sample mean and adding a quadratic term). All models were stratified by recruitment center with the option "strata" from Stata, thus equal coefficients are calculated across strata but with a baseline hazard unique to each stratum. We adjusted *P* values of the multivariable-adjusted associations between 1-SD increments in concentration of individual metabolites and T2D risk using the false discovery rate (FDR)-adjusted procedure to account for the multiple testing (22). To quantify a linear trend, we assigned the median value of each metabolite concentration within each quartile and modeled this variable continuously.

We created a weighted metabolite score combining the glycolysis/gluconeogenesis-related metabolites using the respective coefficients from the multivariable Cox regression model fitted for each individual metabolite (23). We applied the leave-one-out cross-validation approach to obtain unbiased estimates of these models and to avoid overfitting when creating the score (24). In each run, Cox regression models were applied to the all-but-one sample (i.e., the training data set), and the regression coefficient obtained was the weight applied to the remaining 1 sample (i.e., the testing data set) to calculate the score. For metabolites with missing values (hexose diphosphate, 3-phosphoglycerate, phosphoenolpyruvate, and pyruvate) we imputed the values by using the minimum observed value

divided by 2. We also repeated this analysis using a new score with all metabolites except pyruvate and hexose diphosphate to assess the possible influence of the replacement of missing values from these metabolites. We adjusted for the same covariates as previously mentioned. In addition, we adjusted for other metabolites related to glycolysis/gluconeogenesis or the TCA cycle and previously associated with T2D (25–27). Specifically, we adjusted for a branched-chain amino acid score (leucine + isoleucine + valine), aromatic amino acid score (phenylalanine + tyrosine), ratio of glutamine to glutamate, and global arginine bioavailability ratio [arginine/(ornithine + citrulline)].

Some departures from the individual random assignment protocol in a small subset of participants have been reported in the PREDIMED trial (15). As ancillary analyses, we repeated the analyses using robust variance estimators to account for intracluster correlation and we also adjusted for propensity scores predicting randomization to account for small between-group imbalances at baseline.

Using the aforementioned models but with further adjustments for baseline metabolite concentrations of the corresponding metabolite, we also examined the associations between 1-y changes in individual glycolysis/gluconeogenesis- or TCA cycle–related metabolites and T2D risk (using as outcome only cases of T2D occurring after 1 y follow-up). We first calculated the difference between baseline and 1-y concentrations, then normalized this difference using the inverse normal transformation. We applied the same aforementioned procedure to obtain the 1-y weighted metabolite score using the coefficients from Cox regressions for 1-y changes.

In addition, we stratified the analyses by intervention group (control group compared with both MedDiet groups merged together). The likelihood ratio test was used to assess the significance of the 1-df interaction product-term (effect modification in multiplicative scale) between the intervention (MedDiet groups compared with control) and the individual metabolites (continuous).

Finally, we applied multiple linear regression models to examine the associations of quartiles of glycolysis/gluconeogenesis-related metabolites at baseline and 1-y changes with HOMA-IR adjusting for age, sex, intervention group, smoking status, BMI, leisure-time physical activity, hypertension, dyslipidemia, and baseline plasma glucose. Only metabolites previously associated with T2D incidence were included in the analyses.

All statistical analyses were performed using Stata version 15 (Stata Corp), at a 2-tailed α of 0.05.

Results

The CVs were 4.6% for fructose 6-phosphate, 4.5% for fructose 1,6-bis phosphate, 4.0% for 3-phosphoglycerate, 6.3% for phosphoenolpyruvate, 11.5% for pyruvate, 2.9% for lactate, 2.6% for alanine, 3.3% for glycerol-3-phosphate, 1.2% for citrate, 2.5% for aconitate, 1.9% for isocitrate, 2.2% for fumarate, 0.9% for malate, and 2.7% for succinate**.**

Table 1 shows the baseline characteristics of the subset of PREDIMED participants included in our analysis by T2D incidence. Participants who developed T2D were more likely to smoke, had a higher baseline waist circumference and BMI, as well as higher concentrations of fasting glucose at baseline than participants who did not develop T2D during follow-up.

Table 2 shows the HRs and 95% CIs for incident T2D risk according to individual baseline glycolysis/gluconeogenesis-related metabolites. In the multivariable-adjusted models, plasma hexose monophosphate, pyruvate, lactate, alanine, glycerol-3 phosphate, and isocitrate were significantly associated with a higher risk of T2D (23–44% relatively higher risk for each 1-SD increment).

Each 1-SD increment in the weighted score including all metabolites was associated with a 30% (95% CI: 1.12, 1.51) relatively higher risk of T2D (Table 2). Results remained significant when we also adjusted for propensity scores predicting randomization to account for small between-group imbalances at baseline and when we used robust variance estimators to account for intracluster correlations (29%; 95% CI: 3%, 61%). The association became stronger (37%; 95% CI: 18%, 58% per 1-SD increment) when we repeated the analyses with a metabolite score without pyruvate and hexose diphosphate [$P < 0.001$ after FDR correction]. T2D risk was slightly attenuated but still significant when we also adjusted for other T2D-associated metabolites: we calculated a 22% (95% CI: 4%, 44%) higher risk for each 1-SD increment in the score when we also adjusted for branched-chain and aromatic amino acids, ratio of glutamine to glutamate, and the global arginine bioavailability ratio.

Supplemental Table 1 shows the stratified analysis by intervention group for only those metabolites that were significantly associated with T2D. We observed a positive association of hexose monophosphate (as a continuous variable) with T2D in the control group (HR: 1.46; 95% CI: 1.13, 1.87), but no significant association was observed in the MedDiet intervention groups. The P for interaction for the intervention (both MedDiet groups merged compared with the control group) was 0.049 (1 df), but it was nonsignificant after the FDR correction. A nonsignificant trend for an interaction suggesting an increased risk of T2D for higher baseline pyruvate in the MedDiet group but not in the control group was observed, but it became nonsignificant after the FDR correction (P-interaction after FDR correction = 0.076, 1 df).

Baseline HOMA-IR was positively associated with plasma pyruvate, lactate, and alanine (P-trend for quartiles of these metabolites: <0.001, <0.001, and 0.003, respectively). In addition, plasma lactate and alanine were significantly and positively associated as well with 1-y changes in HOMA-IR (P-trend = 0.015 and 0.027, respectively) (**Table 3**).

Our results also indicated a significantly increased risk of T2D associated with 1-y changes in hexose monophosphate, 3-phosphoglycerate, lactate, aconitate, isocitrate, fumarate/maleate, and malate (**Supplemental Table 2**). The strongest associations were observed for lactate and aconitate. Those participants in the upper quartile of 1-y changes in lactate had 3.87-fold higher risk of T2D, and those in the upper quartile of aconitate had 3.16-fold higher risk, than those in the first quartile (HR: 3.87; 95% CI: 2.05, 7.30 and HR: 3.16; 95% CI: 1.76, 5.68, respectively).

A significant association was also found for the 1-y change weighted score of all these metabolites (60% higher risk for each 1-SD increment, HR: 1.60; 95% CI: 1.31, 1.97) (Supplemental Table 2). A consistent association was found when we also adjusted for baseline and 1-y changes in other metabolites

TABLE 1 Baseline participant characteristics according to diabetes status and baseline scores of metabolites[1]

	By diabetes incidence during follow-up		By extreme quartiles of the baseline metabolite score	
	Subcohort[2]	Incident cases	Quartile 1	Quartile 4
n	691	251	204	252
Age, y	66.5 ± 5.7	66.4 ± 5.7	65.7 ± 5.4	66.8 ± 5.7
Women	62.8	55.0	59.8	60.3
Intervention group				
MedDiet + EVOO	30.4	29.9	33.3	27.0
MedDiet + nuts	37.3	33.9	39.7	32.1
Control	32.3	36.3	27.0	40.9
Hypertension	90.9	96.0	90.2	94.4
Dyslipidemia	85.0	79.7	84.3	85.7
Smoking				
Never	60.9	52.6	55.9	57.5
Former	22.6	22.3	20.6	18.3
Current	16.5	25.1	23.5	24.2
Waist circumference, cm	99.5 ± 10.7	103.4 ± 10.0	97.9 ± 11.0	103.3 ± 10.1
BMI, kg/m^2	29.9 ± 3.6	30.8 ± 3.3	29.5 ± 3.8	30.8 ± 3.5
Physical activity, METs-min/d	239 ± 238	249 ± 232	257 ± 249	220 ± 231
Education				
Elementary or lower	75.5	76.5	69.1	75.8
Secondary or higher	24.5	23.5	30.9	24.2
Total energy intake, kcal/d	2277 ± 564	2327 ± 622	2316 ± 593	2268 ± 581
MedDiet score[3]	8.6 ± 1.9	8.5 ± 1.8	8.8 ± 1.7	8.5 ± 2.2
Fasting glucose, mg/dL	99.6 ± 15.2	117.2 ± 17.6	100.2 ± 15.4	108.4 ± 19.2

[1] Values are means ± SDs or percentages unless indicated otherwise. EVOO, extra-virgin olive oil; MedDiet, Mediterranean diet; MET, metabolic equivalent task.

[2] Thirty-seven cases are included in the randomly selected subcohort.

[3] This score is based on the 14-item PREDIMED screener of adherence to the MedDiet.

including branched-chain amino and aromatic amino acids, ratio of glutamine to glutamate, and global arginine availability score (HR: 1.61; 95% CI: 1.29, 2.01 per 1-SD increment) and when we repeated the analyses with a metabolite score without pyruvate and hexose diphosphate (HR: 1.63; 95% CI: 1.36, 1.96 per 1-SD increment).

When these models were stratified by intervention group (**Table 4**), 1-y changes in several metabolites including hexose monophosphate, 3-phosphoglycerate, lactate, and aconitate were also associated with higher T2D risk both in the control and in the MedDiet groups. Citrate, isocitrate, and malate were only associated with higher risk of T2D in the control group, not in the MedDiet intervention groups. The test for interaction was significant for isocitrate and malate, but no longer significant after the FDR correction. One-year changes in the metabolite score were associated with 3.57-fold relatively higher risk of T2D in the control group (95% CI: 1.54, 4.27), whereas no significant associations were observed in the MedDiet groups. However, the interaction was not statistically significant (P-interaction = 0.071).

Discussion

In this prospective nested case-cohort study, we observed that baseline and 1-y changes in fasting plasma concentrations of several glycolysis/gluconeogenesis- and TCA cycle–related metabolites and a global score were associated with higher risk of T2D among participants at high CVD risk. Moreover, 1-y change of this score and some individual metabolites was associated with T2D risk in the control group but not in the MedDiet group, although interactions were not statistically significant after FDR correction. In addition, baseline plasma concentrations of lactate and alanine were associated with increases in HOMA-IR after 1 y.

Because T2D is itself defined by hyperglycemia (28–30), our results may be partly explained by the fact that early dysglycemia usually precedes changes in metabolite concentrations. Sugar-related circulating metabolites were correlated with prediabetes and/or T2D in observational studies (25). The Cooperative Health Research in the Region of Augsburg (KORA) case-control study reported that plasma glucose, mannose, desoxyhexose, and dihexose were higher in T2D cases than in the control group (7). In the Framingham Heart Study Offspring Cohort, glycolysis products increased after a 75-g OGTT (9, 10). That study also reported very modest reductions in circulating concentrations of glucose 1-phosphate, glucose 6-phosphate, fructose 1-phosphate, and fructose 6-phosphate after glucose loads (9).

In our study, both baseline and 1-y changes of plasma lactate concentrations were strongly associated with T2D risk. Previous studies have shown that fasting plasma lactate concentrations are associated with surrogates of insulin resistance and T2D risk (11, 12). Although pancreatic β-cell lines have shown alterations in the glycolytic pathway and TCA metabolism (31), it is unlikely that circulating lactate or pyruvate may have a direct effect in insulin secretion given that the lactate/pyruvate transporter monocarboxylate transporter 1 (MCT1) is specifically disallowed in β-cells (32). However, fasting plasma lactate has been reported as one of the circulating metabolites involved

TABLE 2 Incident type 2 diabetes by baseline glycolysis/gluconeogenesis and TCA cycle fasting plasma metabolites in the PREDIMED trial, 2003–2010[1]

				Adjusted HR (95% CI)					
	n	Type 2 diabetes cases	Adjusted HR per 1-SD increment[2] (95% CI)	Quartile 1	Quartile 2	Quartile 3	Quartile 4	*P*-trend	*P*-trend[3]
Hexose monophosphate[4]	889	251	1.23 (1.07, 1.41)	1.00 (ref)	2.45 (1.62, 3.69)	1.12 (0.73, 1.71)	2.37 (1.58, 3.54)	0.214	0.333
Hexose diphosphate[4]	570	166	1.12 (0.93, 1.33)	1.00 (ref)	0.65 (0.41, 1.03)	1.74 (1.20, 2.54)	0.89 (0.57, 1.41)	0.530	0.619
3-Phosphoglycerate	887	251	1.13 (0.97, 1.32)	1.00 (ref)	1.24 (0.85, 1.82)	1.28 (0.84, 1.94)	1.27 (0.84, 1.92)	0.475	0.619
Phosphoenolpyruvate	885	250	1.13 (0.96, 1.32)	1.00 (ref)	0.87 (0.58, 1.30)	0.77 (0.51, 1.16)	1.40 (0.94, 2.10)	0.045	0.104
Pyruvate	780	238	1.31 (1.11, 1.54)	1.00 (ref)	1.84 (1.21, 2.82)	1.36 (0.88, 2.09)	2.12 (1.37, 3.28)	0.034	0.096
Lactate	889	251	1.26 (1.07, 1.48)	1.00 (ref)	0.92 (0.58, 1.44)	1.70 (1.11, 2.61)	1.66 (1.06, 2.59)	<0.001	<0.001
Alanine	889	251	1.25 (1.08, 1.45)	1.00 (ref)	0.58 (0.37, 0.92)	1.16 (0.78, 1.72)	1.23 (0.83, 1.83)	<0.001	0.001
Glycerol 3-phosphate	889	251	1.44 (1.24, 1.67)	1.00 (ref)	1.18 (0.80, 1.74)	1.29 (0.84, 1.96)	2.74 (1.83, 4.09)	0.002	0.007
Citrate	889	251	1.00 (0.86, 1.17)	1.00 (ref)	0.89 (0.62, 1.27)	0.81 (0.54, 1.22)	0.93 (0.62, 1.40)	0.866	0.866
Aconitate	889	251	1.14 (0.98, 1.33)	1.00 (ref)	1.08 (0.70, 1.68)	1.11 (0.75, 1.65)	1.48 (0.98, 2.23)	0.069	0.138
Isocitrate	889	251	1.17 (1.01, 1.36)	1.00 (ref)	1.36 (0.89, 2.08)	0.95 (0.60, 1.50)	1.58 (1.04, 2.40)	0.023	0.080
Fumarate/maleate[4]	889	251	1.02 (0.88, 1.18)	1.00 (ref)	0.67 (0.43, 1.03)	0.75 (0.51, 1.10)	0.96 (0.64, 1.45)	0.204	0.333
Malate	889	251	1.04 (0.91, 1.19)	1.00 (ref)	0.94 (0.64, 1.39)	1.23 (0.82, 1.85)	1.14 (0.79, 1.65)	0.778	0.837
Succinate	889	251	1.07 (0.93, 1.25)	1.00 (ref)	1.48 (1.03, 2.13)	1.38 (0.96, 2.00)	0.94 (0.60, 1.49)	0.516	0.619
Metabolite score[5]	889	251	1.30 (1.12, 1.51)	1.00 (ref)	1.11 (0.70, 1.75)	1.32 (0.86, 2.01)	1.88 (1.25, 2.83)	0.001	

[1]Models adjusted for age (years), sex (male, female), intervention group (MedDiet + extra-virgin olive oil, MedDiet + nuts), BMI (kg/m^2), smoking (never, current, former), leisure-time physical activity (metabolic equivalent tasks in minutes per day), dyslipidemia, hypertension, and baseline fasting glucose (mean + quadratic term of centered mean) and stratified by recruitment center. TCA, tricarboxylic acid cycle.

[2]An inverse normal transformation was applied to raw values.

[3]False discovery rate–corrected *P* value.

[4]These metabolites were not chromatographically resolved and do not have unique multiple reaction monitoring transitions in MS.

[5]Weighted sum of all metabolites (using regression coefficients as weights after applying the leave-one-out cross-validation approach). Weighted proportional hazards Cox regression models were used.

TABLE 3 Baseline and 1-y changes in HOMA-IR index (95% CIs) by quartiles of baseline glycolysis/gluconeogenesis and TCA cycle metabolites in the PREDIMED trial, 2003–2010[1]

	Baseline HOMA-IR		1-y change of HOMA-IR	
	Adjusted mean difference (95% CI)	*P*-trend	Adjusted mean difference (95% CI)	*P*-trend
Hexose monophosphate				
Quartile 1	0 (ref.)	0.431	0 (ref.)	0.575
Quartile 2	−0.12 (−0.52, 0.28)		−0.40 (−0.91, 0.12)	
Quartile 3	−0.41 (−0.82, 0.01)		−0.26 (−0.80, 0.28)	
Quartile 4	0.10 (−0.30, 0.49)		−0.16 (−0.68, 0.37)	
Pyruvate				
Quartile 1	0 (ref.)	<0.001	0 (ref.)	0.502
Quartile 2	0.12 (−0.35, 0.58)		0.09 (−0.52, 0.69)	
Quartile 3	0.37 (−0.09, 0.83)		0.27 (−0.32, 0.87)	
Quartile 4	0.61 (0.15, 1.07)		0.36 (−0.25, 0.97)	
Lactate				
Quartile 1	0 (ref.)	<0.001	0 (ref.)	0.015
Quartile 2	0.16 (−0.23, 0.56)		0.55 (0.02, 1.09)	
Quartile 3	0.94 (0.55, 1.34)		0.68 (0.14, 1.22)	
Quartile 4	1.03 (0.62, 1.43)		0.70 (0.17, 1.24)	
Alanine				
Quartile 1	0 (ref.)	0.003	0 (ref.)	0.027
Quartile 2	0.24 (−0.18, 0.66)		0.19 (−0.37, 0.75)	
Quartile 3	0.61 (0.20, 1.02)		0.27 (−0.27, 0.80)	
Quartile 4	0.57 (0.16, 0.98)		0.69 (0.15, 1.23)	
Glycerol 3-phosphate				
Quartile 1	0 (ref.)	0.075	0 (ref.)	0.075
Quartile 2	0.31 (−0.10, 0.72)		0.49 (−0.05, 1.04)	
Quartile 3	0.11 (−0.29, 0.52)		0.74 (0.21, 1.28)	
Quartile 4	0.27 (−0.14, 0.67)		0.58 (0.03, 1.12)	

[1]Models adjusted for age (years), sex (male, female), intervention group (MedDiet + extra-virgin olive oil, MedDiet + nuts), BMI (kg/m^2), smoking (never, current, former), leisure-time physical activity (metabolic equivalent tasks in minutes per day), dyslipidemia, hypertension, and baseline fasting glucose. Multivariable linear regression models were used. TCA, tricarboxylic acid.

in insulin resistance and metabolic syndrome phenotypes (6). Increased plasma lactate concentrations have also been reported after the standard 75-g OGTT and hyperinsulinemic-euglycemic clamps, showing differential postchallenge lactatemia in insulin-resistant compared with insulin-sensitive subjects (8, 9, 33–35). Moreover, the increased insulin sensitivity observed after weight loss programs has also been accompanied by reductions in plasma lactate concentrations (36). There is a well-known link between circulating lactate and glucose homeostasis because lactate is a precursor of hepatic gluconeogenesis, potentially enhancing the endogenous glucose production. It has also been shown that plasma lactate transported through MCT1 in the adipose tissue (37) may interfere with insulin action in skeletal muscle (38) and mediate inhibition of lipolysis through the activation of hydroxycarboxylic acid receptor 1 (HCAR1/GPR81) in adipocytes (39). The importance of plasma lactate in metabolism has been reinforced after the observation that this metabolite is the major carbon source to mitochondrial TCA in most of the peripheral tissues (40, 41).

We found that both baseline selected TCA cycle–related metabolites and their 1-y changes were associated with higher T2D risk. Impaired TCA flux in insulin-resistant human skeletal muscle has been suggested as one of the characteristics of the diabetic phenotype (42, 43). Mitochondrial aconitase converts citrate to isocitrate via aconitate, which is a highly sensitive enzyme biomarker of age-related oxidative damage, a process widely linked to hyperglycemia (44). Interestingly, the TCA cycle metabolites isocitrate, aconitate, and malate have been reported to be involved in the metabolomic signature of human aging (45). Both malate and isocitrate are involved in the pyruvate–citrate cycle through malic enzyme oxidizing malate to pyruvate or through the cytosolic isocitrate dehydrogenase converting isocitrate to α-ketoglutarate, and such reactions participate in NAD(P)H production which is critical in the cellular antioxidant defense system. In our study, we found that 1-y changes of isocitrate and malate were only associated with a higher risk of T2D in the control group but not in the MedDiet intervention groups. This finding suggests that the MedDiet could counteract the detrimental effects associated with an increase in these metabolites. In fact, the MedDiet is an antioxidant-rich diet that may prevent cellular aging through a reduced intracellular oxidative stress (46).

Gluconeogenesis from amino acids (mainly via the glucose–alanine cycle) contributes ≤40% of the non-glycogen-derived hepatic glucose production (47–49). Alanine showed the strongest association with HOMA-IR index among 285 candidate metabolites in prepubertal children (50). Alanine is directly connected to pyruvate through a reaction of amino transference catalyzed by alanine aminotransferase (pyruvate is the 2-oxoacid of alanine) and circulating alanine has been proposed as an

TABLE 4 Incident T2D by 1-y changes in glycolysis/gluconeogenesis and TCA cycle metabolites stratified by intervention group in the PREDIMED trial, 2003–2010[1]

	Control group			Mediterranean diet groups (2 groups)				
	n	T2D cases	Adjusted HR per 1-SD increment[2] (95% CI)	*n*	T2D cases	Adjusted HR per 1-SD increment[2] (95% CI)	*P*-interaction	*P*-interaction[3]
Hexose monophosphate	210	58	1.62 (1.15, 2.28)	446	99	1.47 (1.13, 1.90)	0.821	0.945
3-Phosphoglycerate	210	58	2.01 (1.39, 2.91)	445	99	1.44 (1.03, 2.00)	0.876	0.945
Lactate	210	58	2.18 (1.31, 3.63)	446	99	1.63 (1.22, 2.17)	0.899	0.945
Citrate	210	58	1.53 (1.11, 2.11)	446	99	1.14 (0.90, 1.45)	0.118	0.314
Aconitate	210	58	2.14 (1.45, 3.17)	446	99	1.54 (1.15, 2.06)	0.945	0.945
Isocitrate	210	58	2.96 (1.87, 4.68)	446	99	1.13 (0.89, 1.44)	0.025	0.169
Fumarate	209	58	1.33 (0.94, 1.88)	446	99	1.35 (1.05, 1.73)	0.429	0.857
Malate	210	58	1.51 (1.06, 2.17)	446	99	1.03 (0.79, 1.33)	0.042	0.169
Metabolite score[4]	210	58	3.57 (1.54, 4.27)	446	99	1.10 (0.84, 1.44)	0.071	

[1]Models adjusted for baseline metabolites (or metabolite score), age (years), sex (male, female), intervention group (MedDiet + extra-virgin olive oil, MedDiet + nuts), BMI (kg/m^2), smoking (never, current, former), leisure-time physical activity (metabolic equivalent tasks in minutes per day), dyslipidemia, hypertension, and baseline fasting glucose (mean + quadratic term of centered mean) and stratified by recruitment center. TCA, tricarboxylic acid; T2D, type 2 diabetes.

[2]An inverse normal transformation was applied to raw values.

[3]False discovery rate–corrected *P* values.

[4]Weighted sum of all metabolites (using regression coefficients as weights after applying the leave-one-out cross-validation approach). Weighted proportional hazards Cox regression models were used.

indicator of pyruvate (the 2-oxoacid of alanine) production (51). As it is well known, pyruvate is the precursor of lactate through the lactate dehydrogenase reaction. Malate can also be derived from pyruvate through the anaplerotic reaction canalized via oxalacetate through the pyruvate–malate shuttle. One study showed synchronous increments of circulating lactate, pyruvate, alanine, and malate after glucose loads (10). Our results did not show an association between baseline plasma malate and T2D risk but we found an association between 1-y increase of malate and T2D risk in the control group.

Glycerol-3-phosphate, involved in the gluconeogenesis from glycerol, is part of the glycerol-3-phosphate shuttle and a critical intermediate in the synthesis of glycerolipids. The importance of glycerol-3-phosphate in glucose homeostasis is proposed given the observation that overexpression of the glycerol-3 phosphate acyltransferase 1 enzyme converting glycerol-3-phosphate to lysophosphatidic acid causes hepatic insulin resistance (52). In addition, inhibition of glycerol-3-phosphate dehydrogenase by metformin may reduce gluconeogenesis from glycerol and disrupt the cytosolic NAD(H):NAD+ ratio, blocking the use of lactate as a gluconeogenic precursor (53).

Several strengths and limitations of the present study deserve comment. First, we used an efficient case-cohort design nested in a large long-term intervention trial to study a hard clinical endpoint and its association with multiple plasma metabolites quantified by a validated LC–tandem MS platform. Second, the main novelty and uniqueness of the present study is the use of repeated measurements of metabolites after 1 y and the possibility to appraise the effect modification by a well-defined dietary intervention. Third, this is a longitudinal analysis with a relatively long follow-up, a well-characterized population, and we used blinded assessment of incident T2D cases by a clinical adjudication committee. Although the analyses were adjusted for several potential confounders, the possibility of residual or unmeasured confounding cannot be discounted and reduces our ability to draw causal conclusions. Moreover, departures from individual random assignment in a subset of the trial participants could affect our results related with differences between the intervention and control groups (15, 54). However, our results were very similar after using robust estimates of the variance to correct for potential intracluster correlations and adjusting for propensity scores to account for small imbalances in baseline covariables. We acknowledge the limitation derived from the reduced sample size used for pyruvate and hexose diphosphate due to missing values. In addition, a potential technical limitation might be related to possible spurious elevations of lactate or pyruvate (and less likely for other metabolites) because of recent physical activity, the procedure for blood drawing, or preanalytical treatments (51). However, there is no reason to think that these procedures may have differentially affected participants who years later developed T2D and when we repeated the analyses with a metabolite score without pyruvate and hexose diphosphate, the association between the metabolite score and T2D became even stronger. Our findings may not be generalizable to other populations and T2D was a defined secondary endpoint and not the primary endpoint of the PREDIMED trial.

Our results provide a deeper understanding of specific metabolic pathways related to circulating glycolysis/gluconeogenesis and TCA cycle metabolites in relation with insulin resistance and T2D, and how a MedDiet might modulate the association of these metabolites with T2D risk. In addition, it may shed light into the biological interconnections between Mediterranean dietary interventions, changes in metabolomics profiles, and the risk of T2D. Altogether, it may facilitate the development of preventive and early diagnostic strategies for curbing the T2D epidemic and the adverse consequences of diabetes.

In conclusion, we have identified a panel of glycolysis/gluconeogenesis- and TCA cycle–related metabolites that was

significantly associated with T2D risk in a Mediterranean population at high CVD risk

The authors' responsibilities were as follows—MG-F, JLS, MAM-G, ET, FHB, and MR-C: conceived and designed the work; MAM-G, RE, DC, ER, MF, JL, AA-G, CM-B, LS-M, and JS-S: coordinated the subject recruitment at the outpatient clinics and clinical data collection; CBC, CD, and KP: conducted the metabolomics data analysis; MR-C: conducted the statistical analysis and is the guarantor of this work and, as such, takes responsibility for the integrity of the data and the accuracy of the data analysis; MG-F and MR-C: had access to all the data in the study; MG-F, JLS, MAM-G, CB, DW, CR, LL, JL, JS-S, ET, and MR-C: interpreted the data; MG-F, JLS, and MR-C: drafted the manuscript; and all authors : made critical revisions to the manuscript for key intellectual content. All authors read and aproved the final manuscript. The authors report no conflicts of interest.

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