Glycolysis/Gluconeogenesis and TCA Related Metabolites, Mediterranean Diet and Type 2 Diabetes

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Data sharing: The datasets generated and analysed during the current study are not publicly

available due to national data regulations and for ethical reasons, including the possibility that 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 dataset 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.

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ABSTRACT

Background: Glycolysis/gluconeogenesis and tricarboxylic acid cycle (TCA) 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 remains unclear.

Objective: To evaluate the association between baseline and 1-year changes in glycolysis/gluconeogenesis and TCA 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 non-cases in a nested case-cohort study within the PREDIMED Study during median follow-up of 3.8 years. Participants were allocated to MedDiet+extra-virgin olive oil, MedDiet+nuts or control diet. Plasma metabolites were measured using a targeted approach by LC-MS. We tested the associations of baseline and 1-year changes in glycolysis/gluconeogenesis and TCA 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 levels of hexose monophosphate, pyruvate, lactate, alanine, glycerol-3 phosphate, 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 risk of T2D for each 1 SD increment. Baseline lactate and alanine were associated with baseline and 1-year changes of homeostatic model assessment insulin resistance (HOMA-IR). One-year increases in most metabolites and in the weighted

score were associated with higher risk of T2D after 1-year of follow-up. Lower risks were observed in the MeDiet groups in comparison with 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 risk. A MedDiet could counteract the detrimental effects of these metabolites.

INTRODUCTION

Metabolomics is a rapidly evolving discipline that offers a new avenue for identifying novel biomarkers prior to the onset of diabetes beyond classical risk factors (1). Metabolomic studies have revealed that several blood sugars, sugar-related metabolites, components of glycolysis/gluconeogenesis pathway and tricarboxylic acid cycle (TCA) intermediates have been associated with insuin 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 show relevant changes in plasma levels after oral glucose challenges (9,10). Among them, lactate lactate (the end product of anaerobic glycolysis) also showed differential changes in its circulating levels 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 plasma metabolites and prediabetes or T2D, no previous longitudinal study has assessed the association of these metabolites with future T2D incidence in initially non-diabetic 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 relationship between metabolomic profiles composed of gluconeogenesis-pathway metabolites and T2D risk. Therefore, the aim of the present study was to evaluate the association between baseline and 1-year changes in plasma glycolysis/gluconeogenesis-related metabolites and TCA 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 risk.

METHODS

Study design and participants

The present study was a nested case-cohort study within the PREDIMED trial. 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 cardiovascular disease (CVD). At baseline, 7,447 participants aged 55-80 years with high cardiovascular risk, but initially free from diagnosed CVD were allocated to one of three 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); 3) a control group that received advice to follow a low-fat diet (and participants received non-food gifts). Detailed information about the PREDIMED trial was published elsewhere (14,15)).

In the present case-cohort study, we have included all the available incident T2D cases diagnosed during 3.8 years of median follow-up 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=3,541), we selected for the present analysis 889 participants (**Supplemental Figure 1**), including 251 incident T2D cases with available plasma samples and a sub-cohort of 691 randomly selected participants (638 non-cases and 53 overlapping cases). Among the total selected subset of 889 participants, 656 had available blood samples after 1-year of follow-up (499 non-cases and 157 cases that occurred after 1-y of follow-up) and they were included in the 1-year 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 pre-specified secondary endpoint of the trial among participants initially free of diabetes. At baseline, prevalent T2D was identified by clinical 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 two confirmations of fasting plasma glucose ≥7.0 mmol/L or 2-h plasma glucose ≥11.1 mmol/L, after a standard 2-hour 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. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. 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 hours) plasma EDTA samples collected at baseline and at year 1 of intervention. Samples were processed at each recruiting center no later than 2 hours after collection and stored at -80°C. Pairs of samples (baseline and first-year visit) from cases and sub-cohort participants were randomly distributed before being shipped to the Broad Institute in Boston, MA, for metabolomics assays. Using a targeted approach, LC-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-MS platform can be found elsewhere (20).

For this analysis we used plasma levels of metabolites involved in the pathways of glycolysis, gluconeogenesis and TCA metabolites, 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 (see Human Metabolome database HMDB numbers in **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 manuscript we used the general name 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

252 in the measurement of 3-Phosphoglycerate, 4 missing values in phosphoenolpyruvate, 109 in
253 pyruvate and 319 in hexose diphosphate.

254 Participants' triglyceride (TG), total cholesterol, low-density lipoprotein-cholesterol (LDL),
255 and high-density lipoprotein-cholesterol (HDL), were measured using fasting plasma samples
256 at baseline. Serum glucose, triglyceride, total cholesterol and HDL-cholesterol levels were
257 measured using standard enzymatic methods and LDL-cholesterol concentrations were
258 calculated with the Friedewald formula. Plasma glucose was measured using an enzymatic
259 method to convert glucose to 6-phosphogluconate (ADVIA Chemistry Systems, Tarrytown,
260 NY, USA). The intra- and inter-assay coefficients of variation were 1.2 and 1.6. Insulin levels
261 were measured using an immunoenzymometric assay (ADVIA Chemistry Systems) with and
262 intra- and inter-assay coefficient of variation equal to 3.7 and 4.4, respectively. Insulin
263 resistance was calculated by using the HOMA-IR index (HOMA-IR = fasting insulin (μU/mL)
264 × fasting glucose (mmol/L) /22.5).

265 Statistics

266 Individual glycolysis/gluconeogenesis-related metabolite concentrations were normalized and
267 scaled to multiples of 1 SD using the rank-based inverse normal transformation. Weighted
268 proportional hazards Cox regression models using Barlow weights to account for the over-
269 representation of cases, as recommended for case-cohort designs(21) , were applied to estimate
270 HRs and the 95% CIs of T2D comparing participants in each quartile to the lowest quartile as
271 well as per 1SD deviation increment in individual metabolites. Follow-up time was calculated
272 from the date of enrolment to the date of diagnosis of T2D for cases, and to the date of the last
273 visit or the end of the follow-up period for non-cases. Models were adjusted for age, sex,
274 intervention group, smoking, BMI, physical activity, hypertension, dyslipidaemia and baseline
275 plasma glucose (centered on the sample mean and adding a quadratic term). All models were

276 stratified by recruitment center with the option "strata" from Stata, thus equal coefficients are
277 calculated across strata but with a baseline hazard unique to each stratum. We adjusted *P*-
278 values of the multivariable-adjusted associations between 1-SD increment in individual
279 metabolites concentration and T2D risk using the Simes procedure to account for the multiple
280 testing(22). To quantify a linear trend, we assigned the median value of each metabolite
281 concentration within each quartile and modelled this variable continuously.

282 We created a weighted metabolite score combining the glycolysis/gluconeogenesis-related
283 metabolites using the respective coefficients from the multivariable Cox regression model
284 fitted for each individual metabolite (23). We applied the leave-one-out cross-validation
285 (LOOCV) approach to obtain unbiased estimates of these models and to avoid overfitting when
286 creating the score (24). In each run, Cox regression models were applied to all-but-one sample
287 (i.e., the training dataset), and the regression coefficient obtained was the weight applied to the
288 remaining one sample (i.e., the testing dataset) to calculate the score. For metabolites with
289 missing values (hexose diphosphate, 3-phosphoglycerated, phosphoenolpyruvate, and
290 pyruvate) we imputed the values by using the minimum observed value divided by 2. We also
291 repeated this analysis using a new score with all metabolites except pyruvate and hexose
292 diphosphate to assess the possible influence of the replacement of missing values from these
293 metabolites. We adjusted for the same covariates described above. Additionally, we adjusted
294 for other metabolites related to glycolysis/gluconeogenesis or TCA cycle and previously
295 associated with T2D(25-27). Specifically we adjusted for a branched-chain amino acid score
296 (leucine + isoleucine + valine), aromatic amino acid score (phenylalanine + tyrosine), ratio
297 glutamine/glutamate, and global arginine bioavailability ratio (arginine/[ornithine +
298 citrulline]).

299 Some departures from the individual randomization protocol in a small subset of participant
300 have been reported in the PREDIMED trial(15). As ancillary analyses, we repeated the

301 analyses using robust variance estimators to account for intracluster correlation and we
302 additionally adjusted for propensity scores predicting randomization to account for small
303 between-group imbalances at baseline.

304 Using the same models described above but with further adjustments for baseline metabolite
305 concentrations of the corresponding metabolite, we also examined the associations between 1-
306 year changes in individual glycolysis/gluconeogenesis or TCA-related metabolite and T2D risk
307 (using as outcome only cases of T2D occurring after 1-year follow-up). We first calculated the
308 difference between baseline and 1-year levels, and then normalized this difference using the
309 inverse normal transformation. We applied the same procedure described above to obtain the
310 1-year weighted metabolite score using the coefficients from Cox regressions for 1-year
311 changes.

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

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

322 All statistical analyses were performed using Stata version 15 (Stata Corp), at a two-tailed α of
323 0.05.

324

RESULTS

The coefficients of variations 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, 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 by T2D incidence included in our analysis. 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.

The Hazard Ratios (HR) and 95% confidence intervals (CI) for incident T2D risk according to individual baseline glycolysis/gluconeogenesis-related metabolites are shown in **Table 2**. 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% to 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) higher risk of T2D (**Table 2**). Results remained significant when we additionally 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 intra-cluster 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 false discovery rate [FDR] correction). T2D risk was slightly attenuated but still significant when we additionally adjusted for other T2D-associated metabolites: 22% (95% CI: 4%, 44%) higher risk for each 1 SD

increment in the score when we additionally adjusted for branched-chain and aromatic amino acids, glutamine to glutamate ratio and the global arginine bioavailability ratio.

The stratified analysis by intervention group for only those metabolites that were significantly associated with T2D are shown in **Supplemental Table 1**. 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 versus the control group) was 0.049, 1 degree of freedom (df), but it was not significant after the FDR correction. A non-significant trend for an interaction suggesting a higher risk of T2D for baseline pyruvate in the MedDiet group but not in the control group was observed, but it became non-significant after the FDR correction (*P* for interaction after FDR correction = 0.076, 1 df).

Baseline homeostatic model assessment insulin resistance (HOMA-IR) was positively associated with plasma pyruvate, lactate, and alanine (*P* for trend for quartiles of these metabolites: <0.001, <0.001, and 0.003, respectively). Additionally, plasma lactate and alanine were significantly and positively associated as well with 1-year changes in HOMA-IR (*P* for trend 0.015, 0.027, respectively) (**Table 3**).

Our results also indicated a significantly increased risk of T2D associated with one-year 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 one-year 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 (95%CI): 3.87 (2.05, 7.30) and 3.16 (1.76, 5.68), respectively].

A significant association was also found for the one-year changes weighted score of all these metabolites [60% higher risk for each 1 SD increment, HR (95% CI): 1.60 (1.31, 1.97)] (**Supplemental Table 2**). A consistent association was found when we additionally adjusted for baseline and 1-year changes in other metabolites including branched-chain amino and aromatic amino acids, glutamine-to-glutamate ratio and global arginine availability score (HR (95%CI): 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 (95%CI): 1.63 [95% CI: 1.36, 1.96] per 1 SD increment).

When these models were stratified by intervention group (**Table 4**), one-year 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 but 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 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 for interaction = 0.071).

DISCUSSION

In this prospective nested case-cohort study, we observed that baseline and one-year changes in fasting plasma concentrations of several glycolysis/gluconeogenesis and TCA-related metabolites and a global score were associated with higher risk of T2D among participants at high cardiovascular risk. Moreover, one-year change of this score and some individual metabolites was associated with T2D risk in the control group but not in the MedDiet group,

397 although interactions were not statistically significant after FDR correctionIn addition, baseline
398 plasma levels of lactate and alanine were associated with increases in HOMA-IR after one-
399 year.

400 Since T2D is itself defined by hyperglycemia (29,30), our results may be partly explained by
401 the fact that early dysglycemia usually precedes changes in metabolite levels. Sugar-related
402 circulating metabolites were correlated with prediabetes and/or T2D in observational studies
403 (25). The KORA case-control study, reported that plasma glucose, mannose, desoxyhexose and
404 dihexose were higher in T2D cases than in the control group (7). In the Framingham Heart
405 Study Offspring, glycolysis products increased after a 75-gram oral glucose tolerance test
406 (OGTT), (9,10). It was also reported very modest reductions in circulating levels of glucose
407 1-phosphate, glucose 6-phosphate, fructose 1-phosphate and fructose 6-phosphate after glucose
408 loads (9).

409 In our study, both baseline and 1-year changes of plasma lactate levels were strongly associated
410 with T2D risk. Previous studies have shown that fasting plasma lactate levels are associated
411 with surrogates of insulin resistance and T2D risk (11,12). Although pancreatic β-cell lines
412 have shown alterations in the glycolytic pathway and TCA metabolism(31), it is unlikely that
413 circulating lactate or pyruvate may have a direct effect in insulin secretion given that the
414 lactate/pyruvate transporter MCT1 is specifically disallowed in β-cells(32). However, fasting
415 plasma lactate has been reported as one of the circulating metabolites involved in insulin
416 resistance and metabolic syndrome phenotypes (6). Increased plasma lactate levels have also
417 been reported after the standard 75 grams OGTT and hyperinsulinemic-euglycemic clamps,
418 showing differential post-challenge lactatemia in insulin-resistant versus insulin-sensitive
419 subjects(8,9,33-35). Moreover, the increased insulin sensitivity observed after weight loss
420 programs have also been accompanied by reductions in plasma lactate concentrations(36).
421 There is a well-known link between circulating lactate and glucose homeostasis since lactate

422 is a precursor of hepatic gluconeogenesis, potentially enhancing the endogenous glucose
423 production. It has also been shown that plasma lactate transported through MCT1 in the adipose
424 tissue (37) may interfere with insulin action in skeletal muscle (38) and mediate inhibition of
425 lipolysis through the activation of GPR81 receptor in adipocytes (39). The importance of
426 plasma lactate in metabolism has been reinforced after the observation that this metabolite is
427 the major carbon source to mitochondrial TCA in most of the peripheral tissues (40, 41) .

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

444 Gluconeogenesis from amino acids (mainly via the glucose-alanine cycle) contributes up to
445 40% of the non-glycogen-derived hepatic glucose production (47-49). Alanine showed the
446 strongest association with HOMA-IR index among 285 candidate metabolites in pre-pubertal

children (50). Alanine is directly connected to pyruvate through a reaction of amino transference catalyzed by ALT (pyruvate is the 2-oxoacid of alanine) and circulating alanine has been proposed as an 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 showed an association between baseline plasma malate and T2D risk but we found an association between 1-year 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 GPAT1 enzyme converting glycerol-3-phosphate to lysophosphatidic acid causes hepatic insulin resistance (52). Additionally, inhibition of glycerol-3-phosphate dehydrogenase by metformin may reduce gluconeogenesis from glycerol and disrupt cytosolic NADH: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 liquid chromatography-tandem mass spectrometry (LC-MS) platform. Second, the main novelty and uniqueness of the present study is the use of repeated measurements of metabolites after 1-year 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 randomization 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. Additionally, 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 drawn or pre-analytical 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 metabolites in relationship 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-related metabolites that was significantly associated with T2D risk in a Mediterranean population at high cardiovascular risk.

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Table 1. Baseline participant characteristics according to diabetes status and baseline scores of metabolites.

	By diabetes incidence during follow-up		By extreme quartiles of the baseline metabolite score	
	Subcohort[1]	**Incident cases**	**Q1**	**Q4**
n	691	251	204	252
Age (years)	66.5 (5.7)	66.4 (5.7)	65.7 (5.4)	66.8 (5.7)
Sex (% 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)
Body mass index, 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)
Mediterranean diet score[2]	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)

Abbreviations: MedDiet, Mediterranean diet; EVOO, Extra-virgin olive oil; MET, metabolic equivalent. Values are means (SD) or percentages.[1] 37 cases are included in the randomly selected subcohort. [2] This score is based on the 14-item PREDIMED screener of adherence to the Mediterranean Diet.

Table 2. Incident Type 2 diabetes by baseline glycolysis/gluconeogenesis and TCA fasting plasma metabolites in the PREDIMED trial, 2003–2010

	N	Type 2 diabetes Cases	Adjusted[1] HR per 1 SD increment[2] (95% CI)	Adjusted[1] HR (95% CI)				P for trend	P for trend[3]
				Quartile 1	Quartile 2	Quartile 3	Quartile 4		
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] Adjusted for age (years), sex (male, female), intervention group (MedDiet+EVOO, MedDiet+nuts), body mass index (kg/m^2), smoking (never, current, former), leisure-time physical activity (metabolic equivalent tasks in minutes/day), dyslipidemia, hypertension, baseline fasting glucose (mean + quadratic term of centered mean) and stratified by recruitment center. [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 mass spectrometry.[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. Abbreviations: TCA, tricarboxylic acid cycle.

Table 3. Baseline and one-year changes in HOMA-IR Index (95% confidence intervals) by quartiles of baseline glycolysis/gluconeogenesis and TCAmetabolites in the PREDIMED trial, 2003–2010

	Baseline HOMA-IR		1 year change of HOMA-IR	
	Adjusted[1] mean difference (95%CI)	P for trend	Adjusted[1] mean difference (95%CI)	P for trend
Hexose monophosphate				
Q1	0 (ref.)	0.431	0 (ref.)	0.575
Q2	-0.12 (-0.52 , 0.28)		-0.40 (-0.91 , 0.12)	
Q3	-0.41 (-0.82 , 0.01)		-0.26 (-0.80 , 0.28)	
Q4	0.10 (-0.30 , 0.49)		-0.16 (0.68 , 0.37)	
Pyruvate				
Q1	0 (ref.)	<0.001	0 (ref.)	0.502
Q2	0.12 (-0.35 , 0.58)		0.09 (-0.52 , 0.69)	
Q3	0.37 (-0.09 , 0.83)		0.27 (-0.32 , 0.87)	
Q4	0.61 (0.15 , 1.07)		0.36 (-0.25 , 0.97)	
Lactate				
Q1	0 (ref.)	<0.001	0 (ref.)	0.015
Q2	0.16 (-0.23 , 0.56)		0.55 (0.02 , 1.09)	
Q3	0.94 (0.55 , 1.34)		0.68 (0.14 , 1.22)	
Q4	1.03 (0.62 , 1.43)		0.70 (0.17 , 1.24)	
Alanine				
Q1	0 (ref.)	0.003	0 (ref.)	0.027
Q2	0.24 (-0.18 , 0.66)		0.19 (-0.37 , 0.75)	
Q3	0.61 (0.20 , 1.02)		0.27 (-0.27 , 0.80)	
Q4	0.57 (0.16 , 0.98)		0.69 (0.15 , 1.23)	
Glycerol 3-phosphate				
Q1	0 (ref.)	0.075	0 (ref.)	0.075
Q2	0.31 (-0.10 , 0.72)		0.49 (-0.05 , 1.04)	
Q3	0.11 (-0.29 , 0.52)		0.74 (0.21 , 1.28)	
Q4	0.27 (-0.14 , 0.67)		0.58 (0.03 , 1.12)	

1 Adjusted for age (years), sex (male, female), intervention group (MedDiet+EVOO, MedDiet+nuts), body mass index (kg/m2), smoking (never, current, former), leisure-time physical activity (metabolic equivalent tasks in minutes/day), dyslipidemia, hypertension, baseline fasting glucose

Multivariable linear regression models were used

Table 4. Incident Type 2 diabetes by 1-year changes in glycolysis/gluconeogenesis and TCA metabolites stratified by intervention group in the PREDIMED trial, 2003–2010

	Control group			Mediterranean diet groups (2 groups)			P for interaction	P for interaction[3]
	N	Type 2 diabetes Cases	Adjusted HR[1] per 1 SD increment[2] (95% CI)	N	Type 2 diabetes Cases	Adjusted HR[1] per 1 SD increment[2] (95% CI)		
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]	2010	58	3.57 (1.54 , 4.27)	446	99	1.10 (0.84 , 1.44)	0.071	

[1] Adjusted for baseline metabolites (or metabolite score), age (years), sex (male, female), intervention group (MedDiet+EVOO, MedDiet+nuts), body mass index (kg/m^2), smoking (never, current, former), leisure-time physical activity (metabolic equivalent tasks in minutes/day), dyslipidemia, hypertension, baseline fasting glucose (mean + quadratic term of centered mean) and stratified by recruitment center. [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.