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Acylcarnitines and prediction of renal function decline in type 2 diabetes

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ABSTRACT

Introduction We comprehensively investigated whether serum acylcarnitine levels are associated with and predict the decline of glomerular filtration rate (GFR) in type 2 diabetes.

Research design and methods Two cohorts of patients with type 2 diabetes were investigated: a subset of the aggregate Gargano Mortality Study (aGMS, n=575; 9 years of median follow-up; mean age=60.9±9.8; mean diabetes duration=11.6±9.3) as a discovery set from Italy. A sample from the Joslin Kidney Study (JKS, n=252; 10 years of median follow-up; mean age=57.8±5.6; mean diabetes duration=14.2±7.6) was used as an independent validation set with different environmental and ethnic background for some associated metabolites in the aGMS.

Main outcome estimated GFR (eGFR) change over time (mL/min/1.73 m^2/year).

Results Eleven out of the 40 acylcarnitines (by the AbsoluteIDQTM p180 Kit, BIOCRATES) were significantly associated with the rate of eGFR decline after Bonferroni correction. All 11 molecules were internally validated ($p<0.05$). Most of these associations survived the adjustment for several confounders, including age, sex, smoking habit, body mass index, glycated hemoglobin, disease duration, albumin excretion rate, triglycerides, low-density lipoprotein and statins treatment ($p<0.05$). Tiglylcarnitine and methylglutarylcarnitine, but not tetradecenoylcarnitine and hexadecenoylcarnitine, were also associated with eGFR decline in the JKS ($p<0.05$). Using multivariable least absolute shrinkage and selection operator regression analysis, methylglutarylcarnitine, hydroxyvalerylcarnitine, hexenoylcarnitine, decadienylcarnitine, dodecanedioylcarnitine, tetradecadienylcarnitine were independently associated with kidney function decline. The pairwise correlation among these ranged from −0.02 to 0.55. An acylcarnitine score comprising these six molecules improved discrimination ($p<0.01$) and reclassification ($p<0.001$) of two clinical prediction models of GFR decline in diabetes.

Conclusions In patients with type 2 diabetes, four short, three medium and four long-chain acylcarnitines are associated with the rate of kidney function decline. Adding the acylcarnitine score to clinical prediction models improves the identification of individuals who are at greater risk of progression to kidney failure.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ While several metabolites have been found to shape the risk of kidney function loss in type 2 diabetes, the role of circulating acylcarnitines has not been comprehensively addressed.

WHAT THIS STUDY ADDS

⇒ In patients with type 2 diabetes, several acylcarnitines are associated with and improve the prediction of glomerular filtration rate decline.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Selected acylcarnitine may enhance our ability to identify individuals most at risk of progression to renal failure. Future studies on the acylcarnitine pathway's role on kidney function could help uncover new treatments for patients most susceptible to kidney failure.

INTRODUCTION

Type 2 diabetes is burdened by an accelerated decline of glomerular filtration rate (GFR), which worsens quality of life and increases the risk of death.[1–3] Although several strategies to prevent GFR decline are available,[4] these cannot be applied to the large number of individuals with type 2 diabetes. Identifying novel biomarkers of GFR decline in type 2 diabetes could both improve the identification of high-risk patients to be targeted with the existing prevention approaches and point to new pathways of kidney function loss, paving the way for novel interventions for individuals who are not responsive to the currently available strategies.

Interrogating the human metabolome is one of the possible strategies used to identify novel biomarkers in heterogeneous, multifactorial diseases such as chronic renal diseases with kidney function decline.[5] A great help in such modern biomedical research is now given by machine learning models, which can, in fact, identify non-linear relationships

and subtle patterns that traditional statistical methods might miss.[6]

Among several metabolites found to shape the risk of kidney function loss in individuals with type 2 diabetes,[7–16] the role of circulating acylcarnitines, the accumulation of which is proposed as a potential mechanism for the development of renal disease,[8 17] has not been comprehensively addressed. In fact, only a few prospective studies reported that some acylcarnitines are associated with the decline of estimated GFR (eGFR) in type 2 diabetes.[8 10 11]

We studied the relationship between baseline serum levels of 40 acylcarnitines (14 short-chain, 11 medium-chain and 15 long-chain acylcarnitines) and eGFR changes over time. Some of the observed associations were validated in an independent external sample.

We also investigated whether associated acylcarnitines improve the prediction of kidney function loss provided by established clinical models.

RESEARCH DESIGN AND METHODS

Study populations

The study design is illustrated in online supplemental figure S1.

Discovery set; the aggregate Gargano Mortality Study

The aGMS consists of 2140 white people with type 2 diabetes recruited from 2000 to 2011 at the Endocrinology Unit of the Fondazione IRCCS 'Casa Sollievo della Sofferenza', San Giovanni Rotondo (Center Italy), as previously described.[3 18]

The present study was carried out in a subset of 575 representative patients of the aggregate Gargano Mortality Study (aGMS)[3] for whom the decline of eGFR and acylcarnitine measurements data were available.[10]

The study and the informed consent procedures were approved by the local Institutional Ethic Committee Fondazione IRCCS 'Casa Sollievo della Sofferenza', approved on 9 March 2023 Prot N 42/CE. All participants gave written informed consent.

Validation set: the Joslin Kidney Study

This set comprises 252 individuals with type 2 diabetes participating in the Joslin Kidney Study (JKS).[19–21] Individuals included in the present analysis were those JKS participants who were previously included in the metabolomic study by Shah *et al*[21] and had a valid eGFR at baseline and at least one eGFR value during follow-up (up to 10 years). The majority (ie, 80.2%) were white, 8.7% black, 4.0% Latino, 3.6% Asian and the remaining 3.5% of other races. Study protocol and informed consent procedures were approved by the Joslin's Committee on Human Studies.

In this study, the outcome was the rate of eGFR decline over the time, measured as reported in online supplemental methods. Exposures were acylcarnitines assayed as described in online supplemental methods.

Statistical analysis

Patient's baseline characteristics were reported as mean±SD for continuous variables, and as absolute and relative frequencies (percentages) for categorical variables. For skewed continuous variables, median along with range was reported.

In the aGMS, all 40 acylcarnitines were log-transformed and standardized to approximate a normal distribution. For each metabolite, a multiple linear regression model was fitted, using the slope of the eGFR (ie, the rate of eGFR decline) as the dependent variable, the acylcarnitine as the exposure variable and the baseline eGFR as a fixed covariate. The 40 p values of the corresponding regression coefficients (betas) for the metabolite were adjusted for multiple comparisons using the Bonferroni correction (threshold p value being 0.05/40=1.2E-3).

Acylcarnitine that remained statistically significant associated after Bonferroni adjustment was tested in sequential regression models, incorporating additional clinical covariates (ie, baseline age, sex, smoking habit, BMI, HbA1c, disease duration, albumin excretion rate (ACR), triglycerides, low-density lipoprotein (LDL) and statins treatment as adjustment factors.

For metabolites that remained significantly associated after these further adjustments, pairwise correlation of their values was assessed using Pearson correlation coefficients (R). These metabolites were then internally validated as described in online supplemental methods. In order to overcome an overfitting problem, we applied a parsimonious parametric linear regression model including only baseline eGFR and one metabolite as fixed covariates, which substantially limits model complexity and the risk of overfitting.

Internal validation was performed using repeated threefold cross-validation with 10 000 iterations, resulting in 30 000 independent validation folds (sets), allowing a more precise estimation of model performance, particularly in settings where external validation is not feasible.[22]

Model performance was evaluated exclusively on validation sets not used for model training, and the distribution of performance metrics (mean squared error; MSE and R^2) across validation sets was summarized to capture their stability and variability.

In addition, to formally assess potential optimism due to overfitting, we compared the performance of the model estimated in the full sample with the mean cross-validated performance using the SMD, with values <0.50 indicating a small difference. This approach allows quantification of the discrepancy between apparent and internally validated performance.

Finally, the stability of regression coefficients was evaluated by inspecting the distribution of beta estimates across training sets and by computing their bias relative to the coefficient obtained in the full sample. According to Steyerberg, stable regression coefficients and absence of substantial bias are key indicators of limited overfitting and good internal validity.[22]

Potential modifiers of the effect of these metabolites on eGFR decline were investigated, including age at recruitment, gender, smoking habits, BMI, diabetes duration, HbA1c, ACR, triglycerides, LDL and statins treatment. Linear regression models on eGFR decline were performed, incorporating baseline eGFR, the metabolite, the covariate and the interaction term between the metabolite and the covariate.

In the JKS, the association between acylcarnitines and eGFR slope was estimated by including an acylcarnitine x time interaction term in the statistical model.

The least absolute shrinkage and selection operator (LASSO) regularized regression model, with 10-fold cross-validation to determine the optimal λ parameter,[23] was used to select non-redundant variables (λ=0.01), adjusting for baseline eGFR. Finally, a weighted acylcarnitine score using the markers as selected by LASSO was created as previously described.[18] The discrimination provided (ie, area under the ROC curve; AUC-ROC) by the score in predicting eGFR decline (below vs above median value) was tested on top of two previously published clinical models in patients with diabetes. The first model (model 1)[24] comprised sex, age, BMI, HbA1c and ACR. The second model (model 2)[25] comprised age, sex, log2-transformed ACR, total cholesterol, smoking habits, BMI, mean arterial pressure, HbA1c and eGFR. AUC-ROC were compared using the DeLong test.[26] Improvement in discrimination accuracy was measured using the integrated discrimination improvement (IDI). In addition, the category-free net reclassification improvement (cNRI), which examines whether the predicted probabilities of individuals with and without events move in the right direction (upward and downward, respectively) from the base to the new model, was evaluated.[27 28]

The sample of 575 patients achieves >90% power (p=0.05) to detect a regression slope of −0.13 for 1 SD increase of each exposure molecule.

A threshold p value of 1.2E-3 (ie, 0.05/40) was applied after adjustment for multiple comparisons using the Bonferroni correction. A p value <0.05 was used to claim statistical significance for cross-validation and external validation.

All statistical analyses were performed using R software (V.4.3).

Data and resource availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

RESULTS

The clinical features of both aGMS and JKS study patients are summarized in table 1.

Compared with aGMS patients, JKS participants were younger, with longer disease duration and higher baseline eGFR. In addition, in the JKS, there were fewer smokers and more people under various treatments.

Table 1 Clinical features of the study cohorts

	aGMS (N=575)	JKS (N=252)
Women n (%)	275 (47.8)	85 (33.7)
Age at recruitment (years)	60.9±9.8	57.8±5.6
Smokers n (%)	79 (13.7)	17 (6.8)
BMI (kg/m^2)	31.2±6.0	31.5±4.4
Diabetes duration (years)	11.6±9.3	14.2±7.6
HbA1c (%)	8.4±1.9	8.1±1.6
HbA1c (mmol/mol)	68±20.8	64±17.5
Insulin (w/wo other glucose-lowering agents) n (%)	268 (46.6)	198 (78.6)
Lipid-lowering TX n (%)	292 (50.8)	200 (79.4)
Blood pressure-lowering TX (%)	376 (65.4)	217 (86.1)
eGFR (mL/min/1.73 m^2)	77.4±26.1	82.4±21.6
ACR (mg/mmol)	1.56 (0.7–5.0)	2.22 (0.9–9.6)
Annual rate of eGFR decline (mL/min per 1.73 m^2)	−1.32 (−9.5–2.2)	−1.68±0.14

Continuous variables are reported as mean ± standard deviation whereas categorical variables as total frequencies and percentages. Skewed continuous variables are reported as median (range).
ACR, albumin-creatine ratio; aGMS, aggregate Gargano Mortality Study; BMI, body mass index; CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration; eGFR, estimated glomerular filtration rate, calculated using CKD-EPI equation; HbA1c, glycated hemoglobin; JKS, Joslin Kidney Study.

Association between acylcarnitines and eGFR decline in the aGMS

Eleven, out of the 40 acylcarnitines analyzed, were associated with eGFR decline (mL/min per 1.73 m^2), after Bonferroni correction (table 2, model 1; beta estimates ranging from −0.30 to 0.26; p values ranging from 9.4E-8 to 1.0E-3). Two of them (ie, hexenoylcarnitine and dodecanedioylcarnitine) showed an inverse association with eGFR decline (table 2, model 1). Hydroxyvalerylcarnitine, propenoylcarnitine and tiglylcarnitine, all short-chain acylcarnitines, showed the strongest associations. The pairwise correlation among these selected metabolites ranged from −0.74 to 0.92 (online supplemental figure S2).

Cross-validation of the observed associations with eGFR decline

The results of the repeated threefold cross-validation for these 11 metabolites are shown in table 3 and online supplemental figures S3–S13 and clearly indicate successful validation (small/negligible SMD for MSE and for R^2 across all multiple regression models each incorporating a single metabolite).

The MSE and R^2 values obtained from each multiple regression model (one per metabolite) estimated on the

Table 2 Association between acylcarnitines and eGFR decline (mL/min per 1.73 m^2) in the aGMS

	Propenoylcarnitine (C3:1)		Tiglylcarnitine (C5:1)		Methylglutarylcarnitine (C5-M-DC)		Hydroxyvalerylcarnitine C5-OH (C3-DC-M)	
	Beta	P value	Beta	P value	Beta	P value	Beta	P value
Model 1	−0.27	2.0E-6	−0.30	9.0E-6	−0.22	1.0E-3	−0.30	9.4E-8
Model 2	−0.24	1.5E-5	−0.29	9.0E-6	−0.19	4.0E-3	−0.29	1.9E-7
Model 3	−0.21	3.0E-4	−0.26	1.6E-4	−0.13	4.3E-2	−0.26	7.5E-6
Model 4	−0.20	2.0E-3	−0.19	2.0E-2	−0.11	1.1E-1	−0.25	8.3E-5

	Hexenoylcarnitine (C6:1)		Decadienylcarnitine (C10:2)		Dodecanedioylcarnitine (C12-DC)		Tetradecenoylcarnitine (C14:1)	
	Beta	P value	Beta	P value	Beta	P value	Beta	P value
Model 1	0.19	1.0E-3	−0.20	1.0E-3	0.26	1.0E-5	−0.18	1.0E-3
Model 2	0.17	2.0E-3	−0.17	4.0E-3	0.24	3.4E-5	−0.12	4.4E-2
Model 3	0.17	3.0E-3	−0.14	1.8E-2	0.21	3.6E-4	−0.11	6.1E-2
Model 4	0.15	1.0E-2	−0.12	4.0E-2	0.20	1.0E-3	−0.11	6.0E-2

	Tetradecadienylcarnitine (C14:2)		Hexadecenoylcarnitine (C16:1)		Hydroxyhexadecadienylcarnitine (C16:2-OH)	
	Beta	P value	Beta	P value	Beta	P value
Model 1	−0.19	1.0E-3	−0.24	2.1E-5	−0.23	3.4E-5
Model 2	−0.13	2.0E-2	−0.18	2.3E-3	−0.19	1.0E-3
Model 3	−0.13	2.6E-2	−0.14	1.2E-2	−0.16	4.1E-3
Model 4	−0.12	3.0E-2	−0.12	4.9E-2	−0.14	1.7E-2

Linear regression coefficients (per 1 SD increase in each metabolite concentration) were estimated in a model adjusted baseline aGFR.
Model 1=linear regression coefficient (per 1 SD increase in each acylcarnitine concentration) were estimated in model adjusted for baseline eGFR.
Model 2=model 1+general confounders (ie, age, sex, smoking habit and BMI).
Model 3=model 2+diabetes-related variables (ie, diabetes duration and HbA1c) + ACR (albumin/creatinine ratio).
Model 4=model 3+lipids-related variables (ie, triglycerides, low-density lipoproteins and statins treatment).
aGMS, aggregate Gargano Mortality Study; BMI, body mass index; eGFR, estimated glomerular filtration rate; HbA1c, glycated hemoglobin.

full sample closely match those derived from the validation samples. For example, the model incorporating baseline eGFR and propionylcarnitine (online supplemental figure S3) achieved an MSE of 1.797 and an R^2 of 0.047 in the full dataset, compared with a mean MSE of 1.820 and mean R^2 of 0.037 in the validation samples. This resulted in a negligible SMD of 0.08 for MSE and a small SMD of 0.37 for R^2, indicating successful validation. Similar results were observed across all models (online supplemental figures S4–S13).

Multivariable models and subgrouping to gain insights into associations with eGFR decline

All 11 acylcarnitines remained associated with eGFR decline also when adjusted for general confounders (ie, age, sex, smoking habit and adiposity; table 2, model 2). The great majority of them remained statistically significant when additional covariates were also added into the models (table 2, model 3 and model 4). Subgroup analysis showed some evidence of heterogeneity by smoking status, with the associations of eGFR decline with decadienylcarnitine being stronger in smokers than in non-smokers (figure 1, upper panel), and that with hexenoylcarnitine showing the opposite effect (ie, stronger in non-smokers) (figure 1, lower panel).

External validation in the JKS

Four of the 11 acylcarnitines associated with eGFR decline in the aGMS (ie, tiglylcarnitine, methylglutarylcarnitine, tetradecenoylcarnitine and hexadecenoylcarnitine) were also present in the JKS metabolomic dataset. Of these, tiglylcarnitine (beta=−0.38; p=2.0E-2) and methylglutarylcarnitine (beta=−0.50; p=2.0E-3) were confirmed to be associated with eGFR decline.

Prediction analysis

Subsequent LASSO regularized regression with 10-fold cross-validation (λ=0.01, selected via minimum criteria) identified six independent predictors: methylglutarylcarnitine, hydroxyvalerylcarnitine, hexenoylcarnitine, decadienylcarnitine, dodecanedioylcarnitine and tetradecadienylcarnitine. The pairwise correlation among

Table 3 Results of repeated threefold cross-validation of multiple regression models

	Full sample			Validation set		Training set	
	Beta	MSE	R^2	MSE*	R^{2*}	Beta†	Bias‡
Baseline eGFR							
+ C3:1	−0.2675	1.7967	0.0474	1.8199	0.0370	−0.2677	0.0002
+ C5:1	−0.2587	1.8047	0.0432	1.8271	0.0334	−0.2588	0.0002
+ C5-M-DC	−0.2250	1.8294	0.0301	1.8524	0.0201	−0.2257	0.0007
+ C5 OH (C3-DC-M)	−0.3004	1.7783	0.0571	1.8015	0.0466	−0.3005	0.0002
+ C6:1	0.1899	1.8324	0.0285	1.8553	0.0183	0.1930	−0.0031
+ C10:2	−0.2011	1.8313	0.0290	1.8546	0.0183	−0.2016	0.0005
+ C12-DC	0.2562	1.8051	0.0429	1.8279	0.0328	0.2564	−0.0002
+ C14:1	−0.1845	1.8341	0.0275	1.8571	0.0177	−0.1846	0.0001
+ C14:2	−0.1881	1.8326	0.0283	1.8557	0.0183	−0.1882	0.0001
+ C16:1	−0.2393	1.8094	0.0407	1.8334	0.0302	−0.2395	0.0002
+ C16:2-OH	−0.2330	1.8123	0.0391	1.8367	0.0279	−0.2332	0.0002

Cross-validation performed with 10 000 iterations comparing model performance and regression coefficients within each fold against the full sample. Threefold cross-validation with 10 000 iterations, for multiple regression models on eGFR slope with respect to baseline eGFR and selected acylcarnitines: comparison of model performance and regression coefficients within each fold with respect to the full sample.
Beta: the regression coefficient for the acylcarnitine in the model, representing the mean decrease in eGFR for each unit increase in the standardized level.
MSE (mean squared error): The average squared difference between the observed and predicted values from the model.
R^2 (coefficient of determination): a measure of the goodness of fit of the model, ranging from 0 (poor fit) to 1 (perfect fit). It is calculated by dividing the MSE by the variance of the observed outcome values and subtracting the result from 1.
*Means across all validation sets.
†Means across all training sets.
‡Bias is defined as the difference between the slope estimated in the full sample and the mean of the estimated slopes across all training sets.
eGFR, estimated glomerular filtration rate.

these selected metabolites ranged from −0.02 to 0.55, with an inverse correlation observed only between dodecanedioylcarnitine and tetradecadienylcarnitine. A multimarker score, created by summing the standardized serum values of these six acylcarnitines, was strongly and independently associated with eGFR decline in the fully adjusted model 4 (p value=7.0E-6).

Patients were then defined as relatively fast (n=287) or low progressors (n=288) according to individual eGFR decline above or below the cohort's median value (−1.32 mL/min per 1.73 m^2/year). Discrimination ability (AUC) to identify fast progressors was 0.66 (95% CI 0.62 to 0.70), 0.63 (95% CI 0.58 to 0.67) and 0.66 (95% CI 0.62 to 0.70) for the acylcarnitine score, clinical model 1 and clinical model 2, respectively.

The addition of the multimarker score significantly improved the AUC of clinical model 1 (delta AUC=0.06, 95% CI 0.02–0.10; p=1.3E-3), achieved a % relative IDI

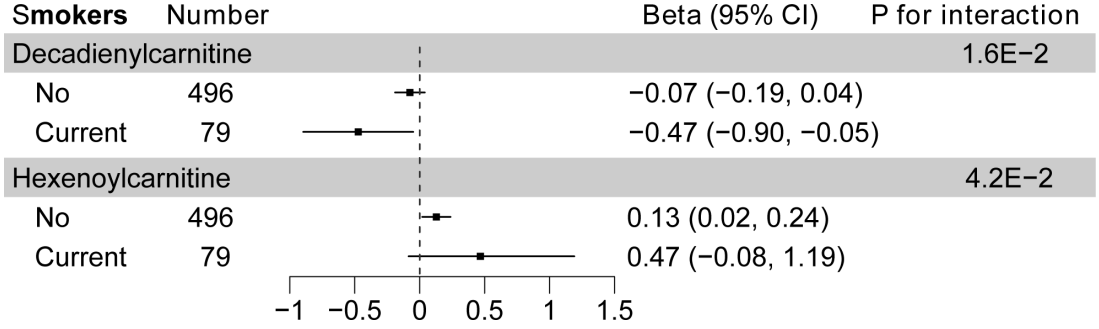

Figure 1 Associations of decadienylcarnitine (upper panel) and hexenoylcarnitine (lower panel) with eGFR decline in the aGMS cohort, stratified by smoking habits (ie, no or current smokers). Linear regression models were used to estimate the regression coefficients (beta) and 95% CI per one SD increase in each acylcarnitine. Models were adjusted for age at recruitment, gender, BMI, diabetes duration, HbA1c, and baseline eGFR. The p value for the metabolite-by-smoking status interaction term is shown for each subgroup. aGMS, aggregate Gargano Mortality Study; BMI, body mass index; eGFR, estimated glomerular filtration rate; HbA1c, glycated hemoglobin.

of 5.86 (95% CI 3.94% to 7.78%, p=2.3E-9) and correctly reclassified patients with a cNRI of 0.39 (95% CI 0.23 to 0.53, p=1.4E-6).

Similarly, the addition of the multimarker score to the clinical model 2, significantly improved the AUC (delta AUC=0.05, 95% CI 0.02 to 0.07, p=1.7E-3), achieved a relative IDI of 4.10% (95% CI 2.49% to 5.71%, p=6.2E-7) and correctly reclassified patients with a cNRI of 0.36 (95% CI 0.20 to 0.52, p=1.4E-5).

DISCUSSION

Previous hypothesis-free metabolomics and lipidomics prospective studies analyzed the possible association between metabolites of lipid origin and the decline of eGFR in type 2 diabetes.[8 10–16] Some of them analyzed these associations through NMR platforms, not including acylcarnitine family and showed no association between any of the lipid fraction metabolites studied and eGFR decline.[12 13 16] Another study utilizing mass spectrometry-based metabolomics and shotgun lipidomics platforms, containing also several acylcarnitines, reported no associations with lipid metabolites.[14] Only two studies reported a significant association between 3 ceramides[15] and 61 lipids (ie, phosphatidylethanolamines, triacylglycerols, diacylglycerols, ceramides and deoxysphingolipids)[11] and a more rapid kidney function decline.

Among all these previous studies, only three reported that some acylcarnitines are associated with the decline of eGFR in type 2 diabetes.[8 10 11] To provide additional data on this topic, we comprehensively addressed the role of short, medium and long-chain acylcarnitines on eGFR decline in people with diabetes. Eleven out of the 40 acylcarnitines assayed were independently associated with the rate of kidney function loss in the discovery cohort. Two of them (ie, hexenoylcarnitine and dodecanedioylcarnitine) showed a protective effect on eGFR decline, while the remaining nine were positively associated with kidney function deterioration over time. The protective effect of hexenoylcarnitine and dodecanedioylcarnitine may indicate an adaptive shift towards an alternative metabolic flux that could be less harmful under conditions of mitochondrial or metabolic stress.[29] Some associations were reinforced by the cigarette smoking habit, a well-known risk factor for kidney disease in type 2 diabetes,[30] thus suggesting that acylcarnitine metabolism and smoking habit shape some common pathway on eGFR decline. All associations were internally validated. Of note, the most strongly associated ones were short-chain acylcarnitines, previously reported associated with incident reduced eGFR in the general population.[31]

Four of these 11 metabolites were present in dataset of the JKS (an independent sample of patients with type 2 diabetes from the USA) and then tested for external validation. Indeed, two of them (ie, tiglylcarnitine and methylglutarylcarnitine) were associated with eGFR decline also in this second set. We cannot exclude that this only partial external validation may be due to ethnic differences and the geographical origin of the discovery and validation cohorts. Indeed, Skupien *et al* reported differences in eGFR slopes between cohorts from Europe and the United States.[32]

We conducted a literature search for possible associations between the identified metabolites and the decline in renal function (ie, eGFR slope) in patients with type 2 diabetes. Only in the paper by Chen *et al* in which a panel of 55 acylcarnitines were studied, 3 (ie, tiglylcarnitine, decadienylcarnitine and hydroxyhexadecadienylcarnitine) out of the 11 associated acylcarnitines were present.[11] Among these, only tiglylcarnitine was associated with eGFR slope in a meta-analysis of 12 891 individuals with type 2 diabetes from China, but not with rapid renal decline or progressive CKD.[11] No data on the role of the other molecules on GFR decline in patients with type 2 diabetes were reported.

Our data showing a significant increase in two C-16 acylcarnitines (ie, hexadecenoylcarnitine and hydroxyhexadecenoylcarnitine) are at odds with previous ones,[8] which described a lower abundance of C16–C20 acylcarnitines associated with kidney function loss in type 2 diabetes American Indians. A possible explanation is that this discrepancy is due to the different genetic, environmental and life style background of the two populations.

In multivariable analysis, all 11 acylcarnitines showed strong associations with eGFR decline. However, many of these metabolites were intercorrelated. By LASSO regression analysis, we selected six molecules for the development of a score, which improved the ability of two established risk prediction models[24 25] to discriminate individuals with accelerated kidney function loss as provided also by the percentage of relative Integrated Discrimination Improvement (rIDI). Furthermore, the addition of the score to the clinical models allowed to reclassify correctly a consistent proportion of individuals.

Our study, therefore, improves the understanding of the role of circulating acylcarnitines on kidney function in type 2 diabetes and suggests that these metabolites may be used in the clinical setting for prediction purposes. Acylcarnitines transport fatty acids from the cytoplasm into the mitochondria, where they undergo beta-oxidation.[33 34] Defective beta-oxidation is involved in the development of renal disease[8 35] and may well explain the biology underlying the associations between serum acylcarnitines and rapid decline of kidney function. Also, inflammation has been reported to link acylcarnitines to eGFR decline.[10]

Strengths of our study include the rigorous study design with quality-controlled metabolomics profiling in all samples utilized. In addition, we used correction for multiple comparisons in the discovery sample and an internal validation utilizing a comprehensive internal validation strategy fully aligned with current methodological recommendations for prediction modeling[22] to robustly confirm our finding. We then further validated some associations in an independent prospective cohort with a different clinical, geographical and ethnic

background.[21] The two cohorts are somewhat different in terms of patient's age, disease duration, baseline eGFR, treatment intensity and, obviously, geographical origin, which further reinforce the credibility of our findings. Notably, different methodologies were used to measure eGFR decline and to analyze the association between acylcarnitines and rate of GFR decline in the external replication, which, if any, have pushed the results towards the null hypothesis. An additional merit of our study is to point acylcarnitines as possible markers to be used for prediction purposes, thus bringing the implementation of precision medicine approach closer. In contrast, the relatively small size of the study cohorts and the lack in the JKS dataset of many metabolites associated in the discovery sample (making only partial our external validation) are recognized as limitations. An additional limitation is that for the same reason, we could not validate the predictive performance of the six metabolite score, a crucial step to ensure that it also works well in different contexts.[36]

Finally, lack of data on diet does not allow us to address the role of an additional important shaper of human metabolome.

In conclusion, we identified several serum acylcarnitines related to the decline of kidney function in patients with type 2 diabetes and suggest that they can be used to improve our ability to predict individuals most at risk. Although targeted metabolite measurement is becoming increasingly feasible and offered by both academic/government labs and industry services with affordable costs, we recognize that before our data become implementable in daily clinical work, the assessment of the six acylcarnitines comprised in the multimarker score needs to be enrolled in a standard clinical chemistry assay.

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Contributors VT and CM conceived the study and wrote the manuscript. VT, AF, MC, AD and CM participated in data analysis and interpretation of results. VT, MM, SDC, HS, AD and CM contributed to data collection. CP and JA supervised the target metabolomics analysis. All authors critically revised the paper and approved its final version. VT and CM are the guarantors of this work and, as such, had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. VT and CM: these authors shared the responsibility to oversee the entire study.

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