Circulating metabolites improve the prediction of renal impairment in patients with type 2 diabetes

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ABSTRACT

Introduction Low glomerular filtration rate (GFR) is a leading cause of reduced lifespan in type 2 diabetes. Unraveling biomarkers capable to identify high-risk patients can help tackle this burden. We investigated the association between 188 serum metabolites and kidney function in type 2 diabetes and then whether the associated metabolites improve two established clinical models for predicting GFR decline in these patients.

Research design and methods Two cohorts comprising 849 individuals with type 2 diabetes (discovery and validation samples) and a follow-up study of 575 patients with estimated GFR (eGFR) decline were analyzed.

Results Ten metabolites were independently associated with low eGFR in the discovery sample, with nine of them being confirmed also in the validation sample (ORs range 1.3–2.4 per 1SD, p values range 1.9x10−2–2.5x10−9). Of these, five metabolites were also associated with eGFR decline (ie, tiglylcarnitine, decadienylcarnitine, total dimethylarginine, decenoylcarnitine and kynurenine) (β range −0.11 to −0.19, p values range 4.8x10−2 to 3.0x10−5). Indeed, tiglylcarnitine and kynurenine, which captured all the information of the other three markers, improved discrimination and reclassification (all p<0.01) of two clinical prediction models of GFR decline in people with diabetes.

Conclusions Further studies are needed to validate our findings in larger cohorts of different clinical, environmental and genetic background.

WHAT IS ALREADY KNOWN ON THIS TOPIC
⇒ In people with type 2 diabetes, reduced glomerular filtration rate (GFR) is common and is one of the leading causes of reduced lifespan; novel biomarker associated with low GFR are needed to improve identification of patients at high risk to be targeted with specific treatments.

WHAT THIS STUDY ADDS
⇒ In type 2 diabetes, tiglylcarnitine and kynurenine are associated with low kidney function and improve the prediction of GFR decline.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY
⇒ Our finding brings one step closer to implementing precision precision for people with diabetes at risk of developing kidney dysfunction.

Unraveling novel biomarkers associated with low GFR can help improve the identification of those patients at highest risk of GFR reduction to be targeted with prevention treatments that will then only need to be implemented in a limited number of patients. Metabolomics is one of the possible strategies used to identify novel biomarkers in several chronic renal diseases with kidney function decline.9

The aim of our study was to investigate (1) the association between 188 serum metabolites and low estimated GFR (eGFR) in patients with type 2 diabetes, (2) to validate the observed associations in an independent sample and finally (3) to confirm the results in a follow-up study of eGFR decline.

Additionally, our second aim was to verify whether the metabolites we found to be associated with eGFR decline in type 2 diabetes improve the prediction of kidney function loss provided by established clinical models.10

In patients with type 2 diabetes, reduced glomerular filtration rate (GFR) is common1 and is one of the leading causes of cardiovascular events2 and reduced lifespan.3–6 As the prevalence of type 2 diabetes increases,7 the burden of impaired kidney function in terms of morbidity and mortality in the general population is expected to increase.

There are several strategies available to prevent and/or delay the decline of kidney function in people with diabetes.8 However, due to the high prevalence of the disease, these strategies cannot be implemented in the entire diabetes population.

Unravelling novel biomarkers associated with low GFR can help improve the identification of those patients at highest risk of GFR reduction to be targeted with prevention treatments that will then only need to be implemented in a limited number of patients. Metabolomics is one of the possible strategies used to identify novel biomarkers in several chronic renal diseases with kidney function decline.9

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RESEARCH DESIGN AND METHODS

Participants

Two cohorts of patients with type 2 diabetes (diagnosed according to American Diabetes Association 2003 criteria) who attended the Endocrinology Unit at IRCCS “Casa Sollievo della Sofferenza”, San Giovanni Rotondo, and who were followed up for 19.7 years were investigated.\(^{12}\)

The Gargano Mortality Study 1 (GMS 1) was used as the discovery cohort.

This sample includes 1028 patients recruited from 2000 to 2010 and followed up until December 2019 for all-cause mortality.\(^{12}\) Circulating metabolites were measured in 326 participants, with the only exclusion criterion being eGFR of 60–69 mL/min/1.73 m\(^2\). Indeed, this extreme phenotype case-control approach (ie, 117 cases with eGFR<60 mL/min/1.73 m\(^2\) and 209 controls with eGFR\(\geq 60\) mL/min/1.73 m\(^2\)) was used in order to increase the likelihood of positive results in the discovery sample.

The Gargano Mortality Study 2 (GMS 2) was used as the validation cohort.

This sample includes 880 patients recruited from 2008 to 2010 and followed up until December 2019 for all-cause mortality.\(^{12}\) As for GMS2, a case-control study for different eGFR levels was created using a subset of the entire cohort, in which circulating metabolites were measured, comprising 162 cases with eGFR<60 mL/min/1.73 m\(^2\) and 361 controls with eGFR\(\geq 60\) mL/min/1.73 m\(^2\). Since this sample was intended to validate associations that had already been observed, the extreme phenotype approach was not used.

eGFR was measured at the time of recruitment for each patient of both discovery and validation cohorts.

Follow-up study

We traced the decline of eGFR in 575 patients (291 from GMS1 and 284 from GMS2) having at least three eGFR records at intervals of 4 months with at least 1.5 years of follow-up.

We measured standardized serum creatinine using the modified kinetic Jaffé reaction (Hitachi 737 autoanalyzer), calibrated to be traceable to an isotope dilution mass spectrometry. GFR was estimated for each patient using the Chronic Kidney Disease Epidemiology Collaboration formula derived from serum creatinine values at baseline and during follow-up.\(^{14}\)

Metabolite quantification and normalization

Metabolite profiles were measured using baseline fasting serum samples that had been stored at –80°C since collection. Metabolite quantification was performed in the Genome Analysis Center at the Helmholtz Zentrum München. The targeted metabolomics approach was based on LC-ESI-MS/MS and FIA-ESI-MS/MS measurements by AbsoluteIDQ p180 Kit (BIOCRATES Life Sciences AG, Innsbruck, Austria). The assay allows simultaneous quantification of 188 metabolites out of 10 µL plasma and includes free carnitine, 40 acylcarnitines (Cx:y), 21 amino acids (19 proteinogenic+citrulline+ornithine), 21 biogenic amines, hexoses (sum of hexoses – about 90%–95% glucose), 90 glycerophospholipids (14 lysophosphatidylcholines (lysoPC) and 76 phosphatidylcholines (PC), and 15 sphingolipids (SM cx:y)). For a full list of all quality-controlled metabolites, see online supplemental table 1. The procedures for sample preparation and mass spectrometric measurements, as well as the metabolite nomenclature, have been described in detail previously.\(^ {15,16}\) Five reference plasma samples and three zero sample Phosphate buffered saline (PBS) were included in each randomized plate.\(^ {17}\)

Data evaluation for quantification of metabolite concentrations and quality assessment was performed with the software MultiQuant V.3.0.1 (Sciex) and the MetIDQ software package, which is an integral part of the AbsoluteIDQ Kit. Metabolite concentrations were calculated using internal standards and reported in µM.

Measurement of circulating cytokines

Circulating levels of 27 cytokines were measured simultaneously in duplicate, using a multiplex detection 27-plex kit from Bio-Rad (online supplemental table 2). The median coefficient of variation was less than 25% for all analyzed cytokines. Data were analyzed as previously described.\(^ {18}\)

Statistical analysis

Patients’ baseline characteristics were reported as mean±SD or median and IQR and frequency and percentage for continuous and categorical variables, respectively. Values of serum metabolites below the limit of detection (LOD) values have been replaced by the LOD itself.

Correlations between metabolites were assessed using the Spearman correlation. All covariates with missing values below 5% were imputed by random forest method.\(^ {19}\) Because of skewed distribution and for comparability between different metabolites, their concentrations were logarithmically transformed and then standardized.

Case-control analyses

To assess the association between the detected serum metabolites levels and low eGFR in the discovery sample (ie, GMS2), Bonferroni adjustment for multiple comparisons was used to determine the significance threshold in unadjusted logistic regression analysis. Because of the potential correlation between metabolites, we next evaluated the independent associations of Bonferroni-survived metabolites using a forward-backward stepwise analysis\(^ {20}\) in a multivariate logistic analysis comprising age at recruitment, sex, smoking habit, body mass index (BMI), HbA1c, eGFR, diabetes duration and ongoing treatments.

Associations were then validated in an independent cohort (ie, GMS 1), considering the fully adjusted model.
Risks were reported as ORs along with their 95% CIs per 1 SD increase of each single metabolite.

Prospective analyses

Repeated-measures, longitudinal, multilevel (mixed-effects) models with follow-up time as a fixed effect were used to compute the annual change in eGFR (measured as mL/min/1.73 m²/year) for each participant. Linear regression models were used to identify the independent biomarker determinants of the annual change in eGFR (mL/min/1.73 m²/year) after accounting for baseline age, sex, smoking habit, BMI, HbA1c, disease duration, ACR (Albumin creatinine ratio), eGFR and ongoing treatments.

The pooled analysis, using data from both GMS 1 and GMS 2, was also adjusted for study cohort factor considered as random effect, so as to have more robust estimates.

Mediation analysis allowing for exposure-mediator interactions was carried out as previously described.²¹ The mediation effect was from linear regression models and was adjusted for study sample, subject’s age, gender, disease duration, BMI, smoking habits, baseline HbA1c, eGFR, ACR, lipid-lowering therapy, insulin therapy and antihypertensive therapy. The 95% CI and p value of the mediation effect were assessed from a statistical contrast defined by jointly modeling both the exposure and the mediator within a single linear model. A p value <0.05 was considered for statistical significance.

Prediction analyses

The discriminative ability of the validated associated metabolites in predicting GFR decline was tested only in the follow-up sample on top of two previously published clinical models in patients with diabetes. The first model (model 1) comprised sex, age, BMI, HbA1c and ACR as variables. The second model (model 2) comprised age, sex, log2-transformed ACR, total cholesterol, smoking habits, BMI, mean arterial pressure, HbA1c and eGFR.

### Table 1: Clinical features of the two independent study cohorts

<table>
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<tr>
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<th>Discovery cohort</th>
<th>Validation cohort</th>
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<tr>
<td></td>
<td>Cases (n=117)</td>
<td>Controls (n=209)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cases (n=162)</td>
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<tr>
<td></td>
<td></td>
<td>Controls (n=361)</td>
</tr>
<tr>
<td><strong>Women (n) (%)</strong></td>
<td>60 (51.3)</td>
<td>80 (38.3)</td>
</tr>
<tr>
<td><strong>Age at recruitment (years)</strong></td>
<td>68.8±7.4</td>
<td>54.6±8.2</td>
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<tr>
<td><strong>Smoking habit (n) (%)</strong></td>
<td>5 (4.2)</td>
<td>53 (25.4)</td>
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<tr>
<td><strong>Diabetes duration (years)</strong></td>
<td>12.6±9.8</td>
<td>9.0±7.8</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>31.9±6.9</td>
<td>31.4±6.2</td>
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<tr>
<td><strong>HbA₁c (%)</strong></td>
<td>8.3±1.7</td>
<td>8.2±1.9</td>
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<tr>
<td><strong>HbA1c (mmol/mol)</strong></td>
<td>67±14.0</td>
<td>66±15.7</td>
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<tr>
<td><strong>eGFR (mL/min/1.73 m²)</strong></td>
<td>48.2±13.1</td>
<td>105.1±8.4</td>
</tr>
<tr>
<td><strong>ACR (mg/mmol)</strong></td>
<td>5.1 (1.2–28.6)</td>
<td>1.2 (0.7–2.8)</td>
</tr>
<tr>
<td><strong>Antihypertensive therapy (n) (%)</strong></td>
<td>112 (95.7)</td>
<td>142 (67.9)</td>
</tr>
<tr>
<td><strong>Insulin therapy (n) (%)</strong></td>
<td>85 (72.6)</td>
<td>92 (44.0)</td>
</tr>
<tr>
<td><strong>Statin therapy (n) (%)</strong></td>
<td>98 (83.6)</td>
<td>148 (70.8)</td>
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Continuous variables were reported as mean±SD, whereas categorical variables as total frequencies and percentages. Skewed variables are presented as median (IQR).

BMI, body mass index; eGFR, estimated glomerular filtration rate.

### Table 2: Clinical features of the follow-up study cohort (n=575)

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<table>
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<tbody>
<tr>
<td><strong>Women (n) (%)</strong></td>
<td>275 (47.8)</td>
</tr>
<tr>
<td><strong>Age at recruitment (years)</strong></td>
<td>60.9±9.7</td>
</tr>
<tr>
<td><strong>Smoking habit (n) (%)</strong></td>
<td>79 (1.7)</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>31.2±5.9</td>
</tr>
<tr>
<td><strong>HbA₁c (mmol/mol)</strong></td>
<td>8.4±1.9</td>
</tr>
<tr>
<td><strong>HbA1c (mmol/mol)</strong></td>
<td>68±15.7</td>
</tr>
<tr>
<td><strong>eGFR (mL/min/1.73 m²)</strong></td>
<td>77.4±26.1</td>
</tr>
<tr>
<td><strong>ACR (mg/mmol)</strong></td>
<td>1.56 (0.7–4.96)</td>
</tr>
<tr>
<td><strong>Antihypertensive therapy (n) (%)</strong></td>
<td>376 (65.4)</td>
</tr>
<tr>
<td><strong>Insulin therapy (n) (%)</strong></td>
<td>268 (46.6)</td>
</tr>
<tr>
<td><strong>Statin therapy (n) (%)</strong></td>
<td>292 (50.8)</td>
</tr>
<tr>
<td><strong>Duration of follow-up (y); py</strong></td>
<td>9.4 (5.0, 11.8)</td>
</tr>
<tr>
<td><strong>Annual eGFR change (mL/min/1.73 m²/year)</strong></td>
<td>1.32 (–2.20, 0.60)</td>
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Continuous variables were reported as mean±SD, whereas categorical variables as total frequencies and percentages. Skewed variables are presented as median (IQR).

ACR, Albumin creatinine ratio; BMI, body mass index; eGFR, estimated glomerular filtration rate.
Multiple $R^2$ and $\epsilon$ statistic were used, respectively, to measure the variance explained by and the discriminatory ability of prediction models without and with the addition of metabolites found to be associated with eGFR decline. Nested linear models were compared using the F-test. Improvement in discrimination was assessed by the delta $\epsilon$ statistic and the relative integrated discrimination improvement (rIDI). In addition, the survival version of the category-free net reclassification improvement (cNRI), which examines whether the predicted probabilities of individuals with and without events move in the right directions (upward and downward, respectively) from the base to the new model, was evaluated. The 95% CIs for discrimination and reclassification measures were computed by bootstrap.

All analyses were performed using R software (R Core Team, 2021) (packages DBI, dplyr, dbplyr, lme4, Matrix, fitdistrplus) and SAS Release V.9.4 (SAS Institute).

Data and resource availability

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

RESULTS

The clinical features of patients from the discovery and validation case-control cohorts, as well as the prospective cohort, are summarized in tables 1 and 2. Patients of the discovery cohort were younger (59.7 vs 62.9 years; $p=0.004$) and more often females (57% vs 49%; $p=0.029$), smokers (17% vs 7.5%; $p<0.001$) and treated with statin (72.3% vs 30.7%; $p<0.001$) and antihypertensive drugs (78.2% vs 59.4%; $p<0.001$). As for study design selection (ie, extreme phenotype approach excluding eGFR of 60–69 mL/min/1.73 m$^2$/year), they have also a higher median eGFR (70.6 vs 62.9 mL/min/m$^2$; $p<0.001$). Of the 188 measured metabolites, 5 (ie, carnosine, DOPA, dopamine, nitrotyrosine and cis-4-hydroxyproline)
were excluded from the analyses in the discovery sample because their value was below the detection limit in more than 80% samples. Also, creatinine data from the metabolomic assay (online supplemental table 1) were not analyzed because serum creatinine values from standard baseline clinical chemistry measurements were available. After Bonferroni correction (threshold \( p \) value being \( 0.05/27=1.8 \times 10^{-3} \)), only the IFN-\( \gamma \) level was associated with renal function decline in a subset of 552 subjects in whom cytokine measurements were available.

Furthermore, eGFR decline increased from tertile 1 (ie, lowest values) to tertile 3 of both tiglylcarnitine and kynurenine levels (\( p \) for trend \(< 0.001 \) and 0.003, respectively) (figure 2A,B), thus providing support for linear associations.

We then investigated the possible role of 27 cytokines related to low-grade inflammation (online supplemental table 2) as mediators of the association of tiglylcarnitine and kynurenine with eGFR decline in a subset of 552 subjects in whom cytokine measurements were available. After Bonferroni correction (threshold \( p \) value being \( 0.05/27=1.8 \times 10^{-3} \)), only the IFN-\( \gamma \)-Inducible protein 10 (IP-10) was associated with tiglylcarnitine and kynurenine (\( \beta \) (SE) and \( p \) values being \(-0.16 \pm 0.03 \); \( p=3.0 \times 10^{-5} \), and \(-0.33 \pm 0.04 \); \( p=2.2 \times 10^{-16} \), respectively). IP-10 was also slightly associated with eGFR decline in our fully adjusted model (\( \beta \) (SE); \( p=0.12 \) (0.06); \( p=4.0 \times 10^{-2} \)). Interestingly, when IP-10 was also added into the model, the associations with eGFR decline of both tiglylcarnitine and kynurenine were attenuated at the point of being no longer significant: \( p \) value becoming 0.08 and 0.14, respectively. Although not reaching a formal statistical significance, results from mediation analyses suggested that some proportion of the total effect of tiglylcarnitine and kynurenine on eGFR decline is mediated by the IP-10 (7.14% (95% CI \(-1.35 \) to 15.63); \( p=0.10 \) and 31.74% (95% CI \(-9.52 \) to 72.99; \( p=0.13 \)).

### Adding metabolites to prediction models of GFR decline

As indicated by the multiple \( R^2 \), the proportion of the eGFR decline variance explained by the combination of tiglylcarnitine and kynurenine, clinical model 1 and clinical model 2, was 4.8%, 6.7% and 10.5%, respectively (online supplemental table 4). Notably, the addition of the two metabolites on top of each clinical model significantly (\( p<0.0001 \) for both) improved the goodness of fit (log-likelihood, online supplemental table 4). Patients were then defined as relatively fast or low progressors according to individual eGFR decline above or below the cohort’s median value. Discrimination ability (\( \varepsilon \) statistic) to identify fast progressors was 0.64 (95% CI 0.59 to 0.68), 0.62 (95% CI 0.58 to 0.67) and 0.67 (95% CI 0.63 to 0.71) for the two metabolites combined together, clinical model 1 and clinical model 2, respectively (table 4). The addition of the two metabolites on top of each clinical model significantly improved both \( \varepsilon \) statistic, IDI and rIDI (table 4). In addition, all eNRI values showed a significant improvement, due to both events and non-events correctly reclassified (table 4).

| Table 3 Metabolites and eGFR slope decline in the follow-up sample (n=575) |
|-----------------------------|-----------------------------|------------|-------------|
| **Metabolite**               | **Linear regression coefficient for annual eGFR slope** | ±SE        | **P value** |
| Tiglylcarnitine             | 0.19                       | 0.06       | 0.003       |
| Decadienylcarnitine         | 0.13                       | 0.06       | 0.030       |
| Total dimethylarginine      | 0.14                       | 0.06       | 0.032       |
| Decenoylcarnitine           | 0.11                       | 0.05       | 0.040       |
| Kynurenine                  | 0.13                       | 0.06       | 0.031       |
| Dodecenooylcarnitine        | 0.11                       | 0.06       | 0.05        |
| Citrulline                  | 0.10                       | 0.06       | 0.10        |
| Pyridoxalpyruv alcaptonine   | 0.10                       | 0.06       | 0.10        |
| Acetyl carnitine            | 0.08                       | 0.06       | 0.18        |

Linear regression coefficients (per 1 SD increase in each metabolite concentration) were estimated in model adjusted for age, sex, smoking habit, BMI, HbA\(_1c\), disease duration ACR, baseline eGFR and ongoing treatments. BMI, body mass index; eGFR, estimated glomerular filtration rate.
DISCUSSION
By using a discovery and validation case-control design, we studied the association between 182 metabolites and renal function in patients with type 2 diabetes. After a further validation in a follow-up study, five biologically plausible metabolites (i.e., tiglylcarnitine, decadienylcarnitine, total dimethylarginine, decenoylcarnitine and kynurenine) were independently and consistently associated with both low eGFR and eGFR decline. Among these, tiglylcarnitine and kynurenine captured all the information of the other three markers and are, therefore, parsimoniously proposed as tools for

Figure 2  Distribution of annual estimated glomerular filtration rate (eGFR) decline by tertiles of tiglylcarnitine and kynurenine. Mean (95% CI) of annual eGFR decline according to baseline tertile of tiglylcarnitine (A) and kynurenine (B) (T1–T3, range in parentheses).
improving the prediction of GFR decline in people with diabetes as discussed below. Importantly, no interaction between basal eGFR and tiglylcarnitine and kynurenine was observed in predicting eGFR decline, thus indicating that both metabolites can be used also in the early stage of kidney disease when the chance of modifying each patient’s clinical trajectory, by means of several protective treatments, are higher. Indeed, these two metabolites improved two well-established risk prediction models of GFR decline in type 2 diabetes.10–11 In detail, it is of note that the percentage of rIDI, a measure of improved discrimination, was definitely higher than the threshold note that the percentage of rIDI, a measure of improved discrimination, was definitely higher than the threshold

<table>
<thead>
<tr>
<th>Prediction models</th>
<th>Discrimination</th>
<th>Reclassification</th>
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<tbody>
<tr>
<td></td>
<td>% C statistic</td>
<td>ΔC statistic</td>
</tr>
<tr>
<td>Tiglylcarnitine+kynurenine</td>
<td>63.7 (59.2 to 68.2)</td>
<td>4.5 (0.01)</td>
</tr>
<tr>
<td>Clinical model 1</td>
<td>62.5 (57.9 to 67.0)</td>
<td>4.5 (0.01)</td>
</tr>
<tr>
<td>Clinical model 1+tiglylcarnitine+kynurenine</td>
<td>67.0 (62.6 to 71.4)</td>
<td>4.5 (0.01)</td>
</tr>
<tr>
<td>Clinical model 2</td>
<td>65.7 (61.2 to 70.1)</td>
<td>4.5 (0.01)</td>
</tr>
<tr>
<td>Clinical model 2+tiglylcarnitine+kynurenine</td>
<td>67.8 (63.5 to 72.2)</td>
<td>2.1 (0.06)</td>
</tr>
</tbody>
</table>

All p values are referred to comparisons versus the same base model (ie, with no metabolites).

eGFR, estimated glomerular filtration rate.

progression to end stage renal disease.31 Acylcarnitines transport fatty acids from the cytoplasm into the mitochondria where they are broken down to produce energy (ie, beta-oxidation).32 Indeed, impaired mitochondrial β-oxidation due to excessive fatty acids accumulation leads to accumulation of short to intermediate-chain acylcarnitines and is proposed as the potential mechanisms underlying the acylcarnitines-mediated development of renal disease.33 34

Kynurenine and its degradation products have been so far associated with CKD only in case control studies in both people with diabetes and in the general population35–41 while the kynurenine-to-tryptophan ratio is the likely consequences of the deleterious effects of kynurenine and kynurenine metabolites on mesangial cells proliferation45 as well as Reactive oxygen species (ROS) production, cell damage and apoptosis in renal tissue.46

Because previous findings suggest that both the acylcarnitine and the kynurenine pathways are strictly correlated to inflammation,45 46 it is conceivable to hypothesize that inflammatory proteins mediate the associations we here describe. Among 27 cytokines we studied as exploratory analysis, only IP-10, belonging to the IFN network,47 was correlated with both tiglylcarnitine and kynurenine and decline of kidney function. Of note, elevated IP-10 levels have been reported in various renal diseases and correlated with disease progression.48 Notably, the associations between the two metabolites and eGFR decline were no longer significant, after adjusting for IP-10, thus suggesting that this cytokine explains part of the mechanism through which tiglylcarnitine and kynurenine affect GFR decline. Although not reaching a formal statistical significance, also mediation analyses were compatible with this possibility which
certainly deserves further larger studies to be investigated in depth.

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 cross-sectional sample. We then validated our finding first in an independent cross-sectional sample and then in a prospective cohort in which also a stepwise analysis was performed to parsimoniously identify the most informative metabolites among those that were validated. In contrast to approaches which are optimal to identify functional clusters of variables that work in synergy (e.g., the principal component analysis), our approach is gold standard to parsimoniously improve prediction models, while preserving interpretability for use in daily clinical work.  

Conversely, we must also recognize several limitations, including the relatively small size of the study cohorts, the arbitrary choice to move the limit for the relatively high eGFR up to ≥70 mL/min/1.73 m², to obtain an ‘extreme phenotype’ in the discovery sample and the fact that they are geographically close to each other, thus leaving open the question of the generalizability of our finding. We also recognize that in the two cross-sectional analyses the direction of causality between metabolites and eGFR cannot be determined. However, this is not the case in the prospective analysis where metabolites were measured at baseline while eGFR decline was repeatedly measured during follow-up and the association between metabolites and kidney function loss was adjusted also for the baseline eGFR. In addition, given our prospective study design aimed at robustly estimate renal function decline by selecting people with at least three eGFR records and at least 1.5 years of follow-up, we cannot exclude a selection bias that reduced the number of people at high risk of mortality in our sample. Also, the robustness of our finding would be more compelling if the selected individuals had more data on eGFR during a longer period follow-up. Finally, we have to recognize that having also urine metabolomics would have improved our study.

In conclusion, in patients with type 2 diabetes, tiglyl-carnitine and kynurenine are reproducible risk factors for reduced kidney function and improve established clinical prediction models of GFR decline. Our finding is the result of a typical effort to implement precision medicine in patients at risk of developing renal dysfunction; however, before it can be applied in the daily clinical work, the two metabolites need to be enrolled in a standard clinical chemistry assay and validated in larger cohorts of different clinical, environmental and genetic background.

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Contributors VT and CM conceived the study and wrote the manuscript and, together with MGS and FPS, designed the protocol. VT, MM, MGS, AF, MC and CM participated in data analysis and interpretation of results. VT, MM, and SD contributed to data collection. LS performed laboratory testing. 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 shared the responsibility to oversee the entire study.

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