Circulating Metabolites Associate With and Improve the Prediction of All-Cause Mortality in Type 2 Diabetes

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Death rate is increased in type 2 diabetes. Unraveling biomarkers of novel pathogenic pathways capable to identify high-risk patients is instrumental to tackle this burden. We investigated the association between serum metabolites and all-cause mortality in type 2 diabetes and then whether the associated metabolites mediate the effect of inflammation on mortality risk and improve ENFORCE (EstimatioN oF mORtality risk in type2 diabetic patiEnts) and RECODe (Risk Equation for Complications Of type 2 Diabetes), two well-established all-cause mortality prediction models in diabetes. Two cohorts comprising 856 individuals (279 all-cause deaths) were analyzed. Serum metabolites ($n = 188$) and pro- and anti-inflammatory cytokines ($n = 7$) were measured. In the pooled analysis, hexanoylcarnitine, kynurenine, and tryptophan were significantly and independently associated with mortality (hazard ratio [HR] 1.60 [95% CI 1.43–1.80]; 1.53 [1.37–1.71]; and 0.71 [0.62–0.80] per 1 SD). The kynurenine-to-tryptophan ratio (KTR), a proxy of indoleamine-2,3-dioxygenase, which degrades tryptophan to kynurenine and contributes to a proinflammatory status, mediated 42% of the significant association between the antiatherogenic interleukin (IL) 13 and mortality. Adding the three metabolites improved discrimination and reclassification (all $P < 0.01$) of both mortality prediction models. In type 2 diabetes, hexanoylcarnitine, tryptophan, and kynurenine are associated to and improve the prediction of all-cause mortality. Further studies are needed to investigate whether interventions aimed at reducing KTR also reduce the risk of death, especially in patients with low IL-13.

In patients with type 2 diabetes, the rate of mortality is almost twice as much as that in individuals without diabetes (1). Unraveling biomarkers capable of pointing to novel pathogenic pathways and identifying high-risk patients suitable for more aggressive management is, therefore, instrumental to tackle this heavy burden.

Few studies have, so far, investigated the role of circulating biomarkers in predicting the risk of mortality in patients with type 2 diabetes (2–7), and even fewer have been focused on serum metabolites (8–11). These latter studies have been limited to only a few metabolites (8,10,11) and/or have not addressed the role of associated metabolites in improving preexisting prediction models

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(8–11). In details, metabolites independently associated with mortality in type 2 diabetes are mostly amino acids (8), fatty acids (10), and choline (11). In the only paper in which a larger number of metabolites were analyzed, also N2,N2-dimethylguanosine, dimethylguanidino valerate, homocitrulline, 1-methyladenosine, acylcarnitine C10:3, urobilin, and hippurate were associated with the mortality rate (9). Unfortunately, the largest metabolomic study on all-cause mortality was performed in the general population and is, therefore, not usable for deriving information in the subset of patients with type 2 diabetes (12).

In this study, we investigated the association between a large number of circulating metabolites and all-cause mortality in individuals with type 2 diabetes. After unraveling and validating some metabolites as robustly and independently associated, we explored whether they mediate the effect of inflammatory cytokines on mortality risk and improve two well-established all-cause mortality prediction models in diabetes: ENFORCE (EstimatioN oF mORtality risk in type2 diabetic patiEnts), a validated user-friendly and freely available risk calculator based on a total of nine variables (13,14), and RECODe (Risk Equation for Complications Of type 2 Diabetes), a well-performing tool, based on a total of 14 variables that have been highly validated in several distinct sets, including both population-based and trial cohorts (15,16).

RESEARCH DESIGN AND METHODS

Participants

Two cohorts of patients with type 2 diabetes (diagnosed according to American Diabetes Association 2018 criteria) from Apulia, Central-Southern Italy were analyzed.

Gargano Mortality Study 1—Discovery Sample

The Gargano Mortality Study 1 (GMS 1) includes 1,028 patients recruited from 2000 to 2005 at the Endocrine Unit of Fondazione Istituto di Ricovero e Cura a Carattere Scientifico "Casa Sollievo della Sofferenza" in San Giovanni Rotondo, followed until December 2014, and has all-cause mortality as the end point. Serum metabolites were assessed in 536 participants (52.1%), constituting the eligible sample for the present analysis.

Gargano Mortality Study 2—Replication Sample

The Gargano Mortality Study 2 (GMS 2) includes 880 patients recruited from 2008 to 2010 at the Endocrine Unit of Fondazione Istituto di Ricovero e Cura a Carattere Scientifico "Casa Sollievo della Sofferenza" in San Giovanni Rotondo, followed until December 2019, and has all-cause mortality as the end point. For this specific analysis, a sample comprising 321 patients participating in an independent substudy of the role of kidney function on mortality rate. No individuals with an estimated glomerular filtration rate (eGFR) in the range of 60 to 69 mL/min/1.73 m^2 were analyzed.

For both studies, the vital status of participants was verified by interrogating the Italian Health Card Database upon data anonymization (https://sistemats1.sanita.finanze.it/wps/portal/) (6). For all studies, the only exclusion criterion was the presence of poor life expectancy for nondiabetes-related diseases (6).

Metabolite Quantification and Normalization

Metabolite profiling was 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 liquid chromatography-electrospray ionization-tandem mass spectrometry and flow injection ionization- electrospray ionization-tandem mass spectrometry measurements by Absolute*IDQ* 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 − ∼90–95% glucose), 90 glycerophospholipids (14 lysophosphatidylcholines [lysoPC] and 76 phosphatidylcholines [PC], and 15 sphingolipids [SMx:y]). For a full list of all quality-controlled metabolites, see Supplementary Table 1. The procedures for sample preparation and mass spectrometric measurements, as well as the metabolite nomenclature, have been described in detail previously (17,18). Three quality control samples (sex-mixed human plasma provided by the manufacturer) and one zero sample (PBS) were included in each randomized plate.

Data evaluation for quantification of metabolite concentrations and quality assessment was performed with the software MultiQuant 3.0.1 (SCIEX) and the Met*IDQ* software package, which is an integral part of the Absolute*IDQ* Kit. Metabolite concentrations were calculated using internal standards and reported in μmol/L.

Measurement of Circulating Cytokines

Serum IL-1β, IL-2, IL-4, IL-6, IL-13, interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α) circulating levels were measured in duplicate, using a multiplex detection 27-plex kit from Bio-Rad. The median coefficient of variation was <25% for all analyzed cytokines. Data were analyzed as previously described (7).

Statistical Analysis

Patients' baseline characteristics are reported as mean ± SD or median and interquartile range for continuous variables and frequency and percentage for categorical variables. Values of serum metabolites below the limit of detection values have been replaced by the limit of detection itself.

Correlations between metabolites were assessed using the Spearman correlation. All covariates with missing values <5% were imputed by random forest method. Because

Table 1—Clinical features of the two independent study cohorts

	GMS 1 (n = 535)	GMS 2 (n = 321)
Male sex	250 (46.7)	183 (57.0)
Age at recruitment (years)	62.9 ± 9.8	59.7 ± 10.3
Smoking habit	40 (7.5)	55 (17.1)
Diabetes duration (years)	11.0 ± 9.0	12.6 ± 9.8
BMI (kg/m^2)	31.1 ± 5.8	31.5 ± 6.4
HbA_{1c} (%)	8.7 ± 1.9	8.2 ± 1.8
HbA_{1c} (mmol/mol)	72.0 ± 15.7	66.0 ± 14.5
eGFR (mL/min/1.73 m^2)	70.6 ± 21.1	83.7 ± 30.3
Antihypertensive therapy	340 (63.4)	251 (78.2)
Insulin therapy	226 (42.2)	137 (42.7)
Statin therapy	164 (30.7)	232 (72.3)
Follow-up (years)	10.0 ± 3.9	8.6 ± 2.6
Follow-up (py)	5,346.2	2,763.9
All-cause death	198 (37.0)	81 (25.2)
Incident rate of all-cause death events (n events per 100 py)* (95% CI)	2.8 (2.4–3.3)	2.2 (1.8–2.8)

Continuous variables are reported as mean ± SD, whereas categorical variables are reported as total frequencies and percentages. eGFR, was calculated using the CKD-EPI equation (50). *Adjusted for age and sex.

of skewed distribution and for comparability between different metabolites, their concentrations were logarithmically transformed and then standardized.

Time variable was defined as the time between the baseline examination and the date of the event (i.e., all-cause mortality) or the date of the last available clinical follow-up for subjects who did not experience the event. The incidence rate for all-cause mortality is expressed as the number of events per 100 person-years (py).

To assess the association between the detected serum metabolites levels and all-cause mortality in the discovery sample (i.e., GMS 1), Bonferroni adjustment for multiple comparisons was used to determine the significance threshold in an unadjusted Cox proportional hazard model. Because of the potential correlation between metabolites, we next evaluated the independent associations of Bonferroni-survived metabolites using a forward-backward stepwise analysis (19) in a fully adjusted model comprising age at recruitment, sex, smoking habit, BMI, glycated hemoglobin A_{1c} (HbA_{1c}), eGFR, diabetes duration, and ongoing treatments.

Associations were then validated in an independent cohort (i.e., GMS 2), considering the fully adjusted model.

When analyses were run in the pooled sample, comprising both GMS 1 and GMS 2, they were also adjusted for study cohort factor considered as a random effect to have more robust estimates. Risks are reported as hazard ratios (HRs) along with their 95% CIs per 1 SD increase of each single metabolite.

Mediation analysis allowing for exposure-mediator interactions, and causal interpretation was carried out as previously described (20). The 95% CI of the mediation effect was computed by bootstrap based on 1,000 resamplings with replacement.

To examine whether the validated associated metabolites increase the accuracy of all-cause mortality prediction models in type 2 diabetes, two different well-established tools were used: ENFORCE (14) and RECODe (15). Discrimination was measured by survival c statistics (21) while improvement in discrimination was assessed by the Δ c statistics and survival version of the relative integrated discrimination improvement (rIDI) (22). 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 (23). The 95% CIs for discrimination and reclassification measures were computed by bootstrap. A P value <0.05 was considered significant. All analyses were performed using SAS 9.4 software (SAS Institute, Cary, NC) and R software packages survival and coxme (R Core Team, 2021).

Data and Resource Availability

The data sets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

RESULTS

Clinical features of patients from GMS 1 and GMS 2 as well as duration of follow-up and number of events are summarized in Table 1. In GMS 1, 198 deaths occurred during follow-up (10.0 ± 3.9 years; 5,346.2 py). In GMS 2,

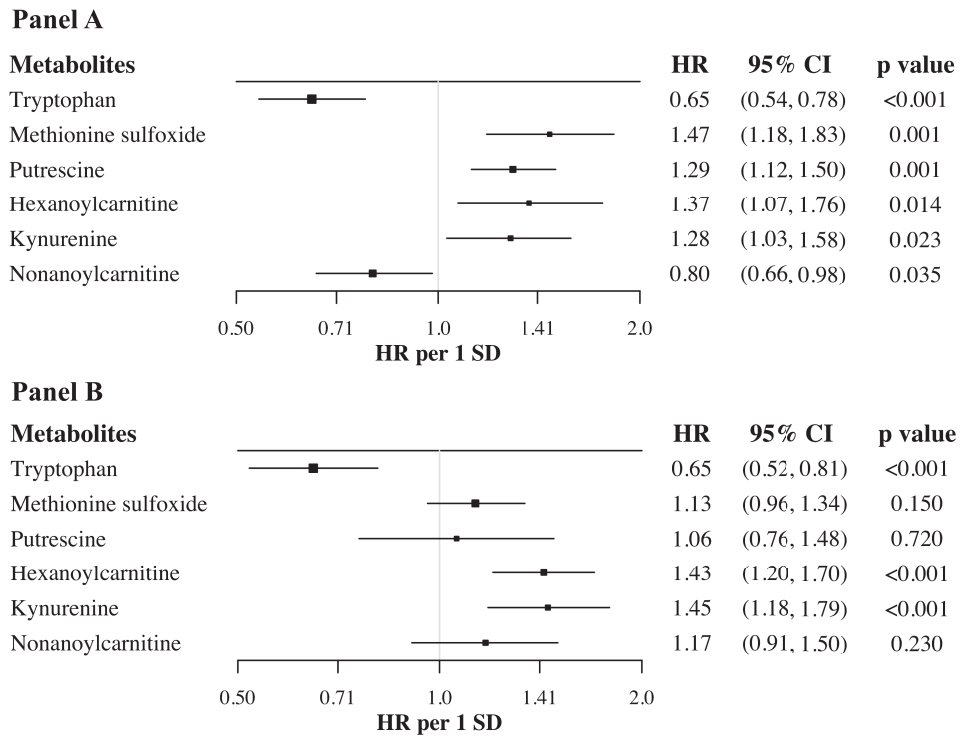

Figure 1— Independent associations between metabolites and all-cause mortality. HRs and 95% CIs for independent associations between metabolites and mortality in GMS 1 (*A*) and GMS 2 (*B*). HRs (per 1 SD increase in each metabolite concentration) were estimated in Cox regression models, adjusting for age at recruitment, sex, smoking habit, BMI, HbA_{1c}, eGFR, diabetes duration, and ongoing treatments.

81 deaths occurred during follow-up (8.6 ± 2.6 years; 2,763.9 py).

Of the 188 metabolites we measured, 5 (i.e., carnosine, L-3,4-dihydroxyphenylalanine, dopamine, nitrotyrosine, and *cis*-4-hydroxyproline) were excluded from the analyses because their value was below the detection limit in >80% samples. Also, creatinine data from the metabolomic assay (Supplementary Table 1) were not analyzed because serum creatinine values from standard baseline clinical chemistry measurements were available and used to compute eGFR.

In the GMS 1, 49 of the 182 metabolites analyzed were significantly associated with all-cause mortality after Bonferroni correction (threshold P value being $0.05/182 = 2.7 \times 10^{-4}$) (Supplementary Table 2).

Among these metabolites, the pairwise correlation ranged from –0.20 to 0.92 (Supplementary Fig. 1). After a stepwise (forward-backward) procedure, six metabolites remained independently associated in a fully adjusted model including age at recruitment, sex, smoking habit, BMI, HbA_{1c}, diabetes duration, eGFR, and ongoing treatments (four with increased and two with decreased risk of all-cause mortality) (Fig. 1*A*). Three of them, belonging to amino acid, biogenic amines, and acylcarnitine superfamilies, were validated in the totally independent GMS 2 cohort (Fig. 1*B*).

When data from the two independent cohorts, comprising 856 individuals and 279 events, were meta-analyzed, the three validated associations, hexanoylcarnitine (HR 1.60, 95% CI 1.43–1.80), kynurenine (HR 1.53, 95% CI 1.37–1.71), and tryptophan (HR 0.71, 95% CI 0.62–0.80) were highly significantly associated with all-cause mortality (all $P < 0.001$, per 1 SD increase), with no difference between male and female participants (P of sex heterogeneity = 0.17, 0.67, and 0.9, respectively). Also the kynurenine-to-tryptophan ratio (KTR), which has been previously associated with metabolic syndrome (24,25), cardiovascular disease (26,27), and mortality (28,29), was associated with all-cause death (HR 1.41, 95% CI 1.21–1.64 per 1 SD increase).

Inflammatory Cytokines, KTR, and All-Cause Mortality

Previous findings suggest that the indoleamine 2,3-dioxygenase (IDO) enzymatic activity and its proxy KTR are stimulated by (30) and are likely to mediate the role of inflammatory cytokines on all-cause death (29). We then measured and investigated the association between several cytokines related to low-grade inflammation and both KTR and all-cause mortality. Five of those we tested (Supplementary Table 3), including IL-4, IL-6, IL-13, INF-γ, and TNF-α, were in fact associated with KTR (see Supplementary Table 4). Of these, four (but not IL-4) were also associated with all-cause mortality in our fully adjusted model (Table 2, left side). Interestingly, when KTR was also added into the model, the associations with

Table 2—Univariable associations between cytokines and all-cause mortality in the pooled sample (n = 810; 256 events)

	Associations with all-cause mortality		Associations with all-cause mortality adjusted also for KTR	
Cytokines	HR (95% CI)	*P*	HR (95% CI)	*P*
IL-6	1.41 (1.26–1.59)	<0.0001	1.44 (1.28–1.62)	<0.0001
IL-13	0.86 (0.74–0.99)	0.036	0.91 (0.79–1.05)	0.21
IFN-γ	1.53 (1.26–1.87)	<0.0001	1.63 (1.33–2.00)	<0.0001
TNF-α	1.41 (1.19–1.67)	<0.0001	1.43 (1.20–1.70)	<0.0001

HRs were estimated in Cox regression models, adjusting for age at recruitment, sex, smoking habit, BMI, HbA_{1c}, eGFR, diabetes duration, ongoing treatments, and study cohort. HRs reflect the risk per 1 SD increase in each cytokine concentration.

increased risk of death of IL-6, IFN-γ, and TNF-α, were virtually identical, while the protective effect of IL-13 was attenuated at the point of being no longer significant (Table 2, right side). Further, mediation analysis showed that a significant and nontrivial proportion (i.e., 42% [95% CI 14–199]) of the association between IL-13 and all-cause mortality went through KTR (Fig. 2).

Adding Metabolites to the ENFORCE and RECODe Mortality Prediction Models

In the pooled sample, discrimination ability (*c* statistic) of hexanoylcarnitine, kynurenine, and tryptophan considered together was 0.71 (95% CI 0.55–0.86) (Table 3). We then tested the effect of adding these three metabolites on top of ENFORCE, a well-performing, validated, and freely available (https://www.operapadrepio.it/enforce/enforce.php) prediction model for 6-year all-cause mortality in patients with type 2 diabetes. To this purpose, GMS 1 and GMS 2, comprising a total of 856 patients and 140 deaths (at 6 years) in which our ENFORCE model was applicable, were used. The addition of the three metabolites on top of ENFORCE resulted in a significant improvement of both *c* statistic and rIDI (Table 3). In addition, cNRI values showed a significant improvement in reclassification, mainly due to nonevents correctly reclassified (Table 3).

The ability of the three metabolites in improving the prediction of all-cause death was also tested in RECODe, a well-performing and validated model for 10-year mortality in patients with type 2 diabetes. To this purpose, a total of 856 patients and 230 deaths (at 10 years) from both cohorts were available. Also in this case, a significant improvement was observed both in discrimination (*c* statistic and rIDI) and reclassification (cNRI) measures (Table 3).

In all, these data consistently show that serum levels of hexanoylcarnitine, kynurenine, and tryptophan improve well-established prediction models of all-cause mortality in patients with type 2 diabetes in terms of both discrimination and reclassification.

DISCUSSION

This study used a discovery and replication design to rigorously evaluate the association between 182 metabolites measured through targeted metabolomics and all-cause mortality in 856 people with type 2 diabetes. Three biologically plausible metabolites (i.e., hexanoylcarnitine, tryptophan, and kynurenine) were independently and consistently associated

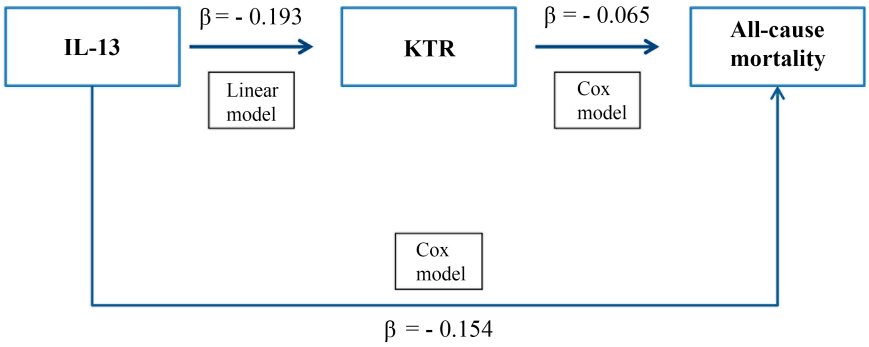

Figure 2—Mediation model showing the role of KTR on the association between IL-13 and all-cause mortality in the pooled sample. Mediation analysis was performed in a fully adjusted model, comprising study cohort, age at recruitment, sex, smoking habit, BMI, HbA_{1c}, diabetes duration, eGFR, and ongoing treatments. β, standardized coefficient of regression. The total effect of IL-13 (β = −0.154) on the outcome partly passes through KTR (β of the KTR-mediated effect of IL-13 = −0.065). The proportion explained by the KTR is equal to 42% (95% CI 14-199) (i.e., 0.065/0.154).

Table 3—Prediction of all-cause mortality by metabolites and by ENFORCE and RECODe (without and with metabolites)

	Discrimination			Reclassification		
Prediction models	*c* statistics (95% CI)	Δ *c* statistics (*P* value)	% rIDI (*P* value)	%1/2 cNRI (*P* value)	% Events (*P* value)	% Nonevents (*P* value)
Hexanoylcarnitine, tryptophan, kynurenine	0.71 (0.55–0.86)					
ENFORCE	0.77 (0.74–0.80)					
ENFORCE + hexanoylcarnitine, tryptophan, kynurenine	0.79 (0.75–0.82)	0.02 (0.01)	14.9 (0.001)	18 (<0.001)	6 (0.18)	30 (<0.001)
RECODe	0.75 (0.72–0.78)					
RECODe + hexanoylcarnitine, tryptophan, kynurenine	0.77 (0.75–0.80)	0.02 (0.005)	18.6 (0.001)	14 (<0.001)	11 (0.001)	18 (<0.001)

All *P* values are referred to comparisons vs. the same base model (i.e., with no metabolites).

with a higher risk of mortality in two independent cohorts of patients with type 2 diabetes. Of note, taking into account eGFR, a strong predictor of mortality (31), did not diminish the relationship between baseline metabolites and death. This indicates that the three associations are independent of renal function, a key point to be addressed when measuring metabolites whose serum concentration is controlled also by renal clearance (32). The same was noticed when BMI was taken into account, thus suggesting that adiposity does not play a major role on the observed associations. Given the strong relationships between the three metabolites and all-cause mortality, it is not surprising that when considered together these markers show a good discrimination ability in predicting the risk of death. More importantly, our data showing that the three metabolites together improve both discrimination and reclassification of two well-established prediction models of all-cause death in type 2 diabetes (14,15) may be of clinical impact.

Increased levels of hexanoylcarnitine, a medium-chain acylcarnitine, which is, along with other members of the acylcarnitines superfamily, a cardiometabolic risk factor in type 2 diabetes (33), has been previously associated with all-cause mortality and cancer-related mortality in men who smoke (34). Accumulation of medium-chain acylcarnitines may be indicative of inefficient β-oxidation of fatty acid as a consequence of altered mitochondrial metabolism, which is known to contribute to both insulin resistance and vascular inflammation (35,36). Tryptophan, an essential amino acid important for protein synthesis (37), has been previously associated with decreased risk of mortality in patients with type 2 diabetes, although with a weaker effect compared with ours (9), while its breakdown product, 5 methoxy-tryptophan along the serotonin pathway, is an anti-inflammatory agent with favorable effects on arterial vessels and renal function (38,39). Kynurenine, a product of tryptophan degradation along the kynurenine pathway, has been associated with risk of cardiovascular events in the general population and in several additional clinical settings (27,40). This pathway, primarily directed toward the production of NAD+ for energy metabolism (41), plays crucial roles in inflammation (41) and when dysregulated is linked to several diseases and disorders (42).

Given the opposite association with mortality rate of tryptophan and kynurenine, it was not surprising that their ratio, KTR, a marker of mortality risk in the general population (29), was associated with the risk of death also in our sample.

Interestingly, KTR is a reliable marker of the IDO activity that degrades tryptophan into kynurenine (41), triggering the homonymous aforementioned deleterious pathway. Furthermore, an increased IDO activity also reduces tryptophan metabolism in the beneficial alternative serotonin pathway. Overall, a shift toward an unhealthy proinflammatory status and subsequent vascular damage is likely to be the final net result of the described alteration of tryptophan metabolism due to IDO overactivity.

IDO is under the control of pro- and anti-inflammatory cytokines, is increased in conditions of low-grade inflammation (30), and is coherently associated with metabolic syndrome (24,25), cardiovascular disease (26,27), and mortality in several clinical sets (28,29). The robust association between KTR and all-cause mortality, as well as the evidence that KTR mediates a nontrivial proportion of the IL-13 antiatherogenic protective effect (43) on mortality risk we here report, is therefore along the same line of previous findings (24–29) and supports the role of KTR (as a proxy of IDO activity) in shaping survival probability (28,29) also in type 2 diabetes. Interestingly, tryptophan supplementation directly or through lifestyle intervention (44) has been reported to prevent and treat cardiovascular disease (45), social behavior, mood and sleep disorders, and several additional chronic diseases (46), possibly by priming the beneficial serotonin pathway.

As said, the addition of hexanoylcarnitine, tryptophan, and kynurenine considered together improves the discrimination ability of both ENFORCE (14) and RECODe (15), two established prediction models of all-cause mortality in patients with type 2 diabetes. Although statistically

significant, the improvement of the *c* statistic is rather small, but it is worth noticing that in already well-performing models, as are those we used here, this index lacks sensitivity in detecting further discrimination improvements (47). It is also important noticing that in both models, the percentage rIDI, also an index of discrimination, is more than twice the threshold requested by international guidelines for adding new biomarkers on top of established prediction models (48). This important statistical and clinical improvement was further reinforced by data from reclassification measures. In fact, adding the three metabolites to ENFORCE and RECODe made it possible to correctly reclassify a consistent proportion of individuals, especially nonevents, thus reducing the risk of overestimation.

Our study has several strengths. We used a rigorous study design with discovery and replication cohorts prospectively analyzed with complete information, including standardized clinical evaluations and mortality validated by death certificates. We also used quality-controlled metabolomics profiling and correction for multiple comparisons. Previous studies of mortality and the metabolome in patients with type 2 diabetes have evaluated only few metabolites (8,10,11) and/or have not addressed the role of mortality-associated metabolites in improving preexisting and established prediction models (8–11). Our study, instead, evaluated a large number of metabolites and discovered new associated markers that improve two well-established and validated prediction models (14,15), thus making our finding implementable in the real-life clinical set.

Conversely, we have to recognize several limitations, including the relatively small size of the cohorts, the fact that they are geographically close to each other, thus limiting the generalizability of our finding, and finally, the lack of data on cause-specific mortality, which does not allow us to address the role of important shapers of life expectancy, including cardiovascular disease and cancer.

In conclusion, in patients with type 2 diabetes, hexanoylcarnitine, tryptophan, and kynurenine are reproducible risk factors for all-cause death and improve established, well-performing prediction models of mortality risk. We believe that a study like ours paves the way for different precision medicine approaches in type 2 diabetes, albeit with different timelines. On the precision prediction side (49), before our data become implementable in daily clinical work, the mortality-associated metabolites need to be enrolled in a standard clinical chemistry assay and validated in larger and less homogeneous cohorts. Conversely, on the treatment side, it is still necessary to investigate whether directing tryptophan metabolism toward the serotonin pathway reduces the risk of death in individuals with diabetes, particularly those with low IL-13 values, before a precision therapeutic approach can be implemented.

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