Circulating metabolites associate with and improve the prediction of all-cause mortality in type 2 diabetes

Running title: Metabolomics and survival in diabetes

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ABSTRACT

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 and RECODe, 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 (HRs, [95%CIs] 1.60, [1.43-1.80]; 1.53, [1.37-1.71]; 0.71, [0.62-0.80] per 1SD). The kynurenine/tryptophan ratio (KTR-a proxy of indoleamine-2,3-dioxygenase which degrades tryptophan to kynurenine and contribute to a pro-inflammatory status) mediated 42% of the significant association between the anti-atherogenic 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.

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In patients with type 2 diabetes the rate of mortality is almost twice as much that in individuals without diabetes (1).

Unraveling biomarkers capable of pointing 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 few metabolites (8, 10, 11), and/or have not addressed the role of associated metabolites in improving pre-existing prediction models (8-11). In details, metabolites independently associated with mortality in type 2 diabetes are mostly aminoacids (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 mortality rate (9). Unfortunately, the largest metabolomic study on all-cause mortality has been carried out 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

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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 totale of nine variables (13, 14) and RECODe's (Risk Equation for Complications Of type 2 Diabetes) a well-performing tool, based on a total of fourteen variables which has 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 (GMS 1) – Discovery sample

The GMS 1 includes 1,028 patients recruited from 2000 to 2005 at the Endocrine Unit of Fondazione IRCCS "Casa Sollievo della Sofferenza" in San Giovanni Rotondo followed until December 2014 and has all-cause mortality as endpoint.

Serum metabolites were assessed in 536 participants (52.1%), constituting the eligible sample for the present analysis.

Gargano Mortality Study 2 (GMS 2) – Replication sample

The GMS 2 includes 880 patients recruited from 2008 to 2010 at the Endocrine Unit of Fondazione IRCCS "Casa Sollievo della Sofferenza" in San Giovanni Rotondo followed until December 2019

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and has all-cause mortality as endpoint. For this specific analysis, a sample comprising 321 patients participating an independent sub-study on the role of kidney function on mortality rate, with no individuals with eGFR in the range of 60-69 ml/min/1.73m^2, was analyzed.

For both studies the vital status of participants was verified by interrogating the Italian Health Card Database upon data anonymization (http://sistemats1.sanita.finanze.it/wps/portal/) (6). For all studies, the only exclusion criterion was the presence of poor life expectancy for non-diabetes-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 LC-ESI-MS/MS and FIA-ESI-MS/MS 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 – about 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

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metabolites, see Supplemental Table 1 in the Supplemental Data. 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 (QC) 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 μM.

Measurement of Circulating Cytokines

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

Statistical analysis

Patients' baseline characteristics were reported as mean ± SD or median and interquartile range 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.

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Correlations between metabolites were assessed using the Spearman correlation. All covariates with missing values below 5% were imputed by random forest method. Because 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 date of the event (i.e., all-cause mortality) or, for subjects who did not experience the event, the date of the last available clinical follow-up. Incidence rate for all-cause mortality was 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, 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.

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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 random effect, so as to have more robust estimates. Risks were reported as HRs along with their 95% CIs per 1SD 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 1000 re-samplings 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 utilized: ENFORCE (14) and RECODe (15). Discrimination was measured by survival *c* statistics (21) while improvement in discrimination was assessed by the delta *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 Release 9.4 (SAS Institute, Cary, NC, USA) and R software (R Core Team, 2021) (packages survival and coxme).

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Data and Resource Availability

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

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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, during follow-up (10.0 ± 3.9 years; 5,346.2 person/year) 198 deaths occurred. In GMS 2, during follow-up (8.6 ± 2.6 years; 2,763.9 person/year), 81 deaths occurred.

Of the 188 metabolites we measured, 5 (i.e., Carnosine, DOPA, Dopamine, Nitrotyrosine and cis-4-Hydroxyproline) were excluded from the analyses because their value was below the detection limit in more than 80% samples. Also, creatinine data from the metabolomic assay (Supplemental 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 out of the 182 metabolites analyzed were significantly associated with all-cause mortality after Bonferroni correction (threshold p value being $0.05/182 = 2.7 \times 10^{-4}$) (Supplemental Table 2).

Among these metabolites, the pairwise correlation ranged from –0.20 to 0.92 (Supplemental Figure 1). After a stepwise (forward-backward) procedure, 6 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, Figure 1, panel A). Three of them, belonging to amino acid, biogenic amines and acylcarnitine super-families, were validated in the totally independent GMS 2 cohort (Figure 1, panel B).

When data from the two independent cohorts, comprising a total of 856 individuals and 279 events, were meta-analyzed the three validated associations, hexanoylcarnitine (HR, 95% CI = 1.60, 1.43-1.80) kynurenine (HR, 95% CI = 1.53, 1.37-1.71) and tryptophan (HR, 95% CI = 0.71, 0.62-0.80) were highly significantly associated with all-cause mortality (all $p < 0.001$, per 1SD increase) with no difference between male and female participants (p of gender heterogeneity = 0.17, 0.67 and 0.9, respectively). Also the kynurenine/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, 95% CI = 1.41, 1.21-1.64 per 1SD increase).

Inflammatory cytokines, KTR and all-cause mortality.

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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 on all-cause death of inflammatory cytokines (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 (Supplemental Table 3), including IL-4, IL-6, IL-13, IFN-γ and TNF-α, were in fact associated with KTR (see Supplemental Table 4). Of these, four (but not IL-4) were also associated with all-cause mortality in our fully adjusted model (Table 2, left panel). Interestingly, when also KTR was added into the model the associations with 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 panel). Further, mediation analysis showed that a significant and non-trivial proportion [i.e., 42% (95% CI: 14%-199%)] of the association between IL-13 and all-cause mortality went through KTR (Figure 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 (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

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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 is a rigorous evaluation of the association between 182 metabolites measured through targeted metabolomics and all-cause mortality in 856 people with type 2 diabetes using a discovery and replication design. Three biologically plausible metabolites (i.e., hexanoylcarnitine, tryptophan and kynurenine) were independently and consistently associated with higher risk of mortality in two independent cohorts of patients with type 2 diabetes. Of note, taking into account eGFR, a strong

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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 cardio-metabolic risk factor in type 2 diabetes (33), has been previously associated with all-cause mortality and cancer-related mortality in smoking men (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 as compared to 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 on inflammation (41) and when dysregulated is linked to several diseases and disorders (42).

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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 towards an unhealthy pro-inflammatory status and subsequent vascular damage is likely to be the final net result of the described alteration of tryptophan metabolism due to IDO over activity.

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 non-trivial proportion of IL-13 anti-atherogenic protective effect (43) on mortality risk we here report, is therefore along the same line of previous findings (24-29) and support 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. Though 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

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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 pre-existing and established prediction models (8-11). Our study, instead, evaluated a large number of metabolites, and discovered new associated markers which 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 standard clinical chemistry assay and validated in larger and less homogeneous cohorts. Conversely, on the treatment side, it is still necessary to investigate whether

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directing tryptophan metabolism towards 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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AUTHOR CONTRIBUTIONS. V.T. and C.M. conceived the study and, together with M.G.S., designed the protocol and wrote the manuscript. M.G.S, M.C., V.T., and C.M. participated in data analysis and interpretation of results. M.M., S.D.C., and V.T. contributed to data collection. L.S. performed laboratory testing. C.P. and J.A. supervised the target metabolomics analysis. All authors critically revised the paper and approved its final version. V.T. and C.M. 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.

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Table 1. Clinical features of the two independent study cohorts

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

Continuous variables were reported as mean ± SD whereas categorical variables as total frequencies and percentages. Skewed variables are presented as median (interquartile range). GMS: Gargano Mortality Study; FMS: Foggia Mortality Study; GHS-prospective: Gargano Heart Study-prospective design; HbA_{1c}: glycated hemoglobin A_{1c}; eGFR, estimated glomerular filtration rate (calculated using the CKD-EPI equation (50)); IR: incidence rate of all-cause death events; py: person/year.

* adjusted for age and sex

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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%IC)	P	HR (95%IC)	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 1SD increase in each cytokine concentration.
KTR: kynurenine/tryptophan ratio; IL-: interleukin; IFN-: interferon; TNF-: tumor necrosis factor.

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

PREDICTION MODELS	DISCRIMINATION			RECLASSIFICATION		
	c statistics (95% CI)	Δ *c* statistics *(p value)*	%rIDI *(p value)*	% ½ cNRI *(p value)*	% Events *(p value)*	% Non-events *(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).

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Figure legends

Figure 1. Independent associations between metabolites and all-cause mortality

Hazard ratios (HRs) and 95% confidence intervals (CIs) for independent associations between metabolites and mortality in GMS 1 (Panel A) and GMS 2 (Panel B)

HRs (per 1SD 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.

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 carried out in a fully adjusted model, comprising study cohort, age at recruitment, sex, smoking habit, BMI, HbA1c, diabetes duration, eGFR and ongoing treatments.

β = standardized coefficient of regression.

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The total effect of IL-13 (β = -0.154) on the outcome partly passes through KTR (β of the KTR-mediated effect of IL13= -0.065). The proportion explained by the KTR is equal to 42% (14%-199%) (i.e., 0.065/0.154).

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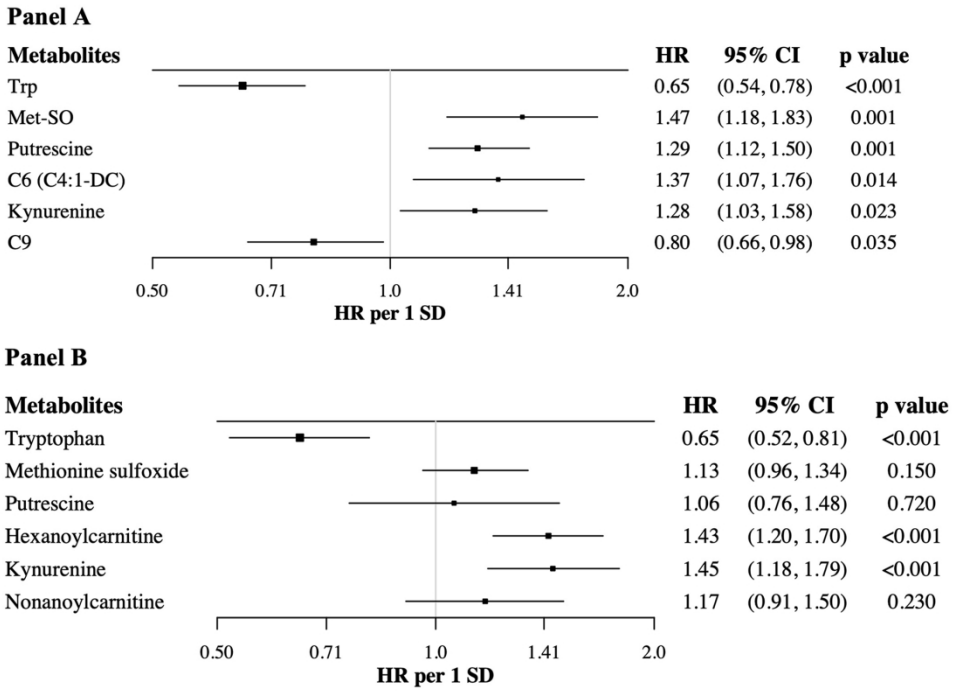

Figure 1. Independent associations between metabolites and all-cause mortality. Hazard ratios (HRs) and 95% confidence intervals (CIs) for independent associations between metabolites and mortality in GMS 1 (Panel A) and GMS 2 (Panel B). HRs (per 1SD increase in each metabolite concentration) were estimated in Cox regression models, adjusting for age at recruitment, sex, smoking habit, BMI, HbA1c, eGFR, diabetes duration and ongoing treatments.

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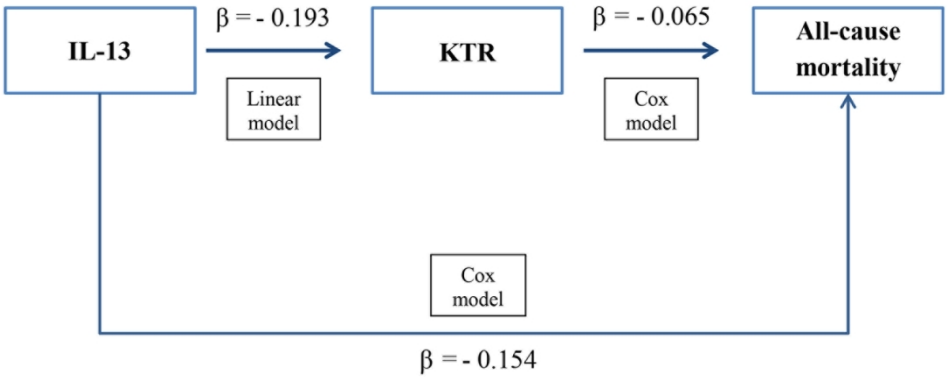

Figure 2. Mediation model showing the role of KTR on the association between IL-13 and all-cause mortality in the pooled sample. Mediation analysis was carried out in a fully adjusted model, comprising study cohort, age at recruitment, sex, smoking habit, BMI, HbA1c, diabetes duration, eGFR and ongoing 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 IL13= -0.065). The proportion explained by the KTR is equal to 42% (14%-199%) (i.e., 0.065/0.154).

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Supplemental Material

Supplemental Table 1: List of metabolites measured with the Absolute*IDQ*® p180 Kit GAC, Helmholtz Zentrum München.

Acylcarnitines (40)			
C0	Carnitine	C10:1	Decenoylcarnitine
C2	Acetylcarnitine	C10:2	Decadienylcarnitine
C3	Propionylcarnitine	C12	Dodecanoylcarnitine
C3:1	Propenoylcarnitine	C12:1	Dodecenoylcarnitine
C3-OH	Hydroxypropionylcarnitine	C12-DC	Dodecanedioylcarnitine
C4	Butyrylcarnitine	C14	Tetradecanoylcarnitine
C4:1	Butenoylcarnitine	C14:1	Tetradecenoylcarnitine
C4-OH (C3-DC)	Hydroxybutyrylcarnitine	C14:1-OH	Hydroxytetradecenoylcarnitine
C5	Valerylcarnitine	C14:2	Tetradecadienylcarnitine
C5:1	Tiglylcarnitine	C14:2-OH	Hydroxytetradecadienylcarnitine
C5:1-DC	Glutaconylcarnitine	C16	Hexadecanoylcarnitine
C5-DC (C6-OH)	Glutarylcarnitine (Hydroxyhexanoylcarnitine)	C16:1	Hexadecenoylcarnitine
C5-M-DC	Methylglutarylcarnitine	C16:1-OH	Hydroxyhexadecenoylcarnitine
C5-OH (C3-DC-M)	Hydroxyvalerylcarnitine (Methylmalonylcarnitine)	C16:2	Hexadecadienylcarnitine
C6 (C4:1-DC)	Hexanoylcarnitine (Fumarylcarnitine)	C16:2-OH	Hydroxyhexadecadienylcarnitine
C6:1	Hexenoylcarnitine	C16-OH	Hydroxyhexadecanoylcarnitine
C7-DC	Pimelylcarnitine	C18	Octadecanoylcarnitine
C8	Octanoylcarnitine	C18:1	Octadecenoylcarnitine
C9	Nonanoylcarnitine	C18:1-OH	Hydroxyoctadecenoylcarnitine
C10	Decanoylcarnitine	C18:2	Octadecadienylcarnitine
Amino Acids (21)			
Ala	Alanine	Lys	Lysine
Arg	Arginine	Met	Methionine
Asn	Asparagine	Orn	Ornithine
Asp	Aspartate	Phe	Phenylalanine
Cit	Citrulline	Pro	Proline
Gln	Glutamine	Ser	Serine
Glu	Glutamate	Thr	Threonine
Gly	Glycine	Trp	Tryptophan
His	Histidine	Tyr	Tyrosine
Ile	Isoleucine	Val	Valine
Leu	Leucine		
Monosaccharides (1)			
Sum of Hexoses (including Glucose)			
Glycerophospholipids (90)			
lysoPC a C14:0	PC aa C34:1	PC aa C42:0	PC ae C38:2
lysoPC a C16:0	PC aa C34:2	PC aa C42:1	PC ae C38:3
lysoPC a C16:1	PC aa C34:3	PC aa C42:2	PC ae C38:4
lysoPC a C17:0	PC aa C34:4	PC aa C42:4	PC ae C38:5
lysoPC a C18:0	PC aa C36:0	PC aa C42:5	PC ae C38:6
lysoPC a C18:1	PC aa C36:1	PC aa C42:6	PC ae C40:1

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lysoPC a C18:2	PC aa C36:2	PC ae C30:0	PC ae C40:2
lysoPC a C20:3	PC aa C36:3	PC ae C30:1	PC ae C40:3
lysoPC a C20:4	PC aa C36:4	PC ae C30:2	PC ae C40:4
lysoPC a C24:0	PC aa C36:5	PC ae C32:1	PC ae C40:5
lysoPC a C26:0	PC aa C36:6	PC ae C32:2	PC ae C40:6
lysoPC a C26:1	PC aa C38:0	PC ae C34:0	PC ae C42:0
lysoPC a C28:0	PC aa C38:1	PC ae C34:1	PC ae C42:1
lysoPC a C28:1	PC aa C38:3	PC ae C34:2	PC ae C42:2
PC aa C24:0	PC aa C38:4	PC ae C34:3	PC ae C42:3
PC aa C26:0	PC aa C38:5	PC ae C36:0	PC ae C42:4
PC aa C28:1	PC aa C38:6	PC ae C36:1	PC ae C42:5
PC aa C30:0	PC aa C40:1	PC ae C36:2	PC ae C44:3
PC aa C30:2	PC aa C40:2	PC ae C36:3	PC ae C44:4
PC aa C32:0	PC aa C40:3	PC ae C36:4	PC ae C44:5
PC aa C32:1	PC aa C40:4	PC ae C36:5	PC ae C44:6
PC aa C32:2	PC aa C40:5	PC ae C38:0	
PC aa C32:3	PC aa C40:6	PC ae C38:1	
Sphingolipids (15)			
SM (OH) C14:1	SM C18:0	SM (OH) C22:1	SM (OH) C24:1
SM C16:0	SM C18:1	SM (OH) C22:2	SM C26:0
SM C16:1	SM C20:2	SM C24:0	SM C26:1
SM (OH) C16:1	SM C22:3	SM C24:1	
Biogenic Amines (21)			
Ac-Orn	Acetylornithine	PEA	Phenylethylamine
ADMA	Asymmetric dimethylarginine	**cis*-OH-Pro	**cis*-4-Hydroxyproline
alpha-AAA	alpha-Aminoadipic acid	*trans*-OH-Pro	*trans*-4-Hydroxyproline
*Carnosine	*Carnosine	Putrescine	Putrescine
**Creatinine	Creatinine	SDMA	Symmetric dimethylarginine
*DOPA	*DOPA	Serotonin	Serotonin
*Dopamine	*Dopamine	Spermidine	Spermidine
Histamine	Histamine	Spermine	Spermine
Kynurenine	Kynurenine	Taurine	Taurine
Met-SO	Methionine sulfoxide	total DMA	Total dimethylarginine
*Nitro-Tyr	*Nitrotyrosine		

* Metabolites excluded from the analyses because more than 80% of the samples had values below the detection limit. **Creatinine excluded from the analyses because serum creatinine values from standard baseline clinical chemistry measurements were already available.

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Supplemental Table 2. Associated metabolites in unadjusted model after Bonferroni correction in the Gargano Mortality Study 1

N	Family	Metabolite		HR	95% CI		p
					LB	UB	
1	Biogenic Amines	SDMA	Symmetric dimethylarginine	2.01	1.74	2.32	$9.1x10^{-22}$
2	Biogenic Amines	total DMA	Total dimethylarginine	1.91	1.67	2.19	$1.3x10^{-20}$
3	Acylcarnitines	C5-DC (C6-OH)	Glutarylcarnitine (Hydroxyhexanoylcarnitine)	1.83	1.61	2.09	$1.7x10^{-20}$
4	Acylcarnitines	C5-M-DC	Methylglutarylcarnitine	1.57	1.42	1.73	$2.7x10^{-18}$
5	Amino acids	Cit	Citrulline	1.82	1.57	2.10	$4.3x10^{-16}$
6	Acylcarnitines	C4	Butyrylcarnitine	1.68	1.46	1.92	$7.0x10^{-14}$
7	Acylcarnitines	C5-OH (C3-DC-M)	Hydroxyvalerylcarnitine (Methylmalonylcarnitine)	1.64	1.43	1.87	$5.7x10^{-13}$
8	Acylcarnitines	C6 (C4:1-DC)	Hexanoylcarnitine	1.85	1.56	2.19	$1.4x10^{-12}$
9	Glycerophospholipids	PC aa C34:4	PC aa C34:4	0.62	0.54	0.72	$2.1x10^{-11}$
10	Acylcarnitines	C5:1-DC	Glutaconylcarnitine	1.60	1.39	1.84	$2.2x10^{-11}$
11	Biogenic Amines	Kynurenine	Kynurenine	1.56	1.37	1.77	$3.1x10^{-11}$
12	Biogenic Amines	Putrescine	Putrescine	1.53	1.35	1.74	$3.7x10^{-11}$
13	Acylcarnitines	C5:1	Tiglylcarnitine	1.63	1.40	1.89	$1.5x10^{-10}$
14	Glycerophospholipids	PC aa C32:2	PC aa C32:2	0.66	0.57	0.75	$1.9x10^{-9}$
15	Biogenic Amines	*trans*-OH-Pro	*trans*-4-Hydroxyproline	1.52	1.33	1.75	$2.1x10^{-9}$
16	Biogenic Amines	Ac-Orn	Acetylornithine	1.57	1.35	1.82	$2.8x10^{-9}$
17	Sphingolipids	SM (OH) C22:1	SM (OH) C22:1	0.67	0.58	0.77	$9.2x10^{-9}$
18	Acylcarnitines	C2	Acetylcarnitine	1.55	1.33	1.79	$1.0x10^{-8}$
19	Acylcarnitines	C5	Valerylcarnitine	1.46	1.28	1.67	$1.3x10^{-8}$
20	Acylcarnitines	C4-OH (C3-DC)	Hydroxybutyrylcarnitine	1.39	1.23	1.57	$8.6x10^{-8}$
21	Acylcarnitines	C10:2	Decadienylcarnitine	1.32	1.19	1.47	$3.9x10^{-7}$
22	Acylcarnitines	C16:1	Hexadecenoylcarnitine	1.40	1.22	1.60	$1.3x10^{-6}$
23	Acylcarnitines	C0	Carnitine	1.38	1.20	1.58	$2.9x10^{-6}$
24	Acylcarnitines	C12:1	Dodecenoylcarnitine	1.36	1.19	1.55	$3.5x10^{-6}$
25	Acylcarnitines	C18:1	Octadecenoylcarnitine	1.33	1.18	1.51	$4.6x10^{-6}$
26	Acylcarnitines	C3	Propionylcarnitine	1.35	1.19	1.54	$4.6x10^{-6}$
27	Biogenic Amines	ADMA	Asymmetric dimethylarginine	1.37	1.20	1.57	$5.4x10^{-6}$
28	Acylcarnitines	C14:1-OH	Hydroxytetradecenoylcarnitine	1.33	1.17	1.51	$7.7x10^{-6}$
29	Acylcarnitines	C14:1	Tetradecenoylcarnitine	1.35	1.18	1.54	$8.3x10^{-6}$
30	Acylcarnitines	C7-DC	Pimelylcarnitine	1.37	1.19	1.57	$8.3x10^{-6}$
31	Amino acids	Asp	Aspartate	1.38	1.20	1.60	$9.2x10^{-6}$
32	Glycerophospholipids	PC aa C36:4	PC aa C36:4	0.72	0.62	0.83	$9.8x10^{-6}$
33	Sphingolipids	SM C24:0	SM C24:0	0.72	0.62	0.83	$1.0x10^{-5}$
34	Glycerophospholipids	PC aa C36:6	PC aa C36:6	0.72	0.62	0.84	$1.3x10^{-5}$
35	Acylcarnitines	C9	Nonanoylcarnitine	1.30	1.15	1.47	$2.1x10^{-5}$
36	Glycerophospholipids	PC aa C36:5	PC aa C36:5	0.72	0.62	0.84	$2.4x10^{-5}$
37	Biogenic Amines	Met-SO	Methionine sulfoxide	1.45	1.22	1.73	$2.4x10^{-5}$
38	Amino acids	Trp	Tryptophan	0.73	0.63	0.85	$4.1x10^{-5}$
39	Glycerophospholipids	lysoPC a C18:2	lysoPC a C18:2	0.76	0.66	0.87	$4.5x10^{-5}$
40	Sphingolipids	SM (OH) C24:1	SM (OH) C24:1	0.77	0.67	0.87	$5.0x10^{-5}$
41	Glycerophospholipids	PC aa C38:5	PC aa C38:5	0.75	0.65	0.86	$6.0x10^{-5}$
42	Glycerophospholipids	PC aa C34:3	PC aa C34:3	0.76	0.66	0.87	$6.8x10^{-5}$
43	Glycerophospholipids	lysoPC a C14:0	lysoPC a C14:0	0.75	0.65	0.87	$9.5x10^{-5}$
44	Biogenic Amines	Spermidine	Spermidine	1.28	1.13	1.45	$1.1x10^{-4}$
45	Acylcarnitines	C14	Tetradecanoylcarnitine	1.27	1.12	1.44	$1.3x10^{-4}$
46	Biogenic Amines	Spermine	Spermine	1.25	1.12	1.41	$1.3x10^{-4}$
47	Acylcarnitines	C12	Dodecanoylcarnitine	1.28	1.13	1.45	$1.3x10^{-4}$
48	Amino acids	Glu	Glutamate	1.32	1.14	1.52	$1.6x10^{-4}$

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49	Acylcarnitines	C14:2	Tetradecadienylcarnitine	1.30	1.13	1.48	$1.6x10^{-4}$

Supplemental Table 3: KTR and cytokines values in the 2 study cohorts

	GMS1 (n=489)	GMS2 (n=321)
KTR	0.04 (0.03–0.05)	0.04 (0.03–0.06)
IL-1 β (pg/mL)	6.6 (4.4–10.9)	8.4 (7.2–9.8)
IL-2 (pg/mL)	26.1 (22.6–32.9)	21.5 (17.8–26.0)
IL-4 (pg/mL)	7.0 (5.2–10.9)	12.5 (9.7–16.7)
IL-6 (pg/mL)	10.3 (7.1–17.6)	12.6 (9.9–17.0)
IL-13 (pg/mL)	7.5 (11.0–29.8)	4.1 (3.3–5.6)
IFNγ (pg/mL)	10.0 (7.3–15.5)	14.6 (11.6–16.8)
TNF-α (pg/mL)	22 (17.9–28.3)	37.1 (17.0–55.0)

Variables are presented as median (interquartile range). GMS: Gargano Mortality Study; KTR: kynurenine/tryptophan ratio; IL-: interleukin; IFN: interferon; TNF-: tumor necrosis factor.

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Supplemental Table 4. Correlation between cytokines and KTR in the pooled sample (n = 810; 256 events)

Cytokines	β (SE)	P
IL-1β	0.06 (0.03)	0.08
IL-2	0.04 (0.03)	0.22
IL-4	-0.13 (0.04)	0.0003
IL-6	0.14 (0.03)	<0.0001
IL-13	-0.25 (0.05)	<0.0001
IFN-γ	0.17 (0.05)	0.001
TNF-α	0.31 (0.06)	<0.0001

All analyses are adjusted for study cohort. Correlations are given as β (SE).
KTR: kynurenine/tryptophan ratio; IL: interleukin; IFN- : interferon; TNF-: tumour necrosis factor.

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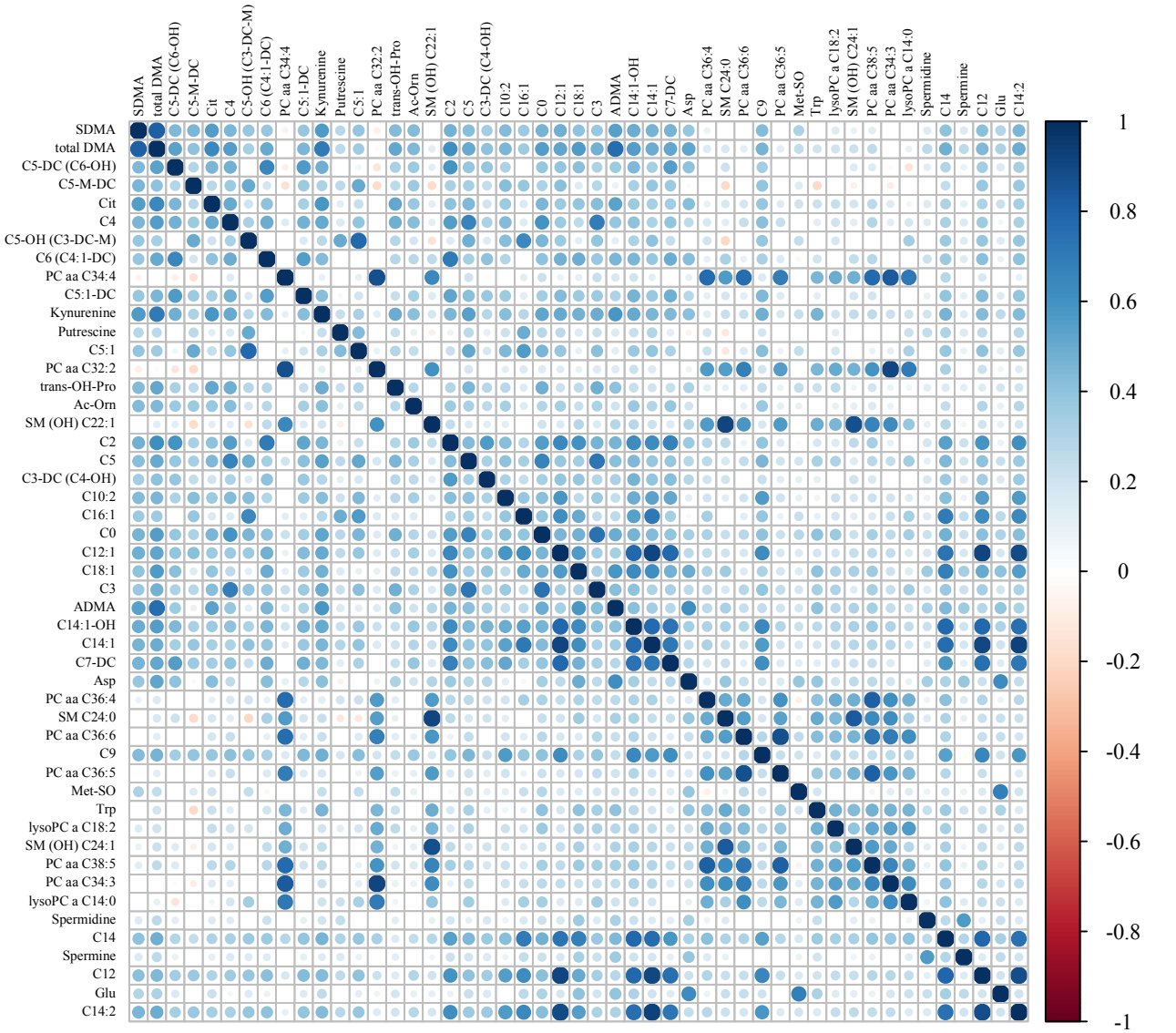
Supplemental Figure 1. Spearman correlation plot between the 49 associated-metabolites in the discovery sample (Gargano Mortality Study 1)



The areas of circles show the absolute value of corresponding correlation coefficients. The stronger the correlation, the more intense the color of the circle. Insignificant correlations are plotted as blank squares.

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