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RESEARCH ARTICLE

Validation in type 2 diabetes of a metabolomic signature of all-cause mortality

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Abstract

Context: Mortality in type 2 diabetes is twice that of the normoglycemic population. Unravelling biomarkers that identify high-risk patients for referral to the most aggressive and costly prevention strategies is needed.

Objective: To validate in type 2 diabetes the association with all-cause mortality of a 14-metabolite score (14-MS) previously reported in the general population and whether this score can be used to improve well-established mortality prediction models.

Methods: This is a sub-study consisting of 600 patients from the "Sapienza University Mortality and Morbidity Event Rate" (SUMMER) study in diabetes, a

prospective multicentre investigation on all-cause mortality in patients with type 2 diabetes. Metabolic biomarkers were quantified from serum samples using high-throughput proton nuclear magnetic resonance metabolomics.

Results: In type 2 diabetes, the 14–MS showed a significant ($p < 0.0001$) association with mortality, which was lower ($p < 0.0001$) than that reported in the general population. This difference was mainly due to two metabolites (histidine and ratio of polyunsaturated fatty acids to total fatty acids) with an effect size that was significantly ($p = 0.01$) lower in diabetes than in the general population. A parsimonious 12-MS (i.e. lacking the 2 metabolites mentioned above) improved patient discrimination and classification of two well-established mortality prediction models ($p < 0.0001$ for all measures).

Conclusions: The metabolomic signature of mortality in the general population is only partially effective in type 2 diabetes. Prediction markers developed and validated in the general population must be revalidated if they are to be used in patients with diabetes.

KEYWORDS
metabolomics, mortality, prognostic models, risk prediction model, type 2 diabetes, validation

1 | INTRODUCTION

The death rate is doubled in type 2 diabetes than in normoglycemic people.[1] Due to the increasing prevalence of the disease,[2] the number of deaths attributable to diabetes is expected to increase further. Unravelling biomarkers that identify high-risk patients for referral to the most aggressive and costly prevention strategies helps tackle this burden.

Few studies have focused on serum metabolites as predictors of all-cause death in type 2 diabetes.[3–7]. Unfortunately, most of these studies[3–6] did not investigate whether the associated metabolites improve well-performing prediction models. Furthermore, it is not known whether serum metabolites, which predict mortality in the general population, are good predictors in people with type 2 diabetes too. We aimed at investigating whether the association with and the ability to predict all-cause mortality in the general population of a 14-metabolite score (14-MS),[8] also known as MetaboHealth, is confirmed in type 2 diabetes. We also investigated whether these metabolites can be used to improve well-established and well-performing mortality prediction models of all-cause mortality in type 2 diabetes: Estimation of Mortality Risk in Type 2 Diabetic Patients (ENFORCE), an user-friendly and freely available risk 9-variable algorithm, which has been validated in several different context,[7,9–11] and Risk Equations for Complications of Type 2 Diabetes (RECODe), a 14–variable algorithm also validated in many distinct cohorts derived from both trial and population-based studies.[12,13] To this aim, the Nightingale Health's metabolomics technology, which is widely exploited in people with type 2 diabetes,[14–16] was used.

2 | MATERIALS AND METHODS

2.1 | Study population

This is a sub-study consisting of 600 patients selected from the "Sapienza University Mortality and Morbidity Event Rate" (SUMMER) study in diabetes, an observational, prospective, multicentre investigation in patients with type 2 diabetes, with all-cause mortality being the primary endpoint.[17] When the present metabolomic study was initiated as of 1 September 2021, 200 patients had died at follow-up and 400 alive patients were randomly chosen as controls, with a minimum follow-up of 4 years, so as to reduce the risk of selection bias. Controls were randomly selected because of the pre-specified aim of improving two well-established prediction models of mortality in patients with type 2 diabetes. Indeed, matched controls would invalidate the use of such models.

2.2 | Metabolic biomarkers methods

Metabolic biomarkers were quantified from serum samples of all 600 individuals using high-throughput proton nuclear magnetic resonance (NMR) metabolomics (Nightingale Health Plc, Helsinki, Finland). The metabolic biomarker assay enables quantification of 250 metabolites from a small volume of serum. Nightingale's blood analysis platform includes both clinically established and emerging biomarkers shown to be medically relevant in large epidemiological studies; indeed, the metabolic profile provides a comprehensive molecular readout of the subject's state of health. More specifically, the analysis provides

simultaneous quantification of routine lipids, lipoprotein subclass profiling with lipid concentrations within 14 subclasses, fatty acid composition, inflammation marker GlycA, and various low-molecular metabolites including amino acids, ketone bodies and gluconeogenesis-related metabolites in molar concentration units. Details of the experimentation and applications of the NMR metabolomics platform have been described previously.[18,19]

2.3 | Statistical methods

Patients' baseline characteristics were reported as mean ± SD (or median and interquartile range) and frequency and percentage for continuous and categorical variables, respectively.

For each subject, a value of one was added to the level of the only three metabolites (citrate, ß-hydroxybutyrate and acetoacetate) being zero in 1, 5 and 27 individuals, respectively. Then, metabolite levels were log-transformed and standardised (mean = 0 and SD = 1) before analyses. Creatinine data from the metabolomic assay were not analysed because serum creatinine values from standard baseline clinical chemistry measurements were available and used to compute eGFR.

Proportional hazards Cox regression model was used to validate the associations of metabolic biomarkers and all-cause mortality using age at blood sampling as the time scale, adjusting for gender. Results were reported as hazard ratios (HR) with 95% confidence intervals (95%CI).

Power analysis was performed with the aim of replicate, with our sample size and number of events, the same prediction accuracy of the 14-MS for all-cause mortality in the general population,[8] using 5-year as time horizon for prediction. The Cochrane Q-test was used to assess the heterogeneity between HR from the two studies.

According to Deelen et al,[8] a 14-MS score plus gender was created, using the weights reported in this previous study.[8] The 5-year all-cause mortality prediction accuracy of the 14-MS was measured by the survival C-statistic.[20]

To examine whether a reweighted (using weights estimated in our study by Cox regression model), parsimonious 12-MS increases the prediction accuracy of all-cause mortality in type 2 diabetes, two different, well-established models (RECODe)[12,13] and ENFORCE[9–11] were used. Predictors included in the two models are reported in the supplementary Table S1.[21]

The time horizon prediction was set at 5 years. Each model was tested without (reference model) and with the addition of 14-MS values. Discrimination was measured by survival C-statistic,[20] the improvement in discrimination by Δ C-statistic,[20] and the survival version of the relative integrated discrimination improvement (rIDI).[22] In addition, the survival version of the category-free net reclassification improvement (cNRI),[22,23] which examines whether the predicted probabilities of individuals with and without events move in the right directions (upward and downward, respectively) from the reference to the enriched model, was evaluated. The 95% CIs for discrimination and reclassification measures were computed by bootstrap.

A p value of less than 0.05 was considered significant. All analyses were performed using SAS Release 9.4 (SAS Institute) and the *R* environment (packages *survC1* and *survIDINRI*).

3 | RESULTS

All the 200 dead patients and 400 random alive patients were selected as of 1 September 2021. One alive patient was removed as not suffering from diabetes. During the metabolomic experiments, six patients died. Overall, the study sample consisted of 599 subjects (206 died, 393 alive) followed up for 4.6 years (range 0.1–6.0). Baseline demographical and clinical characteristics and follow-up are reported in Table 1. Noteworthy, by study design, alive patients had been followed for at least 4 years.

3.1 | Validation in type 2 diabetes

This study has been designed to provide >99% power, assuming a Type I Error of 0.00001, to validate the prediction accuracy of the 14-MS for all-cause mortality reported in the general population.[8]

In people with type 2 diabetes, the 14-MS showed a strong association with all-cause mortality (HR = 1.72, 95%CI = 1.44–2.05) which, however, was significantly lower ($Q = 24.54$, 1 d.f., $p < 0.0001$) than that reported in the general population (HR = 2.73, 95% CI = 2.60–2.86).[8] Coherently, also the C-statistic for prediction of all-cause mortality in type 2 diabetes, though highly significant (0.640, 95% CI = 0.609–0.687, $p < 0.0001$), appeared definitively lower than the C-statistic of 0.837, reported in the general population.[8] Unfortunately, the 95% CI of this latter value was not provided,[8] thus making impossible a formal statistical comparison.

Post-hoc analyses on each of the 14 metabolites were then carried out with the aim to get insights into the worse association with mortality rate of the 14-MS in type 2 diabetes as compared to the general population.[8] As shown in Table 2 (upper part, in bold), 3 metabolites (glycoprotein acetyls, glucose and total lipids in small high-density lipoprotein (HDL)) were individually associated with all-cause mortality, thus indicating that they are established markers of mortality risk transportable from the general population to diabetes. Conversely, 11 metabolites were not significantly associated with mortality rate in our sample. It is of note that the effect size of two of these 11 metabolites (histidine and ratio of polyunsaturated fatty acids to total fatty acids, PUFA/FA) were significantly different from those observed in the general population (p for heterogeneity = 0.01 for both) (Table 2, panel in grey). In detail, while the association with a mortality rate of PUFA/FA ratio was close to the neutral effect, histidine was almost significantly associated ($p = 0.08$) with all-cause mortality towards the opposite direction compared to the general population (Table 2). All

TABLE 1 Patients' baseline demographical and clinical characteristics.

	N = 599
Age (years)	68.7 (10.2)
Female (*n*, %)	230 (38.4%)
Smoker or former smoker (*n*, %)	337 (56.3%)
BMI (kg/m^2)	29.7 (5.1)
Waist (cm)	103.6 (12.9)
T2D first degree family history (*n*, %)	263 (46.1%)
Age at disease onset (years)	56.6 (12.3)
Duration of diabetes (years)	12.0 (9.9)
SBP (mm Hg)	130 (100–200)
DBP (mm Hg)	80 (50–112)
Glycated haemoglobin (%)	6.9 (4.7–14.2)
Total cholesterol (mg/dL)	165 (81–338)
LDL cholesterol (mg/dL)	90 (15–262)
HDL cholesterol (mg/dL)	45 (15–103)
Triglycerides (mg/dL)	121 (30–778)
Creatinine (mg/dL)	0.9 (0.4–7.6)
ACR (mg/mmol)	20.9 (0.1–3572.7)
CKD-EPI (mL/min per 1.73 m^2)	75.2 (4.8–133.4)
Anti-hypertension treatment (*n*, %)	283 (47.2%)
Insulin treatment (*n*, %)	198 (33.1%)
Anti-dyslipidemia treatment (*n*, %)	378 (63.1%)
Previous stroke (*n*, %)	33 (5.5%)
Previous myocardial infarction (*n*, %)	92 (15.4%)
Follow-up (years)	4.6 (0.1–6.0)
Deaths (*n*, %)	206 (34.4%)

Note: Data are mean and standard deviation or median and range or percentage.

Abbreviations: ACR, urinary albumin-to-creatinine ratio; BMI, body mass index; CKD-EPI, estimated glomerular filtration rate calculated by Chronic Kidney Disease-Epidemiology Collaboration equation; CVD, cardiovascular disease; DBP, diastolic blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SBP, systolic blood pressure.

this makes, unlikely, a false negative finding and that these two metabolites play a role in the subset of individuals with type 2 diabetes.

Finally, the lack of association with a mortality rate of the remaining 9 metabolites (Table 2, lower part, in italics) can well be a false negative result. In fact, none of them showed significant heterogeneity as compared to the general population. In addition, six of them lacked enough power (i.e., <80%) to reach formal statistical significance with an effect size equal to that reported in the general population[8] (Table 2), thus further increasing the possibility of a false-negative finding.

3.2 | Improvement of well-stablished mortality prediction models in type 2 diabetes

Finally, a reweighted, parsimonious 12-MS (i.e. lacking of histidine and the PUFA/FA ratio) added on top of RECODe and ENFORCE, significantly improved the 5-year prediction discrimination (Table 3, see delta C-statistic and IDI) and correctly reclassified a non-negligible proportion of patients both with and without events (Table 3, see cNRI).

4 | DISCUSSION

Our study reports that a 14–MS showing a good discrimination in predicting all-cause mortality in the general population[8] is significantly associated with and also predicts the risk of death in the clinical setting of type 2 diabetes. However, as compared to data reported in the general population,[8] a statistically significant HR reduction of 46% on a log scale and a relevant C-statistic decrease from 0.837 to 0.648 was observed in people with diabetes. The worse association and predicting performance in people with diabetes is due to the fact that 2 of the 14 metabolites (i.e., histidine and the PUFA/FA ratio) not only were not formally associated with mortality rate in people with type 2 diabetes but also had an effect size that was significantly different from that observed in the general population. These two metabolites appear to be, therefore, truly specific risk markers for the general population that cannot be used in people with diabetes. Conversely, glycoprotein acetyls, glucose and total lipids in small HDL were similarly associated with all-cause mortality in people with diabetes and in the general population and are therefore useable in both clinical sets. Finally, no robust words can be said for the remaining 9 metabolites for which lack of association with mortality risk in type 2 diabetes is likely a false negative result. Importantly, when a parsimonious 12-MS (i.e., lacking the 2 specific metabolites of the general population) was added to two well-performing predictive models, a significant improvement was observed in both discrimination (% rIDI above the threshold required by the international guidelines for valuable new biomarkers[24] and in the correct reclassification of a large proportion of individuals, mainly non-events.

In this context, the rather small, though statistically significant improvement of the C-statistic is not surprizing given that this index lacks sensitivity in detecting discrimination improvements in prediction models that are already well-performing,[25] as are both RECODe and ENFORCE.

The biology underlying the relationship between the 14 metabolites and all-cause mortality in patients with type 2 diabetes is beyond the scope of our work. Certainly, understanding why metabolites that are established markers of mortality risk in the general population are not such in the context of type 2 diabetes is intriguing and could provide relevant insight into the specific pathways that shape the likelihood of survival in people with diabetes.

TABLE 2 Association between all-cause mortality in type 2 diabetes and 14 previous metabolites associated with mortality in the general population.

Biomarker	Full name	From the present study		From Deelen J et al.[8]	Heterogeneity	Power in
		HR (95%CI)	*p*-value	HR (95%CI)	*p*-value	our study
GlycA	**Glycoprotein acetyls**	**1.39 (1.16–1.65)**	**0.0003**	**1.32 (1.27–1.38)**	**0.6019**	**0.98**
Glc	**Glucose**	**1.26 (1.07–1.48)**	**0.0047**	**1.16 (1.13–1.19)**	**0.3143**	**0.57**
S-HDL-L	**Total lipids in small HDL**	**0.81(0.66–1.00)**	**0.0553**	**0.87 (0.84–0.90)**	**0.5353**	**0.52**
His	Histidine	1.19 (0.98–1.45)	0.0804	0.93 (0.90–0.96)	0.0145	0.18
PUFA/FA	Ratio of polyunsaturated fatty acids to total fatty acids (%)	1.04 (0.83–1.3)	0.7501	0.78 (0.75–0.80)	0.0137	0.95
Alb	*Albumin*	*0.81 (0.65–1.01)*	*0.0653*	*0.89 (0.87–0.92)*	*0.4101*	*0.39*
Phe	*Phenylalanine*	*1.19 (0.98–1.44)*	*0.0767*	*1.13 (1.09–1.17)*	*0.6054*	*0.42*
VLDL-D	*Mean diameter for VLDL particles*	*0.85 (0.66–1.10)*	*0.2225*	*0.85 (0.80–0.90)*	*0.9952*	*0.65*
Lac	*Lactate*	*1.10 (0.93–1.30)*	*0.2461*	*1.06 (1.03–1.10)*	*0.6418*	*0.13*
Val	*Valine*	*0.87 (0.59–1.27)*	*0.4714*	*0.87 (0.82–0.92)*	*0.9986*	*0.52*
Leu	*Leucine*	*0.85 (0.52–1.41)*	*0.5359*	*0.82 (0.76–0.89)*	*0.8718*	*0.81*
AcAce	*Acetoacetate*	*1.02 (0.86– 1.19)*	*0.8570*	*1.08 (1.05–1.11)*	*0.4595*	*0.20*
Ile	*Isoleucine*	*0.97 (0.64–1.47)*	*0.8730*	*1.23 (1.14–1.32)*	*0.2652*	*0.84*
XXL-VLDL-L	*Total lipids in chylomicrons and extremely large VLDL*	*0.99 (0.72–1.37)*	*0.9563*	*0.80 (0.75–0.85)*	*0.2001*	*0.89*

Note: Heterogeneity *p*-value refers to Cochrane Q-test, used to assess the heterogeneity between hazard ratios from the two studies. In bold are metabolites that are significantly associated with all-cause mortality in our study. In grey are metabolites whose association with all-cause mortality is significantly heterogeneous from that reported in Deleen J et al (ref). In italics are non-significantly associated metabolites.

Abbreviations: 95%CI, 95% Confidence Interval; HR, Hazard Ratio.

TABLE 3 5-year all-cause mortality prediction accuracy and reclassification measures.

Prediction models	Discrimination				Reclassification		
	C-statistic (95%CI)	Δ C-statistic (95%CI)	IDI (*p*-value)	rIDI% (*p*-value)	cNRI (*p*-value)	cNRI in events % (*p*-value)	cNRI in non-events % (*p*-value)
12-MS	0.687 (0.651–0.723)						
RECODe	0.721 (0.686–0.757)						
RECODe + 12-MS	0.743 (0.715–0.772)	0.023 (0.010–0.038)	0.019 ($p = 0.012$)	10.60% ($p = 0.011$)	0.398 ($p < 0.0001$)	1.0 ($p = 0.930$)	38.8 ($p < 0.0001$)
ENFORCE	0.704 (0.650–0.744)						
ENFORCE + 12-MS	0.739 (0.708–0.770)	0.036 (0.017–0.055)	0.014 ($p = 0.020$)	9.61% ($p = 0.022$)	0.443 ($p < 0.0001$)	1.9 ($p = 0.852$)	42.4 ($p < 0.0001$)

Note: This table reports the discrimination ability (C-statistic) of the different models in predicting 5-year all-cause mortality. Both the RECODe and the ENFORCE models were tested without and with the addition of 12-MS. The improvement provided by the addition of 12-MS was investigated by examining both the improvement in discrimination (Δ C-statistic, IDI and rIDI) and reclassification (cNRI, overall and in events and non-events, separately) ability. The latter investigates whether the predicted probabilities of individuals with and without events move in the right directions (upward and downward, respectively) from reference to the enriched model.

Abbreviations: 12-MS, 12-metabolite score; cNRI, continuous net reclassification improvement; IDI, integrated discrimination improvement; rIDI, relative integrated discrimination improvement.

We do acknowledge that our study has some limitations. First, the sample size we studied is relatively small, especially in comparison to the previous paper using the same metabolomics platform.[8] Therefore, negative findings in a post-hoc analysis of some metabolites should be interpreted with caution because of the possible risk of false negative findings due to insufficient statistical power. In addition, our results are based only on Italian white people, thus leaving open the question of the generalisability of our findings. All this to say that other larger studies on different populations are needed to confirm our present findings. Additional limitations are the lack of information on cardiovascular mortality as well as on several complications and comorbidities which affect the risk of mortality in people with type 2 diabetes. Finally, we do acknowledge that our study does not provide insights into the mechanisms underlying the observed associations between metabolites and all-cause mortality. Further work, ranging from in vivo pathophysiological and intervention studies to in vitro cellular and molecular investigations, is needed to unravel the mechanisms through which these markers influence mortality.

In conclusion, our study indicates that the metabolomic signature for all-cause mortality reported in the general population[8] is only partially active in patients with diabetes. This suggests that metabolomic markers developed and validated for prediction purposes in the general population must be revalidated if they are to be used in people with diabetes. Further studies focusing on type 2 diabetes are therefore needed to fully uncover the metabolomic signatures of all-cause mortality that are specific to these patients, with the ultimate goal of better identifying those who are at the highest risk and therefore in need of a more aggressive and timely treatment.

AUTHOR CONTRIBUTIONS

Massimiliano Copetti as part of the committee of the "SUMMER study in diabetes", designed this specific study, performed statistical analyses, interpreted results, drafted the manuscript. Marco Giorgio Baroni, Raffaella Buzzetti, Maria Gisella Cavallo, Efiso Cossu, Paola D'Angelo, Salvatore De Cosmo, Frida Leonetti, Susanna Morano, Lelio Morviducci, P.P., Giuseppe Pugliese, Sabrina Prudente as part of the committee of the "SUMMER study in diabetes" supervised patient recruitment and/or manipulation of biological samples and read and assisted in writing the manuscript. Vincenzo Trischitta as the PI of the committee of the "SUMMER study in diabetes", conceived and designed this specific the study, interpreted results, drafted the manuscript. Massimiliano Copetti and Vincenzo Trischitta are the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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CONFLICT OF INTEREST STATEMENT

No potential conflicts of interest relevant to this article were reported.

DATA AVAILABILITY STATEMENT

The SUMMER study database is available upon request from the own Institutional Biologic Specimen and Data Repository (https://www.summerstudyindiabetes.it).

ETHICS STATEMENT

The SUMMER study in diabetes was registered at ClinicalTrials.gov, NCT02311244; URL (https://clinicaltrials.gov/ct2/show/NCT02311244?termZSUMMER&rankZ5). The study will be conducted in accordance with the Declaration of Helsinki. The study protocol has been approved by the coordinating centre's Ethic Committee (Comitato Etico "Sapienza," Prot.n. 782/2014) and thereafter by the Ethics Committee of each centre outside the Umberto I "Sapienza" University Hospital, in Rome. Participants will be asked to give a written informed consent for participating in the study and will be then assigned a centre-specific progressive code.

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PEER REVIEW

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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