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# The association between plasma metabolites and future risk of all-cause mortality

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**Background.** Metabolite profiles provide snapshots of the overall effect of numerous exposures accumulated over life courses, which may lead to health outcomes in the future.

**Objective.** We hypothesized that the risk of all-cause mortality is linked to alterations in metabolism earlier in life, which are reflected in plasma metabolite profiles. We aimed to identify plasma metabolites associated with future risk of all-cause mortality.

**Methods.** Through metabolomics, 110 metabolites were measured in 3833 individuals from the Malmö Diet and Cancer—Cardiovascular Cohort (MDC-CC). A total of 1574 deaths occurred within an average follow-up time of 22.2 years. Metabolites that were significantly associated with all-cause mortality in MDC-CC were replicated in 1500 individuals from Malmö Preventive Project re-examination (MPP), among whom 715 deaths occurred within an average follow-up time of 11.3 years.

**Results.** Twenty two metabolites were significantly associated with all-cause mortality in MDC-CC, of which 13 were replicated in MPP. Levels of trigonelline, glutamate, dimethylglycine, C18-1-carnitine, C16-1-carnitine, C14-1-carnitine, and 1-methyladenosine were associated with an increased risk, while levels of valine, tryptophan, lysine, leucine, histidine, and 2-aminoisobutyrate were associated with a decreased risk of all-cause mortality.

**Conclusion.** We used metabolomics in two Swedish prospective cohorts and identified replicable associations between 13 metabolites and future risk of all-cause mortality. Novel associations between five metabolites—C18-1-carnitine, C16-1-carnitine, C14-1-carnitine, trigonelline, and 2-aminoisobutyrate—and all-cause mortality were discovered. These findings suggest potential new biomarkers for the prediction of mortality and provide insights for understanding the biochemical pathways that lead to mortality.

**Keywords:** all-cause mortality, association, metabolite, metabolomics

## Introduction

The biological pathways contributing to mortality risk are complex and, to a large extent, unknown. Variations in genetic structures and environmental exposures can lead to differences in several metabolic processes, which influence the risk of mortality [1]. However, identifying all the relevant factors and capturing their flux in the life courses is challenging [1]. Plasma metabolomics, as an overview read-out of the metabolism of an indi-

vidual, can identify metabolites that are involved in the processes influencing our lifespan [2]. In large cohorts, the associations between metabolites and long-term mortality risk may either be driven by certain diseases or represent more generic mortality-related metabolic variations [2–8]. These associations may be different in different age groups or be sex specific or vary by causes of mortality.

Using metabolomics as a tool to understand mechanisms leading to mortality or predict lifespan, some studies have established metabolite

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profiles associated with all-cause mortality [9–12] and longevity [1, 13]. However, many of them were limited by either narrow metabolite coverage [3, 5, 10, 11] or, to some extent, small sample sizes [6, 9, 12]. Additionally, because of differences in study populations, analytical methods, and statistical analyses [2], the findings from previous studies were not perfectly concordant [9–12].

In the present study, we used a liquid chromatography–mass spectrometry platform to quantify 110 plasma metabolites at baseline in 3833 individuals from the Malmö Diet and Cancer—Cardiovascular Cohort (MDC-CC), and mortality was recorded in the follow-up. This study aimed to identify which metabolites' concentrations are associated with future risk of all-cause mortality. Metabolites with significant associations were further examined in 1500 individuals from Malmö Preventive Project (MPP) re-examination. The replicable metabolites were also explored for whether their associations with mortality were different between sex, age groups, or causes of death.

**Methods**

*Study populations*

The Malmö Diet and Cancer (MDC) study is a cohort study carried out in the 1990s to investigate the relationship between diet and subsequent cancer risk in Malmö's population [14, 15]. Between 1991 and 1996, around 17,000 women and 11,000 men were recruited [14]. In a cardiovascular cohort subset of MDC (MDC-CC) that aimed to study the epidemiology of carotid artery disease [6], among the 5405 participants who fasted, a random sample of 3833 participants was selected [5]. Their citrate plasma samples were obtained and stored at −80°C for later metabolic analyses [2]. A total of 1574 deaths occurred within the average follow-up time of 22.2 years.

MPP, carried out in 1974, aimed to screen a large strata of Malmö's adult population to identify individuals with a high risk of developing diseases for preventive interventions [16, 17]. Between 1974 and 1992, approximately 11,000 females and 22,000 males participated in the project [17]. Between 2002 and 2006, all participants who were alive were invited for the re-examination of cardiometabolic risk factors, and their overnight-fasting EDTA plasma samples were collected and

stored at −80°C for later analyses [2]. A total of 5386 individuals from this re-examination were randomly selected as a sample in previous studies [6, 7]. From this sample, a case-control cohort was constructed by analyzing metabolites in all participants who either had type 2 diabetes (T2D) at baseline ($N = 451$), developed T2D ($N = 204$), or developed coronary artery disease (CAD; $N = 384$) before 31 December 2013. Controls were randomly selected as participants without T2D at baseline or during follow-up ($N = 496$) [7]. A total of 715 deaths occurred within the average follow-up time of 11.2 years.

The ethics committee of Lund University approved the study protocols of MDC-CC and MPP re-examination, and all participants provided written informed consent.

*Endpoint definitions and biochemical measurements*

T2D was defined as a fasting plasma glucose of 7.0 mmol/L or being on antidiabetic medication or being diagnosed with T2D by physicians or a registration in local or national Swedish diabetes registries [2, 6]. CAD was defined as fatal or non-fatal myocardial infarction or coronary revascularization (coronary artery bypass surgery and percutaneous intervention) or death due to ischemic heart diseases [7]. Prevalence of baseline T2D and CAD were defined as being diagnosed with T2D or CAD prior to baseline examinations.

Information regarding demographic characteristics, socio-economic factors, lifestyle, medications and treatments, previous and current diseases was elicited by self-administered questionnaires [15–17]. Anthropometric factors, such as height, weight, and hip and waist circumference were measured in physical examinations and body mass index (BMI) was calculated [15–17]. Blood pressure was measured with a sphygmomanometer in the supine position after 10 min of rest [2, 17]. Fasting glucose, cholesterol, high-density lipoprotein cholesterol (HDL), triglycerides (TG), and serum creatinine were examined based on the standard procedures at the Department of Clinical Chemistry at Malmö University Hospital [2, 6, 7]. Low-density lipoprotein cholesterol (LDL) was estimated with the Friedewald equation [7]. The estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) creatinine equation [18].

*Analytical procedure and data processing*

A UHPLC-QTOF-MS System (Agilent Technologies 1290 LC, 6550 MS, Santa Clara, CA, USA) was used for the profiling of plasma metabolites and was described in detail [19]. To be brief, plasma samples stored at −80°C were thawed and extracted by addition of six volumes of extraction solution, which consisted of 80:20 methanol/water containing 18 stable isotope-labeled internal standards that were purchased from Cambridge Isotope Laboratories (Andover, MA, USA) and Toronto Research Chemicals (Toronto, ON, Canada). Extracted samples were separated on an Acquity UHPLC BEH Amide column (1.7 $\mu$m, 2.1 mm × 100 mm, Water Corporation, Milford, MA, USA). After separation, samples were analyzed in positive ion mode in batches of 180 samples. Quality control samples, which were pooled plasma, were injected into every eight analytical samples and run at the beginning of each batch, which aimed to capture analytical drift and condition the column to maintain high repeatability. Also, the quality control samples were used to calculate the technical variation of the measurements (Table S1). By matching MS/MS fragmentation with the Human Metabolome Database [20] and METLIN [21] or by matching fragment ions to putative molecular fragments or using synthetic standards, metabolites were annotated at level 1 or 2 according to the Metabolomics Standards Initiative [22]. Information about all annotated metabolites was included in Table S1. Data processing was done by using Agilent Profinder B.06.00 (Agilent Technologies, Santa Clara, CA, USA) to integrate metabolite features. Using low-order nonlinear locally estimated smoothing functions to create correction curves, metabolites' intensities were normalized based on the measurement of metabolites in quality control samples [23]. The performance of the normalization method was assessed by comparison to normalization using the 18 available internal standards, indicating good coherence (Figure S1).

*Statistical analysis*

R (version.4.0.2) was used for all statistical analyses. The metabolite data were centered to zero and unit variance scaled before analysis. In MDC-CC, the association between each metabolite's plasma concentration and future risk of all-cause mortality was analyzed by Cox proportional hazards models. Model 1 was adjusted for sex and age. Model 2 was additionally adjusted for BMI, fasting glucose, waist circumference, LDL cholesterol, high-density lipoprotein cholesterol (HDL), cholesterol, TG, systolic blood pressure (SBP), diastolic blood pressure (DBP), having antihypertensive treatment (AHT) or not, having lipid-lowering treatment (LLT) or not, smoking status, the prevalence of baseline T2D, and CAD. Association was considered significant at a false discovery rate (FDR)–adjusted *p*-value of <0.05. The identified all-cause-mortality-related metabolites were further examined in MPP re-examination by the same Cox proportional hazards models. Since metabolite levels are known to be associated with both kidney function and mortality [24], an extra sensitivity analysis that made an additional adjustment for eGFR based on Model 2 was performed on replicable all-cause mortality-related metabolites in both MDC-CC and MPP re-examination. Due to the skewed distributions of metabolites, Spearman's correlation tests were used to analyze the intercorrelations between the replicable metabolites.

Linear regressions were used to examine the relationships between all-cause-mortality-related metabolites and age. The associations between replicable metabolites and all-cause mortality were further examined in males and females in both MDC-CC and MPP re-examination. The associations between replicable metabolites and mortality due to cancer, cardiovascular diseases (CVD), and other causes were also examined in both studies.

## Results

The general characteristics of the two selected samples from MDC-CC and MPP re-examination are presented in Table 1. The participants in MPP re-examination (70.0 years) were older than in MDC-CC (57.7 years). A total of 71.4% of participants in MPP re-examination were male, which was higher than in MDC-CC (41.3%). A higher baseline prevalence of T2D was shown in MPP re-examination (30.1%) than MDC-CC (4.90%). In MDC-CC, 1574 deaths occurred within the average follow-up time of 22.2 years. In MPP re-examination, 715 deaths occurred within the average follow-up time of 11.2 years.

### In MDC-CC, 22 metabolites were associated with future risk of all-cause mortality

Among the 110 metabolites in MDC-CC, 38 showed significant associations (FDR-adjusted *p*-value <0.05) with all-cause mortality in the sex

**Table 1.** *General characteristics of participants in the Malmö Diet and Cancer–Cardiovascular Cohort (MDC-CC) and Malmö Preventive Project (MPP) re-examination*

| Variables | MDC-CC (N = 3833) | | MPP re-examination (N = 1500) | |
|---|---|---|---|---|
| | Mean (SD) | N (%) | Mean (SD) | N (%) |
| Number of deaths | | 1574 (41.1) | | 715 (52.6) |
| Average survival years | 22.2 (±5.87) | | 11.2 (±3.98) | |
| Age (years) | 57.7 (±5.99) | | 70.0 (±6.00) | |
| Sex (female) | | 2250 (58.7) | | 429 (28.6) |
| BMI (kg/m$^2$) | 25.7 (±3.90) | | 27.7 (±4.39) | |
| Waist circumference (cm)[a] | 83.5 (±12.7) | | 97.2 (±12.5) | |
| Fasting glucose (mmol/L) | 5.20 (±1.38) | | 6.12 (±1.51) | |
| TG (mmol/L) | 1.31 (±0.64) | | 1.31 (±0.67) | |
| HDL (mmol/L) | 1.39 (±0.37) | | 1.33 (±0.40) | |
| LDL (mmol/L) | 4.16 (±0.99) | | 3.65 (±1.00) | |
| Cholesterol (mmol/L) | 6.15 (±1.08) | | 5.58 (±1.10) | |
| SBP (mmHg) | 142 (±19.1) | | 148 (±21.8) | |
| DBP (mmHg) | 86.9 (±9.46) | | 84.6 (±11.5) | |
| Smoking[b] | | 1011 (27.2) | | 305 (20.3) |
| Having AHT | | 623 (16.3) | | 599 (39.9) |
| Having LLT[c] | | 86 (2.24) | | 243(16.2) |
| Prevalence of baseline T2D | | 188 (4.90) | | 451(30.1) |
| Prevalence of baseline CAD | | 82 (2.14) | | 78 (5.20) |
| eGFR (umol/dl)[d] | 76.60 (±13.72) | | 66.27 (±15.89) | |

Abbreviations: AHT, antihypertensive treatment; BMI, body mass index; CAD, coronary artery disease; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; LLT, lipid-lowering treatment; N, number of participants; SBP, systolic blood pressure; SD, standard deviation; TG, triglycerides; T2D, type 2 diabetes.
[a]One missing value of waist circumference in MPP re-examination.
[b]A hundred and eleven missing values of smoking in MDC-CC.
[c]A hundred and six missing values of having LLT in MPP re-examination.
[d]Forty seven missing values of eGFR in MDC and 70 missing values of eGFR in MPP re-examination.

and age-adjusted Cox proportional hazards models (Fig. 1). When model 2 was adjusted for age, sex, BMI, fasting glucose, waist circumference, LDL, HDL, cholesterol, TG, SBP, DBP, AHT, LLT, smoking status, the prevalence of baseline T2D, and CAD, 22 metabolites in MDC-CC were significantly associated with future risk of all-cause mortality (Table 2).

**In MPP re-examination, 13 all-cause mortality related metabolites were replicable**

The associations between the 22 metabolites and all-cause mortality were further examined in MPP re-examination (Table S2). Thirteen of the 22 metabolites showed significant association (valine, tryptophan, trigonelline, lysine, leucine, histidine, glutamate, dimethylglycine, C18-1-carnitine, C16-

1-carnitine, C14-1-carnitine, 2-aminoisobutyrate [AIB] , and 1-methyladenosine). Figure 2 shows that the directions of the associations in MDC-CC and MPP re-examination were consistent. Trigonelline, glutamate, dimethylglycine, C18-1-carnitine, C16-1-carnitine, C14-1-carnitine, and 1-methyladenosine were associated with an increased risk of future all-cause mortality. Valine, tryptophan, lysine, leucine, histidine, and AIB were inversely associated with future risk of all-cause mortality. The effect of all the 13 metabolites on future risk of all-cause mortality was stronger in MPP re-examination than in MDC-CC. Sensitivity analyses showed that the associations between all-cause mortality and all 13 metabolites in MDC (N = 3786) and 12 metabolites in MPP (N = 1430) re-examination remained significant after adjusting for eGFR, except trigonelline (Table S3).
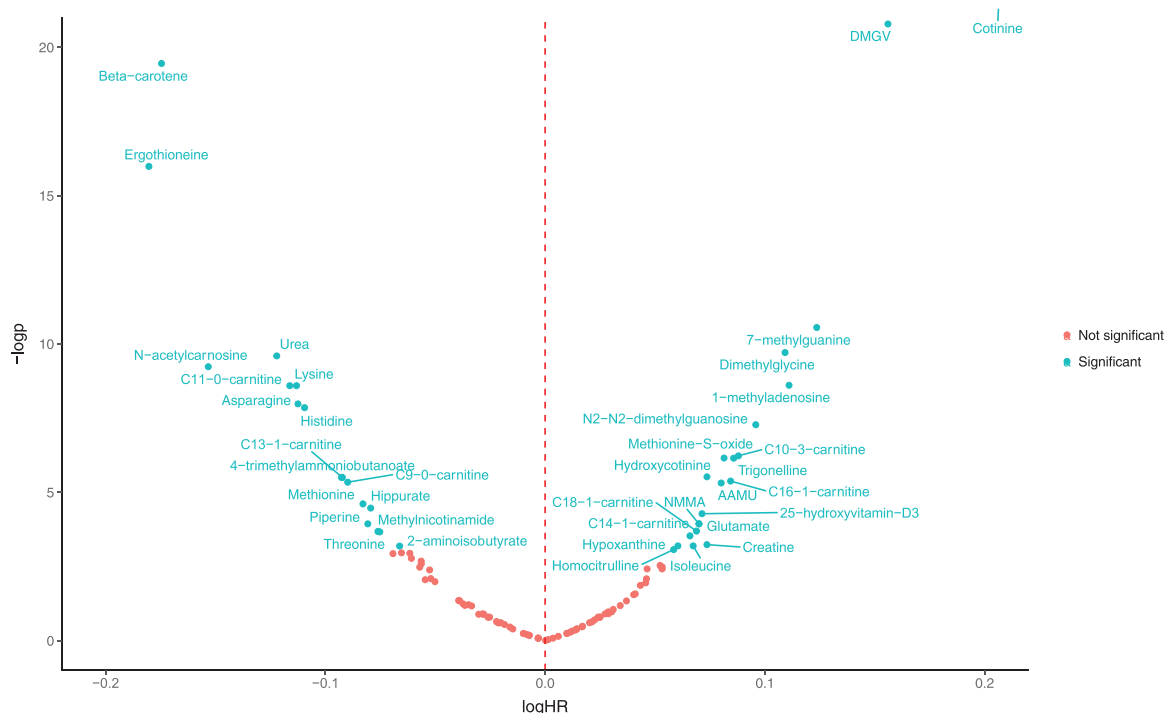
**Fig. 1** *Associations between the plasma levels of 110 metabolites at baseline and future risk of all-cause mortality in participants from the Malmö Diet and Cancer–Cardiovascular Cohort (n = 3833). Using Cox regression model 1 (adjusted age and sex), logHR is the 10log of hazard ratio (per standard deviation increment of metabolite) and −log p is the negative 10log of false discovery rate (FDR)–adjusted p-value. The association is considered significant when the FDR-adjusted p-value is <0.05.*

*Spearman correlation tests for the 13 metabolites*

Spearman correlation tests were used to analyze the intercorrelations between the 13 metabolites (Figure S2). In both studies, there were very strong positive correlations ($p > 0.25$) between five amino acids (valine, tryptophan, lysine, leucine, and histidine). Very strong correlations ($p > 0.5$) were also observed between C18-1-carnitine, C16-1-carnitine, and C14-1-carnitine.

*Relationships between the 13 metabolites and sex*

When stratified by sex (Fig. 3), in MDC-CC, 10 out of the 13 metabolites in males and six out of the 13 metabolites in females were significantly associated with future risk of all-cause mortality. Sex was only recognized as an effect modifier in the association between histidine and future risk of all-cause mortality and the association was stronger in males than in females. In MPP re-examination, all 13 metabolites in males and six metabolites in females were significantly associated with future

risk of all-cause mortality. No effect modification was found in MPP re-examination.

*Relationships between the 13 metabolites and age*

Multivariate-adjusted linear regressions examined the relationship between baseline age and the 13 metabolites in both MDC-CC and MPP re-examination (Figure S3). Dimethylglycine, 1-methyladenosine, trigonelline, C18-1-carnitine, C16-1-carnitine, and C14-1-carnitine consistently increased with age. Tryptophan, histidine, valine, leucine, and AIB consistently decreased with age, but some such decreases were not significant in middle-aged individuals. Lysine and glutamate appeared to increase up to a certain age and then decrease.

*Relationships between the 13 metabolites and cause-specific mortalities*

Out of the 13 metabolites, five in MDC-CC and four in MPP re-examination were associated with cancer mortality; seven metabolites in MDC-CC

**Table 2.** *Hazard ratios (per standard deviation increment of metabolite), 95% confidence intervals, and p-values of the plasma levels of the 22 metabolites that are associated with all-cause mortality in the Malmö Diet and Cancer–Cardiovascular Cohort (n = 3833). The Cox regressions were adjusted for multivariate (age, body mass index, fasting glucose, waist circumference, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, cholesterol, triglycerides, systolic blood pressure, diastolic blood pressure, having antihypertensive treatment or not, having lipid-lowering treatment or not, smoking status, prevalence of baseline type 2 diabetes, and prevalence of baseline coronary artery disease)*

| Metabolites | Hazard ratio (95% confidence interval) | p-value |
|---|---|---|
| Cotinine | 1.20 (1.14–1.26) | <0.0001 |
| Ergothioneine | 0.85 (0.80–0.91) | <0.0001 |
| Urea | 0.90 (0.85–0.94) | <0.0001 |
| N-acetylcarnosine | 0.87 (0.81–0.93) | <0.0001 |
| Beta-carotene | 0.90 (0.85–0.95) | 0.0004 |
| C16-1-Carnitine | 1.10 (1.04–1.16) | 0.0004 |
| 1-Methyladenosine | 1.10 (1.04–1.15) | 0.0005 |
| C18-1-Carnitine | 1.09 (1.04–1.15) | 0.0005 |
| Dimethylglycine | 1.09 (1.04–1.14) | 0.0008 |
| Lysine | 0.91 (0.87–0.97) | 0.0011 |
| 2-Aminoisobutyrate | 0.91 (0.86–0.96) | 0.0012 |
| Histidine | 0.92 (0.87–0.97) | 0.0016 |
| 4-Trimethylammoniobutanoate | 0.92 (0.87–0.97) | 0.0019 |
| Tryptophan | 0.92 (0.87–0.97) | 0.0024 |
| Leucine | 0.91 (0.86–0.97) | 0.0043 |
| Valine | 0.92 (0.87–0.98) | 0.0046 |
| Methionine | 0.93 (0.88–0.98) | 0.0054 |
| C5-0-Carnitine | 0.93 (0.88–0.98) | 0.0055 |
| Glutamate | 1.07 (1.02–1.13) | 0.0057 |
| C14-1-Carnitine | 1.07 (1.02–1.13) | 0.0058 |
| Trigonelline | 1.07 (1.02–1.13) | 0.0061 |
| C3-0-Carnitine | 0.93 (0.88–0.98) | 0.0064 |

and 11 metabolites in MPP re-examination were associated with CVD mortality; and seven metabolites in MDC-CC and 11 metabolites in MPP re-examination were associated with other-cause mortality (Table 3).

## Discussion

The key finding of the present study was that circulating levels of 13 metabolites were associated with future risk of all-cause mortality. Importantly, these associations were replicable in two independent cohorts, comprising over 5000 individuals with different baseline ages, sex distributions, and follow-up times. While five of the associations were novel findings, eight metabolites have previously been associated with mortality, further indicating the generalizability of our findings. These findings

may shed light on potential biochemical pathways that lead to mortality.

*Long-chain acyl-carnitines, trigonelline, and AIB*

Among the 13 metabolites that showed significant associations with all-cause mortality, the associations of five metabolites—C18-1-carnitine, C16-1-carnitine, C14-1-carnitine, AIB, and trigonelline—were novel findings.

In fatty-acid oxidation (FAO), carnitine transports long-chain fatty acids across the mitochondrial inner membrane for $\beta$ oxidation by transforming from long-chain acyl-CoA and carnitine to CoA and long-chain acyl-carnitine (LCAC) (e.g., C18-1-carnitine, C16-1-carnitine, C14-1-carnitine) [25, 26]. Accumulation of acyl-carnitines in cytoplasm might result from FAO disruption and
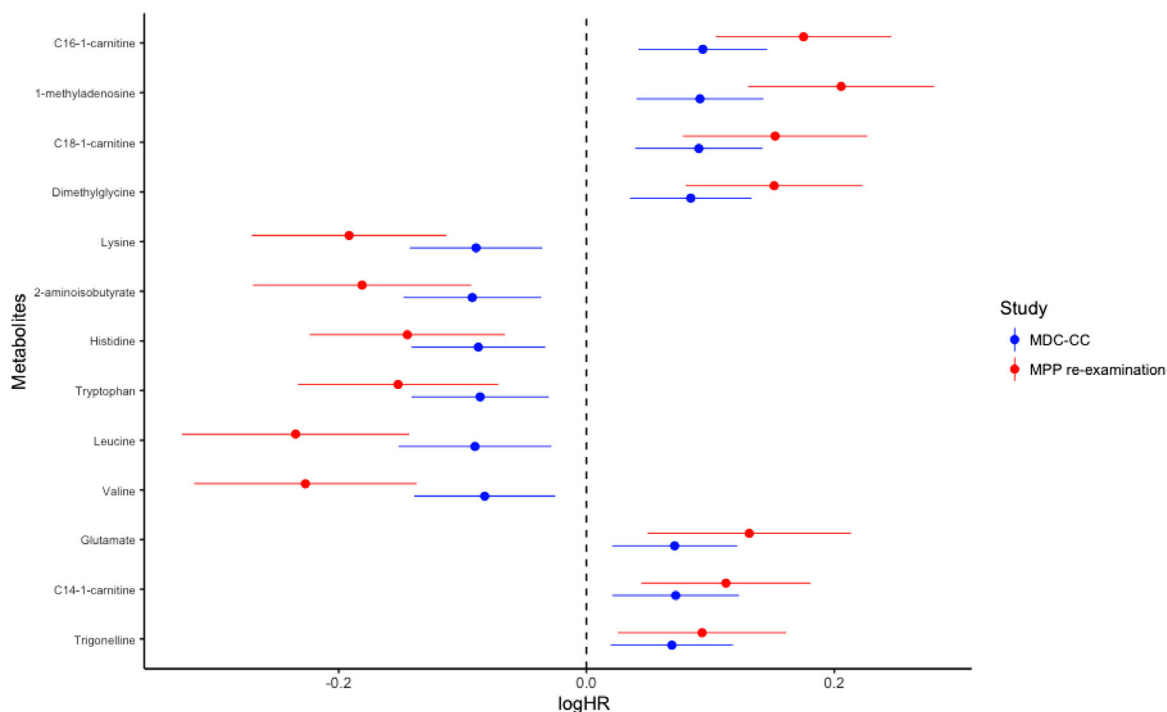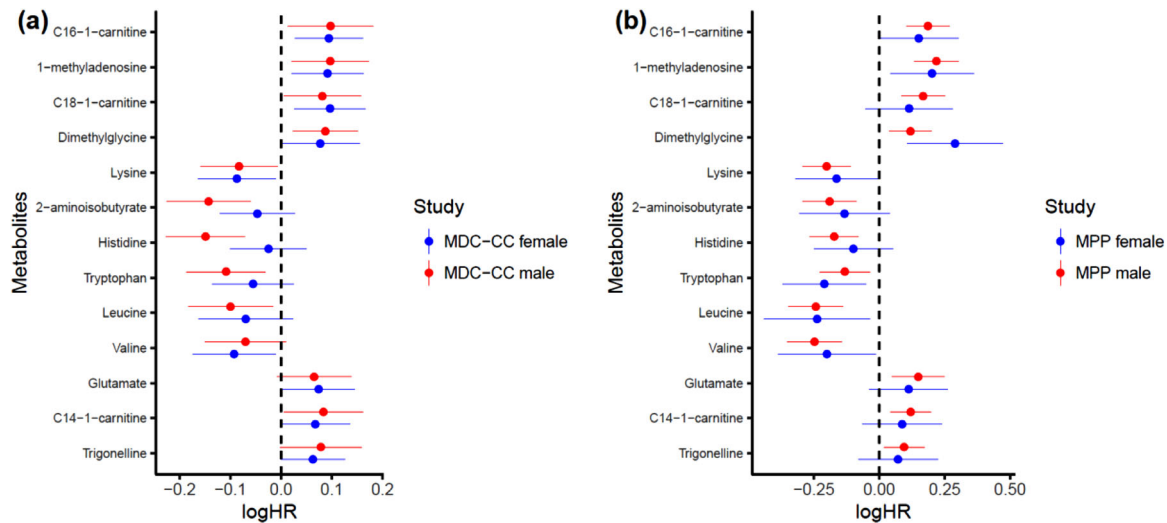
**Fig. 2** *Associations between the plasma levels of the 13 identified metabolites at baseline and future risk of all-cause mortality in the Malmö Diet and Cancer–Cardiovascular Cohort (MDC-CC) (n = 3833) and Malmö Preventive Project (MPP) re-examination (n = 1500). LogHR is the 10log of hazard ratio (per standard deviation increment of metabolite). The error bar denotes 95% standard deviation of the 10log of hazard ratio. The Cox regressions were adjusted for multivariate (age, sex, body mass index, fasting glucose, waist circumference, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, cholesterol, triglycerides, systolic blood pressure, diastolic blood pressure, having antihypertensive treatment or not, having lipid-lowering treatment or not, smoking status, prevalence of baseline type 2 diabetes, and prevalence of baseline coronary artery disease).*

dysfunctional mitochondrial metabolism [27–29], which leads to a large accumulation of acyl-carnitine in plasma [28]. It has been shown that the accumulation of LCAC in the cytoplasm could inhibit the exchange of sodium and calcium ions and lead to membrane instability [30]. One study using the data from MDC-CC has found an association between LCAC accumulation and the development of atrial fibrillation [29]. Another study, using multicohort metabolic analysis, has identified a specific acyl-carnitine, C10-1-carnitine, that could be used as a biomarker for atrial fibrillation [31]. It has also been reported that higher levels of LCAC were associated with an increased risk of cardio-vascular death and acute myocardial infarction in patients with angina pectoris [32]. Additionally, an association between an increased risk of all-cause hospitalization and higher levels of LCAC has been observed in patients with end-stage heart failure [33]. The results of the present study showed that

higher levels of LCACs were significantly associated with higher all-cause mortality risk. One could speculate that mitochondrial dysfunction, leading to increased LCAC in the circulation [34], could be the factor driving the association between LCAC and increased risk of atrial fibrillation, myocardial infarction, and mortality.

In this study, we showed that the coffee biomarker trigonelline [35, 36] is associated with an increased risk of all-cause mortality. This is not in line with previous findings of inverse associations between trigonelline and liver cancer mortality and liver disease mortality [37]. Moreover, inverse associations have been shown between coffee intake and all-cause and cardiovascular mortality [38] and trigonelline has been suggested as a therapeutic agent for cardiovascular disorders [39]. However, several metabolites that were associated with coffee intake, such as caffeine,

**Fig. 3** *Associations between the plasma levels of the 13 identified metabolites at baseline and future risk of all-cause mortality in males and females in (a) the Malmö Diet and Cancer–Cardiovascular Cohort (MDC-CC) (n = 3833) and (b) Malmö Preventive Project (MPP) re-examination (n = 1500). LogHR is the 10 log of hazard ratio (per standard deviation increment of metabolite). The error bar denotes 95% standard deviation of the 10 log of hazard ratio. The Cox regressions were adjusted for multivariate (age, body mass index, fasting glucose, waist circumference, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, cholesterol, triglycerides, systolic blood pressure, diastolic blood pressure, having antihypertensive treatment or not, having lipid-lowering treatment or not, smoking status, prevalence of baseline type 2 diabetes, and prevalence of baseline coronary artery disease).*

**Table 3.** *Hazard ratios (per standard deviation increment of metabolite) and p-values of the plasma levels of the 13 metabolites that are associated with cause-specific mortality (cancer, cardiovascular diseases [CVD], and other mortality) in the Malmö Diet and Cancer–Cardiovascular Cohort (MDC-CC) (n = 3833) and Malmö Preventive Project (MPP) (n = 1500). The Cox regressions were adjusted for multivariate (age, body mass index, fasting glucose, waist circumference, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, cholesterol, triglycerides, systolic blood pressure, diastolic blood pressure, having antihypertensive treatment or not, having lipid-lowering treatment or not, smoking status, prevalence of baseline type 2 diabetes, and prevalence of baseline coronary artery disease)*

| | MDC-CC | | | MPP re-examination | | |
|---|---|---|---|---|---|---|
| Metabolites | Cancer | CVD | Other | Cancer | CVD | Other |
| C16-1-Carnitine | 1.11 | 1.10* | 1.11* | 0.96 | 1.25*** | 1.37*** |
| 1-Methyladenosine | 1.07 | 1.12* | 1.11* | 1.12 | 1.25*** | 1.30*** |
| C18-1-Carnitine | 1.10* | 1.10* | 1.09 | 0.91 | 1.22*** | 1.33*** |
| Dimethylglycine | 1.08 | 1.10* | 1.08 | 1.01 | 1.24** | 1.32*** |
| Lysine | 0.92 | 0.99 | 0.84*** | 0.86* | 0.80*** | 0.83** |
| 2-Aminoisobutyrate | 0.93 | 0.85** | 0.94 | 0.97 | 0.75*** | 0.81** |
| Histidine | 0.90* | 0.98 | 0.87** | 0.83* | 0.86* | 0.91 |
| Tryptophan | 0.94 | 1.00 | 0.82*** | 0.90 | 0.86* | 0.82** |
| Leucine | 0.90* | 0.96 | 0.89* | 0.81* | 0.78*** | 0.81** |
| Valine | 0.90* | 0.98 | 0.88* | 0.81* | 0.79*** | 0.81** |
| Glutamate | 1.09* | 1.11* | 1.02 | 1.11 | 1.17 | 1.22* |
| C14-1-Carnitine | 1.05 | 1.10 | 1.09 | 0.96 | 1.16** | 1.21** |
| Trigonelline | 1.08 | 1.13** | 1.01 | 1.14 | 1.08 | 1.09 |

*p < 0.05.
**p < 0.01.
***p < 0.001.

paraxanthine, theophylline, and 5-acetylamino-6-amino-3-methyluracil [40], were not associated with all-cause mortality in this study, suggesting that the association between trigonelline and mortality may be driven by other factors. Our sensitivity analysis indicated that, although overall our findings were not strongly driven by kidney function, the association between trigonelline and mortality could potentially be the exception. Further studies are needed to clarify the possible pathways between trigonelline and all-cause mortality.

AIB [41], which is often used as a probe for testing placental nutrient transport, has not been associated with mortality in previous studies [41–44]. However, this study showed that AIB was inversely associated with all-cause mortality. Further investigations are needed to clarify the biochemical functions of AIB.

### Identified metabolites that were consistent with previous studies

Among the 13 metabolites that showed significant associations with all-cause mortality, eight metabolites' associations were consistent with previous findings, which strengthened the credibility of this study.

Among them, six are amino acids. Two branch chain amino acids (BCAAs), leucine and valine, were inversely associated with all-cause mortality. Although this is consistent with previous studies [45], BCAAs have also been reported as having positive associations with the incidence of T2D [46] and CAD [47]. A study of middle-aged and elderly individuals has shown that BCAAs were positively associated with the fat-free mass index [48]. Higher levels of BCAAs may imply higher age-related muscle loss [7] and therefore elder biological age. This may confound the relationship between BCAAs and all-cause mortality. Levels of lysine [1, 12], tryptophan [12, 13], and histidine [11, 12] have previously been associated with longevity or inversely associated with all-cause mortality. The results of this study further strengthened these conclusions. Moreover, in MDC-CC, the strength of association differed between gender and histidine showed a stronger protective effect in males than in females. Glutamate has not been associated with mortality in previous studies but has frequently been associated with an increased risk of both T2D [7, 49, 50] and CAD [7, 51], possibly driving the association observed in the present study. Levels of both dimethylglycine [52, 53] and 1-methyladenosine [6] have previously been associated with an increased risk of all-cause mortality.

### Associations with mortality are not driven by specific causes of death

Among the 13 metabolites consistently associated with mortality, the associations in males and females were consistent. Some metabolites, such as LCACs, dimethylglycine, 1-methyladenosine, trigonelline, tryptophan, and histidine, seemed to consistently increase or decrease with age, which suggests that they may be markers of biological aging, while other metabolites, such as lysine and glutamate, may be influenced by age in a more complex nonlinear way. No specific cause of death was driving the associations. Notably, although several metabolites, such as glutamate [7, 50–52], leucine [46], lysine [1, 12], and LCAC [28–34], have been associated with increased risk of CVD, the associations with mortality were not driven specifically by cardiovascular causes in middle-aged individuals. This suggests that these metabolites may be linked to mortality risks through other pathways.

Our findings showed that the associations between metabolites and all-cause mortality in middle-aged individuals were present more than 20 years prior to death. For 13 metabolites, these associations were replicated in a cohort of elderly individuals with shorter follow-up time, indicating that these associations are not age specific but rather generalizable to different age spans. For the nine metabolites associated with mortality only in MDC, further studies are needed to confirm whether these metabolites are only predictors for future lifespan in middle-aged individuals.

### Strengths and limitations

This study measured a relatively wide range of metabolites in two large prospective population-based cohorts with a long follow-up period. It not only replicated the associations between eight metabolites and all-cause mortality discerned in previous research but also identified novel associations between five metabolites and all-cause mortality. However, as an observational study, it can only report on associations but cannot prove a causal link. Additionally, for sampling, the participants from MPP re-examination were not randomly selected, which led to the higher prevalence rate of T2D and CADs in MPP re-examination.

## Conclusion

Our study identified novel and replicable associations between five metabolites—C18-1-carnitine, C16-1-carnitine, C14-1-carnitine, AIB, and trigonelline—and all-cause mortality. These metabolites could be potential new biomarkers for predicting mortality risk. Further research is needed to understand the biochemical mechanisms underlying the relationships between these metabolites and mortality.

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## Conflict of interest

There is no conflict of interest to disclose.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1**. The explained variance (R squared) for each of the 18 metabolites comparing the two normalization strategies (LOESS correction and internal standard normalization).

**Figure S2**. Intercorrelations (spearman correlation) between the 13 identified metabolites that are significantly associated with future risk of all-cause mortality in Malmö Diet and Cancer-Cardiovascular Cohort (MDC-CC) (n=3833) and Malmö Preventive Project (MPP) re-examination (n=1500). (a) MDC-CC (b) MPP re-examination.

**Figure S3**. Linear relationships between the plasma levels of the 13 identified metabolites at baseline and age in Malmö Diet and Cancer-Cardiovascular Cohort (MDC-CC) (n=3833) and Malmö Preventive Project (MPP) re-examination (n=1500). The linear regressions were adjusted for multivariate (sex, BMI, fasting glucose, waist circumference, LDL, HDL, cholesterol, TG, SBP, DBP, having AHT or not, having LLT or not, smoking status, prevalence of baseline T2D, prevalence of baseline CAD).

**Table S1**. Metabolites Measured in Malmö Diet and Cancer-Cardiovascular Cohort (MDC-CC) and Replicated in Malmö Preventive Project (MPP) Re-examination

**Table S2**. Hazard Ratios (per standard deviation increment of metabolite), 95% Confidence Intervals and P Values of the Plasma Levels of the 22 Metabolites that are associated with all-cause mortality in Malmö Preventive Project (MPP) re-examination (n=1500). The Cox Regressions were Adjusted for Multivariate (age, BMI, fasting glucose, waist circumference, LDL, HDL, cholesterol, TG, SBP, DBP, having AHT or not, having LLT or not, smoking status, prevalence of baseline T2D, prevalence of baseline CAD)

**Table S3**. Hazard Ratios (per standard deviation increment of metabolite) and 95% Confidence Intervals of the Plasma Levels of the 13 Metabolites that are associated with all-cause mortality in Malmö Diet and Cancer-Cardiovascular Cohort (MDC-CC) (n=3833) and Malmö Preventive Project (MPP) re-examination (n=1500). The Cox Regressions were Adjusted for Multivariate (age, BMI, fasting glucose, waist circumference, LDL, HDL, cholesterol, TG, SBP, DBP, having AHT or not, having LLT or not, smoking status, prevalence of baseline T2D, prevalence of baseline CAD and eGFR) ∎