Untargeted high-resolution plasma metabolomic profiling predicts outcomes in patients with coronary artery disease

Anurag Mehta, Emory University
Chang Liu, Emory University
Aditi Nayak, Emory University
Ayman S. Tahhan, Emory University
Yi-An Ko, Emory University
Devinder S. Dhindsa, Emory University
Jeong Hwan Kim, Emory University
Salim S. Hayek, University of Michigan
Laurence Sperling, Emory University
Puja Kiran Mehta, Emory University

Only first 10 authors above; see publication for full author list.

Journal Title: PLOS ONE
Volume: Volume 15, Number 8
Publisher: Public Library Science | 2020-08-18, Pages e0237579-e0237579
Type of Work: Article | Final Publisher PDF
Publisher DOI: 10.1371/journal.pone.0237579
Permanent URL: https://pid.emory.edu/ark:/25593/vgzc7

Final published version: http://dx.doi.org/10.1371/journal.pone.0237579

Copyright information:

© 2020 Mehta et al.

This is an Open Access work distributed under the terms of the Creative Commons Attribution 4.0 International License (https://creativecommons.org/licenses/by/4.0/).

Accessed November 30, 2022 5:20 AM EST
Untargeted high-resolution plasma metabolomic profiling predicts outcomes in patients with coronary artery disease

Anurag Mehta1, Chang Liu1,2, Aditi Nayak1, Ayman S. Tahhan1, Yi-An Ko1,2, Devinder S. Dhindsa3, Jeong Hwan Kim1, Salim S. Hayek1, Laurence S. Sperling1, Puja K. Mehta1, Yan V. Sun3,5, Karan Uppal6, Dean P. Jones6, Arshed A. Quyyumi1*

1 Emory Clinical Cardiovascular Research Institute, Division of Cardiology, Department of Medicine, Emory University School of Medicine, Atlanta, Georgia, 2 Department of Epidemiology, Rollins School of Public Health, Emory University, Atlanta, Georgia, 3 Department of Biostatistics and Bioinformatics, Rollins School of Public Health, Emory University, Atlanta, Georgia, 4 Division of Cardiology, Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, Michigan, 5 Atlanta VA Health Care System, Decatur, Georgia, 6 Division of Pulmonary, Allergy, Critical Care and Sleep Medicine, Department of Medicine, Emory University School of Medicine, Atlanta, Georgia

*aquyyum@emory.edu

Objectives

Patients with CAD have substantial residual risk of mortality, and whether hitherto unknown small-molecule metabolites and metabolic pathways contribute to this risk is unclear. We sought to determine the predictive value of plasma metabolomic profiling in patients with CAD.

Approach and results

Untargeted high-resolution plasma metabolomic profiling of subjects undergoing coronary angiography was performed using liquid chromatography/mass spectrometry. Metabolic features and pathways associated with mortality were identified in 454 subjects using metabolome-wide association studies and Mummichog, respectively, and validated in 322 subjects. A metabolomic risk score comprising of log-transformed HR estimates of metabolites that associated with mortality and passed LASSO regression was created and its performance validated. In 776 subjects (66.8 years, 64% male, 17% Black), 433 and 357 features associated with mortality (FDR-adjusted q < 0.20); and clustered into 21 and 9 metabolic pathways in first and second cohorts, respectively. Six pathways (urea cycle/amino group, tryptophan, aspartate/asparagine, lysine, tyrosine, and carnitine shuttle) were common. A metabolomic risk score comprising of 7 metabolites independently predicted mortality in the second cohort (HR per 1-unit increase 2.14, 95%CI 1.62, 2.83). Adding the score to a model of clinical predictors improved risk discrimination (delta C-statistic 0.039, 95%CI -0.006, 0.086; and Integrated Discrimination Index 0.084, 95%CI 0.030, 0.151) and reclassification (continuous Net Reclassification Index 23.3%, 95%CI 7.9%, 38.2%).
Conclusions
Differential regulation of six metabolic pathways involved in myocardial energetics and systemic inflammation is independently associated with mortality in patients with CAD. A novel risk score consisting of representative metabolites is highly predictive of mortality.

Introduction
Patients with established coronary artery disease (CAD) are at a high risk of mortality and CAD is the leading cause of death in the United States. [1] The current paradigm of risk assessment among patients with CAD involves ascertainment of high-risk clinical characteristics that are known to portend adverse outcomes. [2] This approach is imperfect and does not provide information regarding pathobiological factors responsible for the increased ‘residual’ disease risk observed among some patients with CAD. [3] Additionally, the precise metabolic pathways underlying this risk of adverse outcomes are not well elucidated. Improved understanding of these pathways may help provide key insights into complex disease mechanisms, personalize risk assessment by identifying novel risk markers that prognosticate outcomes, and potentially discern targets for therapeutic interventions in this high-risk patient population.

The emerging role of metabolomics profiling in this conceptual framework has been recently described in a scientific statement from the American Heart Association (AHA). [4] Metabolomics is the systematic study of small-molecule metabolites across biological systems, and the metabolome constitutes the final downstream product of many regulatory complexes (genome → transcriptome → proteome → metabolome) that are proximal to a disease phenotype. [4] Technical advances in targeted metabolomics data mapping to biological pathways has recently provided important insights into the pathobiology of CAD. For example, one study demonstrated a strong association of arginine and its downstream metabolites ornithine and citrulline, key substrates in the nitric oxide synthesis pathway, with major adverse cardiovascular events among patients with CAD. [5] Another recent study showed that significant alteration in metabolism of phospholipids, amino acids, short-chain acylcarnitines, and primary bile acids is associated with CAD severity among patients undergoing coronary angiography. [6]

Nevertheless, there is a paucity of studies evaluating the prognostic utility of untargeted metabolomics among patients with CAD. Untargeted metabolomics is an approach that focuses on global detection and relative quantitation of small-molecule metabolites to understand both known and unknown metabolic changes that accompany disease states. [7] Herein, we used untargeted high-resolution plasma metabolomic profiling to identify metabolic pathways associated with mortality among patients with CAD undergoing cardiac catheterization. We have additionally created and internally validated a novel metabolomic risk score to predict mortality risk in our cohort.

Materials and methods
Study population
We studied subjects enrolled in the Emory Cardiovascular Biobank—an ongoing prospective registry of patients undergoing cardiac catheterization for evaluation of suspected or known CAD at three Emory Healthcare affiliated hospitals in Atlanta, Georgia, USA. [8] In the
current study we evaluated subjects that underwent untargeted high-resolution plasma metabolomic profiling at different time points and were part of two distinct cohorts. Subjects in the first cohort were enrolled in the years 2004 to 2005, underwent plasma metabolomic profiling in 2012, and were sex/race propensity matched 1:1 for the presence or absence of all-cause mortality during follow-up. Subjects in the second cohort were enrolled in the years 2004 to 2011, underwent metabolomic profiling in 2016, and were sex/race propensity matched 1:2 for the presence or absence of all-cause mortality during follow-up.

Participants in both cohorts were interviewed to collect information about demographic characteristics, medical history, and behavioral habits as previously described. [8] The prevalence of hypertension, diabetes, heart failure (HF), peripheral artery disease (PAD), stroke, and prior coronary artery bypass grafting (CABG) was determined by physician diagnosis and/or treatment. [8] Medical records and International Classification of Diseases (ICD)-9 diagnostic codes were reviewed to confirm self-reported medical history. Weight and height were measured at enrollment and body mass index (BMI) was calculated by dividing weight (in kilogram) by height (in meters)-square. Left ventricular ejection fraction (LVEF) was obtained from medical records and estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration equation. [9] Serum high-sensitivity C-reactive protein (hs-CRP), plasma high-sensitivity cardiac Troponin-I (hs-cTnI), and serum N-terminal prohormone of brain natriuretic peptide (NT-proBNP) levels were measured using the using a sandwich immunoassay (in mg/L, FirstMark Inc., San Diego, CA), Alere NT-proBNP (in pg/mL, Abbott Laboratories, Inc.), and STAT Troponin-I assays (in pg/mL, Abbott Laboratories, Inc.), respectively. All patients were stable at the time of enrollment and those with myocardial infarction, defined using international criteria, [10] were also included if clinically stable.

Subjects with data available regarding age, sex, and race, and prospective follow-up for all-cause mortality were included (n = 494 for first and 440 for second cohort); while those with cardiac transplantation, history of non-ischemic cardiomyopathy, and follow-up duration less than 30 days were excluded (n = 40 and 118, respectively). Thus, 454 subjects in the first cohort and 322 subjects in the second cohort were analyzed. This study complies with the Declaration of Helsinki and was approved by the institutional review board at Emory University (Atlanta, Georgia) under IRB00000343. All subjects provided written informed consent at the time of enrollment.

**High-resolution metabolomic profiling**

All subjects underwent an overnight fast before blood collection and untargeted high-resolution metabolomic profiling using liquid-chromatography mass spectrometry (LC/MS) was performed using standardized techniques after thawing each subject’s plasma stored at -80°C. [11–17] Plasma samples were run in a randomized order in batches of 20 and three technical replicates were analyzed for each sample in a sequential manner. Plasma aliquots (65 μL) were treated with 130 μL acetonitrile (2:1 volume/volume) containing 3.5 μL of an internal isotopic standard mix and placed on ice for 30 minutes. [14–17] The internal standard mix consisted of 14 stable isotopic chemicals, [14] which cover a broad range of chemical properties represented in small molecules: [13C6]-d-glucose, [15N]-indole, [2-15N]-l-lysine dihydrochloride, [13C7]-l-glutamic acid, [13C7]-benzoic acid, [3,4-13C2]-cholesterol, [15N]-l-tyrosine, [trimethyl-13C3]-caffeine, [15N2]-uracil, [3,3-13C2]-cystine, [1,2-13C2]-palmitic acid, [15N,13C5]-l-methionine, [15N]-choline chloride, and 2’-deoxyguanosine-15N5,13C10-5’-monophosphate. Samples were analyzed using a Thermo LTQ Velos Orbitrap high-resolution (60,000 mass resolution) mass spectrometer (Thermo Fisher Scientific, San Diego, California)
and C18 column chromatography (Higgins Analytical Inc., Targa, 2.1 × 10 cm) in positive ionization mode with a scanning m/z range of 85–2000 over 10 minutes. [11, 18] Elution was obtained with a formic acid/acetonitrile gradient at a flow rate of 0.35 mL/min for the initial 6 minutes and 0.50 mL/min for the remaining 4 minutes. The first 2-minute period consisted of 5% solution A (2% volume/volume formic acid in water), 60% water, and 35% acetonitrile. This was followed by a 4-min linear gradient to 5% solution A, 0% water, and 95% acetonitrile. The final 4-minute period was maintained at 5% solution A and 95% acetonitrile. For quality control and assurance, pooled reference plasma was run before and after each batch of 20 samples. The average Pearson correlation coefficient and coefficient of variation (%) within the quality cohort (QC) samples for the first cohort were 0.96 and 11.3%, respectively. The corresponding values within the QC samples for the second cohort were 0.95 and 10.5%, respectively. In addition, principal component analysis (PCA) of the QC and study samples was performed to evaluate batch-effects (S1 Fig). Although the pairwise correlation between QC samples were high in both cohorts, batch-effects were observed and therefore we included batch as a covariate in the regression models as described below. Ion dissociation mass spectrometry (LC-MS/MS) analysis was performed using the same protocols as LC/MS using high purity N\textsubscript{2} at normalized collision energy of 35%. The tandem mass spectrometry data were processed using the \texttt{xcmsFragments} function in XCMS, [19–21] and the experimental spectra were compared with in-silico fragmentation using MetFrag. [22]

### Data processing

Raw data files were processed into the computable document format (.cdf) format using Xcalibur file converter software (Thermo Fisher, San Diego, California). The adaptive processing software package, apLCMS (available at http://web1.sph.emory.edu/apLCMS/), designed for use with high-resolution mass spectrometry data, was used for noise removal and feature extraction, alignment, and quantification. [23] All metabolic features were identified using a unique combination of m/z and retention time. The mean feature intensity value was used for analysis, which was calculated from the mean of non-zero readings for each feature of every subject. A feature was retained for further analysis if at least 80% of subjects had non-zero intensity reading. After exclusion, the zero mean intensity values were treated as truly zero intensities. All intensity values were log\textsubscript{2} transformed [log\textsubscript{2}(m+1), m = feature intensity], mean centered and scaled by standard deviation. A total of 6,781 features entered analysis in the first cohort and 8,714 in the second cohort.

### Follow-up and outcomes

Study subjects were prospectively followed for the primary outcome of all-cause mortality and the secondary outcome of cardiovascular death. Outcome censoring was performed at 3 years and follow-up data was obtained by annual phone contact, electronic medical record review, and data from the social security death index and state records. [8] Cardiovascular death was defined as death attributable to an ischemic cardiovascular cause like fatal MI, stroke or sudden death secondary to a presumed cardiovascular cause in this high-risk population and cardiovascular death events were adjudicated by two cardiologists who were blinded to study data. [24]

### Statistical analyses

Subject characteristics were reported as number (proportion) for categorical variables and means (standard deviation) for continuous variables. The differences between first and second cohort subjects were assessed using two sample t-tests for continuous variables and Chi-square
or Fisher’s exact tests for categorical variables as appropriate. The characteristics of cases and controls in the first and second cohorts were also compared.

**Metabolome-wide association studies**

Metabolome-wide association study (MWAS) was carried out for first and second cohort subjects separately by fitting individual Cox proportional hazards regression models for each feature with time to all-cause mortality (censored at 3 years) as the dependent variable and the feature intensity value as the independent variable. Cox models were adjusted for age, sex (male vs female), and race (black vs other). Batch effect was accounted for by treating batches as a categorical covariate in Cox models. Multiple hypothesis testing correction was performed using the Benjamini-Hochberg False Discovery Rate (FDR) method. [25]

**Metabolite annotation**

Metabolite annotation was performed using a combination of computational methods, LC-MS/MS, and comparison of retention time with authentic standards. Computational metabolite annotation was performed using the R package xMSannotator (available at https://sourceforge.net/projects/xmsannotator/). xMSannotator uses a multilevel clustering procedure based on correlation of features, retention time, mass defect, isotope/adduct patterns, and network and pathway associations for categorizing database matches into different confidence levels. [26] Confidence levels range from zero to three, designating annotations from no to high confidence, which reduces the risk of false annotations and allows prioritization of computationally derived annotations for further experimental evaluation and confirmation using MS/MS and authentic standards. [26] Annotations with confidence score 2 or above from xMSannotator were targeted for MS/MS evaluation and the retention times were compared with an in-house library of previously confirmed metabolites. Metabolite identification levels were assigned using an identification scheme adapted from Schymanski et al. [27] A list of our in-house database of metabolites confirmed using MS/MS and authentic standards has been previously published. [11]

**Metabolic pathway analyses**

In the first cohort, features associated with all-cause mortality that had an FDR-adjusted q-value < 0.20 in MWAS were chosen as target features that entered pathway analysis in Mummichog (version 1.0.7). [28] The same approach was used in the second cohort in a separate analysis. For both first and second cohort pathway analyses, all features from first (n = 6,781) and second cohorts (n = 8,714) were utilized as the feature reference pool. The Mummichog output of significant pathways (p-value < 0.05) identified in first and second cohorts was compared.

**Metabolomic score derivation**

The metabolomic risk score was created by identifying common features in both cohorts that were associated with all-cause mortality and passed the FDR-adjusted threshold of q-value < 0.20 in MWAS. We allowed for an m/z difference of 10 ppm and a retention time difference of 40 seconds when identifying common features. These common metabolites were selected as candidates for creating a metabolomic risk score and Least Absolute Shrinkage and Selection Operator (LASSO) regression was utilized to further sub-select metabolites and avoid redundancy. [29] We evaluated the association of these sub-selected metabolites with circulating hs-CRP, hs-cTnI, and NT-proBNP levels using linear regression models adjusted for age, sex, race, and batch effect.
The hazard ratio (HR) estimate for the association of each feature selected after LASSO regression with all-cause mortality in the first cohort was natural log-transformed and the ln (HR) (i.e., coefficient estimate) of each feature was treated as its weight. The weighted metabolomic risk score was calculated as the sum of each feature’s intensity multiplied by its respective weight. The weighted score was mean centered, scaled by standard deviation, and categorized by its median and tertile cutoffs.

**Metabolomic score performance**

The metabolomic risk score’s performance for predicting the primary outcome was first tested in the second cohort. Kaplan-Meir curves were utilized to evaluate the survival probability of subjects belonging to risk score categories by at the median and tertile cutoffs. Cox proportional hazards regression models adjusted for age, sex, race, and batch effect were utilized to test the score’s predictive value in the second cohort. As a sensitivity analysis, age was dichotomized at 75 years and Cox models were adjusted for current smoking, eGFR below 60 ml/min/1.73 m$^2$, diagnosis of hypertension, diabetes, HF, PAD, stroke, and prior CABG. These variables were chosen because a recent study from the Thrombolysis In Myocardial Infarction (TIMI) group showed that a simple integer-based scheme using these predictors can stratify atherothrombotic risk in a secondary prevention population. [2] As an exploratory analysis, we further adjusted Cox models for log-transformed circulating levels of hs-CRP, hs-cTnI, and NT-proBNP.

The score’s all-cause mortality risk calibration capability in the second cohort was studied using a calibration plot and Hosmer-Lemeshow chi-square test. The risk discrimination and reclassification capabilities were studied in context of two baseline models: a) a model comprising of age, sex, and race; and b) a model comprising of age (dichotomized at 75 years), current smoking, eGFR below 60 ml/min/1.73 m$^2$, hypertension, diabetes, HF, PAD, stroke, and prior CABG. Change c-statistic, Integrated Discrimination Index (IDI), and continuous Net Reclassification Index (NRI) after adding the metabolomic score to the two baseline models was evaluated. The features were further normalized using the 'limma' package prior to conducting these analyses. [30] The metabolomic score’s performance for predicting the secondary outcome of cardiovascular death was tested using the same approach outline above. Finally, the score’s performance was evaluated in the combined cohort as well. The workflow for this study is displayed in S2 Fig and the STROBE statement for this study is provided in S1 Table. All analyses were performed using R version 3.5.1 (R Foundation for Statistical Computing, Vienna, Austria). All relevant data are within the manuscript and its Supporting Information files.

**Results**

**Baseline characteristics**

The mean age of 454 subjects in the first cohort was 67.5 years, 65% were male, and 16% were black (Table 1). The second cohort included 322 subjects, with mean age of 65.9 years, 63% were male, and 19% were black (Table 1). Subjects in both cohorts had similar demographic characteristics, cardiovascular risk factor burden, prevalence of PAD, stroke, HF, and statin use. However, subjects in the first cohort had a higher prevalence of prior CABG and acute myocardial infarction (MI) at the time of enrolment (Table 1). The baseline characteristics of cases and controls in the first and second cohorts are described in S2 Table.

**Metabolome-wide association studies**

A total of 6,781 metabolites were analyzed in the first cohort MWAS and at a raw p-value threshold of <0.05, 948 metabolites were independently associated with all-cause mortality.
After adjusting for age, sex, and race. After FDR correction, 433 metabolites remained significantly associated with all-cause mortality (298 with HR < 1 and 135 with HR > 1) at the q-value threshold of < 0.20. The Manhattan plots for this analysis are depicted in Fig 1A and 1B. The MWAS analysis was performed separately in the second cohort using 8,714 metabolites. At a raw p-value threshold of < 0.05, 934 metabolites independently associated with the primary outcome (719 with HR < 1 and 215 with HR > 1) after adjusting for age, sex, and race. After FDR correction, 357 metabolites (299 with HR < 1 and 57 with HR > 1) passed the q-value threshold of < 0.20 (Fig 1C and 1D).

Metabolic pathway analyses

Metabolic pathway analysis was conducted in the first cohort by using the 433 metabolites of significance as input features in Mummichog. Twenty-one metabolic pathways associated with all-cause mortality and the urea cycle/amino group metabolism pathway was the most significant (Fig 2 and S3 Table). In the second cohort, 357 metabolites of significance were used as input features and 9 metabolic pathways associated with all-cause mortality, among which the tryptophan metabolism pathway was the most significant (Fig 2 and S3 Table). On comparing significant metabolic pathways between the first and second cohorts there were six common metabolic pathways that were associated with all-cause mortality—urea cycle/amino group metabolism, tryptophan metabolism, aspartate/asparagine metabolism, lysine metabolism, tyrosine metabolism, and carnitine shuttle pathway (Fig 2).

Metabolomic risk score derivation

Among the 433 and 357 metabolites that passed the FDR-adjusted q-value threshold of 0.20 during MWAS in the first and second cohorts, respectively, we observed that 24 metabolites associated with...
all-cause mortality were common between the two cohorts. These metabolites were selected for LASSO regression and a total of 7 metabolites which were not correlated with each other were selected as components of the metabolomic risk score (Table 2). Among these 7 metabolites two were annotated as creatinine [M+H] (13C) (m/z 115.0693, rt 55 s) and N8-Acetylspermidine (m/z 188.1755, rt 51 s). Three metabolites were associated with an increased hazard of mortality and four metabolites were associated with a decreased hazard of mortality in both the cohorts (Table 2).

The association of these 7 metabolites with cardiovascular biomarkers was explored using linear regression models (S4 Table). The metabolites associated with an increased mortality hazard were also associated with higher circulating levels of hs-cTnI and NT-proBNP, and vice versa (S4 Table). However, the associations with hs-CRP were inconsistent.

Metabolomic risk score performance
The metabolomic risk score’s values ranged from -6.203 to 4.933 with median, lower tertile, and upper tertile cut-off values of -0.075, -0.394, and 0.255, respectively. Subjects in the second
Fig 2. Bubble plot for metabolic pathways associated with all-cause mortality in the first and second cohorts. The color intensity of each bubble is inversely proportional to the p-value for the association of a metabolic pathway with all-cause mortality. Twenty-one pathways in the first cohort and nine pathways in the second cohort were associated with all-cause mortality. Six metabolic pathways were common between the two cohorts—urea cycle/amino group metabolism, tryptophan metabolism, aspartate/asparagine metabolism, lysine metabolism, tyrosine metabolism, and carnitine shuttle pathway.

Table 2. Unique metabolic features comprising the metabolomic risk score.

| m/z (ppm) | rt (s) | HR  | 95% CI          | q-value | HR  | 95% CI          | q-value |
|-----------|-------|-----|-----------------|---------|-----|-----------------|---------|
| 115.0693  | 55    | 1.62| (1.33, 1.98)    | 0.001   | 1.58| (1.22, 2.03)    | 0.052   |
| 188.1755  | 51    | 2.27| (1.57, 3.29)    | 0.004   | 3.01| (1.63, 5.56)    | 0.051   |
| 207.1106  | 65    | 1.74| (1.42, 2.13)    | <0.001 | 1.77| (1.35, 2.32)    | 0.018   |
| 444.6726  | 55    | 0.74| (0.61, 0.90)    | 0.076   | 0.81| (0.69, 0.95)    | 0.198   |
| 559.2977  | 388   | 0.65| (0.50, 0.83)    | 0.047   | 0.66| (0.49, 0.89)    | 0.170   |
| 1050.6578 | 412   | 0.76| (0.63, 0.92)    | 0.107   | 0.73| (0.61, 0.87)    | 0.060   |
| 1078.627  | 412   | 0.74| (0.63, 0.88)    | 0.038   | 0.72| (0.60, 0.87)    | 0.058   |

Abbreviations: HR = hazard ratio, CI = confidence interval, m/z = mass-to-charge, rt = retention time; s = seconds. Metabolite identified as *Creatinine[M+H] (13C) and N8-Acetylspermidine

https://doi.org/10.1371/journal.pone.0237579.t002
cohort with a score greater than the median experienced an increased risk of all-cause mortality as compared to those with a score below the median (Fig 3A). This difference was primarily driven by subjects in the highest tertile group that were at a significantly higher risk of the primary outcome as compared with their counterparts in the lower two tertiles (Fig 3B). Similar findings were observed in the combined cohort (Fig 3C and 3D) as well as for the secondary outcome of cardiovascular death (S3A–S3D Fig).

The metabolomic risk score was associated with all-cause mortality in the second cohort with a HR of 2.26 (95% CI 1.7, 2.90; \( p < 0.001 \)) per 1-unit increase, after adjusting for age, sex, race, and batch effect (Table 3). Subjects with a metabolomic score value above the median

https://doi.org/10.1371/journal.pone.0237579.g003
were at a three-fold risk of all-cause mortality as compared to those with score value below the median (Table 3). Age above 75 years was the only risk factor independently associated with all-cause mortality (HR 2.12, 95% CI 1.21, 3.71; p = 0.009) in the model containing variables evaluated in the recent TIMI study. Nevertheless, the risk score retained its independent predictive value after multivariable adjustment (Table 3). Similarly, the risk score was associated with the primary outcome in the combined cohort after adjusting for age, sex, race, and batch effect (Table 3). Current smoking was the only risk factor independently associated with all-cause mortality (HR 2.29, 95% CI 1.38, 3.80; p = 0.001) in the multivariable adjusted model. The risk score retained its independent predictive value after multivariable adjustment in the combined cohort as well (Table 3). Furthermore, adjustment for hs-CRP, hs-cTnI, and NT-proBNP levels did not attenuate this association (S5 Table). The metabolomic risk score was also associated with the secondary outcome of cardiovascular death in both the second and combined cohorts (S6 Table).

The risk score was well-calibrated in the second cohort with Hosmer-Lemeshow chi-square p = 0.84 (S4 Fig). Furthermore, adding the risk score to the baseline model comprising of age, sex, and race as well as the model containing TIMI risk factors resulted in a nominal improvement in C-statistic in the second cohort (Table 4). However, in the combined cohort addition of the risk score to both models resulted in a significant improvement in the C-statistic (Table 4). Additionally, the risk score resulted in a significant improvement in IDI and a significant change in continuous NRI for both models in the second and combined cohorts (Table 4). Similar findings were observed for the secondary outcome as well (S7 Table).

### Discussion

We report two important findings in this study of subjects with CAD undergoing untargeted high-resolution plasma metabolomic profiling. First, differential regulation of six validated metabolic pathways was associated with all-cause mortality in our cohort. Second, a novel metabolomic risk score was created using seven unique metabolites that was highly predictive of adverse cardiovascular outcomes, improved risk reclassification, and added incremental risk discriminatory value to a risk factor model comprising of traditional cardiovascular risk factors.
Our results indicate that six metabolic pathways (urea cycle/amino group metabolism, tryptophan metabolism, aspartate/asparagine metabolism, lysine metabolism, tyrosine metabolism, and the carnitine shuttle) involved in myocardial energetics and inflammation are associated with mortality among patients with CAD. CAD is a complex condition characterized by inflammation, arterial remodeling and metabolic reprogramming at the level of cardiac myocytes. [31, 32] In this regard, clinical second of the associated metabolic phenotype provides an opportunity to elucidate the end-products of genetic and environmental factors that drive clinical outcomes among patients with CAD. [33] Herein, we have used computational pathway analyses to investigate mechanistic biological pathways that are associated with increased mortality in our cohort. By shifting the focus to metabolite clusters with a collective biological function, our analytical technique reduces false positive findings, improves reproducibility between independent studies, and elucidates potential culprit pathways for preclinical focus. [28, 34]

Under normal conditions, the myocardium derives 50–70% of its energy requirement from mitochondrial β-oxidation of fatty acids. [35–37] However, during ischemia, energy demand-supply mismatch and intracellular acidification results in a metabolic shift to anaerobic, non-glycolytic amino acid substrates that are able to replenish Kreb’s cycle intermediates through a process known as anaplerosis. [38–42] We found that key metabolic pathways—the carnitine shuttle, lysine, aspartate and asparagine, and urea cycle metabolism, representative of this ischemic metabolic adaptation are associated with all-cause mortality in our cohort. Acylcarnitines are lipid intermediates that shuttle fatty acids into mitochondria for β-oxidation, [43] and several prior studies have demonstrated that impaired carnitine shuttle function and elevated acylcarnitine levels predict adverse cardiovascular outcomes and death in CAD patients. [44–47] We were able to validate these findings in our cohort in addition to other novel metabolites.

Our results also indicate that differential regulation of lysine, aspartate and asparagine, and urea cycle is associated with mortality among patients with CAD. 6-N-trimethyllysine (TML) is a precursor in carnitine biosynthesis, [48, 49] however few studies have focused on the role of lysine metabolism in prognosticating CAD outcomes. Loland and colleagues showed that baseline TML levels are associated with angiographic CAD progression in a study of 183

| Table 4. Improvement in all-cause mortality risk discrimination and risk reclassification indices with metabolomic risk score. |
|-------------------------------------------------|-------------------------------------------------|
| Second cohort                                  | Combined cohort                                 |
| Estimate (95% CI)                              | p-value                                         |
| Estimate (95% CI)                              | p-value                                         |
| Baseline C-statistic                           | 0.618 (0.527, 0.710)                            | 0.574 (0.515, 0.634) |
| Delta C-statistic                              | 0.066 (-0.002, 0.135)                           | 0.104 (0.045, 0.163) |
| IDI                                             | 0.097 (0.059, 0.143)                            | 0.097 (0.059, 0.143) |
| NRI                                             | 21.2% (5.8%, 35.3%)                             | 26.3% (18.2%, 35.0%) |
| Baseline C-statistic                           | 0.678 (0.608, 0.748)                            | 0.653 (0.604, 0.703) |
| Delta C-statistic                              | 0.039 (-0.006, 0.086)                           | 0.047 (0.016, 0.078) |
| IDI                                             | 0.084 (0.030, 0.151)                            | 0.084 (0.030, 0.151) |
| NRI                                             | 23.3% (7.9%, 38.2%)                             | 19.9% (11.2%, 29.4%) |

* Model 1 consists of age, sex, and race.  
* Model 2 consists of age (dichotomized at 75 years), current smoking, hypertension, diabetes, HF, PAD, stroke, prior CABG, and eGFR (dichotomized at 60 ml/min/1.73 m²). Abbreviations: CI = confidence interval, IDI = Integrated Discrimination Index, and NRI = Net Reclassification Index.

https://doi.org/10.1371/journal.pone.0237579.t004
patients. Aspartate and asparagine are key metabolic sinks for excess Kreb’s cycle intermediates produced during anaplerosis. Preclinical studies have shown that inhibition of aspartate utilization reduces ischemic resilience, and that inclusion of aspartate in cardioplegic solutions improves post-ischemic recovery. Nevertheless, these findings have not yet been translated into prognostication of adverse outcomes in CAD patients. The urea cycle functions to excrete excess ammonia generated from amino acid catabolism. Prior studies by Shah et al. and Amin et al. demonstrated that patients with CAD differentially regulate urea cycle metabolism compared to healthy controls.

Dysregulated tyrosine and tryptophan metabolic pathways were also predictive of mortality in our cohort. Interferon gamma (IFN-γ), an inflammatory cytokine that plays a key role in the development and progression of CAD regulates tryptophan and tyrosine metabolism. Tryptophan is catabolized to kynurenine by Indoleamine 2,3-Dioxygenase, a rate limiting enzyme that is induced by IFN-γ. The resultant high kynurenine:tryptophan ratio has previously been shown to correlate with the presence of CAD, and to predict major coronary events and all-cause mortality in stable CAD patients. Tyrosine, a precursor for catecholamine biosynthesis, is synthesized from phenylalanine via phenylalanine hydroxylase (PAH). Oxidative stress in IFN-γ stimulated macrophages reduces PAH bioavailability, diminishing tyrosine production. In a large cohort of CAD patients, Murr et al. showed that elevated Phenylalanine:Tyrosine ratios correlate with high sensitivity CRP levels, a well-established biomarker of adverse cardiovascular outcomes. Alterations in catecholamine biosynthesis and consequent neurohormonal dysregulation could represent an additional mechanism by which dysregulated tyrosine metabolism affects mortality in CAD patients.

From a risk prediction perspective, it is well established that patients with stable CAD vary in their risk of mortality and personalizing risk assessment by identifying those at high-risk of adverse outcomes has been an area of active research interest. The TIMI risk score for secondary prevention predicts adverse outcomes among patients with known atherosclerotic vascular disease. This risk score consists of nine easily measurable clinical variables (age, current smoking, hypertension, diabetes, heart failure, stroke, prior coronary artery bypass grafting, peripheral artery disease, and chronic kidney disease) and has been shown to perform well in external validation studies. However, this approach does not identify individual plasma metabolites or the dysregulated metabolic pathways associated with mortality in this high-risk patient population. In addition to identifying the six significant metabolic pathways described above, we identified a unique subset of seven metabolites that was identified using stringent statistical criteria and these were used to create a novel metabolomic risk score. This risk score independently predicted mortality with each absolute unit increase in its value. This independent association was not attenuated after adjusting for cardiovascular biomarkers that represent systemic inflammation (hs-CRP), subclinical myocardial injury (hs-cTnI), or myocyte stretch (NT-proBNP), and can be useful for prognosticating outcomes among patients with CAD. Furthermore, adding the risk score to a model containing TIMI risk factors improved risk discrimination and reclassification indices. Once externally validated, this novel risk score may serve as an important tool for personalizing risk assessment among patients with CAD.

**Strengths and limitations**

Strengths of our study include a high-risk patient population analyzed as two independent cohorts. Our untargeted high-resolution plasma metabolomic profiling technique is unique and the measurement of each metabolite in triplicate make our observations robust. Finally,
the significant metabolic pathways and individual metabolites identified in our study along with the novel metabolomic risk score were internally validated.

Limitations of the study include a modest sample size and the lack of annotation for several unique metabolites associated with mortality. However, we have ascertained validated metabolic pathways that contain these metabolites and thus used them in our analysis. Our cohort consists of subjects living in Southeastern US and thus our findings may not be generalizable to other geographical regions. Most importantly, our findings pertaining to metabolic pathways and the risk predictive value of metabolomic risk need replication in external cohorts.

Conclusions

The differential regulation of six metabolic pathways involved in myocardial energetics and inflammation, identified using untargeted high-resolution plasma metabolomic profiling is associated with all-cause mortality among patients with CAD. A novel metabolomic risk score consisting of metabolites associated with all-cause mortality in our cohort is highly predictive of mortality and holds promise for serving as a tool that prognosticates outcomes in this high-risk patient population.

Supporting information

S1 Fig. Principal component analysis plots of study samples and quality control samples.
(DOCX)

S2 Fig. Study design.
(DOCX)

S3 Fig. A. Kaplan–Meier survival curves for cardiovascular death among second cohort subjects stratified using metabolomic risk score categories above and below median. B. Kaplan–Meier survival curves for cardiovascular death among second cohort subjects stratified using metabolomic risk score categories of highest, middle, and lowest tertile. C. Kaplan–Meier survival curves for cardiovascular death among all subjects stratified using metabolomic risk score categories above and below median. D. Kaplan–Meier survival curves for cardiovascular death among all subjects stratified using metabolomic risk score categories of highest, middle, and lowest tertile.
(DOCX)

S4 Fig. Metabolomic risk score calibration in the second cohort.
(DOCX)

S1 Table. STROBE statement.
(DOCX)

S2 Table. Baseline characteristics of cases and controls in the first and second cohorts.
(DOCX)

S3 Table. Metabolic pathways associated with all-cause mortality in the first and second cohorts.
(DOCX)

S4 Table. Association of significant metabolites and metabolomic risk score with cardiovascular biomarkers.
(DOCX)
S5 Table. Association of metabolomic risk score with death after adjustment for cardiovascular biomarkers in the second and combined cohorts.

(DOCX)

S6 Table. Association of metabolomic risk score with cardiovascular death in the second and combined cohorts.

(DOCX)

S7 Table. Improvement in cardiovascular death risk discrimination and risk reclassification indices with metabolomic risk score.

(DOCX)

Acknowledgments

The authors would like to acknowledge the Emory Cardiovascular Biobank participants and study coordinators

Author Contributions

Conceptualization: Anurag Mehta, Yi-An Ko, Salim S. Hayek, Karan Uppal, Dean P. Jones, Arshed A. Quyyumi.

Data curation: Anurag Mehta, Chang Liu, Ayman S. Tahhan, Devinder S. Dhindsa, Jeong Hwan Kim, Salim S. Hayek, Laurence S. Sperling, Puja K. Mehta, Yan V. Sun, Karan Uppal, Dean P. Jones, Arshed A. Quyyumi.

Formal analysis: Chang Liu, Aditi Nayak, Yi-An Ko, Karan Uppal, Dean P. Jones.

Funding acquisition: Dean P. Jones, Arshed A. Quyyumi.

Investigation: Anurag Mehta, Devinder S. Dhindsa, Jeong Hwan Kim, Salim S. Hayek, Laurence S. Sperling, Puja K. Mehta, Yan V. Sun, Karan Uppal, Dean P. Jones, Arshed A. Quyyumi.

Methodology: Anurag Mehta, Chang Liu, Aditi Nayak, Ayman S. Tahhan, Yi-An Ko, Puja K. Mehta, Yan V. Sun, Karan Uppal, Dean P. Jones.

Project administration: Arshed A. Quyyumi.

Resources: Arshed A. Quyyumi.

Software: Chang Liu, Karan Uppal.

Supervision: Arshed A. Quyyumi.

Writing – original draft: Anurag Mehta, Chang Liu, Aditi Nayak, Ayman S. Tahhan, Arshed A. Quyyumi.

Writing – review & editing: Yi-An Ko, Devinder S. Dhindsa, Jeong Hwan Kim, Salim S. Hayek, Laurence S. Sperling, Puja K. Mehta, Yan V. Sun, Karan Uppal, Dean P. Jones.

References

1. Benjamin EJ, Muntner P, Alonso A, Bittencourt MS, Callaway CW, Carson AP, et al. Heart Disease and Stroke Statistics-2019 Update: A Report From the American Heart Association. Circulation. 2019; 139(10):e56–e66. https://doi.org/10.1161/CIR.0000000000006699 PMID: 30700139

2. Bohula EA, Bonacca MP, Braunwald E, Aylward PE, Corbalan R, De Ferrari GM, et al. Atherothrombotic Risk Stratification and the Efficacy and Safety of Vorapaxar in Patients With Stable Ischemic Heart
2. Patel KV, Pandey A, de Lemos JA. Conceptual Framework for Addressing Residual Atherosclerotic Cardiovascular Disease Risk in the Era of Precision Medicine. Circulation. 2018; 137(24):2551–53. https://doi.org/10.1161/CIRCULATIONAHA.118.035289 PMID: 29643058

3. Cheng S, Shah SH, Corwin EJ, Fiehn O, Fitzgerald RL, Gerszten RE, et al. Potential Impact and Study Considerations of Metabolomics in Cardiovascular Health and Disease: A Scientific Statement From the American Heart Association. Circ Cardiovasc Genet. 2017; 10(2).

4. Tang WH, Wang Z, Cho L, Brennan DM, Hazen SL. Diminished global arginine bioavailability and increased arginine catabolism as metabolic profile of increased cardiovascular risk. J Am Coll Cardiol. 2009; 53(22):2061–7. https://doi.org/10.1016/j.jacc.2009.02.036 PMID: 19477356

5. Fan Y, Li Y, Chen Y, Zhao YJ, Liu LW, Li J, et al. Comprehensive Metabolomic Characterization of Coronary Artery Diseases. J Am Coll Cardiol. 2016; 68(12):1281–93. https://doi.org/10.1016/j.jacc.2016.06.044 PMID: 27634119

6. Schrimpe-Rutledge AC, Codreanu SG, Sherrod SD, McLean JA. Untargeted Metabolomics Strategies-Challenges and Emerging Directions. J Am Soc Mass Spectrom. 2016; 27(12):1897–905. https://doi.org/10.1007/s13361-016-1469-y PMID: 27624161

7. Ko YA, Hayek S, Sandesara P, Samman Tahhan A, Quyyumi A. Cohort profile: the Emory Cardiovascular Biobank (EmCAB). BMJ Open. 2017; 7(12):e018753. https://doi.org/10.1136/bmjopen-2017-018753 PMID: 29288185

8. Levey AS, Stevens LA, Schmid CH, Zhang YL, Castro AF 3rd, Feldman HI, et al. A new equation to estimate glomerular filtration rate. Ann Intern Med. 2009; 150(9):604–12. https://doi.org/10.7326/0003-4819-150-9-200905050-00006 PMID: 19414839

9. Thygesen K, Alpert JS, Jaffe AS, Simoons ML, Chaitman BR, White HD, Writing Group on the Joint ESC/ACCF/AHA/WHFT/HeFT/IdS, Thygesen K, Alpert JS, White HD, et al. Third universal definition of myocardial infarction. Eur Heart J. 2012; 33(20):2551–67. https://doi.org/10.1093/eurheartj/ehs184 PMID: 22922414

10. Go YM, Walker DI, Liang Y, Uppal K, Soltow QA, Tran V, et al. Reference Standardization for Mass Spectrometry and High-resolution Metabolomics Applications to Exposure Research. Toxicol Sci. 2015; 148(2):531–43. https://doi.org/10.1093/toxsci/kfv198 PMID: 26358001

11. Johnson JM, Yu T, Strobel FH, Jones DP. A practical approach to detect unique metabolic patterns for personalized medicine. Analyst. 2010; 135(11):2864–70. https://doi.org/10.1039/c0an00333f PMID: 20838665

12. Osborn MP, Park Y, Parks MB, Burgess LG, Uppal K, Lee K, et al. Metabolome-wide association study of neovascular age-related macular degeneration. PLoS One. 2013; 8(8):e72737. https://doi.org/10.1371/journal.pone.0072737

13. Soltow QA, Strobel FH, Mansfield KG, Wachtman L, Park Y, Jones DP. High-performance metabolic profiling with dual chromatography-Fourier-transform mass spectrometry (DC-FTMS) for study of the exposome. Metabolomics. 2013; 9(1 Suppl):S132–S43. https://doi.org/10.1007/s11306-011-0332-1 PMID: 26229523

14. Frediani JK, Jones DP, Tukvadze N, Uppal K, Sanikidze E, Kipiani M, et al. Plasma metabolomics in human pulmonary tuberculosis disease: a pilot study. PLoS One. 2014; 9(10):e108854. https://doi.org/10.1371/journal.pone.0108854

15. Burgess LG, Uppal K, Walker DI, Roberson RM, Tran V, Parks MB, et al. Metabolome-Wide Association Study of Primary Open Angle Glaucoma. Invest Ophthalmol Vis Sci. 2015; 56(8):5020–8. https://doi.org/10.1017/iogs.2015.23529995

16. Mitchell SL, Uppal K, Williamson SM, Liu K, Burgess LG, Tran V, et al. The Carnitine Shuttle Pathway is Altered in Patients With Neovascular Age-Related Macular Degeneration. Invest Ophthalmol Vis Sci. 2018; 59(12):4978–85. https://doi.org/10.1167/iovs.18-25137 PMID: 30326066

17. Roede JR, Uppal K, Park Y, Lee K, Tran V, Walker D, et al. Serum metabolomics of slow vs. rapid motor progression Parkinson’s disease: a pilot study. PLoS One. 2013; 8(10):e77629. https://doi.org/10.1371/journal.pone.0077629 PMID: 24167579

18. Smith CA, Want EJ, O’Maille G, Abagyan R, Siuzdak G. XCMS: processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. Anal Chem. 2006; 78 (3):779–87. https://doi.org/10.1021/ac051437y PMID: 16448051

19. Tautenhahn R, Bottcher C, Neumann S. Highly sensitive feature detection for high resolution LC/MS. BMC Bioinformatics. 2008; 9:504. https://doi.org/10.1186/1471-2105-9-504 PMID: 19040729
21. Benton HP, Want EJ, Ebbels TM. Correction of mass calibration gaps in liquid chromatography-mass spectrometry metabolomics data. Bioinformatics. 2010; 26(19):2488–9. https://doi.org/10.1093/bioinformatics/btq441 PMID: 20671148

22. Ruttkies C, Schymanski EL, Wolf S, Hollender J, Neumann S. MetFrag relaunched: incorporating strategies beyond in silico fragmentation. J Cheminform. 2016; 8:3. https://doi.org/10.1186/s13321-016-0115-9 PMID: 26834843

23. Yu T, Park Y, Johnson JM, Jones DP. apLCMS—adaptive processing of high-resolution LC/MS data. Bioinformatics. 2009; 25(15):1930–6. https://doi.org/10.1093/bioinformatics/btp291 PMID: 19414529

24. Patel RS, Ghasemzadeh N, Eapen DJ, Sher S, Arshad S, Ko YA, et al. Novel Biomarker of Oxidative Stress Is Associated With Risk of Death in Patients With Coronary Artery Disease. Circulation. 2016; 133(4):361–9. https://doi.org/10.1161/CIRCULATIONAHA.115.019790 PMID: 26673559

25. Benjamin Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society Series B (Methodological). 1995; 57(1):289–300.

26. Uppal K, Walker DI, Jones DP. xMSannotator: An R Package for Network-Based Annotation of High-Resolution Metabolomics Data. Anal Chem. 2017; 89(2):1063–67. https://doi.org/10.1021/acs.analchem.6b01214 PMID: 27977166

27. Schymanski EL, Jeon J, Gulde R, Fenner K, Ruff M, Singer HP, et al. Identifying small molecules via high resolution mass spectrometry: communicating confidence. Environ Sci Technol. 2014; 48(4):2097–8. https://doi.org/10.1021/es5002105 PMID: 24476540

28. Li S, Park Y, Duraisingham S, Strobel FH, Khan N, Soltow QA, et al. Predicting network activity from high throughput metabolomics. PLoS computational biology. 2013; 9(7):e1003123. https://doi.org/10.1371/journal.pcbi.1003123 PMID: 23861661

29. Tibshirani R. Regression Shrinkage and Selection via the Lasso. Journal of the Royal Statistical Society Series B (Methodological). 1996; 58(1):267–88.

30. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res. 2015; 43(7):e47. https://doi.org/10.1093/nar/gkv007 PMID: 25605792

31. Libby P, Theroux P. Pathophysiology of coronary artery disease. Circulation. 2005; 111(25):3481–8. https://doi.org/10.1161/CIRCULATIONAHA.105.537878 PMID: 15983262

32. Heusch G, Libby P, Gersh B, Yellon D, Bohm M, Lopaschuk G, et al. Cardiovascular remodelling in coronary artery disease and heart failure. Lancet. 2014; 383(9932):1933–43. https://doi.org/10.1016/S0140-6736(14)60107-0 PMID: 24831770

33. Holmes E, Wilson ID, Nicholson JK. Metabolic phenotyping in health and disease. Cell. 2008; 134(5):714–7. https://doi.org/10.1016/j.cell.2008.08.026 PMID: 18775301

34. Chong J, Soufan O, Li C, Caraus I, Li S, Bourque G, et al. MetaboAnalyst 4.0: towards more transparent and integrative metabolomics analysis. Nucleic Acids Res. 2018; 46(W1):W486–W94. https://doi.org/10.1093/nar/gky310 PMID: 29762782

35. Lopaschuk GD, Ussher JR, Holmes CD, Jaswal JS, Stanley WC. Myocardial fatty acid metabolism in health and disease. Physiological reviews. 2010; 90(1):207–90. https://doi.org/10.1152/physrev.00015.2009 PMID: 20086077

36. Fillmore N, Mori J, Lopaschuk G. Mitochondrial fatty acid oxidation alterations in heart failure, ischaemic heart disease and diabetic cardiomyopathy. British journal of pharmacology. 2014; 171(8):2080–90. https://doi.org/10.1111/bph.12475 PMID: 24147975

37. Folmes CD, Sohaw D, Clanachan AS, Lopaschuk GD. High rates of residual fatty acid oxidation during mild ischemia decrease cardiac work and efficiency. Journal of molecular and cellular cardioiology. 2009; 47(1):142–48. https://doi.org/10.1016/j.yjmcc.2009.03.005 PMID: 19303418

38. Taegtmeyer H, Harinstein ME, Gheorghiade M. More than bricks and mortar: comments on protein and amino acid metabolism in the heart. The American journal of cardiology. 2008; 101(11):S3–S7.

39. Drake KJ, Sidarov VY, McGuinness OP, Wasserman DH, Wikswo JP. Amino acids as metabolic substrates during cardiac ischemia. Experimental Biology and Medicine. 2012; 237(12):1369–78. https://doi.org/10.1258/ebm.2012.012025 PMID: 23354395

40. McDonald TF, MacLeod D. Metabolism and the electrical activity of anoxic ventricular muscle. The Journal of physiology. 1973; 229(3):559–82. https://doi.org/10.1113/jphysiol.1973.sp010154 PMID: 4693674

41. Des Rosiers C, Labarthe F, Lloyd SG, Chatham JC. Cardiac anaplerosis in health and disease: food for thought. Cardiovascular research. 2011; 90(2):210–19. https://doi.org/10.1093/cvr/cvr055 PMID: 21398307
42. McDonald TF, MacLeod DP. DNP-induced dissipation of ATP in anoxic ventricular muscle. J Physiol. 1973; 229(3):583–99. https://doi.org/10.1113/jphysiol.1973.sp010155 PMID: 4266423

43. Reuter SE, Evans AM. Carnitine and acylcarnitines. Clinical pharmacokinetics. 2012; 51(9):553–72. https://doi.org/10.1007/BF03261931 PMID: 22804748

44. Rizza S, Copetti M, Rossi C, Cianfarani M, Zucchelli M, Luzi A, et al. Metabolomics signature improves the prediction of cardiovascular events in elderly subjects. Atherosclerosis. 2014; 232(2):260–64. https://doi.org/10.1016/j.atherosclerosis.2013.10.029 PMID: 24468136

45. Shah SH, Sun J-L, Stevens RD, Bain JR, Muehlbauer MJ, Pieper KS, et al. Baseline metabolomic profiles predict cardiovascular events in patients at risk for coronary artery disease. American heart journal. 2012; 163(5):844–50. e1. https://doi.org/10.1016/j.ahj.2012.02.005 PMID: 22607863

46. Shah SH, Bain JR, Muehlbauer MJ, Stevens RD, Crosslin DR, Haynes C, et al. Association of a peripheral blood metabolic profile with coronary artery disease and risk of subsequent cardiovascular events. Circulation: Cardiovascular Genetics. 2010; 3(2):207–14. https://doi.org/10.1161/CIRCGENETICS.109.852814 PMID: 20173117

47. Strand E, Pedersen ER, Svingen GF, Olsen T, Bjernadal B, Karlsson T, et al. Serum acylcarnitines and risk of cardiovascular death and acute myocardial infarction in patients with stable angina pectoris. Journal of the American Heart Association. 2017; 6(2):e003620. https://doi.org/10.1161/JAHA.116.003620 PMID: 28159823

48. Bremer J. Carnitine—metabolism and functions. Physiol Rev. 1983; 63(4):1420–80. https://doi.org/10.1152/physrev.1983.63.4.1420 PMID: 6361812

49. Strijsbos K, Vaz FM, Distel B. Enzymology of the carnitine biosynthesis pathway. IUBMB Life. 2010; 62(5):357–62. https://doi.org/10.1002/iub.323 PMID: 20306513

50. Leland KH, Bleie Ø, Borgeraas H, Strand E, Ueland PM, Svardal A, et al. The association between progression of atherosclerosis and the methylated amino acids asymmetric dimethylarginine and trimethyllysine. PLoS One. 2013; 8(5):e64774. https://doi.org/10.1371/journal.pone.0064774 PMID: 23734218

51. Julia P, Young H, Buckberg G, Kofsky E, Bugyi H. Studies of myocardial protection in the immature heart. II. Evidence for importance of amino acid metabolism in tolerance to ischemia. The Journal of thoracic and cardiovascular surgery. 1990; 100(6):888–95. PMID: 22469111

52. Suleiman M, Dihmis W, Caputo M, Angelini G, Bryan A. Changes in myocardial concentration of glutamate and aspartate during coronary artery surgery. American Journal of Physiology -Heart and Circulatory Physiology. 1997; 272(3):H1063–H69.

53. Rosenkranz ER. Substrate enhancement of cardioprotective solution: experimental studies and clinical evaluation. The Annals of thoracic surgery. 1995; 60(3):797–800. https://doi.org/10.1016/0003-4975(95)00456-U PMID: 7677536

54. Nelson DL, Lehniger AL, Cox MM. Lehniger principles of biochemistry: Macmillan; 2008.

55. Amin AM, Mostafa H, Arif NH, Kader M, Hay YK. Metabolomics profiling and pathway analysis of human plasma and urine reveal further insights into the multifactorial nature of Coronary Artery Disease (CAD). Clin Chim Acta. 2019; 493:112–22. https://doi.org/10.1016/j.cca.2019.02.030 PMID: 30826371

56. Robertson A-KL, Rudling M, Zhou X, Gorelik L, Flavell RA, Hansson GK. Disruption of TGF-β signaling in T cells accelerates atherosclerosis. The Journal of clinical investigation. 2003; 112(9):1342–50. https://doi.org/10.1172/JCI18607 PMID: 14568988

57. Ait-Oufella H, Salomon BL, Potteaux S, Robertson A-KL, Gourdy P, Zoll J, et al. Natural regulatory T cells control the development of atherosclerosis in mice. Nature medicine. 2006; 12(2):178. https://doi.org/10.1038/nm1343 PMID: 16462800

58. Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. Nature. 2011; 473(7347):317. https://doi.org/10.1038/nature10146 PMID: 21593864

59. Libby P, Lichtman AH, Hansson GK. Immune effector mechanisms implicated in atherosclerosis: from mice to humans. Immunity. 2013; 38(6):1092–104. https://doi.org/10.1016/j.immuni.2013.06.009 PMID: 23809160

60. Chen Y, Guillemin GJ. Kynurenine pathway metabolites in humans: disease and healthy states. International Journal of Tryptophan Research. 2009; 2:JTR. S2097.

61. Murr C, Grammer TB, Kleber ME, Meinitzer A, März W, Fuchs D. Low serum tryptophan predicts higher mortality in cardiovascular disease. European journal of clinical investigation. 2015; 45(3):247–54. https://doi.org/10.1111/eji.12402 PMID: 25586781

62. Werner ER, Bitterlich G, Fuchs D, Hausen A, Reibnegger G, Szabo G, et al. Human macrophages degrade tryptophan upon induction by interferon-gamma. Life sciences. 1987; 41(3):273–80. https://doi.org/10.1016/0024-3205(87)90149-4 PMID: 3110526

63. Weiss G, Murr C, Zoller H, Haun M, Widner B, Ludescher C, et al. Modulation of neopterin formation and tryptophan degradation by Th1- and Th2-derived cytokines in human monocycte cells. Clinical and
experimental immunology. 1999; 116(3):435. https://doi.org/10.1046/j.1365-2249.1999.00910.x PMID: 10361231

64. Munn DH, Shafizadeh E, Attwood JT, Bondarev I, Pashine A, Mellor AL. Inhibition of T cell proliferation by macrophage tryptophan catabolism. Journal of Experimental Medicine. 1999; 189(9):1363–72. https://doi.org/10.1084/jem.199.9.1363 PMID: 10224276

65. Hwu P, Du MX, Lapointe R, Do M, Taylor MW, Young HA. Indoleamine 2, 3-dioxygenase production by human dendritic cells results in the inhibition of T cell proliferation. The Journal of Immunology, 2000; 164(7):3596–99. https://doi.org/10.4049/jimmunol.164.7.3596 PMID: 10725715

66. Terness P, Bauer TM, Röse L, Duffer C, Watzlik A, Simon H, et al. Inhibition of allogeneic T cell proliferation by indoleamine 2, 3-dioxygenase-expressing dendritic cells: mediation of suppression by tryptophan metabolites. Journal of Experimental Medicine. 2002; 196(4):447–57. https://doi.org/10.1084/jem.20020052 PMID: 12186837

67. Wirleitner B, Rudzite V, Neurauter G, Murr C, Kainins U, Erglis A, et al. Immune activation and degradation of tryptophan in coronary heart disease. European journal of clinical investigation. 2003; 33(7):550–54. https://doi.org/10.1046/j.1365-2362.2003.01186.x PMID: 12814390

68. Pedersen ER, Midttun Ø, Ueland PM, Schartum-Hansen H, Seifert R, Igland J, et al. Systemic markers of interferon-γ-mediated immune activation and long-term prognosis in patients with stable coronary artery disease. Arteriosclerosis, Thrombosis, and Vascular Biology. 2011; 31(3):698–704. https://doi.org/10.1161/ATVBAHA.110.219329 PMID: 21183733

69. Koppel JD. Phenylalanine and tyrosine metabolism in chronic kidney failure. The Journal of nutrition. 2007; 137(6):1586S–90S.

70. Fuchs D, Baier-Bitterlich G, Wede I, Wachter HJCHMS. Reactive oxygen and apoptosis. 1997; 34:139–68.

71. Nathan CF, Murray HW, Wiebe M, Rubin BY. Identification of interferon-γ as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. Journal of Experimental Medicine. 1983; 158(3):670–89. https://doi.org/10.1084/jem.158.3.670 PMID: 6411853

72. Mur C, Grammer TB, Meinitzer A, Kleber ME, März W, Fuchs D. Immune activation and inflammation in patients with cardiovascular disease are associated with higher phenylalanine to tyrosine ratios: the ludwigshafen risk and cardiovascular health study. Journal of amino acids. 2014; 2014.

73. Koenig W. High-sensitivity C-reactive protein and atherosclerotic disease: from improved risk prediction to risk-guided therapy. International journal of cardiology. 2013; 168(6):5126–34. https://doi.org/10.1016/j.ijcard.2013.07.113 PMID: 23978367

74. Shitara J, Ogita M, Wada H, Tsuboi S, Endo H, Doi S, et al. Clinical impact of high-sensitivity C-reactive protein during follow-up on long-term adverse clinical outcomes in patients with coronary artery disease treated with percutaneous coronary intervention. Journal of cardioiology. 2019; 73(1):45–50. https://doi.org/10.1016/j.jcc.2018.06.002 PMID: 30001869

75. Yin J, Wang Y, Hu H, Li X, Xue M, Cheng W, et al. P2X7 receptor inhibition attenuated sympathetic nerve sprouting after myocardial infarction via the NLRP3/IL-1β pathway. Journal of cellular and molecular biology. 2017; 21(11):2695–710. https://doi.org/10.1111/jccm.13185 PMID: 28470940

76. Yang N, Cheng W, Hu H, Xue M, Li X, Wang Y, et al. Atorvastatin attenuates sympathetic hyperinnervation together with the augmentation of M2 macrophages in rats postmyocardial infarction. Cardiovascular therapeutics. 2016; 34(4):234–44. https://doi.org/10.1111/1755-5922.12193 PMID: 27149420

77. Gomes A, Correia G, Coelho M, Araújo JR, Pinho MJ, Teixeira AL, et al. Dietary unsaturated fatty acids differently affect catecholamine handling by adrenal chromaffin cells. The Journal of nutritional biochemistry. 2015; 26(5):563–70. https://doi.org/10.1016/j.jnutbio.2014.12.009 PMID: 25727966

78. Mok Y, Ballew SH, Bash LD, Bhatt DL, Boden WE, Bonaca MP, et al. International Validation of the Thrombolysis in Myocardial Infarction (TIMI) Risk Score for Secondary Prevention in Post-MI Patients: A Collaborative Analysis of the Chronic Kidney Disease Prognosis Consortium and the Risk Validation Scientific Committee. J Am Heart Assoc. 2018; 7(14).

79. Puymirat E, Bonaca M, Fumery M, Tea V, Aissaoui N, Lemesles G, et al. Atherothrombotic risk stratification after acute myocardial infarction: The Thrombolysis in Myocardial Infarction Risk Score for Secondary Prevention in the light of the French Registry of Acute ST Elevation or non-ST Elevation Myocardial Infarction registries. Clin Cardiol. 2019; 42(2):227–34. https://doi.org/10.1002/clc.23131 PMID: 30536449

80. Rickers PM. High-sensitivity C-reactive protein, inflammation, and cardiovascular risk: from concept to clinical practice to clinical benefit. Am Heart J. 2004; 148(1 Suppl):S19–26. https://doi.org/10.1016/j.ahj.2004.04.028 PMID: 15211329

81. McCarthy CP, Raber I, Chapman AR, Sandoval Y, Apple FS, Mills NL, et al. Myocardial Injury in the Era of High-Sensitivity Cardiac Troponin Assays: A Practical Approach for Clinicians. JAMA Cardiol. 2019.
82. Hall C. NT-ProBNP: the mechanism behind the marker. J Card Fail. 2005; 11(5 Suppl):S81–3. https://doi.org/10.1016/j.cardfail.2005.04.019 PMID: 15948107

83. McCarthy CP, McEvoy JW, Januzzi JL Jr, Biomarkers in stable coronary artery disease. Am Heart J. 2018; 196:82–96. https://doi.org/10.1016/j.ahj.2017.10.016 PMID: 29421018