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A Diabetes-Predictive Amino Acid Score and Future Cardiovascular Disease

Martin Magnusson[1,2][*], Gregory D. Lewis[3][*], Ulrika Ericson[4], Marju Orho-Melander[5], Bo Hedblad, [2,6], Gunnar Engström[2], Gerd Östling[2], Clary Clish[7], Thomas J. Wang, [8,9][†] Robert E. Gerszten, and [4,8,9][†] Olle Melander[2,6][†]

[*],[†] denotes equal author contributions

Author Affiliations:

[1]Department of Cardiology, Skane University Hospital, Malmö, Sweden; [2]Department of Clinical Sciences, Lund University, Malmö, Sweden; [3]Center for Immunology and Inflammatory Diseases, Massachusetts General Hospital, Harvard Medical School, Boston, MA; [4]Department of Clinical Sciences, Nutrition Epidemiology, Lund University, Malmö, Sweden; [5]Department of Clinical Sciences, Diabetes and Cardiovascular Disease–Genetic Epidemiology, Lund University, Malmö, Sweden; [6]Center of Emergency Medicine, Skane University Hospital, Malmö, Sweden; [7]The Broad Institute of MIT and Harvard, Cambridge, MA; [8]Cardiology Division, Massachusetts General Hospital, Harvard Medical School, Boston, MA; and [9]Cardiovascular Research Center, Massachusetts General Hospital, Harvard Medical School, Boston, MA

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Address for Correspondence:

Martin Magnusson, MD, PhD. Department of Cardiology, Entrance 35, floor 2, Skane University Hospital, SE 205 02 Malmö, Sweden. email: martin.magnusson@med.lu.se.

Telephone: +46-40-337274, Fax: +46-40-336209

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ABSTRACT:

Aims: We recently identified a metabolic signature of 3 amino acids (tyrosine, phenylalanine and isoleucine) that strongly predicts diabetes development. As novel modifiable targets for intervention are needed to meet the expected increase of cardiovascular disease (CVD) caused by the diabetes epidemic, we investigated whether this diabetes predictive amino acid score (DM-AA-score) predicts development of CVD and its functional consequences.

Methods and results: We performed a matched case-control study derived from the population-based Malmö Diet and Cancer Cardiovascular Cohort (MDC-CC), all free of CVD. During 12 years of follow-up, 253 individuals developed CVD and were matched for age, sex and Framingham risk score with 253 controls. Amino acids were profiled in baseline plasma samples using liquid chromatography-tandem mass spectrometry and relationship to incident CVD was assessed using conditional logistic regression. We further examined whether the amino acid score also correlated with anatomical (intima-media thickness and plaque formation) and functional (exercise-induced myocardial ischemia) abnormalities.

Compared to the lowest quartile of the DM-AA-score, the odds ratio (95% confidence interval) for incident CVD in subjects belonging to quartiles 2, 3 and 4 was 1.27 (0.72-2.22), 1.96 (1.07-3.60) and 2.20 (1.12-4.31) (P_{trend}= 0.010) after multivariate adjustment. Increasing quartile of the DM-AA-score was cross-sectionally related to carotid intima-media thickness (P_{trend}=0.037) and with presence of at least one plaque larger than 10 mm^2 (P_{trend}=0.001). As compared to the lowest quartile of the DM-AA-score, the odds ratio (95% confidence interval) for inducible ischemia in subjects belonging to quartiles 2, 3 and 4 was 3.31 (1.05-10.4), 4.24 (1.36-13.3), 4.86 (1.47-16.1) (P_{trend}= 0.011).

Conclusions: This study identifies branched chain and aromatic amino acids as novel markers of CVD development and as an early link between diabetes and CVD susceptibility.

INTRODUCTION

Diabetes and its cardiovascular complications remain major causes of morbidity and mortality and the prevalence of diabetes continues to increase in epidemic proportions. [1] Although much effort has been put forth to prevent diabetes related complications by more intensive glucose lowering therapies, the results regarding cardiovascular disease (CVD) complications have been disappointing. [2, 3] This stresses the importance of identifying new pathophysiological pathways to explain the diabetes-related increase in CVD risk.

Using high throughput plasma metabolite profiling, we recently demonstrated that plasma levels of five branched-chained and aromatic amino acids (leucine, valine, isoleucine, tyrosine and phenylalanine), and in particular a score of three of these amino acids (tyrosine, phenylalanine, and isoleucine), strongly predict the risk of future type 2 diabetes. Abnormalities in these amino acids were observed up to 12 years before disease onset in two different populations, and prediction was independent of glucose levels and other established diabetes risk factors. Individuals belonging to the top versus the bottom quartile of the score based on tyrosine, phenylalanine and isoleucine (DM-AA-score) had a 4-6 fold increased risk of developing diabetes later in life. [4] Even though the relationship between the amino acids and diabetes risk was independent of measures of insulin resistance, the strong cross-sectional correlations with insulin resistance suggested that elevated levels might signal not only increased risk of future diabetes but also increased risk of CVD. In this context and given the strong but largely unexplained link between diabetes and CVD susceptibility, we tested whether the DM-AA-score is associated with subclinical measures of atherosclerosis and future development of CVD. We also evaluated whether the DM-AA-score predicted the functional consequences of CVD by examining the DM-AA-score in an independent group of subjects undergoing exercise tolerance testing to diagnose myocardial ischemia.

METHODS

Study samples

The Malmö Diet and Cancer (MDC) study is a population-based, prospective epidemiologic cohort of 28,449 persons enrolled between 1991 and 1996. From this cohort, 6,103 persons were randomly selected to participate in the MDC Cardiovascular Cohort, (MDC-CC) which was designed to investigate the epidemiology of carotid artery disease. [5, 6] Of MDC-CC participants, fasting plasma samples were available in 5,400 subjects of whom we excluded 143 subjects who had CVD prior to the baseline exam. Of the remaining subjects, 680 had missing values on one or more covariates used in the multivariate models, leaving 4577 subjects eligible for inclusion in the nested case-control study. During a mean follow-up time of 12.2 (2.3) years, 364 first incident CVD (myocardial infarction or stroke) events occurred. We matched incident CVD cases with CVD free control subjects based on gender, age (± 1 year) and Framingham risk score [7] (<0·1 % difference in 10 year estimated risk) and also required that the follow-up time of the control was at least as long as that of the corresponding incident CVD case. These criteria resulted in successful matching of 253 incident CVD cases with 253 control subjects.

In cross sectional analyses we used the incident CVD case-control material from MDC-CC (n=506) and added MDC-CC subjects included in the incident diabetes case-control recently published. [4] From this added study sample (n=326) we excluded 6 subjects with CVD prior to the baseline exam. Furthermore, 35 subjects were included in both studies, resulting in a total of 791 subjects free from prevalent CVD for cross sectional analyses of DM-AA-score in relation to intima-media thickness (IMT) of the common carotid artery (CCA). Finally, from June 1992, classification of plaque burden from the common carotid artery up to and including the bulb and 1 cm of the internal carotid artery was introduced in the MDC-CC

using a 6-graded plaque score. Out of the 791 subjects, 564 had been classified using this 6-graded plaque score.

A distinct cohort of subjects who underwent exercise stress testing with myocardial perfusion imaging at Massachusetts General Hospital (MGH) underwent metabolic profiling. Patients who underwent pharmacological testing were excluded. All patients with inducible ischemia were selected for metabolic profiling (cases, N=83) in addition to a control cohort consisting 83 subjects of similar age and gender who did not have evidence of inducible ischemia. The study protocols were approved by the Institutional Review Boards of Lund University, Sweden and the Massachusetts General Hospital. All study participants provided written informed consent.

Metabolic profiling

Given the relatively high analytical demands of LC-MS profiling we were not able to analyze the entire cohort; instead we used a matched case-control approach to maximize efficiency. Plasma metabolites were profiled in EDTA plasma samples drawn at the baseline exam in the MDC-CC, and drawn immediately before the start of exercise testing in the MGH exercise cohort. Samples were stored at -80˚ C and profiled using liquid chromatography-tandem mass spectrometry (LC-MS) as described in detail previously. [4] Formic acid, ammonium acetate, LC-MS grade solvents, and valine-d8 were purchased from Sigma-Aldrich (St. Louis, MO). The remainder of the isotopically-labeled analytical standards were purchased from Cambridge Isotope Labs, Inc (Andover, MA). Plasma samples (10 µL) were prepared for LC-MS analyses via protein precipitation with the addition of 9 volumes of 74.9:24.9:0.2 v/v/v acetonitrile/methanol/formic acid containing two additional stable isotope-labeled internal standards for valine-d8 and phenylalanine-d8. The samples were centrifuged (10 min, 10,000 rpm, 4ºC) and the supernatants were injected directly. For all isotope standards used, peak areas were >2 orders of magnitude above the lower limit of quantification (as defined as a

discrete peak 10-fold greater than noise) and fell well within the linear range of the dose-response relationship.

LC-MS data were acquired using a 4000 QTRAP triple quadrupole mass spectrometer (Applied Biosystems/Sciex; Foster City, CA) that was coupled to a multiplexed LC system comprised of two 1200 Series pumps (Agilent Technologies; Santa Clara CA) and an HTS PAL autosampler (Leap Technologies; Carrboro, NC) equipped with 2 injection ports and a column selection valve. The two pumps were similarly configured for hydrophilic interaction chromatography (HILIC) using 150 x 2.1 mm Atlantis HILIC columns (Waters; Milford, MA) and with the same mobile phases (mobile phase A: 10 mM ammonium formate and 0·1% formic acid, v/v; mobile phase B: acetonitrile with 0.1% formic acid, v/v). Multiplexing was used to enable the measurement of 61 metabolite transitions divided between the 2 LC systems, and each sample was injected once on each. Each column was eluted isocratically with 5% mobile phase A for 1 minute followed by a linear gradient to 60% mobile phase A over 10 minutes. MS analyses were carried out using electrospray ionization (ESI) and multiple reaction monitoring (MRM) scans in the positive ion mode. Declustering potentials and collision energies were optimized for each metabolite by infusion of reference standards prior to sample analyses. The dwell time for each transition was 30 ms, the ion spray voltage was 4.5 kV, and the source temperature was 425ºC. Internal standard peak areas were monitored for quality control and individual samples with peak areas differing from the group mean by more than 2 standard deviations were re-analyzed. MultiQuant software (Version 1.1; Applied Biosystems/Sciex; Foster City, CA) was used for automated peak integration and metabolite peaks were manually reviewed for quality of integration and compared against a known standard to confirm identity.

Clinical assessment

Clinical characteristics of the study populations at the baseline exam are shown in Table 1 (MDC) and Supplementary Table 1 (MGH). MDC and MGH participants underwent similar standardized medical histories with physical examinations and laboratory assessments. Blood pressure was obtained after 10 minutes of rest in the supine position in MDC, and upright on the treadmill immediately prior to exercise in the MGH exercise cohort. We calculated the body mass index (BMI) as weight in kilograms divided by the square of the height in meters. Hypertension was defined as systolic blood pressure (SBP) ≥ 140 mmHg or diastolic blood pressure (DBP) ≥ 90 mmHg, or use of AHT. Diabetes was defined as whole fasting blood glucose >109 mg/dl (>6.0 mmol/L), a self-reported physician diagnosis of diabetes, or use of anti-diabetic medication. Cigarette smoking was elicited by a self-administered questionnaire, with current cigarette smoking defined as any use within the past year. In the MDC cohort, we measured fasting insulin, fasting total cholesterol, fasting HDL cholesterol, and fasting triglycerides according to standard procedures at the Department of Clinical Chemistry, University Hospital Malmö. LDL cholesterol was calculated according to Friedewald's formula. C-reactive protein was measured by high-sensitivity assay (Roche Diagnostics, Basel, Switzerland). We used the Homeostatic Model Assessment of insulin resistance (HOMA-index). [8]

Classification of cardiovascular events

The procedure for case retrieval has been described previously. [6, 9] Briefly, the Swedish National Hospital Discharge and Cause of Death Registries (The National Board of Health and Welfare) [10] and the Stroke registry of Malmo [11] were used. The ascertainment of cases and validity of these registries has been shown to be high. [10, 11] A CVD event was defined as fatal or nonfatal myocardial infarction on the basis of *International Classification of Diseases* 9th and 10th Revisions (ICD9 and ICD10) codes 410 and I21, respectively, fatal or nonfatal stroke defined using codes 430, 431, 434 and 436 (ICD9) and I60, I61, I63, and I64 (ICD10),

or death attributable to CHD on the basis of codes 412 and 414 (ICD9) or I22-I23 and I25 (ICD10), whichever came first. Follow-up extended to January 1st 2007.

Carotid IMT and plaque score

Using B-mode ultrasound, the right carotid artery was scanned within a predefined window of 3 cm of the distal CCA, the bifurcation and 1 cm of the internal and external carotid artery. IMT of the CCA was measured "off-line" in the far wall according to the leading edge principle, using a specially designed computer-assisted image analyzing system [12] and defined as the mean IMT-values of a 1 cm distance just proximal to the bulb. [6] In addition, we assessed a semi-quantitative six-graded carotid plaque score with 6 levels , where 0 = no plaques or wall thickenings (defined as focal intima-media thickness (IMT) >1·2 mm); 1 =one small plaque (<10 mm^2) or wall thickening (IMT > 1.2 mm); 2 = two or more small plaques (<10 mm^2); 3 = one plaque >10 mm^2; 4 = one plaque >10 mm^2 plus one or more small plaques (<10 mm^2); 5 = two or more plaques >10 mm^2, one circumferent plaque, or one plaque causing more than 50% stenosis. Moderate to severe atherosclerosis was defined as plaque score ≥ 3, i.e. at least one plaque >10 mm^2 as previously described [9].

Dietary assessment

Dietary information was collected with an interview-based, modified diet history method that combined (i) a 7-day menu-book for registration of meals that varies from day to day (usually lunch and dinner meals), cold beverages and nutrient supplements, and (ii) a 168-item questionnaire for assessment of consumption frequencies and portion sizes of regularly eaten foods that were not covered by the menu-book. Finally, (iii) a 45-minute interview completed the dietary assessment. The diet assessment method has been described in detail elsewhere. [13, 14]

Intakes of the following food groups were examined in this study: protein rich foods of animal origin except dairy products (meat, poultry, fish/shellfish and egg) (g), dairy products (milk,

yoghurt, sour milk and cheese) (portions) and cereal products (bread, breakfast cereals, pasta and rice) (portions). The food intake was converted to intakes of total energy (kcal) and protein (g) using the MDC nutrient database where the majority of the nutrient information comes from PC-KOST2-93 from the National Food Administration in Uppsala, Sweden. The relative validity of the MDC diet method was evaluated 1984-85. [15, 16]

Classification of ischemia

Symptoms, heart rate, blood pressure, and a 12-lead ECG were recorded before the test, midway through each stage, and during recovery. The maximum effort stress test was terminated if there was physical exhaustion, severe angina, a >2-mm horizontal or downsloping ST-segment depression, a ≥20-mm Hg fall in systolic blood pressure, or sustained ventricular arrhythmia. Duration of the stress test, metabolic equivalents achieved, peak heart rate, and peak blood pressure were recorded. If the patient developed angina during the test, the timing, quality (typical versus atypical), and effect on the test (limiting or non-limiting) were noted. The maximal horizontal or down-sloping ST-segment changes were recorded in each ECG lead.

A stress-rest imaging protocol was used. [99]Technitium was administered at peak stress, and imaging was performed soon thereafter. Thirty minutes following exercise testing a second injection was administered, and repeated imaging was performed. Quantitative analysis of perfusion was performed to calculate reversible and fixed perfusion defects. Patients with a reversible perfusion defect in one or more territories were selected as cases, and those without any perfusion defect were selected as controls. Left ventricular ejection fraction was calculated with the use of commercially available software. [17]

Statistics

All variables with skewed distributions were log transformed prior to analysis. Group-wise differences in continuous variables were compared using a paired t-test. In the MDC, we

performed conditional logistic regression analyses to test for differences in individual amino acids and DM-AA-score between incident CVD cases and matched controls and adjusted for age, gender, smoking, BMI, SBP, AHT, diabetes status at baseline and incident diabetes , LDL, HDL, triglycerides (log transformed), hsCRP (log transformed) and HOMA index (log transformed).

All amino acids were first log transformed and thereafter scaled to multiples of 1 standard deviation [SD]. The Cronbach´s alpha for the standardized values of the log transformed levels of the three amino acids was 0.71 The amino acid combination was modeled according to the formula z-score of log X1 + z-score of log X2 + z-score of log X3 with Xj denoting the value for the jth amino acid. This score was thereafter scaled to multiples of 1 SD (DM-AA-score). The DM-AA-score were also analyzed as quartiles of their distributions.

To test for cross sectional relationship between the DM-AA-score and measures of subclinical atherosclerosis, we used linear regression (IMT (log transformed) and logistic regression (presence of moderate to severe atherosclerosis) and adjusted for age, gender, smoking, BMI, SBP, AHT, diabetes, LDL, HDL, triglycerides (log transformed), hsCRP (log transformed) and HOMA index (log transformed). Correlations between dietary variables and the DM-AA-score were adjusted for total energy intake and age.

To test the relationship between the DM-AA-score and measures of inducible myocardial ischemia, the DM-AA-score was analyzed as a continuous variable (log transformed and scaled to standard deviation [SD] of 1) by logistic regression (presence of inducible ischemia) and adjusted for age, gender, BMI, diabetes, and creatinine. The performance of the DM-AA-score in predicting inducible myocardial ischemia was assessed by calculating the sum of standardized biomarker values weighted according to their corresponding beta coefficients from a regression model containing covariates and those indicated metabolites, and then

entering the weighted value of the score into a separate logistic regression model. In order to make effect estimates previously reported between the DM AA score and diabetes mellitus [4] comparable to those between the DM AA score and CVD, we chose identical categorization in this study (i.e. quartiles), therefore values were grouped into quartiles, and tested using a class variable to estimate the odds ratio for each quartile. A p-value for trend was obtained by entering the quartile into the model as an ordinal variable.

Analyses were performed using SPSS Windows version 19·0 or SAS version 9·1·3 (SAS Insitute, Cary, NC) and a two-tailed P value of <0·05 was considered statistically significant.

RESULTS

Amino acid score and incident CVD

Characteristics of the study participants in the MDC nested case control sample, as well as the MDC population used in the cross-sectional studies and the MGH exercise testing sample are shown in Table 1 and Supplementary Table 1. As a result of the matching procedure in MDC, there were no differences between incident CVD cases and controls with respect to age and sex and the risk factor pattern was similar (Table 1). Also, apart from anti-diabetic medication, (only 12 subjects had DM-medication in the CVD-case group and none in the control group) there were no significant differences in medication intake (i.e. AHT, lipid lowering medication and trombocyte aggregating inhibitory medication (data not shown) between cases and control. In the MGH exercise sample, subjects with inducible myocardial ischemia were older, had higher body mass index (BMI) and antihypertensive treatment (AHT) medications and more frequent diabetes (Supplementary Table 1). Sample values for each variable included in the DM-AA-score (Mean/SD for the log-transform values of tyrosine, isoleucine and phenylanalaine) in incident CVD cases and controls have been accounted for in Supplementary Table 2. Baseline standardized values of the DM-AA-score

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were significantly higher in CVD cases compared to controls (mean (SD); 0.08 (0.99) vs. -0.08 (1.01), p = 0.038).

Each 1 SD increase of the DM-AA-score was associated with approximately a 27% increased risk of future CVD. Quartile analyses revealed linear increases of CVD risk with an approximately 2-fold increase in risk of future CVD in the top versus bottom quartile of the DM-AA-score after multivariate adjustment that included not only CVD risk factors but also measures of insulin resistance and other risk factors for diabetes (Table 2). All three of the amino acids had directionally consistent associations with incident CVD, though no individual amino acid was statistically significant [(odds ratio= 1.25, 95% confidence interval (0.98-1.61) for isoleucine, 1.19 (0.95-1.48) for tyrosine and 1.25 (0.95-1.63) for phenylalanine)]. In the analysis of the individual amino acids composing the DM-AA-score and their associations with incident CVD the β-coefficient (SE) were 0.225 (0.127) for isoleucine, 0.220 (0.137) for phenylanaline and 0.170 (0.114) for tyrosine.

Cross sectional relationships between amino acid score and measures of carotid atherosclerosis

In order to test whether the relationship between the DM-AA-score and future CVD events is mediated by anatomical carotid abnormalities known to be predictive of CVD (IMT) [9] and atherosclerotic lesion formation we then performed cross-sectional multivariate adjusted analyses in CVD free individuals between the DM-AA-score and measures of subclinical disease. IMT increased significantly across quartiles of the DM-AA-score (P_{trend} = 0.037) (Table 3). Similarly, each SD increase of DM-AA-score was associated with an approximately 1.4-fold increased risk of having moderate to severe atherosclerosis and the top versus bottom quartile of the scores was associated with an approximately 2.6-fold increased risk of moderate to severe carotid atherosclerosis (Table 4). [9]

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DM-AA-score and dietary intake

The DM-AA-score was weakly positively correlated with protein intake (r=0.074, P=0.037). Whereas the DM-AA-score was positively correlated with intake of animal protein sources except milk and other dairy products (r=0.093, P=0.009), it was significantly inversely correlated with intake of milk and other dairy products (r=-0.080, P=0.025). However, the DM-AA-score remained significantly associated with incident CVD after adjustment for total protein intake as well as for intake of animal protein sources (data not shown). When intake for dairy protein was entered on top of all other covariates in the conditional regression model, the predictive capacity of the DM-AA-score was slightly attenuated; odds ratio= 1.26, 95% confidence interval (0.99-1.60), p=0.060.

DM-AA Score and inducible myocardial ischemia:

To determine if the metabolite score was related to functional consequences of CVD, we examined the relationship of the DM-AA-score to exercise-induced myocardial ischemia in a distinct cohort of 166 subjects referred for diagnostic exercise stress testing at a tertiary care center. Each 1 SD increase of the DM-AA-score was associated with an approximately 80% increased risk of inducible myocardial ischemia. As compared to the lowest quartile of the DM-AA-score, the top quartile of the score was associated with a nearly 5-fold odds ratio for inducible myocardial ischemia (Table 5).

DISCUSSION

Here we show for the first time that the identical score of three branched chain and aromatic amino acids, recently shown to predict diabetes in an ambulatory population, also predicts future CVD during long term follow-up. Notably, the prospective relationship was

independent of both CVD and diabetes risk factors, suggesting that disturbed amino acid metabolism may represent a novel modifiable link between diabetes and CVD.

Several prior studies have demonstrated cross-sectional associations between established coronary artery disease (CAD) and altered levels of amino acids [18, 19] One study [18] found an association between CAD and a principal components-derived pattern of several constituents that included some components in the DM-AA-score and most recently Shah and co workers published prospective data accounting for an a principal components-derived pattern of several branched chain amino acids (valine, leucine, methionine, tyrosine, isoleucine and phenylalanine) significantly associated with a lower hazard ration for mortality. But interestingly, no significant association was seen for this principal components-derived pattern and incident CVD. Also, the study population differed from ours in that all patients had cardiac catheterization procedure (with an approximately 60 % prevalence of 1-3 vessel disease), giving a high burden of prevalent CAD. [20] In addition, Tyr-O-sulfate in its multimeric form has been shown to be protective in a myocardial infarction model in the pigs, however since it was a multimeric form of tyrosine and sulfate it is hard to conclude that similar anticoagulation effects would be achieved with tyrosine alone. [21] In the current study, in an ambulatory population without CVD at baseline examination, we found cross-sectional associations between the DM-AA-score (derived from tyrosine, isoleucine and phenylalanine) and the presence of subclinical carotid atherosclerosis, as well as with thicker IMT, and exercise-induced myocardial ischemia. This suggests that the association between DM-AA-score and future development of CVD is mediated, at least in part, by propensity for atherosclerosis. However, despite the fact that elevated fasting plasma levels of the DM-AA-score occurred several years before onset of CVD, it remains to be shown whether or not the association is causal. In the perspective that insulin resistance is associated with incident diabetes and as well as with CVD [22, 23] it is interesting that studies of branched chain amino

acid supplementation in both experimental studies [24, 25] and in humans [26-28] indicate that amino acids may directly promote insulin resistance, possibly via disruption of insulin signaling in skeletal muscle. The underlying cellular mechanisms may include activation of the mTOR, JUN and IRS1 signaling pathways in skeletal muscle. [24, 25] In fact, recent studies have revealed that ribosomal protein S6 kinase 1 (S6K1), an effector of mTOR, is sensitive to both insulin and nutrients, including amino acids and that amino acids also negatively affect insulin signaling through mTOR/S6K1 phosphorylation of IRS1. [29]

An obvious factor that could alter amino acid levels in plasma is diet. In fact, two out of the three amino acids in the DM-AA-score are essential (isoleucine and phenylalanine), whereas tyrosine is a conversion product of phenylalanine. We observed relatively weak relationships between protein intake, dietary sources of protein and the DM-AA-score relation to incident CVD remained virtually unaltered after adjustment for these dietary variables. Still, as high milk and dairy consumption has been shown to be associated with reduced risk of both diabetes and CVD [30] it was interesting to observe that in contrast to other protein sources, intake of milk and dairy products was significantly negatively correlated to levels of the DM-AA-score. However, and in contrast, a recent study showed that ingestion of a rapidly absorbed extract derived from whey protein (consisting of 18 different amino acids, including tyrosine, phenylalanine and isoleucine) improved endothelium-dependent dilation in older adults by a mechanism independent of changes in circulating vasoactive compounds. [31] Still, since this extract consisted of 18 different amino acids, [31] controlled intervention studies testing whether a diet with low levels of isoleucine, tyrosine and phenylalanine alone may improve glucose tolerance and reduce CVD risk, are warranted.

There was a more than two-fold increase of CVD risk in the top versus the bottom quartile of the DM-AA-score after adjustment for CVD and diabetes risk factors, an effect estimate comparable to one major traditional CVD risk factor. In times when the incidence rates of

smoking, hypertension and hypercholesterolemia have stagnated, the rapidly increasing proportion of obesity and diabetes in the population has been pointed out as the main CVD threat during the 21st century. In this context, the strong association between the DM-AA-score and both incident diabetes [4] and incident CVD during long term follow-up may prove to be particularly important for more accurate identification of individuals at high cardiometabolic risk and thus candidates for primary preventive interventions. Due to the matching procedure of our incident CVD cases and controls, which included Framingham risk score, the average 10 year risk of both cases and controls was 8%, i.e. putting the study population at intermediate CVD risk, a segment in whom risk stratification is particularly important in guiding pharmacological primary preventive decisions.

An important strength of the current study is the use of a well-characterized prospective cohort with more than 5,000 participants that has been followed longitudinally for CVD incidence for decades using nation-wide registers with 100% coverage and high proven accuracy. [10, 11] From this cohort, we used a matched case-control approach to maximize efficiency. Given the relatively high analytical demands of LC-MS profiling, studies in the entire cohort must remain the subject of future investigation. However, all individuals were free of CVD at the time the when the fasting blood samples were collected, and matching for age, gender and Framingham risk score as well as adjustment for CVD and diabetes risk factors at baseline minimized potential confounding contributions.

We used the MDC to test a specific hypothesis based on our prior published work, and then extended our analyses to study exercise-induced ischemia to examine functional aspects of amino acid metabolism. However, even though there was a significant difference in the DM-AA-score between cases and control the distribution of the amino acids did overlap, thus it's premature to make claims of clinical application of this DM-AA-score before our data has been replicated in additional cohorts to further evaluate the strengths of the associated

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findings. Also, due to the high analytic demands as well and costs for metabolic analysis, to date, a widespread use of this diabetes and CVD -predictive amino acid score, might meet difficulties.

Finally, we must note that despite the association of the amino acid score with CVD that was statically independent of prevalent diabetes at baseline, incident diabetes, as well as measurements of insulin resistance, it is impossible to fully exclude the possibility that the association of the DM-AA-score with CVD is affected by other biological properties of diabetes per se.

There is an unmet clinical need for circulating biomarkers of myocardial ischemia. The ability of the DM-AA-score to differentiate between subjects with and without inducible myocardial ischemia after adjustment for CAD risk factors indicates that these metabolites predict functional consequences of CAD (i.e. ischemia). This finding may reflect a relationship between the DM-AA-score and higher CAD burden that predisposes patients to ischemia (65% (54/83) of the subjects were found to have multivessel disease at coronary catheterization). Alternatively, systematic maladaptive metabolic processing of branched-chain and aromatic amino acids reflected in this score may also signal less efficient myocardial substrate utilization that predisposes to ischemia.

In summary, our findings show that a score of fasting plasma levels of isoleucine, tyrosine and phenylalanine, recently shown to predict diabetes development, predicts CVD events during long term follow-up most likely through increased propensity of atherosclerosis. This score also predicts inducible myocardial ischemia in an at-risk clinical cohort undergoing diagnostic exercise testing.

CONFLICTS OF INTEREST

None declared.

SUPPLEMENTARY MATERIAL

Table S1. Baseline Characteristics of Exercise Testing Sample

Table S2. Baseline Values of the Individual Amino Acids (log transformed) in the DM-AA-score

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Table 1: Baseline Characteristics of Matched Case-Control and Cross-sectional Samples

	Incident CVD cases	Controls	Cross sectional sample
n	253	253	791
Age (years)	60.4 (5.1)	60.4 (5.1)	59.2 (5.6)
Sex (% women)	49.2	47.4	51.3
Current smoker, n (%)	32.8	32.4	30.3
BMI* (kg/m2)	26.6 (4.2)	26.2 (4.2)	27.0 (4.5)
Systolic BP (mmHg)	149.1 (18.5)	148.6 (19.1)	147.4 (18.7)
High blood pressure medication, n (%)	23.1	24.6	23.4
$F^{\dagger}$-Glucose (mmol/L)	5.2 (1.1)	5.6 (2.1)	5.4 (1.4)
$F^{\dagger}$-Insulin (microU/mL)‡	7.0 (6.0)‡	7.0 (5.0)‡	8.0 (6.0)‡
$F^{\dagger}$-Triglycerides (mmol/L)‡	1.2 (0.8)‡	1.3 (0.9)‡	1.3 (0.9)‡
$F^{\dagger}$-Total-Cholesterol	6.3 (1.0)	6.2 (1.1)	6.3 (1.1)
$F^{\dagger}$-Low density lipoprotein	4.4 (0.9)	4.3 (1.0)	4.3 (1.0)
$F^{\dagger}$-High density lipoprotein	1.4 (0.7)	1.5 (0.7)	1.3 (0.6)
Hypertension, n (%)	80.8	75.8	76.9
Diabetes (%)	14.9§	7.4§	7.2

*Body mass index, †Fasting. Values are displayed as mean (SD) or medians (IQR)‡ or frequency in percent. § The proportion of individuals with diabetes at baseline was statistically significantly higher in the incident CVD cases group compared to controls, p=0.007.

Table 2. DM-AA-score and Risk of Future CVD

	DM-AA-score*	P-values
DM-AA score as a continuous variable		
Per SD increment	1.27 (1.01-1.61)	0.045
Metabolite as a categorical variable		
First quartile	1.0 (Referent)	–
Second quartile	1.27 (0.72-2.22)	0.415
Third quartile	1.96 (1.07-3.60)	0.030
Fourth quartile	2.20 (1.12-4.31)	0.022
P for trend	0.010	

Values are odds ratios (95% confidence intervals) for incident CVD, from conditional logistic regressions (n=506).
Regressions are adjusted for age, sex, smoking, SBP, AHT, LDL, HDL, diabetes status at baseline and incident diabetes, BMI, logHOMA, logtriglycerides, logCRP.
* Score of Isoleucine, Tyrosine and Phenylalanine .
Values of DM-AA-score within quartiles (mean, (SD)): q1: -1.25 (0.59), q2: -0.33 (0.18), q3: 0.35 (0.20) and q4: 1.22 (0.48)

Table 3. Multivariate adjusted analysis of the relation between quartiles of DM-AA-score and logIMT

	Quartiles of score of Isoleucine, Tyrosine, Phenylalanine	
	Regression Coefficient (SE)*	P value
Model with quartile group		
Group 1 (lowest baseline values)	Referent	—
Group 2	-0.006 (0.008)	0.884
Group 3	0.016 (0.009)	0.075
Group 4 (highest baseline values)	0.015 (0.009)	0.096
P for trend	*0.006 (0.003)*	*0.037*
DM-AA score as a continuous variable	0.005 (0.003)	0.104

Values are beta-coefficients (standard errors) and the model is adjusted for age, sex, smoking, SBP, AHT, LDL, HDL, diabetes status at baseline examination, BMI, logHOMA, logtriglycerides, logCRP (n=791).

Table 4. DM-AA-score and risk of Moderate to Severe Atherosclerosis at Baseline Examination

	DM-AA-score*	P-values
DM-AA score as a continuous variable		
Per SD increment	1.41 (1.13-1.76)	0.002
Metabolite as a categorical variable		
First quartile	1.0 (Referent)	
Second quartile	1.14 (0.66-1.96)	0.631
Third quartile	1.68 (0.97-2.94)	0.067
Fourth quartile	2.62 (1.43-4.81)	0.002
P for trend	0.001	

Values are odds ratios (95% confidence intervals) for prevalent moderate to severe atherosclerosis adjusted for age, sex, smoking, SBP, AHT, LDL, HDL, diabetes status at baseline examination, BMI, logHOMA, logtriglycerides, logCRP.
 (n=564).
* Score of Isoleucine, Tyrosine and Phenylalanine.
Values of DM-AA-score within quartiles (mean, (SD)): q1: -1.27 (0.59), q2: -0.30 (0.18), q3: 0.34 (0.19) and q4: 1.23 (0.51)

Table 5. DM-AA-score and Risk of Exercise Induced Myocardial Ischemia

	DM-AA-score*
DM-AA score as a continuous variable	
Per SD increment	1.80 (1.20-2.70)
P	0.005
Metabolite as a categorical variable	
First quartile	1.0 (Referent)
Second quartile	3.31 (1.05-10.43)
Third quartile	4.24 (1.6-13.25)
Fourth quartile	4.86 (1.47-16.09)
P for trend	0.011

Values are odds ratios (95% confidence intervals) for inducible myocardial ischemia. Regressions are adjusted for age, sex, diabetes status, BMI, and creatinine.
* Score of Isoleucine, Tyrosine and Phenylalanine.

Supplementary Table 1: Baseline Characteristics of Exercise Testing Sample

	Exercise-induced Ischemia cases	No Exercise-induced Ischemia controls	P-value
n	83	83	
Age (years)	67.0 (11.2)	61.6 (7.5)	0.003
Sex (% women)	11	10	0.798
Current smoker, n (%)	3.6	9.6	0.009
BMI* (kg/m^2)	29.4 (4.6)	28.0 (4.0)	0.071
Systolic BP (mmHg)	129.5 (18.3)	130.4 (15.4)	0.890
High blood pressure medication, n (%)	97.5	69.9	<0.001
Hypertension, n (%)	97.5	74.6	<0.001
Diabetes (%)	34.9	16.9	0.008
Creatinine (mg/dL)	1.23 (0.41)	1.14 (0.29)	0.095

Values are displayed as mean (SD). *Body mass index

Supplementary Table 2: Baseline Values of the Individual Amino Acids (log transformed) in the DM-AA-score

Amino Acid	Cases (n=253)	Controls (n=253)	All (n=506)
Isoleucin	0.052 (1.019)	-0.077 (0.977)	0.048 (0.104)
Tyrosine	0.054 (0.960)	-0.076 (1.021)	0.017 (0.102)
Phenylalanine	0.058 (1.078)	-0.074 (0.893)	-0.032 (0.090)

Values are displayed as mean (SD).