

# A diabetes-predictive amino acid score and future cardiovascular disease

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## Aims

We recently identified a metabolic signature of three 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 (IMT) and plaque formation] and functional (exercise-induced myocardial ischaemia) abnormalities. Compared with 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) ( $P_{\text{trend}} = 0.010$ ), respectively, after multivariate adjustment. Increasing quartile of the DM-AA score was cross-sectionally related to carotid IMT ( $P_{\text{trend}} = 0.037$ ) and with the presence of at least one plaque larger than 10 mm<sup>2</sup> ( $P_{\text{trend}} = 0.001$ ). Compared with the lowest quartile of the DM-AA score, the odds ratio (95% confidence interval) for inducible ischaemia in subjects belonging to quartiles 2, 3, and 4 was 3.31 (1.05–10.4), 4.24 (1.36–13.3), and 4.86 (1.47–16.1) ( $P_{\text{trend}} = 0.011$ ), respectively.

## Conclusion

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.

## Keywords

Metabolomics • Amino acids • Diabetes • Cardiovascular disease

## Introduction

Diabetes and its cardiovascular complications remain major causes of morbidity and mortality, and the prevalence of diabetes continues to increase in epidemic proportions.<sup>1</sup> 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.<sup>2,3</sup> 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-chain and aromatic amino acids (leucine, valine, isoleucine, tyrosine, and phenylalanine), and in particular a score of three of these amino acids

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(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 vs. 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.<sup>4</sup> 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 ischaemia.

## Methods

### Study samples

The Malmö Diet and Cancer (MDC) study is a population-based, prospective epidemiological cohort of 28 449 persons enrolled between 1991 and 1996. From this cohort, 6103 persons were randomly selected to participate in the MDC Cardiovascular Cohort (MDC-CC), which was designed to investigate the epidemiology of carotid artery disease.<sup>5,6</sup> Of MDC-CC participants, fasting plasma samples were available in 5400 subjects of whom we excluded 143 subjects who had CVD prior to the baseline examination. 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 ( $\pm 1$  year), and Framingham risk score<sup>7</sup> ( $<0.1\%$  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 study recently published.<sup>4</sup> From this added study sample ( $n = 326$ ), we excluded six subjects with CVD prior to the baseline examination. 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 CCA up to and including the bulb and 1 cm of the internal carotid artery was introduced in the MDC-CC, using a six-graded plaque score. Out of the 791 subjects, 564 had been classified using this six-graded plaque score.

A distinct cohort of subjects who underwent exercise stress testing with myocardial perfusion imaging at the Massachusetts General Hospital (MGH) underwent metabolic profiling. Patients

who underwent pharmacological testing were excluded. All patients with inducible ischaemia 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 ischaemia.

The study protocols were approved by the Institutional Review Boards of Lund University, Sweden and the MGH. All study participants provided written informed consent.

### Metabolic profiling

Given the relatively high analytical demands of liquid chromatography–tandem mass spectrometry (LC-MS) profiling, we were not able to analyse 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 examination in the MDC-CC, and drawn immediately before the start of exercise testing in the MGH exercise cohort. Samples were stored at  $-80^{\circ}\text{C}$  and profiled using LC-MS as described in detail previously.<sup>4</sup> Formic acid, ammonium acetate, LC-MS grade solvents, and valine-d8 were purchased from Sigma-Aldrich (St Louis, MO, USA). The remainder of the isotopically labelled analytical standards were purchased from Cambridge Isotope Labs, Inc. (Andover, MA, USA). Plasma samples (10  $\mu\text{L}$ ) were prepared for LC-MS analyses via protein precipitation with the addition of nine volumes of 74.9:24.9:0.2 v/v/v acetonitrile/methanol/formic acid containing two additional stable isotope-labelled internal standards for valine-d8 and phenylalanine-d8. The samples were centrifuged (10 min, 10 000 r.p.m.,  $4^{\circ}\text{C}$ ) 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.

Liquid chromatography–tandem mass spectrometry data were acquired using a 4000 QTRAP triple-quadrupole mass spectrometer (Applied Biosystems/Sciex; Foster City, CA, USA) that was coupled to a multiplexed LC system comprised of two 1200 Series pumps (Agilent Technologies; Santa Clara, CA, USA) and an HTS PAL autosampler (Leap Technologies; Carrboro, NC, USA) equipped with two injection ports and a column selection valve. The two pumps were similarly configured for hydrophilic interaction chromatography (HILIC) using  $150 \times 2.1$  mm Atlantis HILIC columns (Waters; Milford, MA, USA) 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 two LC systems, and each sample was injected once on each. Each column was eluted isocratically with 5% mobile phase A for 1 min, followed by a linear gradient to 60% mobile phase A over 10 min. MS analyses were carried out using electrospray ionization and multiple reaction monitoring 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  $425^{\circ}\text{C}$ . Internal standard peak areas were monitored for quality control, and individual samples with peak areas differing from the group mean by  $>2$  SD were re-analysed. The MultiQuant software (Version 1.1; Applied Biosystems/Sciex; Foster City, CA, USA) 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.

**Table 1** Baseline characteristics of matched case-control and cross-sectional samples

	Incident CVD cases	Controls	Cross-sectional sample
<i>n</i>	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, <i>n</i> (%)	32.8	32.4	30.3
BMI <sup>a</sup> (kg/m <sup>2</sup> )	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, <i>n</i> (%)	23.1	24.6	23.4
Fasting glucose (mmol/L)	5.2 (1.1)	5.6 (2.1)	5.4 (1.4)
Fasting insulin (μU/mL)	7.0 (6.0) <sup>b</sup>	7.0 (5.0) <sup>b</sup>	8.0 (6.0) <sup>b</sup>
Fasting triglycerides (mmol/L)	1.2 (0.8) <sup>b</sup>	1.3 (0.9) <sup>b</sup>	1.3 (0.9) <sup>b</sup>
Fasting total cholesterol	6.3 (1.0)	6.2 (1.1)	6.3 (1.1)
Fasting low-density lipoprotein	4.4 (0.9)	4.3 (1.0)	4.3 (1.0)
Fasting high-density lipoprotein	1.4 (0.7)	1.5 (0.7)	1.3 (0.6)
Hypertension, <i>n</i> (%)	80.8	75.8	76.9
Diabetes (%)	14.9 <sup>c</sup>	7.4 <sup>c</sup>	7.2

<sup>a</sup>Body mass index. Values are displayed as mean (SD) or medians (IQR)<sup>b</sup> or frequency in per cent. <sup>c</sup>The proportion of individuals with diabetes at baseline was statistically significantly higher in the incident CVD cases group compared with controls,  $P = 0.007$ .

## Clinical assessment

Clinical characteristics of the study populations at the baseline examination are shown in *Table 1* (MDC) and Supplementary material online, *Table S1* (MGH). MDC and MGH participants underwent similar standardized medical histories with physical examinations and laboratory assessments. Blood pressure was obtained after 10 min 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 metres. Hypertension was defined as systolic blood pressure (SBP)  $\geq 140$  mmHg or diastolic blood pressure  $\geq 90$  mmHg, or use of antihypertensive treatment (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).<sup>8</sup>

## Classification of cardiovascular events

The procedure for case retrieval has been described previously.<sup>6,9</sup> Briefly, the Swedish National Hospital Discharge and Cause of Death Registries (The National Board of Health and Welfare)<sup>10</sup> and the Stroke Registry of Malmö<sup>11</sup> were used. The ascertainment of cases and validity of these registries has been shown to be high.<sup>10,11</sup> 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 1 January 2007.

## Carotid intima-media thickness 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. Intima-media thickness of the CCA was measured 'off-line' in the far wall, according to the leading edge principle, using a specially designed computer-assisted image-analysing system<sup>12</sup> and defined as the mean IMT-values of a 1 cm distance just proximal to the bulb.<sup>6</sup> In addition, we assessed a semi-quantitative six-graded carotid plaque score with six levels, where 0 = no plaques or wall thickenings (defined as focal IMT  $>1.2$  mm); 1 = one small plaque ( $<10$  mm<sup>2</sup>) or wall thickening (IMT  $>1.2$  mm); 2 = two or more small plaques ( $<10$  mm<sup>2</sup>); 3 = one plaque  $>10$  mm<sup>2</sup>; 4 = one plaque  $>10$  mm<sup>2</sup> plus one or more small plaques ( $<10$  mm<sup>2</sup>); 5 = two or more plaques  $>10$  mm<sup>2</sup>, one circumferent plaque, or one plaque causing  $>50\%$  stenosis. Moderate-to-severe atherosclerosis was defined as plaque score  $\geq 3$ , i.e. at least one plaque  $>10$  mm<sup>2</sup> as previously described.<sup>9</sup>

## 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 vary from day to day (usually lunch and dinner meals), cold beverages, and nutrient supplements; (ii) a 168-item questionnaire for the assessment of consumption frequencies and portion sizes of regularly eaten foods that were not covered by the menu book; and finally, (iii) a 45 min interview completed the dietary assessment. The diet assessment method has been described in detail elsewhere.<sup>13,14</sup>

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 for 1984–85.<sup>15,16</sup>

## Classification of ischaemia

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  $\geq 20$  mmHg fall in SBP, 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 vs. 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.<sup>99</sup> Technetium 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.<sup>17</sup>

## 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), hs-C-reactive protein (log-transformed), and HOMA index (log-transformed).

All amino acids were first log-transformed and thereafter scaled to multiples of 1 SD. 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 modelled according to the formula:  $z\text{-score of } \log X_1 + z\text{-score of } \log X^2 + z\text{-score of } \log X_3$ , with  $X_j$  denoting the value for the *j*th amino acid. This score was thereafter scaled to multiples of 1 SD (DM-AA score). The DM-AA score were also analysed 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), hs-C-reactive protein (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 ischaemia, the DM-AA score was analysed as a continuous variable (log-transformed and scaled to SD of 1) by logistic regression (presence of inducible ischaemia) and adjusted for age,

gender, BMI, diabetes, and creatinine. The performance of the DM-AA score in predicting inducible myocardial ischaemia 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<sup>4</sup> comparable with 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 Institute, Cary, NC, USA), and a two-tailed *P*-value <0.05 was considered statistically significant.

## Results

### Amino acid score and incident cardiovascular disease

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 material online, *Table S1*. 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 thrombocyte aggregating inhibitory medication (data not shown) between cases and control. In the MGH exercise sample, subjects with inducible myocardial ischaemia were older, had higher BMI and AHT medications and more frequent diabetes (Supplementary material online, *Table S1*). Sample values for each variable included in the DM-AA score (mean/SD for the log-transformed values of tyrosine, isoleucine, and phenylalanine) in incident CVD cases and controls have been accounted for in Supplementary material online, *Table S2*. Baseline standardized values of the DM-AA score were significantly higher in CVD cases compared with 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 two-fold increase in risk of future CVD in the top vs. 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

**Table 2** DM-AA score and risk of future cardiovascular disease

	DM-AA score <sup>a</sup>	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-value for trend	0.010	

Values are odds ratios (95% confidence intervals) for incident cardiovascular disease, 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, log(HOMA), log(triglycerides), and log(C-reactive protein).

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).

<sup>a</sup>Score of isoleucine, tyrosine, and phenylalanine.

CVD the  $\beta$ -coefficient (SE) were 0.225 (0.127) for isoleucine, 0.220 (0.137) for phenylalanine, 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)<sup>9</sup> 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. Intima-media thickness increased significantly across quartiles of the DM-AA score ( $P_{\text{trend}} = 0.037$ ) (Table 3). Similarly, each SD increase of DM-AA score was associated with an  $\sim 1.4$ -fold increased risk of having moderate-to-severe atherosclerosis, and the top vs. bottom quartile of the scores was associated with an  $\sim 2.6$ -fold increased risk of moderate-to-severe carotid atherosclerosis (Table 4).<sup>9</sup>

### 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

**Table 3** Multivariate-adjusted analysis of the relation between quartiles of DM-AA score and log(intima-media thickness)

	Quartiles of score of isoleucine, tyrosine, phenylalanine	
	Regression coefficient (SE) <sup>a</sup>	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-value 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, log(HOMA), log(triglycerides), and log(C-reactive protein) ( $n = 791$ ).

**Table 4** DM-AA score and risk of moderate-to-severe atherosclerosis at baseline examination

	DM-AA score <sup>a</sup>	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-value 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, log(HOMA), log(triglycerides), and log(C-reactive protein).  $n = 564$ .

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).

<sup>a</sup>Score of isoleucine, tyrosine, and phenylalanine.

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 ischaemia

To determine whether the metabolite score was related to functional consequences of CVD, we examined the relationship of the DM-AA score to exercise-induced myocardial ischaemia in a distinct cohort of 166 subjects referred for diagnostic exercise

**Table 5 DM-AA score and risk of exercise-induced myocardial ischaemia**

	DM-AA score <sup>a</sup>
DM-AA score as a continuous variable	
Per SD increment	1.80 (1.20–2.70)
P-value	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-value for trend	0.011

Values are odds ratios (95% confidence intervals) for inducible myocardial ischaemia. Regressions are adjusted for age, sex, diabetes status, BMI, and creatinine.

<sup>a</sup>Score of isoleucine, tyrosine, and phenylalanine.

stress testing at a tertiary care centre. Each 1 SD increase of the DM-AA score was associated with an ~80% increased risk of inducible myocardial ischaemia. Compared with the lowest quartile of the DM-AA score, the top quartile of the score was associated with a nearly five-fold odds ratio for inducible myocardial ischaemia (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.<sup>18,19</sup> One study<sup>18</sup> 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 *et al.*<sup>20</sup> published prospective data accounting for a principal components-derived pattern of several branched-chain amino acids (valine, leucine, methionine, tyrosine, isoleucine, and phenylalanine) significantly associated with a lower hazard ratio 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 ~60% prevalence of 1–3-vessel disease), giving a high burden of prevalent CAD. 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.<sup>21</sup> 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 ischaemia. 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 the 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,<sup>22,23</sup> it is interesting that studies of branched-chain amino acid supplementation in both experimental studies<sup>24,25</sup> and in humans<sup>26–28</sup> indicate that amino acids may directly promote insulin resistance, possibly via disruption of insulin signalling in skeletal muscle. The underlying cellular mechanisms may include activation of the mTOR, JUN, and IRS1 signalling pathways in skeletal muscle.<sup>24,25</sup> 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 signalling through mTOR/S6K1 phosphorylation of IRS1.<sup>29</sup>

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 and 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,<sup>30</sup> 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.<sup>31</sup> Still, since this extract consisted of 18 different amino acids,<sup>31</sup> 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 vs. the bottom quartile of the DM-AA score after adjustment for CVD and diabetes risk factors, an effect estimate comparable with one major traditional CVD risk factor. In times when the incidence rates of smoking, hypertension, and hypercholesterolaemia have stagnated, the rapidly increasing proportion of obesity and diabetes in the population has been pointed out as the main CVD threat during the twenty-first century. In this context, the strong association between the DM-AA score and both incident diabetes<sup>4</sup> 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. Owing 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 which 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 5000 participants that has been followed longitudinally for CVD incidence for decades, using nation-wide registers with 100% coverage and high proven accuracy.<sup>10,11</sup> 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 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 ischaemia to examine functional aspects of amino acid metabolism. However, even though there was a significant difference in the DM-AA score between cases and controls, the distribution of the amino acids did overlap; thus, it is premature to make claims of clinical application of this DM-AA score before our data have been replicated in additional cohorts to further evaluate the strengths of the associated findings. Also, owing to the high analytic demands as well as 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 ischaemia. The ability of the DM-AA score to differentiate between subjects with and without inducible myocardial ischaemia after adjustment for CAD risk factors indicates that these metabolites predict functional consequences of CAD (i.e. ischaemia). This finding may reflect a relationship between the DM-AA score and higher CAD burden that predisposes patients to ischaemia [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 ischaemia.

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 ischaemia in an at-risk clinical cohort undergoing diagnostic exercise testing.

## Supplementary material

Supplementary material is available at *European Heart Journal* online.

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