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Threonine in Coronary Artery Disease: Insights From Metabolic Syndrome Amino Acid Profiling

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Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Background: Metabolic syndrome (MeS) amplifies cardiovascular risk, but molecular markers that distinguish between MeS and coronary artery disease (CAD) remain limited. The aim of this study was to determine whether targeted amino acid profiling can identify CAD-specific signals independent of MeS and suggest translational biomarkers for risk stratification.

Material/Methods: In a prospective single-center cohort, we quantified 48 serum amino acids by liquid chromatography-tandem mass spectrometry in 65 fasting adults undergoing planned evaluation for chronic coronary syndrome. Quality control comprised blanks, reference sera, internal standards, and retention-time monitoring. Coronary artery disease was adjudicated angiographically in a reference hemodynamic center. Participants were grouped by metabolic syndrome (n=25) versus controls (n=40) and further stratified by CAD status. Group differences were assessed nonparametrically (2-sided α=0.05).

Results: The MeS group exhibited a distinct signature versus controls, with lower histidine, ethanolamine, and tryptophan, and higher cystine and proline (all $P \leq 0.03$). The CAD prevalence was 60% in MeS versus 35% in controls (P=0.051). Across MeS strata, threonine was associated most robustly with CAD: concentrations were higher in angiography-confirmed CAD irrespective of MeS, with significant pairwise differences among noMeS+noCAD, MeS+noCAD, noMeS+CAD, and MeS+CAD groups (P=0.03-0.048). Other MeS-linked amino acids were not consistently associated with CAD. Multivariable adjustment was not performed due to the limited sample size.

Conclusions: Targeted amino-acid profiling revealed redox- and immune-linked perturbations in MeS and identified threonine as a potential exploratory signal of CAD independent of MeS in our exploratory, limited-sample-size study. If validated, threonine may refine CAD risk stratification beyond traditional risk factors and MeS-related metabolic changes and inform mechanistic studies of amino-acid metabolism in atherogenesis.

Keywords: Cardiovascular Diseases • Coronary Disease • Glucose Intolerance • Thienamycins

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Introduction

Metabolic syndrome (MeS) is defined as the coexistence of abdominal obesity, high blood pressure, dyslipidemia, and impaired glucose metabolism, all of which predispose to increased cardiovascular and diabetic risk. The development of cardiovascular disease in this context is driven not only by classical risk factors but also by chronic low-grade inflammation, immune dysregulation, and hemodynamic stress, which together accelerate atherosclerotic plaque formation and destabilization [1,2]. In particular, prothrombotic activation triggered by a naive immune response involving neutrophils can amplify vascular injury, linking metabolic disturbances with heightened vascular risk [3]. Among possible atherosclerosis-triggering factors, metabolic derangements and environmental factors are gaining recent attention [2,4,5]. MeS has been observed to significantly increase mortality in patients with stable coronary artery disease (CAD) [6]. Whereas current guidelines recommend vigorous control of all MeS components, the syndrome itself has not been clearly classified as an autonomous cardiovascular risk factor. A healthy lifestyle, combined with pharmacological interventions, is regarded as a mainstay of therapy to improve outcomes in MeS patients [7]. A previous report highlighted the role of endocan, an endothelial dysfunction marker, in the MeS population, suggesting underlying molecular mechanisms beyond traditional risk factors [8]. Atherosclerosis is regarded as the result of activated pathways of naive and acquired inflammatory responses in diabetic, dyslipidemic, and hypertensive patients. Comprehensive metabolomic analysis has revealed that these processes are far more complex than previously assumed, with a distinct metabolic fingerprint that distinguished atherosclerotic patients from the control group [9].

Metabolomics provides a promising approach to unravel cardiovascular disease (CVD) by enabling the discovery of novel risk biomarkers and revealing hidden pathophysiological mechanisms. Abnormal amino acid metabolism is increasingly recognized as a potential driver of cardiovascular pathology. For instance, reduced circulating histidine levels have been associated with adverse metabolic profiles and elevated cardiovascular risk, whereas genetic variants linked to higher histidine concentrations have been shown to reduce the risk of CAD [10]. In a previous report [11], the concept of targeted proteomics analysis for cardiovascular event risk prediction was proposed. The correlation between carotid disease and several proteins was noted in the Cardiovascular Health Study (CHS) and the Atherosclerosis Risk in Communities Study (ARIC) [12]. Amino acid profiling may redefine pathological conditions by identifying possible derangements in hemostasis that could be corrected.

The aim of the prospective, exploratory, single-center analysis was to investigate a possible association between coronary risk in MeS patients and amino acid profiling by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). To the best of our knowledge, this is the first study to demonstrate that targeted amino acid profiling can distinguish metabolic alterations specific to MeS and CAD, and to identify threonine as a potential novel biomarker of CAD independent of metabolic syndrome.

Material and Methods

Patients

Patients with chronic coronary syndrome were enrolled in a prospective study. All patients were hospitalized in the internal medicine and hypertensiology department in 2024 in a planned manner. The enrollment criteria included stable anginal symptoms on exertion. Demographic characteristics, clinical data, and laboratory results were collected, followed by blood sampling and subsequent serum analysis to quantify concentrations of 48 amino acids. Patients with acute coronary syndromes, significant valvular pathologies, or presenting a history of cardiovascular interventions were not included in the analysis. Dietary restrictions, any supplementation, and food allergies were considered contraindications to study enrollment.

Methods

Blood samples were collected on admission after a minimum of 12 h of fasting. The laboratory tests were followed by transthoracic echocardiography performed by an experienced team of echocardiographers and coronary angiography in a reference hemodynamics center.

Blood Sample Analysis for Amino Acids

After a 12-h overnight fast, venous blood was collected on admission and centrifuged, and the serum was aliquoted and stored at -80°C until analysis. Thirty-nine different amino acid concentrations were determined by LC-MS/MS using the MassChrom Amino Acid Analysis kit (Chromsystems Instruments & Chemicals GmbH, Germany). For preparation, 25 µL of serum was combined with 50 µL of the reconstituted internal standard and 400 µL of the precipitation reagent supplied in the kit. After vortexing for 30 s, the mixture was centrifuged at 16000×g for 5 m. Then, the supernatant was transferred to autosampler vials. Lyophilized calibrators and quality controls were reconstituted according to the manufacturer's instructions and processed identically to patient samples. The sample order was randomized prior to LC-MS/MS acquisition. Analyses were performed on a 5500+ QTRAP triple quadrupole mass spectrometer (Sciex, Framingham, MA, USA) coupled with an Agilent 1260 Infinity II HPLC system (Agilent Technologies, Santa Clara, USA). Chromatographic separation

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Table 1. Mobile phase gradient conditions for the determination of the full amino acid panel.

Time (min)	Mobile phase A (%)	Mobile phase B (%)	Flow (mL/min)
0.00	100	0	0.8
0.50	100	0	0.8
1.00	89	11	0.8
6.30	89	11	0.8
12.30	83	17	0.8
14.80	68	32	0.8
14.90	0	100	0.8
16.40	0	100	0.8
16.50	0	100	0.3
17.40	0	100	0.3
17.50	0	100	0.8
17.60	0	100	0.8
17.70	100	0	0.8
19.30	100	0	0.8
19.80	100	0	1.8
20.40	100	0	1.8
20.50	100	0	0.8

was performed on the MassChrom analytical column supplied with the kit. Mobile phase A and mobile phase B were prepared according to the manufacturer's instructions. The binary gradient program is detailed in **Table 1**.

The column temperature was held at 25°C, and the injection volume was set at 5 µL. The mass spectrometer operated in positive electrospray ionization (ESI+) mode with multiple reaction monitoring (MRM) transitions optimized for each amino acid. Data were acquired in Analyst software (AB Sciex v.1.7.3), with retention times checked and adjusted prior to quantification to ensure correct peak assignments. Integration, processing, and concentration calculations were performed in SciexOS (AB Sciex v.3.3.0.12027). Calibrators (multi-point) and quality control (QC) materials at 2 levels were included in each analytical batch. QC samples were analyzed at the beginning, middle, and end of each sequence to monitor accuracy and reproducibility. All analytes passed QC acceptance criteria. Carryover was assessed by blank injections following the highest calibrator. The intra-assay coefficient of variation, calculated from repeated QC analyses, was 4.98%.

Statistical Analysis

After the normality of the distribution of variables was tested with the Shapiro-Wilk test, the median (IQR) was used to describe the variables that did not follow the normal distribution. We used the *t* test and the Mann-Whitney test to compare the variables between the groups. Statistical analysis was performed using JASP (Version 0.14.1, University of Amsterdam, Netherlands, 2020). Significance set at $P<0.05$.

Results

Of the 65 patients, 25 (11 men [44%]; median age 68 [range, 61-69] years) met the criteria for metabolic syndrome [1] and comprised group 1 (MeS). The remaining 40 patients (24 men [60%]; median age 67 [range, 62-74] years) were allocated to group 2 (Control). Among clinical characteristics, arterial hypertension ($P=0.09$) and diabetes mellitus ($P<0.001$) diagnosis, followed by body mass index, differentiated the 2 groups ($P=0.04$), as presented in **Table 2**. The laboratory results, followed by the amino acid serum concentrations, were compared between the 2 groups (**Table 3**).

Significant differences in circulating levels of histidine ($P=0.03$), ethanolamine ($P=0.007$), cystine ($P=0.004$), proline ($P=0.03$), and tryptophan ($P=0.02$) were noted between the MeS and the Control groups in our analysis.

Coronary Artery Disease Risk

All patients enrolled in the study underwent coronary angiographies. There were 15 (60%) and 14 (35%) patients with angiographically confirmed coronary disease, defined as coronary

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Table 2. Demographic and clinical characteristics of the analyzed groups.

Parameters	Group 1 (MeS) n=25	Group 2 (Control) n=40	*P*
Demographic:			
Age (median (Q1-Q3) (years)	68 (61-69)	67 (62-74)	0.68
Sex (male) (%)	11 (44.0)	24 (60.0)	0.21
Weight (median (Q1-Q3) (kg)	80 (73-90)	84 (70-91)	0.70
Height (median (Q1-Q3) (cm)	163 (157-170)	170 (160-175)	0.04
BMI (median (Q1-Q3) (kg//m^2)	30.4 (27.8-34.3)	27.2 (25.1-28.8)	0.011
Clinical:			
Arterial hypertension (n (%))	23 (92.0)	23 (57.5)	0.009
Diabetes mellitus (n (%))	14 (56.0)	3 (7.5)	<0.001
Dyslipidemia (n (%))	24 (96.0)	33 (82.5)	0.35
COPD (n (%))	2 (8.0)	6 (15.0)	0.36
Smoking (n (%))	2 (8.0)	5 (12.5)	0.70
Positive family history (n (%))	5 (20.0)	6 (15.0)	0.78
Pharmacotherapy:			
B-blockers (n (%))	20 (80.0)	20 (50.0)	0.02
ACE-I (n (%))	12 (48.0)	12 (30.0)	0.15
ARB (n (%))	6 (24.0)	8 (20.0)	0.71
Ca-blockers (n (%))	6 (24.0)	9 (22.5)	0.90
Statins (n (%))	21 (84.0)	24 (60.0)	0.79
High-dose statin therapy (n (%))*	10 (40.0)	17 (42.5)	0.18
Diuretics (n (%))	2 (8.0)	5 (12.5)	0.58
Metformin (n (%))	14 (56.0)	3 (7.5)	<0.001
SGLT2i (n (%))	2 (8.0)	1 (2.5)	0.32

ACE-I – angiotensin converting enzyme inhibitor; ARB – angiotensin receptor blocker; BMI – body mass index; COPD – chronic obstructive pulmonary disease; cm – centimeter; kg – kilogram; MeS – metabolic syndrome; m^2 – square meter; n – number; SGLT2i – sodium glucose linked transporter 2 inhibitor; Q – quartile. * Atorvastatin daily dose at least 40 mg or Rosuvastatin 20 mg/day.

Table 3. Laboratory results and amino acid analyses in the MeS and Control groups.

Parameters	Group 1 (MeS) n=25	Group 2 (Control) n=40	p
Peripheral blood count:			
WBC (×10^9/L) (median (Q1-Q3)	7.9 (6.4-10.3)	6.5 (6.0-7.4)	0.06
Hb (mmol/L) (median (Q1-Q3)	8.5 (8.2-9.2)	9.0 (8.4-9.3)	0.29
Hct (%) (median (Q1-Q3)	41 (39-45)	43 (40-45)	0.29
Plt (×10^9/L) (median (Q1-Q3)	238 (201-266)	223 (175-313)	0.83
Kidney function			
Creatinine (umol/L) (median (Q1-Q3)	76 (62-112)	83 (68-92)	0.98
Liver tests			
ALT (IU/L) (median (Q1-Q3)	22 (20-31)	28 (23-51)	0.09
HF tests			
BNP (pg/ml) (median (Q1-Q3)	402 (223-678)	202 (107-504)	0.31
Myocardia tests			
CK-MB (ng/ml) (median (Q1-Q3)	2.1 (1.2-4.7)	1.5 (1.2-2.2)	0.16
Lipogram:			
Total cholesterol (mmol/dl) (median (Q1-Q3)	4.1 (3.0-4.7)	4.1 (3.3-5.3)	0.28
HDL (mmol/dL) (median (Q1-Q3)	1.0 (0.9-1.2)	1.4 (1.2-1.6)	<0.001
HDL <40 mg/dl (n (%)	23 (92)	11 (27.5)	<0.001
LDL (mmol/dL) (median (Q1-Q3)	2.1 (1.4-2.8)	2.1 (1.4-3.2)	0.98
Triglycerides (mmol/dL) (median (Q1-Q3)	2.1 (1.4-2.8)	1.1 (0.8-1.3)	0.005
Triglycerides >150 mg/dl (%)	15 (60)	8 (20)	<0.001
Uric acid (umol/L) (median (Q1-Q3)	333 (266-385)	337 (307-373)	0.75
Lipoprotein (a) (mg/L) (median (Q1-Q3)	2.8 (2.2-8.6)	3.9 (2.1-6.0)	0.32

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Table 3 continued. Laboratory results and amino acid analyses in the MeS and Control groups.

Parameters	Group 1 (MeS) n=25	Group 2 (Control) n=40	p
Amino acids analysis (uM) (median (Q1-Q3):			
1-Metylhistidine	7.6 (5.3-10.7)	6.5 (5.4-8.6)	0.41
Alpha-aminobutyric acid	20.0 (17.0-26.5)	21.4 (18.6-28.7)	0.58
Alanine	396.6 (355.2-472.9)	410.5 (359.7-443.8)	0.94
Allo-isoleucine	2.1 (1.4-2.8)	1.7 (1.4-2.1)	0.14
Asparagine	45.3 (41.9-46.8)	46.6 (42.6-52.7)	0.16
Glutamine	589.4 (541.4-614.9)	603 (504.8-650.2)	0.73
Histidine	87.9 (72.1-95.6)	93.7 (87.1-113.7)	0.03
Taurine	153.1 (108.8-168.2)	133.0 (101.9-160.1)	0.29
Beta-aminoisobutyric acid	2.7 (2.2-4.5)	2.4 (1.4-3.5)	0.08
Arginine	80.9 (73.0-91.1)	88.8 (75.5-101.3)	0.32
Aspartic acid	26.9 (20.6-31.7)	22.6 (18.3-33.0)	0.82
Ethanolamine	9.1 (8.4-9.5)	9.9 (9.3-11.9)	0.007
Glycine	251.4 (208.7-290.5)	240 (198.9-276.2)	0.35
Lysine	198.5 (160.7-218.0)	193.4 (171.8-218.0)	0.86
Sarcosine	1.5 (1.1-1.8)	1.3 (1.1-1.7)	0.63
Valine	244.7 (209.6-270.2)	247.4 (226.7-271.3)	0.87
Beta-alanine	2.8 (2.4-3.5)	2.9 (2.4-3.8)	0.64
Citrulline	34.0 (29.3-36.9)	35.1 (32.7-38.0)	0.37
Cystine	61.0 (56.2-72.4)	52.2 (44.3-61.8)	0.004
Homocystine	Below LOD	Below LOD	N/A
Hydroxylisine	Below LOD	Below LOD	N/A
Leucine	136.7 (118.3-158.9)	140.8 (122.1-148.2)	0.89
Serine	140.7 (117.5-156.0)	137.6 (118.0-150.9)	0.93
Threonine	106.8 (91.9-137.9)	100.6 (91.3-122.1)	0.58
Tyrosine	62.3 (57.3-77.8)	70.0 (62.6-79.3)	0.29
4-hydroxyproline	7.9 (7.0-13.6)	13.2 (9.6-15.4)	0.07
Gamma-aminobutyric acid	Below LOD	Below LOD	N/A
Methionine	22.9 (20.2-26.9)	26.0 (21.8-27.6)	0.26
Phenomyalanine	71.5 (64.0-81.3)	75.9 (68.6-87.4)	0.23
Amino acids analysis (uM) (median (Q1-Q3):			
Phosphoethanolamine	Below LOD	Below LOD	N/A
Pipecolic acid	1.4 (1.0-1.8)	1.4 (1.0-1.7)	0.85
Saccharopine	Below LOD	Below LOD	N/A
3-methylhistidine	12.2 (6.5-19.8)	10.7 (6.0-23.6)	0.96
Adenosyl-homocysteine	Below LOD	Below LOD	N/A
Alpha-aminoadipic acid	1.1 (0.9-1.8)	1.0 (0.8-1.2)	0.84
Anserine	Below LOD	Below LOD	N/A
Argininosuccinic acid	Below LOD	Below LOD	N/A
Glutamic acid	78.6 (66.8-101.7)	74.4 (60.0-102.9)	0.89
Proline	187.2 (178.1-223.5)	172. (160.5-188.4)	0.03
Tryptophane	52.3 (45.8-60.3)	61.1 (50.9-68.3)	0.02

ALT – alanine aminotransferase; BNP – brain natriuretic peptide; CK-MB creatine kinase MB; dL – deciliter; IU – units; HDL – high-density lipoprotein; L -liter; LDL – low-density lipoprotein; MeS – metabolic syndrome; mmol – millimole; umol – micromole; n – number; N/A – not applicable; ng – nanogram; pg – picogram; Q – quartile.

lumen narrowing at least 50%, in the MeS and Control groups, respectively (*P*=0.051).

To disentangle MeS from CAD-related effects, serum amino acids were first compared across the 4 subgroups defined by the Mes×CAD cross-classification (noMeS+noCAD vs MeS+noCAD vs NoMeS+CAD vs MeS+CAD). The possible differences in serum amino acid concentration in relation to coronary disease were analyzed as presented in **Table 4**.

Threonine did not differ by MeS status but was consistently higher in patients with CAD within both MeS strata, indicating a CAD-specific signal. The significant differences regarding the threonine serum concentration were noted between

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Table 4. Amino acid concentrations in subgroups.

Amino acids analysis (uM) (median (Q1-Q3))	NoMeS+ noCAD n=26 (1)	MeS+ noCAD n=10 (2)	NoMeS+ CAD n=14 (3)	MeS+ CAD n=15 (4)	*P* 1 vs 2	*P* 1 vs 3	*P* 1 vs 4	*P* 2 vs 3	*P* 2 vs 4	*P* 3 vs 4
1-Metylhistidine	6.2 (4.8-9.1)	6.5 (5.4-15.7)	6.8 (6.4-8.4)	9.2 (5.5-10.6)	0.61	0.26	0.32	0.59	0.85	0.40
Alpha-aminobutyric acid	21.5 (18.6-26.8)	22.9 (18.2-27.1)	21.1 (18.8-28.2)	20.0 (16.4-25.5)	0.82	0.88	0.57	0.84	0.85	0.71
Alanine	403 (355-443)	392 (372-446)	411 (381-494)	419 (348-514)	0.93	0.38	0.53	0.47	0.64	0.74
Allo-isoleucine	1.6 (1.4-1.9)	1.9 (1.4-2.3)	1.9 (1.5-2.2)	2.5 (1.4-2.9)	0.57	0.32	0.07	0.95	0.26	0.25
Asparagine	46.3 (41.8-51.1)	42.4 (38.0-46.8)	47.0 (46.0-58.7)	46.0 (44.3-47.2)	0.19	0.14	0.79	0.03	0.16	0.10
Glutamine	574 (501-626)	598 (574-610)	622 (585-671)	589 (537-632)	0.93	0.20	0.58	0.172	0.94	0.27
Histidine	92 (82-101)	84 (71-91)	96 (91-117)	88 (76-101)	0.09	0.19	0.39	0.03	0.36	0.08
Taurine	146 (108-161)	149 (90-157)	112 (97-135)	160 (121-172)	0.99	0.28	0.36	0.51	0.42	0.17
Beta-aminoisobutyric acid	2.5 (1.4-4.4)	2.7 (1.5-4.9)	2.2 (1.2-3.3)	2.7 (2.5-4.4)	0.69	0.48	0.09	0.44	0.45	0.06
Arginine	82.1 (66.1-103.1)	77.5 (69.6-96.8)	88.9 (83.3-97.4)	83.0 (76.2-89.2)	0.61	0.56	0.88	0.15	0.52	0.23
Aspartic acid	24.8 (18.9-34.9)	27.9 (26.7-31.6)	20.5 (18.4-25.5)	22.3 (18.9-31.0)	0.99	0.26	0.51	0.17	0.64	0.23
Ethanolamine	9.8 (9.4-11.2)	9.2 (8.5-9.5)	10.0 (9.3-12.4)	9.1 (8.3-9.5)	0.13	0.68	0.02	0.17	0.64	0.053
Glycine	235 (207-277)	277 (219-305)	252 (195-276)	249 (208-272)	0.30	0.99	0.74	0.29	0.50	0.75
Isoleucine	Below LOD	58.8 (53.5-63.3)	Below LOD	63.9 (47.5-74.6)	N/A	N/A	N/A	0.70	1.00	0.95
Lysine	193 (166-217)	198 (172-222)	210 (183-220)	199 (153-221)	0.72	0.44	1.00	0.66	0.68	0.49
Sarcosine	1.4 (1.1-1.8)	1.2 (0.9-1.7)	1.3 (1.2-1.4)	1.7 (1.3-1.9)	0.26	0.56	0.27	0.55	0.144	0.10
Valine	246 (224-270)	245 (238-256)	252 (232-277)	245 (237-256)	0.93	0.81	0.99	0.80	0.98	0.88
Beta-alanine	2.9 (2.6-4.0)	3.1 (2.3-3.5)	2.8 (2.4-3.5)	2.8 (2.4-3.2)	0.69	0.65	0.66	1.00	1.00	0.81
Citrulline	35.5 (33.8-37.8)	32.9 (30.5-34.9)	32.9 (30.5-37.3)	34.2 (29.2-43.0)	0.03	0.13	0.72	0.67	0.37	0.53
Cystine	51.8 (33.1-61.1)	60.7 (57.0-70.7)	54.4 (48.0-66.9)	63.2 (57.0-77.4)	0.07	0.56	0.01	0.19	0.81	0.09
Homocystine	Below LOD	Below LOD	Below LOD	Below LOD	N/A	N/A	N/A	N/A	N/A	N/A

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Amino acids analysis (uM) (median (Q1-Q3))	NoMeS+ noCAD n=26 (1)	MeS+ noCAD n=10 (2)	NoMeS+ CAD n=14 (3)	MeS+ CAD n=15 (4)	*P* 1 vs 2	*P* 1 vs 3	*P* 1 vs 4	*P* 2 vs 3	*P* 2 vs 4	*P* 3 vs 4
Hydroxylysine	Below LOD	Below LOD	Below LOD	Below LOD	N/A	N/A	N/A	N/A	N/A	N/A
Leucine	142 (117-146)	134 (119-144)	140 (132-148)	149 (103-161)	0.99	0.67	0.53	0.59	0.85	0.95
Serine	136 (119-150)	133 (111-151)	134 (113-152)	141 (124-167)	0.41	0.98	0.50	0.59	0.26	0.51
Threonine	95 (87-114)	95 (81-105)	115 (102-150)	134 (94-140)	0.59	0.04	0.03	0.04	0.048	0.59
Tyrosine	68.5 (62.7-75.5)	64 (56-75)	70.5 (61.4-79.5)	62 (58-82)	0.34	0.82	0.53	0.37	0.77	0.62
4-hydroxyproline	12.9 (7.8-15.4)	7.5 (7.3-8.2)	13.4 (11.0-14.3)	9.2 (6.9-15.1)	0.19	0.72	0.39	0.04	0.61	0.20
Gamma-aminobutyric acid	Below LOD	Below LOD	Below LOD	Below LOD	N/A	N/A	N/A	N/A	N/A	N/A
Methionine	23.6 (20.1-26.7)	21.5 (19.0-23.4)	28.2 (26.1-29.9)	25.4 (22.0-27.7)	0.26	0.003	0.36	0.003	0.09	0.10
Phenylalanine	74.8 (67.7-84.9)	73.2 (62.3-78.8)	78.9 (73.0-87.6)	66.8 (64.1-86.2)	0.33	0.34	0.60	0.21	0.89	0.42
Phosphoethanolamine	Below LOD	Below LOD	Below LOD	Below LOD	N/A	N/A	N/A	N/A	N/A	N/A
Pipecolic acid	1.4 (1.0-1.7)	1.2 (0.9-1.6)	1.5 (1.1-1.9)	1.4 (1.0-1.9)	0.82	0.36	0.58	0.24	0.50	0.75
Saccharopine	Below LOD	Below LOD	Below LOD	Below LOD	N/A	N/A	N/A	N/A	N/A	N/A
3-methylhistidine	9.6 (5.1-19.7)	11.6 (5.6-18.0)	12.8 (8.2-26.9)	14.9 (8.4-20.4)	0.96	0.19	0.58	0.44	0.68	0.85
Adenosyl-homocysteine	Below LOD	Below LOD	Below LOD	Below LOD	N/A	N/A	N/A	N/A	N/A	N/A
Alpha-aminoadipic acid	0.3 (0.8-1.2)	0.3 (0.8-1.3)	0.3 (0.9-1.2)	1.2 (0.9-1.8)	0.83	1.00	0.27	0.89	0.64	0.35
Anserine	Below LOD	Below LOD	Below LOD	Below LOD	N/A	N/A	N/A	N/A	N/A	N/A
Argininosuccinic acid	Below LOD	Below LOD	Below LOD	Below LOD	N/A	N/A	N/A	N/A	N/A	N/A
Glutamic acid	75.6 (59.4-103.5)	82 (54-98)	73.4 (61.9-96.8)	74 (67-101)	0.99	0.79	0.97	0.98	0.98	0.72
Proline	171 (160-181)	180 (153-197)	180 (169-211)	196 (186-243)	0.40	0.19	0.004	0.70	0.08	0.11
Tryptophane	60.7 (50.1-66.4)	51.8 (46.7-59.7)	64.5 (51.8-70.7)	52.3 (43.3-59.9)	0.13	0.35	0.12	0.08	0.94	0.09

MeS+CAD – metabolic syndrome and coronary artery disease; MeS+noCAD – metabolic syndrome and no coronary artery disease; N/A – not applicable; noMeS+CAD – no metabolic syndrome and coronary artery disease; noMeS+noCAD – no metabolic syndrome and no coronary artery disease; um – micromole; Q – quartile.

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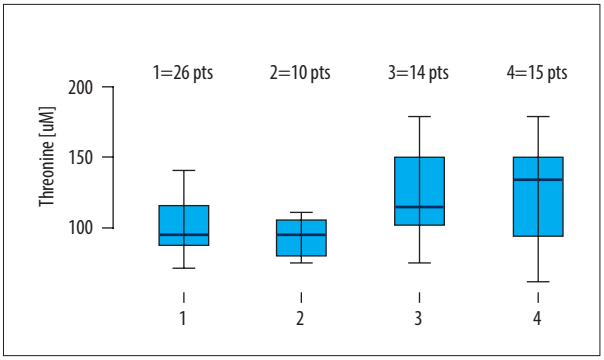

Figure 1. Differences in threonine concentration between analyzed groups. 1 – no MeS+no CAD (n=26); 2 – MeS+no CAD (n=10);3 – no MeS+CAD (n=14);4 – MeS+CAD (n=15). Significant differences noted between groups: 1-3 (*P*=0.039), 1-4 (*P*=0.030), 2-3 (*P*=0.040), 2-4 (*P*=0.048). CAD – coronary artery disease; MeS – metabolic syndrome.

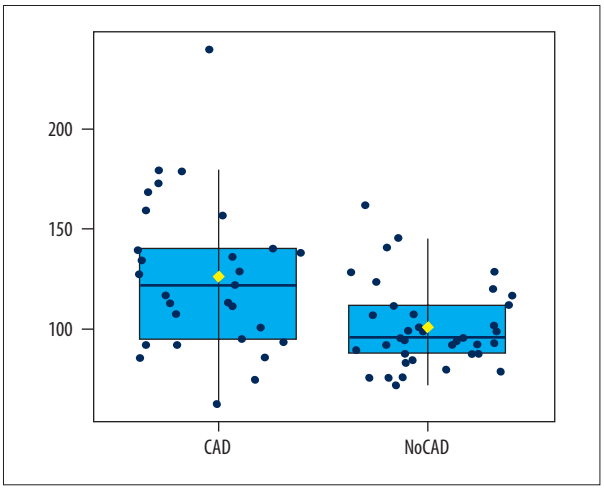

Figure 2. Distribution of threonine in CAD (n=29) and noCAD groups (n=36). CAD – coronary artery disease.

the subgroups (noMeS+noCAD vs MeS+noCAD vs NoMeS+CAD vs MeS+CAD) (**Figure 1**).

No meaningful effect modification by MeS was apparent, which justified a pooled evaluation of threonine with respect to CAD. Accordingly, in the combined CAD vs noCAD comparison, threonine was significantly associated with CAD (*P*=0.0012) (**Figure 2**) and showed moderate discriminative ability (AUC=0.714) (**Figure 3**).

Discussion

In this preliminary prospective study, we applied targeted amino acid profiling as a non-traditional risk factor analysis in patients with CAD, with and without MeS. Our findings demonstrate that

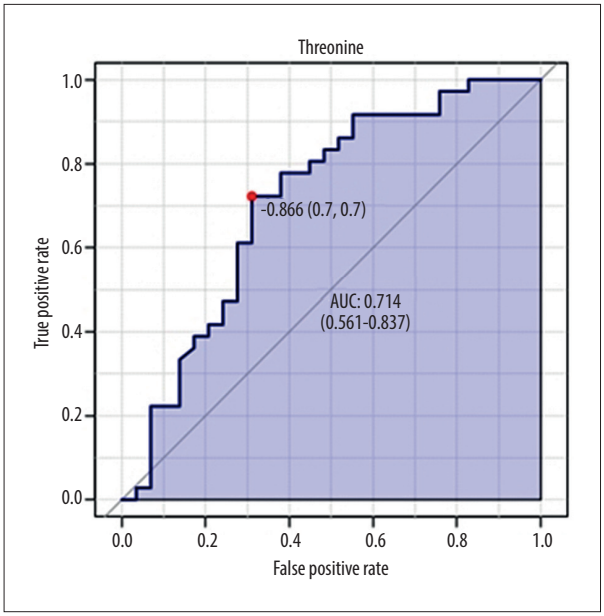

Figure 3. ROC curve for threonine discriminating CAD vs noCAD. The AUC is 0.714 (95% CI: 0.561-0.837). AUC – area under the curve; CAD – coronary artery disease; CI – confidence interval; ROC – receiver-operating characteristic.

amino acid profiles can differentiate metabolic alterations associated with MeS and CAD, highlighting both established and novel biomarkers of cardiovascular risk. Compared with controls, patients with MeS exhibited lower circulating levels of histidine, tryptophan, and ethanolamine, and higher levels of cystine and proline. These amino acids are involved in redox balance, inflammation, and structural metabolism [13]. Histidine, for example, is a precursor of carnosine, a dipeptide with antioxidant and anti-glycation properties. Low histidine levels have been consistently observed in obesity and MeS, correlating with systemic inflammation and oxidative stress [13]. Tryptophan depletion in MeS likely reflects increased catabolism via the indoleamine 2,3-dioxygenase (IDO)-kynurenine pathway, driven by chronic immune activation [14,15]. Low tryptophan and elevated kynurenine/tryptophan ratios have been associated with endothelial dysfunction and higher cardiovascular risk [16]. The increase in cystine, the oxidized form of cysteine, highlights systemic oxidative stress as cystine/cysteine redox potential is a sensitive marker of redox balance [17,18]. Proline elevation has been reported in insulin-resistant states, a condition known to substantially increase the risk of CAD [19]. Reduced ethanolamine may reflect disrupted phospholipid metabolism, as described in obesity and type 2 diabetes [19,20].

As MeS is regarded as a significant contributor to cardiovascular morbidity, we performed a subanalysis. The high prevalence of angiographically-confirmed coronary disease in the MeS group was noted. The most striking and novel finding of our study is that threonine levels were elevated in patients with

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angiographically confirmed CAD, regardless of MeS status. Unlike histidine or tryptophan, threonine did not differentiate between patients with and without MeS, suggesting a CAD-specific association. Threonine is one of the essential amino acids, with a varied role in atherosclerosis, its inverse association with atherogenic lipid profiles is postulated [21], and its involvement in pro-atherogenic signaling pathways regulated by serine/threonine kinases has been reported [22]. Phosphorylation of the threonine in glycogen synthase/kinase-3 (GSK-3) activates this enzyme family that influences lipid metabolism, macrophages' polarization, and inflammatory activation, and can be considered as a potential therapeutic target [23]. In contrast, GSK-3 deficiency can inhibit neovascularization during atherosclerosis progression [24]. Elevated threonine may therefore reflect dysregulation of these metabolic and signaling pathways. Interestingly, higher threonine levels were observed in chronic kidney disease [25], one of the pathophysiological conditions associated with increased coronary disease prevalence.

The plasma proteomics analysis suggested by Kraaijenhof et al [26] was identified as a potential predictor that, when integrated with traditional risk factors, improves risk stratification and treatment decisions in coronary disease and accelerates plaque progression prediction. Currently, the understanding of coronary disease pathophysiology has been evaluated, indicating the inflammatory backgrounds combined with omics profiling, including the role of amino acid metabolism but significant knowledge gaps remain that should be addressed in future analyses to improve diagnostic tools and allow an individualized precision approach [27].

These findings provide several clinical insights. First, they support the concept that amino acid profiling can reveal pathophysiological processes beyond traditional risk factors. The distinct profile in MeS patients underscores the role of redox imbalance and immune dysregulation in cardiovascular risk. Second, the identification of threonine as a candidate biomarker of CAD, independent of MeS, is novel and may improve risk stratification if validated in larger cohorts.

A limitation of this study was the limited number of White patients, and the presented results will require further investigation in a larger-scale cohort.

We strongly believe that the results of our primary study may suggest novel pathophysiological mechanisms of atherosclerosis formation, independent of standard clinical factors and based on amino acid analysis.

Conclusions

In our discovery-oriented, preliminary study, we observed differences in amino acid profiles in MeS patients with chronic coronary disease, including altered concentrations of histidine, ethanolamine, cystine, proline, and tryptophan. Circulating threonine may be considered a novel exploratory signal of coronary disease risk, independent of metabolic syndrome. If validated, threonine may refine CAD risk stratification beyond traditional factors and MeS-related metabolic changes and inform mechanistic studies of amino-acid metabolism in atherogenesis

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Department and Institution Where Work Was Done

The work was done at the Department of Inorganic and Analytical Chemistry, Poznań University of Medical Sciences, Poznań, Poland.

Ethic Statement

The study was approved by the Institutional Review Board of Poznań University of Medical Sciences (protocol code 113/21, November 6, 2021). Informed consent was obtained from all participants.

Declaration of Figures' Authenticity

All figures submitted have been created by the authors who confirm that the images are original with no duplication and have not been previously published in whole or in part.

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