ORIGINAL ARTICLE

Metabolomic signature of arterial stiffness in male patients with peripheral arterial disease

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Arterial stiffness is an independent determinant of cardiovascular risk and a marker of subclinical organ damage. Metabolomics may facilitate identification of novel low-molecular cardiovascular risk factors. The aim of the present study was to compare metabolic signatures and functional–biochemical characteristics of patients with peripheral arterial disease (PAD) and clinically healthy subjects. We studied 42 men with symptomatic PAD (aged 66 ± 7 years) and 46 healthy men (aged 66 ± 8 years). Aortic pulse wave velocity (aPWV) was assessed by applanation tonometry using the Sphygmocor device. Metabolic profiling was performed with high-performance liquid chromatography and mass spectrometry. Serum oxidized low-density lipoprotein (oxLDL) level was measured by enzyme-linked immunosorbent assay. The aPWV as well as serum levels of lactate, free carnitine and 11 amino acids including tyrosine were higher among the patients with PAD. In contrast, serum levels of pyruvate, citrate, α -ketoglutarate, aconitate and cysteine were higher in the control group. In multiple regression models, aPWV was independently determined by log-tyrosine and log-oxLDL in the patients ($R^2 = 0.61$; $P < 0.001$) and by age, log-pyruvate and log-oxLDL in the controls ($R^2 = 0.52$; $P < 0.001$). Our study describes for the first time significant differences in metabolomic signature of patients with advanced atherosclerosis compared with clinically healthy controls. The aPWV is independently associated with serum levels of tyrosine and oxLDL in the patients with PAD and is related to pyruvate and oxLDL levels in the control group. The measurement of low-molecular metabolites, which are related to changes in vascular phenotypes, may lead to identification of novel vascular risk markers.

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INTRODUCTION

Cardiovascular diseases (CVDs) are the leading cause of mortality in developed countries.¹ However, their molecular etiology is still poorly understood as CVD develop slowly and are affected by multiple risk factors. The strong relationship of CVD with cholesterol and triglyceride levels has been recognized for many years.² However, apart from these associations, there is a strong possibility that integration of metabolomic profile with arterial functional parameters and data regarding atherogenic nature of low-density lipoprotein (LDL) atherogenicity may reveal novel vascular risk markers.

Arterial stiffness (AS) is a major determinant of cardiovascular risk and contributes to progression of atherosclerosis.³ Recent research indicates that aortic pulse wave velocity (aPWV), which is regarded as the current gold standard measure of AS, improves prediction of cardiovascular mortality beyond conventional risk factors.³ Arterial stiffening may impair distal tissue perfusion, thus affecting multiple metabolic pathways.⁴ Furthermore, decreased small artery elasticity is

an independent predictor of all-cause and cardiovascular mortality in patients with symptomatic peripheral arterial disease (PAD).⁵

The establishment of the nature and spectrum of metabolic dysbalances in CVD is an intriguing objective. It has been demonstrated that decreased serum levels of citrate, oxaloacetate and argininosuccinate are independently associated with inducible ischemia in the patients with coronary artery disease (CAD).⁶ A large prospective study has demonstrated that decreased arginine levels predict major adverse cardiovascular events in subjects undergoing cardiac catheterization.⁷ Apart from metabolic alterations, abnormal oxidation of LDL is an important step in initiation and progression of atherosclerosis.⁸ Recent research suggests that oxidized low-density lipoprotein (oxLDL) levels are increased in patients with coronary atherosclerosis⁸ and PAD.⁹ Recently, it has been shown that oxLDL is independently associated with 10-year risk of CAD events and improves the reclassification capacity of Framingham-derived risk functions.¹⁰

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Angiographic score (AngSc) provides information about the location and severity of atherosclerotic lesions¹¹ and is associated with AS⁹ in patients with PAD. The AngSc is negatively correlated with maximum treadmill walking distance¹² and ankle-brachial pressure index (ABPI),¹³ which is a functional measure of the severity of PAD.¹⁴ Furthermore, AngSc is an independent risk factor for major amputation in diabetic subjects with PAD.¹⁵

Previous studies have shown that PAD is associated with metabolic dysbalances¹⁶ and AS.¹⁷ However, the relationship between AS and serum low-molecular weight metabolites and oxLDL in the patients with PAD has not been studied. Thus, the aim of the present study was to compare the pattern of metabolic signatures and functional-biochemical characteristics of PAD patients and clinically healthy individuals.

MATERIALS AND METHODS

Study population

The study population comprised 42 men (mean age 66 ± 7 years) with symptomatic PAD. The patients were enrolled from the Department of Vascular Surgery, Tartu University Hospital, Estonia. The patients had stage II ($n=31$), III ($n=8$) or IV ($n=3$) of chronic ischemia as defined by Fontaine: stage II = intermittent claudication; stage III = leg pain at rest; stage IV = focal tissue necrosis or gangrene. Patients were excluded in case they had had myocardial infarction, coronary revascularization or cerebrovascular events during the previous 6 months, earlier revascularization procedures at the lower limb, atrial fibrillation, diabetes mellitus, malignancies and renal failure (estimated glomerular filtration rate (eGFR) $< 60 \text{ ml min}^{-1}$ per 1.73 m^2). Overall, 28 (67%) patients with hypertension and 7 (17%) patients with CAD were included in the study. Twenty-two patients received pentoxifylline, 14 patients used aspirin, 8 patients were treated with statins, 9 patients were treated with angiotensin-converting enzyme inhibitors, 11 patients used calcium channel blockers, 6 patients were treated with angiotensin-receptor blockers, 5 patients used β -blockers and 2 patients received diuretics.

A total of 46 clinically healthy men (mean age 66 ± 8 years) were recruited by a family physician. The exclusion criteria for the control group were as follows: CAD, cardiac arrhythmias or valve pathologies, cerebral or peripheral atherosclerotic disease, diabetes mellitus, malignancies, renal failure (eGFR $< 60 \text{ ml min}^{-1}$ per 1.73 m^2) and known inflammatory conditions. The control subjects did not use any medications regularly. The study complies with the Declaration of Helsinki. The study protocol was approved by the Ethics Committee of the University of Tartu. All subjects provided written informed consent.

Hemodynamic measurements

Brachial blood pressure (BP) and heart rate were registered in the supine position from the left arm using an automated digital oscillometric BP monitor (OMRON M4-I; Omron Healthcare Europe, Hoofddorp, The Netherlands). Pulse wave analysis (SCOR Px, 7.0; AtCor Medical, Sydney, NSW, Australia) was then used to generate a corresponding central waveform using a generalized transfer function, which has been prospectively validated for assessment of ascending aortic BP.¹⁸ Augmentation index (AIx), mean arterial pressure (MAP), central systolic BP (CSBP) and central diastolic BP (CDBP) were determined by pulse wave analysis. Aortic and brachial pulse wave velocities (aPWV and bPWV, respectively) was measured by the foot-to-foot method, using the same device as described previously.⁹

The ABPI was estimated using the Bidirectional Doppler MD 6 (DE; Hokanson, Bellevue, WA, USA). Systolic BP was measured bilaterally over the brachial, tibialis posterior and dorsalis pedis arteries. The higher systolic BP of the dorsalis pedis or the posterior tibial artery was used for calculation of the ABPI.¹⁹

Angiographic score

All the patients were examined with digital subtraction angiography (Axiom Artis; Siemens Medical Solutions, Forchheim, Germany) of the aorta and the

arteries of the lower extremities using the standard technique via the femoral approach at the Department of Radiology, Tartu University Hospital, Estonia, as was described in detail previously.⁹

Low-molecular weight metabolites

The metabolites were quantified on a Shimadzu Prominence (Shimadzu, Kyoto, Japan) HPLC and QTRAP 3200 (AB Sciex, Framingham, MA, USA) mass spectrometry tandem. For all metabolites, a standard curve of known concentrations consisting of five or six concentrations within two orders of magnitude around the expected concentrations was built. Quantification of metabolites in samples was based on these standard curves and carried out automatically with the mass spectrometry controller software Analyst 1.4.2. Unless specified otherwise, all the needed chemicals were from Sigma-Aldrich (Munich, Germany).

The protocol for amino acids and acylcarnitines was based on the work of Schultze *et al.*²⁰ The internal standards were from Cambridge Isotope Laboratories (Tewksbury, MA, USA). In brief, 100 μl plasma was mixed with 20 μl internal standards, dried, derivatized with 60 μl 3 M HCl/butanol at 65 °C for 15 min, dried and resolved in 100 μl acetonitrile:water (1:1). Fifteen microliters was injected into the mass spectrometry. The acylcarnitines were analyzed as precursors of $m/z=85$ fragments. Amino acids were analyzed by multiple reaction monitoring (MRM) scan with the following transitions: [²H₃] leucine 191/89, [²H₂]ornithine 191/72, [²H₃]methionine 209/107, [²H₄]alanine 150/48, [²H₅]phenylalanine 228/126, [²H₆]valine 182/80, [²H₄, ¹³C] arginine 236/75, [²H₂]citrulline 234/115, [²H₃]glutamate 263/87, [¹³C₆]tyrosine 244/142, [¹⁵N, ¹³C] glycine 134/78, [²H₃]aspartate 249/147, ornithine 189/70, arginine 231/70, glycine 132/76, citrulline 232/113, alanine 146/44, asparagine 189/144, aspartate 246/144, glutamine 203/84, glutamate 260/84, histidine 212/110, leucine 188/86, lysine 203/84, methionine 206/104, phenylalanine 222/120, proline 172/70, tryptophan 261/244, serine 162/60, threonine 176/74, tyrosine 238/136, valine 174/72, cysteine 353/130, 206/104 and hydroxyproline 189/87. Ionization was performed at 4500 V and 400 °C, declustering potential was set to 40 V and collision energy to 38 V.

For hydroxy acid analysis, 5 μl plasma was mixed with 35 μl (500 μM [²H₄] succinic acid and [²H₄]malonic acid in methanol). The samples were centrifuged for 15 min at 10 000 g and 20 μl was injected. An HILIC (Luna 5 μm HILIC 200 A, 150 $\times$ 3 mm²; Phenomenex, Torrance, CA, USA) column was used with a flow rate of 0.2 ml min⁻¹ and the eluents used were: A—5 mM ammonium formate in water and B—5 mM ammonium formate in methanol. The gradient was 5 min isocratic 95% eluent B, gradiental decline to 5% eluent B within 15 min and 5 isocratic flow of 5% eluent B. MRM transitions in negative polarization mode were [²H₄]succinic acid 121/77, [²H₄] malonic acid 106/59, citrate 191/111, 191/87, 191/129, α -oxoglutarate 145/101, 145/57, pyruvate 87/43, succinate 117/73, malonic acid 103/41, β -hydroxybutyrate 103/59, *cis*-aconitic acid 173/85, 173/129, 173/111 and oxaloacetate 131/87, 145/101. If multiple MRM transitions were used for a metabolite, their correlation with each other was confirmed and the average value of concentrations calculated from each MRM ion pair was taken. Ionization was performed at -4500 V and 200 °C, declustering potential was set to -20 V and collision energy to -10 to -30 V.

Statistical analysis

Software STATISTICA (version 10.0 for Windows; StatSoft, Tulsa, OK, USA) was used for all statistical analyses. Categorical variables are presented as proportions and continuous variables as a mean $\pm$ s.d. Log transformation was used for the skewed variables. As there was significant difference in BP between the groups, aPWV and bPWV have been adjusted for MAP. Multiple regression analysis was used to evaluate the independent determinants of aPWV. *P*-values < 0.05 were regarded as statistically significant.

RESULTS

Characteristics of the study population

The demographic and clinical characteristics of the participants are summarized in Table 1. The PAD patients presented with higher peripheral and central BP, aPWV, bPWV, AIx@75, glucose,

Table 1 Hemodynamic and biochemical characteristics of the study participants

Characteristic	PAD patients (n = 42)	Controls (n = 46)	P-value
Age (years)	66 ± 7	66 ± 8	0.78
BMI (kg m ⁻²)	25.9 ± 3.7	25.7 ± 4.7	0.84
MAP (mm Hg)	103.7 ± 13.1	92.6 ± 6.4	<0.001
PSBP (mm Hg)	148.8 ± 18.9	126.4 ± 7.5	<0.001
PDBP (mm Hg)	79.7 ± 9.4	75.2 ± 6.1	0.01
CSBP (mm Hg)	134.6 ± 17.4	117 ± 9.1	<0.001
CDBP (mm Hg)	80.6 ± 10	76 ± 6.2	0.01
Heart rate (beats min ⁻¹)	63 ± 11	60.7 ± 8.8	0.13
AIx@75 (%)	26.5 ± 7.1	18.6 ± 6.1	<0.001
aPWV (m s ⁻¹) ^a	9.9 ± 1.6	8.5 ± 0.7	<0.001
bPWV (m s ⁻¹) ^a	8.9 ± 0.6	8.6 ± 0.3	<0.001
Angiographic score	26.5 ± 8.3	ND	ND
ABPI	0.4 ± 0.3	1.2 ± 0.1	<0.001
Glucose (mmol l ⁻¹)	5.7 ± 0.7	5.3 ± 0.4	0.004
Total cholesterol (mmol l ⁻¹)	6 ± 1.1	5.5 ± 1.2	0.08
LDL-cholesterol (mmol l ⁻¹)	4.2 ± 1.1	3.9 ± 1.2	0.27
HDL-cholesterol (mmol l ⁻¹)	1.2 ± 0.3	1.3 ± 0.3	0.36
Triglycerides (mmol l ⁻¹)	1.7 ± 0.6	1.2 ± 0.5	<0.001
hsCRP (mg l ⁻¹) ^b	2.6 (1.4–5.6)	1 (0.5–1.7)	<0.001
eGFR (ml min ⁻¹ per 1.73 m ²)	97.8 ± 25.9	95.1 ± 18.6	0.68
oxLDL (U l ⁻¹)	69 (55–98)	41.9 (36–54.4)	<0.001

Abbreviations: ABPI, ankle brachial pressure index; AIx@75, augmentation index, corrected for a heart rate of 75 beats per minute; aPWV, aortic pulse wave velocity; BMI, body mass index; bPWV, brachial pulse wave velocity; CDBP, central diastolic blood pressure; CSBP, central systolic blood pressure; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; MAP, mean arterial pressure; oxLDL, oxidized low-density lipoprotein; PAD, peripheral arterial disease; PDBP, peripheral diastolic blood pressure; PSBP, peripheral systolic blood pressure. Entries in bold represent statistically significant differences.

^aaPWV and bPWV have been adjusted for MAP.

^bIndicates medians and interquartile ranges.

triglycerides and high-sensitivity C-reactive protein. There were no significant differences in age, body mass index (BMI), heart rate, LDL, low-density lipoprotein or eGFR.

The amino-acid levels are presented in Table 2. Serum levels of aspartate, glutamate, glycine, histidine, leucine, methionine, ornithine, phenylalanine, serine, threonine and tyrosine were significantly higher in the patients with atherosclerosis. By contrast, the circulating level of cysteine was higher in the controls. Next, we compared the levels of several key metabolites and acylcarnitines (Table 3). The PAD patients had higher circulating levels of 7-ketocholesterol, lactate and free carnitine. Serum levels of aconitate, α -ketoglutarate, citrate and pyruvate were higher in the clinically healthy subjects.

Relationship between AS and low-molecular-weight metabolites

The aPWV correlated significantly with log-phenylalanine ($r=0.66$, $P<0.001$), log-tyrosine ($r=0.56$, $P<0.001$), log-serine ($r=0.31$, $P=0.04$), glucose ($r=0.51$, $P<0.001$), AngSc ($r=0.32$, $P=0.04$), AIx@75 ($r=0.35$, $P=0.03$), bPWV ($r=0.67$, $P<0.001$), eGFR ($r=-0.39$, $P=0.01$) and log-oxLDL ($r=0.43$, $P=0.005$) for the patients with PAD. In the control group, aPWV was correlated to age ($r=0.48$, $P=0.001$), BMI ($r=0.37$, $P=0.01$), bPWV ($r=0.42$, $P=0.004$), log-oxLDL ($r=0.41$, $P=0.04$) and log-pyruvate ($r=0.54$, $P<0.001$). In multiple regression models, aPWV was determined by log-tyrosine and log-oxLDL in the patients ($R^2=0.61$; $P<0.001$) and by age, log-pyruvate and log-oxLDL in the controls ($R^2=0.52$; $P<0.001$) (Table 4). These associations remained statistically

significant after adjustment for BMI, ABPI, AngSc, cholesterol, triglycerides, high-sensitivity C-reactive protein, eGFR, glucose and heart rate.

The bPWV was correlated with AIx@75 ($r=0.41$, $P=0.01$), log-phenylalanine ($r=0.34$, $P=0.03$) and log-tyrosine ($r=0.4$, $P=0.001$) in the patient group. In the controls, bPWV was related to AIx@75 ($r=0.39$, $P=0.01$). The AIx@75 showed correlation with CSBP ($r=0.33$, $P=0.04$), MAP ($r=0.31$, $P=0.046$) and histidine ($r=-0.33$, $P=0.04$) in the patient group. In the apparently healthy subjects, AIx@75 was correlated with log-succinate ($r=0.52$, $P<0.001$). However, the associations of bPWV and AIx@75 with metabolic intermediates lost significance after adjustment for confounders.

Associations of phenylalanine, tyrosine and pyruvate with other variables

The log-phenylalanine correlated with age, alanine, asparagine, citrulline, glutamine, glucose, glycine, lysine, methionine, ornithine, log-serine, log-lactate, log-tyrosine and log-oxLDL in the PAD patients, whereas log-phenylalanine showed correlation with BMI, total cholesterol, LDL, log-oxLDL, alanine, asparagine, citrulline, cysteine, glutamine, log-lactate, lysine, methionine, ornithine and log-serine in the control group (Table 5). The log-tyrosine correlated with age, ABPI, PSBP, PDBP, CSBP, CDBP and glucose in patients with atherosclerosis. The log-tyrosine was associated with total cholesterol in the controls. The log-pyruvate was correlated with log-oxLDL, log-citrate, log-lactate, alanine, log- α -ketoglutarate, asparagine, cysteine, glutamine, glycine, lysine, ornithine and log-serine in the patient group. The log-pyruvate showed correlation with log-oxLDL, log- α -ketoglutarate and log-lactate in the apparently healthy subjects.

DISCUSSION

Metabolomic profiling of various pathological conditions is a new promising area in translational medicine.²¹ Recently, we have shown that oxLDL added power to discriminate survival time of patients with PAD.⁵ In the current study, we performed extensive metabolic profiling in the patients with established diagnosis of PAD as compared with clinically healthy individuals. We have shown that aPWV is independently associated with tyrosine and oxLDL in the patients with PAD. Moreover, aPWV was independently related to age, pyruvate and oxLDL in the control group. To our knowledge, this is the first study which comprehensively analyzed serum low-molecular-weight metabolites and investigated the relationship of AS with metabolic intermediates in patients with PAD in comparison with clinically healthy subjects.

Several studies have examined metabolic changes in subjects with atherosclerosis. A recent study has demonstrated that a pattern of chemical signals arising from fatty acid chain protons of lipoproteins and the choline head group protons of phospholipids indicates worse prognosis in patients with PAD.¹⁶ However, the latter study used nuclear magnetic resonance-based metabolic profiling and the majority of analytes within the profile remained unidentified. We extend these findings by showing that serum levels of phenylalanine, tyrosine, aspartate, glutamate, glycine, histidine, leucine, methionine, serine, ornithine, glucose, lactate and free carnitine were higher in the patient group. Higher levels of amino acids and glucose may reflect an increase in the size of the amino-acid pool (via both proteolysis and suppressed production of metabolites, such as amino-acid derivatives) and increased gluconeogenesis in the patient group. Interestingly, the levels of alanine and glutamine were similar but the level of ornithine

was different between the study groups. This could be explained by the increased proteolysis in PAD patients and the use of alanine as well as glutamine in nitrogen metabolism, which resulted in altered balance between the levels of pyruvate and alanine, as well as between the levels of glutamine and glutamate, respectively. Elevated lactate levels

indicate both diminished activity of lactate–glucose cycle as well as decreased aerobic glycolysis, which could be related to the impairment of distal tissue perfusion in the patients with atherosclerosis. However, the exact mechanisms of biochemical alterations in atherosclerosis cannot be clarified in this cross-sectional study.

Recent research indicates that medium- and long-chain acylcarnitines predict cardiovascular events in elderly subjects.²² By contrast, we did not find significant differences in serum levels of acylcarnitines between the study groups. However, Rizza *et al.*²² determined serum acylcarnitines by MRM, whereas we used precursor scan approach. Furthermore, the mean age of the subjects in the aforementioned study was 85 years,²² but in our study the mean age of the subjects was 66 years. Hence, accumulation of acylcarnitines, which could be indicative of inefficient β -oxidation and mitochondrial dysfunction, is affected by aging.

We have demonstrated that the patients with PAD had higher values of free carnitine as compared with the controls. Recent studies have highlighted the importance of carnitine derivatives in the atherogenesis. Animal models indicate that dietary L-carnitine promotes atherosclerosis through the increase synthesis of trimethylamine-N-oxide and the suppression of reverse cholesterol transport.²³ In a large cohort of patients undergoing cardiac evaluation, elevated plasma levels of L-carnitine were independently associated with major cardiovascular events.²³ However, the causal role of L-carnitine in arterial stiffening and atherosclerosis cannot be explained by our cross-sectional study.

A large study in patients undergoing coronary catheterization has demonstrated that diminished serum arginine and high citrulline levels are associated with higher risk of cardiovascular events during the first 3 years after angiography.⁷ These results are intriguing, as arginine increases endothelial NO production.²⁴ However, in our study, there were no differences in arginine or citrulline levels between the PAD patients and the controls. Differences between the results of

Table 2 Serum amino-acid levels of the study participants

Characteristic	PAD patients (n = 42)	Controls (n = 46)	P-value
Alanine ($\mu\text{mol l}^{-1}$)	156.3 $\pm$ 57.2	138.9 $\pm$ 44.4	0.11
Arginine ($\mu\text{mol l}^{-1}$)	164.4 $\pm$ 75	157.3 $\pm$ 41.3	0.57
Asparagine ($\mu\text{mol l}^{-1}$)	100.9 $\pm$ 48.4	96.4 $\pm$ 24.7	0.58
Aspartate ($\mu\text{mol l}^{-1}$)	319.9 $\pm$ 353.3	21.1 $\pm$ 21.9	<0.001
Citrulline ($\mu\text{mol l}^{-1}$)	183 $\pm$ 75	193 $\pm$ 64	0.29
Cysteine ($\mu\text{mol l}^{-1}$)	8.8 $\pm$ 9.2	15.3 $\pm$ 6.8	<0.001
Glutamine ($\mu\text{mol l}^{-1}$)	670.4 $\pm$ 253.6	643.1 $\pm$ 187.2	0.56
Glutamate ($\mu\text{mol l}^{-1}$)	613.5 $\pm$ 631.7	141.1 $\pm$ 127.2	<0.001
Glycine ($\mu\text{mol l}^{-1}$)	404.6 $\pm$ 149.2	320.6 $\pm$ 109.3	0.003
Histidine ($\mu\text{mol l}^{-1}$)	180.5 $\pm$ 48.9	156.1 $\pm$ 36.1	0.01
Hydroxyproline ($\mu\text{mol l}^{-1}$)	76 $\pm$ 25.1	69.7 $\pm$ 19.5	0.19
Leucine ($\mu\text{mol l}^{-1}$)	154.4 $\pm$ 38.3	131.4 $\pm$ 33.7	0.004
Lysine ($\mu\text{mol l}^{-1}$)	409.2 $\pm$ 122.1	371.9 $\pm$ 95.8	0.11
Methionine ($\mu\text{mol l}^{-1}$)	51.9 $\pm$ 13	42.6 $\pm$ 11.8	<0.001
Ornithine ($\mu\text{mol l}^{-1}$)	297.9 $\pm$ 103.7	208.5 $\pm$ 57	<0.001
Phenylalanine ^a ($\mu\text{mol l}^{-1}$)	141.1 (98.8–181)	108 (95.4–121)	0.004
Proline ($\mu\text{mol l}^{-1}$)	1268.5 $\pm$ 399.4	1123.3 $\pm$ 381	0.08
Serine ^a ($\mu\text{mol l}^{-1}$)	249 (183–291)	175.5 (152–223)	<0.001
Threonine ($\mu\text{mol l}^{-1}$)	22.1 $\pm$ 33.7	10.8 $\pm$ 16.7	0.047
Tryptophan ($\mu\text{mol l}^{-1}$)	26.3 $\pm$ 10.9	26.1 $\pm$ 8.6	0.91
Tyrosine ^a ($\mu\text{mol l}^{-1}$)	33.1 (23–37)	24.7 (20–31)	0.01
Valine ^a ($\mu\text{mol l}^{-1}$)	102.5 (87.8–135)	96 (84.4–109)	0.12

Abbreviation: PAD, peripheral arterial disease.

Entries in bold indicate statistically significant differences.

^aIndicates medians and interquartile ranges.

Table 3 Acylcarnitines, hydroxy acids and other metabolic parameters of the study participants

Characteristic	PAD patients (n = 42)	Controls (n = 46)	P-value
Aconitate ($\mu\text{mol l}^{-1}$) ^a	4.5 (3.3–6.9)	6.8 (5.4–8.8)	0.003
α -Ketoglutarate ($\mu\text{mol l}^{-1}$) ^a	6.2 (2.1–8.6)	8.8 (6.8–10)	0.002
β -Hydroxybutyrate ($\mu\text{mol l}^{-1}$) ^a	1.2 (0.6–6.4)	2.1 (1.3–6.8)	0.26
Citrate ($\mu\text{mol l}^{-1}$) ^a	286.1 (233.3–426)	442.1 (393–542)	<0.001
Citrulline ($\mu\text{mol l}^{-1}$)	183.8 $\pm$ 75.4	192.9 $\pm$ 64.2	0.54
7-Ketocholesterol ($\mu\text{mol l}^{-1}$) ^a	2.3 (1.8–24.2)	2.1 (1.8–2.6)	0.046
Lactate ($\mu\text{mol l}^{-1}$) ^a	823 (700.5–963.5)	571.3 (459–769)	<0.001
Malonate ($\mu\text{mol l}^{-1}$) ^a	32.8 (24.8–49.3)	1.7 (0.9–5.1)	0.15
Oxaloacetate ($\mu\text{mol l}^{-1}$)	35.6 $\pm$ 23.3	33.3 $\pm$ 27.2	0.67
Pyruvate ($\mu\text{mol l}^{-1}$) ^a	40.6 (25.1–52.6)	45.8 (35.9–65.3)	0.02
Succinate ($\mu\text{mol l}^{-1}$) ^a	14.7 (11.1–17.9)	14.5 (10.8–16.6)	0.43
Free carnitine (C0) ($\mu\text{mol l}^{-1}$) ^a	40.1 (31.5–50.1)	28.9 (21.5–38.1)	<0.001
Acetylcarnitine (C2) ($\mu\text{mol l}^{-1}$) ^a	14.5 (12.5–19.3)	17.2 (11.3–22.6)	0.47
Propionylcarnitine (C3) ($\mu\text{mol l}^{-1}$) ^a	0 (0–3.3)	0 (0–1.8)	0.68
Butyrylcarnitine (C4) ($\mu\text{mol l}^{-1}$) ^a	2.5 (0.1–7.1)	1.7 (0.1–5.9)	0.13
Pentanoylcarnitine (C5) ($\mu\text{mol l}^{-1}$) ^a	0.7 (0–3.3)	0.1 (0–3)	0.79
Hexanoylcarnitine (C6) ($\mu\text{mol l}^{-1}$) ^a	0.4 (0–3.1)	0.2 (0–3.3)	0.77
Octanoylcarnitine (C8) ($\mu\text{mol l}^{-1}$) ^a	2 (0–3.5)	2.5 (0–5.6)	0.46
Decanoylcarnitine (C10) ($\mu\text{mol l}^{-1}$) ^a	2.9 (0.9–4.9)	3.4 (0–6.5)	0.55
Tetradecanoylcarnitine (C14) ($\mu\text{mol l}^{-1}$) ^a	0.5 (0.2–1)	0.5 (0.1–0.9)	0.46
Octadecanoylcarnitine (C18) ($\mu\text{mol l}^{-1}$) ^a	0.1 (0–0.3)	0 (0–0.2)	0.16

Abbreviation: PAD, peripheral arterial disease.

Entries in bold indicate statistically significant differences.

^aIndicates medians and interquartile ranges.

the studies could be explained by the fact that arginine metabolism is mainly related to coronary rather than peripheral atherosclerosis. However, it should be emphasized that from three tightly related compounds of the urea cycle (ornithine, citrulline and arginine), the level of ornithine was significantly higher in the PAD patients. Another possible explanation is the difference in study designs. Tang *et al.*⁷ studied alterations of arginine bioavailability in a longitudinal study,

whereas we examined associations of metabolic signature and AS in a cross-sectional study. Therefore, our findings should be verified in prospective studies.

We have demonstrated that aPWV is independently associated with tyrosine and phenylalanine levels in the PAD patients. Indeed, the dominating amount of phenylalanine is converted into tyrosine. It could be that the relationship between tyrosine and AS is affected by alterations in glucose metabolism. Consistent with this hypothesis, both aPWV and tyrosine were correlated with serum glucose in the patients with PAD. A large prospective study has reported that serum levels of aromatic (e.g., tyrosine) and branched-chain amino acids predict the development of diabetes in normoglycemic subjects.²⁵ Furthermore, the same study group has shown that a metabolic cluster containing phenylalanine, tyrosine and isoleucine was independently associated with intima-media thickness and exercise-induced myocardial ischemia in subjects free of CVD.²⁶ In addition, each 1 s.d. increase in the diabetes-predictive amino-acid score was associated with approximately a 27% increased risk of future CVD.²⁶ Interestingly, in a large cohort of patients undergoing cardiac catheterization a panel of amino acids containing phenylalanine, tyrosine, isoleucine, leucine, valine and methionine was protective against all-cause mortality.²⁷ However, in the latter study 61% of patients had hemodynamically significant coronary stenoses,²⁷ whereas Magnusson *et al.*²⁶ studied clinically healthy subjects. The precise role

Table 4 Multiple regression analysis for PAD patients and the control subjects with aPWV adjusted for MAP as the dependent variable

	Regression coefficient	s.e.	P-value
Patients^a			
Log-tyrosine	0.53	0.12	<0.001
Log-oxLDL	0.34	0.1	0.002
Age (years)	0.21	0.12	0.09
Controls^b			
Age (years)	0.42	0.11	<0.001
Log-pyruvate	0.36	0.12	0.003
Log-oxLDL	0.31	0.11	0.01

Abbreviations: aPWV, aortic pulse wave velocity; MAP, mean arterial pressure; oxLDL, oxidized low-density lipoprotein; PAD, peripheral arterial disease.

Entries in bold represent statistically significant differences.

^aR² value=0.61; P<0.001; n=42.

^bR² value=0.52; P<0.001; n=46.

Table 5 Correlation coefficients of phenylalanine and tyrosine for the PAD patients and for the control subjects

Characteristic	PAD patients			Controls		
	Log-Phe	Log-Tyr	Log-Pyr	Log-Phe	Log-Tyr	Log-Pyr
Age (years)	0.42**	0.49**	0.19	0.1	-0.08	0.18
BMI (kg m ⁻²)	0.2	-0.12	0.18	0.38**	0.2	0.25
ABPI	-0.26	-0.31*	0.13	0.1	0.05	-0.12
AngSc	0.33*	0.64**	-0.18	ND	ND	ND
PSBP (mm Hg)	0.3	0.43**	0.01	0.17	0.15	0.05
PDBP (mm Hg)	0.27	0.4**	0.01	0.09	0.21	0.1
CSBP (mm Hg)	0.24	0.36*	-0.13	0.16	0.11	-0.13
CDBP (mm Hg)	0.28	0.33*	-0.05	0.08	0.2	0.11
Alanine (μmol l ⁻¹)	0.56**	0.18	0.42**	0.68**	0.1	-0.01
Log-AKG	0.27	0.1	0.79**	0.17	0.02	0.33*
Asparagine (μmol l ⁻¹)	0.39*	0.16	0.7**	0.68**	-0.03	-0.04
Log-citrate	0.24	0.11	0.52**	0.1	-0.01	0.07
Citrulline	0.32*	0.16	0.15	0.63**	0.01	-0.1
Cysteine	0.17	0.01	0.42**	0.56**	-0.1	-0.16
Glucose (mmol l ⁻¹)	0.54**	0.42**	0.15	-0.04	0.13	-0.03
Glutamine (μmol l ⁻¹)	0.34*	0.04	0.41**	0.72**	-0.16	-0.11
Glycine (μmol l ⁻¹)	0.45**	0.14	0.5**	0.26	0.2	-0.14
Log-lactate	0.35*	0.01	0.64**	0.31*	0.04	0.36*
LDL (mmol l ⁻¹)	0.06	0.23	0.02	0.35*	0.28	0.03
Lysine (μmol l ⁻¹)	0.36*	0.08	0.44**	0.75**	-0.05	-0.1
Methionine (μmol l ⁻¹)	0.41**	0.07	0.23	0.76**	-0.21	-0.1
Ornithine (μmol l ⁻¹)	0.53**	0.2	0.46**	0.56**	0.1	-0.12
Log-oxLDL	0.32*	0.12	0.33*	0.37*	0.1	0.32*
Log-serine	0.57**	0.29	0.59**	0.37*	0.01	-0.16
Total chol (mmol l ⁻¹)	0.05	0.27	-0.03	0.36*	0.3*	0.02
Log-Tyr	0.54**	ND	0.02	0.14	ND	0.03

Abbreviations: ABPI, ankle brachial pressure index; AKG, α-ketoglutarate; AngSc, angiographic score; BMI, body mass index; Chol, cholesterol; CSBP, central systolic blood pressure; CDBP, central diastolic blood pressure; LDL, low-density lipoprotein; ND, not determined; oxLDL, oxidized low-density lipoprotein; PAD, peripheral arterial disease; PDBP, peripheral diastolic blood pressure; PSBP, peripheral systolic blood pressure; Phe, phenylalanine; Pyr, pyruvate; Tyr, tyrosine.

Entries in bold indicate statistically significant correlations between the parameters.

*P<0.05, **P<0.01.

of aromatic and branched-chain amino acids remains to be elucidated in future studies.

Our findings revealed that AngSc is correlated with serum levels of phenylalanine and tyrosine. Accordingly, the results of animal experiments²⁸ and human studies²⁹ indicate that myocardial infarction is associated with increased levels of phenylalanine. It could be hypothesized that the association of phenylalanine with the extent of atherosclerosis is influenced by high-grade oxidative stress. It has been demonstrated that decreased levels of reduced glutathione and elevated levels of phenylalanine promote oxidative stress.³⁰ We have shown that serum phenylalanine is related to oxLDL in the patient group. Consistently, it has been demonstrated that plasma total antioxidant reactivity is significantly lower in patients with phenylketonuria and it is inversely correlated with phenylalanine levels.³¹ Alternatively, the relationship between severity grade of atherosclerosis and aromatic amino acids could be affected by BP. As tyrosine is a precursor to dopamine, epinephrine and norepinephrine,³² it could be hypothesized that tyrosine modulates BP through the synthesis of catecholamines. In our study, tyrosine level was correlated with peripheral and central BP in the patient group.

We have shown that aPWV is associated with serum pyruvate in the control group. As pyruvate is an important intermediate in glucose catabolism, this association could indicate that glycolysis is related to AS in clinically healthy subjects. Moreover, serum pyruvate was correlated with oxLDL in the controls. It might be that high-grade oxidative stress is a possible link between AS and alterations in pyruvate metabolism in apparently healthy individuals. However, the precise mechanisms underlying these associations remain to be elucidated in further studies.

There are several limitations in the current study. First, the major limitation of our study is its cross-sectional design. Therefore, we cannot infer causal relationship between AS, low-molecular-weight metabolites and atherosclerosis. Our findings should be confirmed in future longitudinal studies. Second, as the sample size was relatively small, additional studies using a larger patient population should be conducted to identify the impact of low-molecular-weight metabolites on arterial stiffening. Third, all the study participants were men. As there might be differences in metabolic intermediate levels between men and women, our results cannot be extrapolated to women. Fourth, we did not collect dietary information of the study participants. However, blood samples were taken after overnight fasting to minimize the impact of diet on the serum metabolite levels. Finally, as the sample size was relatively small, additional studies using a larger patient population should be conducted to identify the impact of low-molecular-weight metabolites on arterial stiffening.

In conclusion, our results suggest that tyrosine and oxLDL might be associated with AS in patients with PAD, whereas pyruvate and oxLDL could influence AS in clinically healthy subjects. Potentially, metabolic profiling may elucidate pathways that affect arterial stiffening in patients with atherosclerosis and in apparently healthy individuals. Identification of low-molecular-weight metabolites, which are related to changes in vascular phenotype, may lead to improved cardiovascular risk prediction based on arteriobolomic parameters (i.e., metabolomic and arterial wall phenotype).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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