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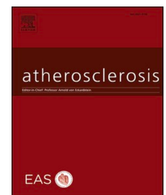
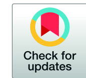


Metabolic profiling links cardiovascular risk and vascular end organ damage



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ABSTRACT

Background and aims: An untargeted metabolomics approach allows for a better understanding and identification of new candidate metabolites involved in the etiology of vascular disease. We aimed to investigate the associations of cardiovascular (CV) risk factors with the metabolic fingerprint and macro- and microvascular health in an untargeted metabolomic approach in predefined CV risk groups of aged individuals.
Methods: The metabolic fingerprint and the macro- and microvascular health from 155 well-characterized aged (50–80 years) individuals, based on the EXAMIN AGE study, were analysed. Nuclear magnetic resonance spectroscopy was used to analyse the metabolic fingerprint. Carotid-femoral pulse wave velocity and retinal vessel diameters were assessed to quantify macro- and microvascular health.
Results: The metabolic fingerprint became more heterogeneous with an increasing number of risk factors. There was strong evidence for higher levels of glutamine [estimate (95% CI): −14.54 (−17.81 to −11.27), $p < 0.001$], glycine [−5.84 (−7.88 to −3.79), $p < 0.001$], histidine [−0.73 (−0.96 to −0.50), $p < 0.001$], and acetate [−1.68 (−2.91 to −0.46), $p = 0.007$] to be associated with a lower CV risk profile. Tryptophan, however, was positively associated with higher CV risk [0.31 (0.06–0.56), $p = 0.015$]. The combination of *a priori* defined CV risk factors explained up to 45.4% of the metabolic variation. The metabolic fingerprint explained 20% of macro- and 23% of microvascular variation.
Conclusions: Metabolic profiling has the potential to improve CV risk stratification by identifying new underlying metabolic pathways associated with atherosclerotic disease development, from cardiovascular risk to metabolites, to vascular end organ damage.

1. Introduction

Cardiovascular (CV) diseases are still the main cause of non-communicable deaths worldwide. The mechanistic pathways of how classical CV risk factors, such as hypertension, diabetes, obesity, hypocholesteraemia or low cardiorespiratory fitness (CRF), affect macro- and microvascular health are not fully understood.

Vascular health can be assessed in the macro- or microcirculation as a subclinical biomarker of vascular end organ damage. Pulse wave velocity (PWV) is the gold standard to quantify arterial stiffness in the macrocirculation. Central PWV has the potential to improve CV risk prediction with a reclassification rate between 13 and 15% [1,2], compared to standard CV risk assessment, and is a reliable predictor for CV risk in the general population [1]. An increase of 1 m/s in central PWV has previously been associated with a 15% higher CV risk and all-cause mortality [1]. Static retinal vessel analysis (SVA) is a non-invasive approach to investigate microvascular health [3]. Narrowing of central retinal arteriolar (CRAE) and widening of central

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retinal venular diameter equivalents (CRVE) have previously been associated with stroke [4], coronary heart disease [5] and higher CV mortality [6] and seem therefore to be predictive for long-term CV outcomes [7,8]. Retinal vessel analysis has been shown to have added value for CV risk stratification of stroke [8] and CV events [7] on top of classical risk factors.

Metabolites drive various processes affecting vascular health, such as inflammation, glycolysis and fluid balance [9,10]. Interestingly, the metabolome is influenced by both genome and exposome [9]. Thus, metabolic phenotyping seems to have the potential to provide insights into multiple molecular mechanisms underlying vascular health that contribute to the pathogenesis of CV disease [11,12]. Previous studies reported improved accuracy for CV risk stratification by additional analysis of the metabolic profile [10,13]. A recent scientific statement from the American Heart Association highlighted the potential of metabolomics in CV health and disease [14]. Our study, for the first time, aimed to investigate associations of CV risk factors with the metabolic profile and vascular health in an untargeted metabolomics approach in predefined CV risk groups of aged individuals.

2. Materials and methods

2.1. Study design

This cross-sectional study investigated the metabolic profile from a well-characterized aged cohort (EXAMIN AGE study [15]). One-hundred and fifty-five aged (50–80 years) men and women with and without CV risk factors were included in this study. Blood sampling and extensive clinical phenotyping were performed between 2016 and 2018 at the Department of Sport, Exercise and Health in Basel, Switzerland. Participants were asked to refrain from exercise 24 h, and from alcohol and caffeine consumption 12 h prior to the examination. Blood sampling as well as vascular phenotyping were performed under fasting conditions. Metabolites were analysed in 2020 at the Gottfried Schatz Research Center for Cell Signaling, Metabolism and Aging, Molecular Biology and Biochemistry, Medical University of Graz. Researchers who analysed blood samples as well as vascular parameters were blinded to group allocation or any other sample characteristics. The study was registered at ClinicalTrials.gov (NCT02796976) and was conducted in accordance to the principles stated in the Helsinki Declaration [16]. This study was approved from the Ethics Committee of Northwest and Central Switzerland (EKNZ 2015-351). All individuals signed a written informed consent before the first measurement took place.

2.2. Inclusion and exclusion criteria

The EXAMIN AGE study population was characterised by healthy individuals and CV risk patients between 50 and 80 years. Healthy individuals were defined by the absence of any of the CV risk factors described below. CV risk patients were inactive ($\leq$one MET per week) and had at least two CV risk factors. Risk factors were defined as obesity, hypertension, diabetes, active smoking status, low high-density-lipoprotein (HDL) or high low-density-lipoprotein (LDL) levels or hypertriglyceridemia, or intake of medication to treat these risk factors (Supplementary Table S1). Participants were separated into four CV risk groups. Participants in group I were healthy and had no CV risk factors except age (low CV risk), group II had two CV risk factors, group III had three CV risk factors, and group IV had $>$ three CV risk factors. Individuals with decompensated pulmonary or chronic inflammatory disease, macular degeneration, glaucoma or any chronic eye disease or compromising orthopaedic problems were excluded. A study protocol with detailed descriptions of the inclusion and exclusion criteria and the phenotyping process has been published previously [15]. The PROCAM scoring system was used to calculate the ten-year risk for acute coronary events [17].

2.3. Blood sampling

Trained medical staff withdrew blood samples by venepuncture of the cubital fossa of the right or left arm. Serum was isolated and stored at -80 °C after centrifugation by 3000g for 10 min.

2.4. Reagents

Sodium phosphate, dibasic (Na_2HPO_4), sodium hydroxide, hydrochloric acid (32% m/v), and sodium azide (NaN_3) were obtained from VWR International (Darmstadt, Germany). 3(trimethylsilyl) propionic acid-2,2,3,3-d_4 sodium salt (TSP) was obtained from Alfa Aesar (Karlsruhe, Germany). Deuterium oxide (D_2O) was obtained from Cambridge Isotopes laboratories (Tewksbury, MA). Deionized water was purified using in-house Milli-Q Advantage Water Purification System from Millipore (Schwalbach, Germany). The phosphate NMR buffer solution was prepared by dissolving 5.56 g of anhydrous Na_2HPO4, 0.4 g of TSP, and 0.2 g NaN_3, in 400 mL of D_2O and adjusted to pH 7.4 with 1 M NaOH and HCl. Upon addition of D_2O to a final volume of 500 mL, the pH was re-adjusted to pH 7.4 with 1 M NaOH and HCl.

2.5. Metabolic phenotyping by NMR spectroscopy

To remove proteins and to quench enzymatic reactions in the samples, 200 μL serum was mixed with 400 μL methanol and stored at -20 °C for 1 h until further processing. Afterwards, the samples were spun at 17,949 relative centrifugal force at 4 °C for 30 min. Supernatants were lyophilized and 500 μL of NMR buffer in D_2O was added to the samples and transferred to 5 mm NMR tubes. All NMR experiments were performed at 310 K on an AVANCE™ Neo Bruker Ultrashield 600 MHz spectrometer equipped with a TXI probe head. The 1D CPMG (Carr-Purcell_Meiboom_Gill) pulse sequence (cpmgpr1d, 512 scans, 73,728 points in F1, 11,904.76 HZ spectral width, 512 transients, recycle delays 4 s) with water suppression using pre-saturation, was used for ^{1}H 1D NMR experiments [18]. Bruker Topspin version 4.0.2 was used for NMR data acquisition. The spectra for all samples were automatically processed (exponential line broadening of 0.3 Hz), phased, and referenced using trisodium phosphate (TSP) at 0.0 ppm using Bruker Topspin 4.0.2 software (Bruker GmbH, Rheinstetten, Germany).

2.6. Statistical analysis

Spectra pre-processing and data analyses have been carried out using the state-of-the-art data analysis pipeline proposed by the group of Prof. Jeremy Nicholson at Imperials College London using Matlab® scripts and MetaboAnalyst 4.0 [19]. NMR data were imported to Matlab® vR2014a (Mathworks, Natick, Massachusetts, United States), regions around the water, TSP, and remaining methanol signals excluded, and probabilistic quotient normalization was performed to correct for sample metabolite dilution. To identify differences in metabolic profiles, multivariate statistical analysis was performed in Matlab® and MetaboAnalyst, 4.0 and included Principal Component Analysis (PCA), Orthogonal-Partial Least Squares - Discriminant Analysis (O-PLS-DA), and all associated data consistency checks and 7-fold cross-validation. Stated concentrations correspond to normalized concentrations after probabilistic quotient normalization. Metabolite identification was carried out using Chenomx NMR Suite 7.6 (Chenomx Inc., Edmonton, AB, Canada) and reference compounds. Quantification of metabolites was carried out by signal integration of normalized spectra. As we focussed on polar metabolites, we removed lipoproteins using methanol extraction to reduce signal overlap and to obtain high-quality metabolite data for untargeted metabolomics. Lipids remaining after the extraction were combined to total lipoprotein-bound lipids for further analysis. Reduced NMR spectra revealed altered serum concentrations of different metabolites that were further analysed using univariate statistics. Further use of analysis of variance and linear regression models and other statistical approaches are described in the Supplementary materials.

2.7. Vascular health

The methods to assess PWV using applanation tonometry as a marker of large artery stiffness and analysis of retinal vessel diameters as a microvascular biomarker have previously been described in the published study protocol [15]. A detailed description of the assessments can be found in the Supplementary materials.

3. Results

Three hundred and thirty-seven participants were screened for eligibility. One hundred and eighty-two participants were excluded, n = 174 due to in- and exclusion criteria and n = 8 declined to participate. One hundred and fifty-five participants were included to investigate the association of CV risk factors with the metabolic fingerprint and macro- as well as microvascular health. Seventy-three participants were allocated to CV risk group I, 30 to risk group II, 27 to risk group III, and 25 to risk group IV. Sample characteristics for the whole cohort and separated for the four CV risk groups are described in Table 1. Risk groups differed in several CV risk factors, physical activity levels and CRF as well as macro- and microvascular health. Risk group I was characterized by the lowest risk and group IV by the highest CV risk profile (Table 1). Risk factor distribution is described in Supplementary Table S1.

3.1. Metabolites and cardiovascular risk

Using untargeted NMR spectroscopy, the metabolic fingerprints of serum samples were determined. PCA and Partial Least Squares-Discriminant Analysis (PLS-DA) of serum samples showed that the serum metabolic profiles gradually changed with the increasing number of risk factors (Fig. 1A). While the metabolic profile of individuals with a small number of risk factors was more homogenous, the profile became more heterogeneous with increasing numbers of risk factors. When comparing differences in metabolic fingerprint between serum samples of individuals with different numbers of risk factors, O-PLS-DA revealed increased clustering of patient samples with increasing correlation coefficients R^2Y up to 0.534 and Q^2 of 0.442 ($p < 0.01$) (Fig. 1B and C). Reduced NMR spectra revealed altered serum metabolites in different numbers of risk factors (Fig. 1D) and indicated that the serum levels of lipoprotein-bound lipids, acetate, glutamine, glycine, histidine, phenylalanine, and tryptophan significantly changed. These six metabolites and total lipoprotein-bound lipids were further analysed using univariate statistics. Branched-chain amino acids valine, leucine, and isoleucine have been reported to be associated with CV risk [20,21], but have not been observed to be significantly different in our study sample.

Five (glutamine, glycine, histidine, tryptophan, acetate) metabolites showed statistically significant group differences between the CV risk groups (Fig. 2). Risk group I (low risk) showed higher glutamine, glycine and histidine concentrations compared to risk group II, III and IV. In addition, tryptophan levels were lower in risk group I compared to risk group III and acetate concentration was higher in risk group I compared to risk group IV (Fig. 2). There was strong evidence for higher levels of glutamine [estimate (95% CI): −14.54 (−17.81 to −11.27), $p < 0.001$], glycine [−5.84 (−7.88 to −3.79), $p < 0.001$], histidine [−0.73 (−0.96 to −0.50), $p < 0.001$], and acetate [−1.68 (−2.91 to −0.46), $p = 0.007$] to be associated with a lower CV risk profile. Tryptophan, however, was positively associated with higher CV risk [0.31 (0.06–0.56), $p = 0.015$]. There was no evidence for an association of phenylalanine [-0.35 (−0.97 to 0.28), $p = 0.274$] and total lipoprotein-bound lipids [3.55 (−1.17 to 8.28), $p = 0.139$] with CV risk (Fig. 2).

Table 1
Sample characteristics.

	Whole population mean (SD)	Risk group I mean (SD)	Risk group II mean (SD)	Risk group III mean (SD)	Risk group IV mean (SD)	*p*
Sex (f/m)	84/71	43/30	20/10	14/13	7/18	0.015
Age (years)	59 (7)	59 (7)	58 (7)	58 (6)	59 (7)	0.444
Height (cm)	169 (8)	169 (8)	166 (8)	169 (7)	172 (7)	0.295
Body mass (kg)	82 (18)	67 (9)	91 (16)	96 (13)	98 (12)	<0.001
BMI (kg/m^2)	29 (6)	23 (3)	33 (5)	34 (4)	33 (3)	<0.001
WC (cm)	99 (17)	86 (8)	108 (13)	113 (12)	114 (8)	<0.001
Fat mass (kg)	29 (13)	18 (7)	39 (10)	39 (11)	37 (10)	<0.001
Muscle mass (kg)	30 (6)	27 (5)	29 (7)	32 (7)	34 (7)	<0.001
Rest sys. BP (mmHg)	130 (15)	128 (15)	133 (15)	132 (17)	132 (13)	0.112
Rest dia. BP (mmHg)	84 (10)	79 (8)	86 (11)	87 (8)	89 (10)	<0.001
24 h sys. BP (mmHg)	125 (10)	120 (7)	128 (10)	130 (11)	131 (11)	<0.001
24 h dia. BP (mmHg)	79 (7)	76 (6)	79 (7)	82 (9)	82 (8)	<0.001
Fasting glucose (mmol/l)	5.3 (1.4)	4.7 (0.5)	5.2 (0.7)	5.4 (0.9)	6.8 (2.7)	<0.001
Triglyceride (mmol/l)	1.42 (0.91)	0.99 (0.30)	1.43 (0.57)	1.47 (0.53)	2.60 (1.52)	<0.001
HDL (mmol/l)	1.6 (0.5)	1.8 (0.4)	1.3 (0.3)	1.4 (0.3)	1.2 (0.3)	<0.001
LDL (mmol/l)	3.1 (0.8)	3.0 (0.8)	3.3 (0.5)	3.1 (0.7)	3.1 (1.1)	0.547
Hs-CRP (mg/l)	2.6 (3.5)	1.4 (2.2)	3.1 (3.4)	4.0 (5.7)	3.9 (2.8)	<0.001
PROCAM score	36 (10)	30 (8)	39 (9)	42 (9)	44 (10)	<0.001
10 years risk (%)	6.5 (6.4)	3.3 (2.8)	7.6 (5.7)	9.8 (7.7)	11.7 (8.7)	<0.001
Activity and fitness						
Steps per day (n)	10,082 (4385)	11,728 (4677)	8836 (4270)	8659 (3086)	8309 (3113)	<0.001
Distance per day (m)	6628 (3118)	7910 (3400)	5471 (2660)	5550 (2176)	5434 (2137)	<0.001
Fast walking (min)	1190 (1019)	1579 (1167)	897 (753)	879 (748)	735 (624)	<0.001
FQPA (METs)	12.5 (26.4)	25.0 (34.4)	1.1 (2.2)	2.5 (4.0)	0.4 (1.3)	<0.001
VO_2peak (ml/min/kg)	31 (9)	36 (9)	26 (4)	26 (5)	25 (4)	<0.001
Vascular health						
CRAE (μm)	174 (14)	176 (14)	173 (17)	170 (14)	173 (12)	0.179
CRVE (μm)	213 (17)	207 (15)	219 (18)	219 (17)	219 (12)	<0.001
AVR	0.82 (0.06)	0.85 (0.05)	0.79 (0.05)	0.78 (0.04)	0.79 (0.04)	<0.001
PWV (m/s)	7.8 (1.6)	7.2 (1.5)	7.8 (1.1)	8.4 (1.4)	8.9 (1.7)	<0.001

BMI, body mass index; WC, waist circumference; BP, blood pressure; HDL, high-density lipoprotein, LDL, low-density lipoprotein; Hs-CRP, high-sensitive C-reactive protein; FQPA, Freiburg questionnaire of physical activity; METs, metabolic equivalents; VO_2peak, peak oxygen uptake; CRAE, central retinal arteriolar equivalent; CRVE, central retinal venular equivalent; AVR, arteriolar-to-venular diameter ratio; PWV, pulse wave velocity; SD, standard deviation; *p*, level of significance for overall group differences.

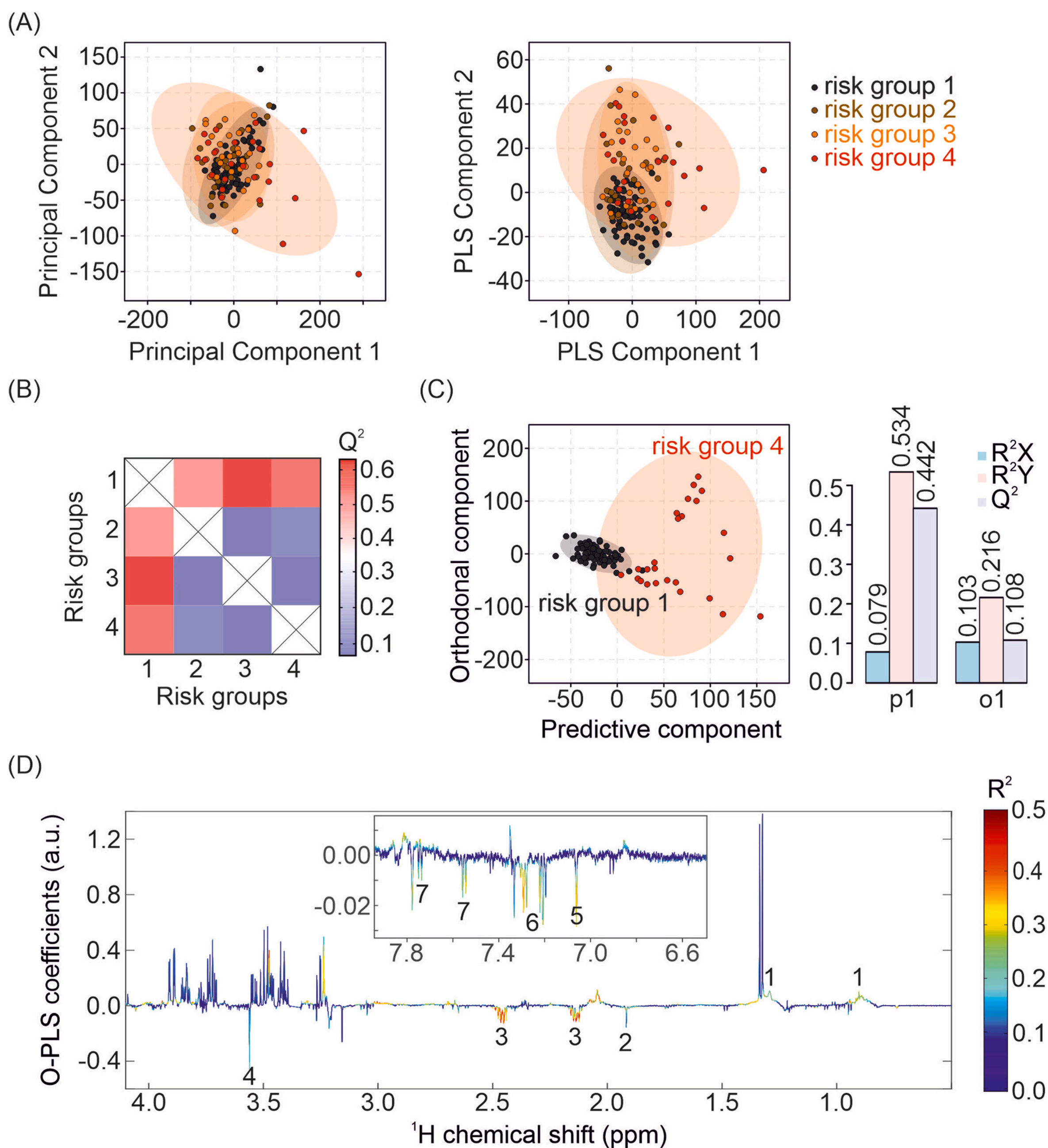

Fig. 1. NMR enables identification of metabolites associated with CV risk profile.
(A) Principal component analysis and partial least squares-discriminant analysis plots of 155 serum samples. (B) Heatmap showing orthogonal-partial least squares - discriminant analysis derived (O-PLS-DA) Q^2 for pairwise comparisons of serum samples. (C) O-PLS-DA plot of serum samples of risk group I and risk group IV, including cross validation. (D) The reduced nuclear magnetic resonance spectroscopy spectrum reveals altered components in normalized serum samples. Positive covariance corresponds to component present at increased concentrations, whereas negative covariance corresponds to decreased component concentration. Predictivity of the model is represented by R^2. 1 = lipoprotein-bound lipids, 2 = acetate, 3 = glutamine, 4 = glycine, 5 = histidine, 6 = phenylalanine, 7 = tryptophan.

The combination of *a priori* defined CV risk factors (obesity, hypertension, diabetes, hypercholesterolemia, smoking and low CRF) together explained 45.43%, 37.78%, 38.15%, 9.82%, 15.75%, 14.76%, and 17.34% of the variation of glutamine, glycine, histidine, tryptophan, phenylalanine, acetate, and total lipoprotein-bound lipids, respectively. The adjusted R^2 of metabolites and CV risk factors are described separately in Table 2.

Correlation coefficients and 95% confidence intervals of correlations between metabolites and sample characteristics are described in Table 3. Obesity [body mass, BMI, waist circumference (WC), and fat mass] was associated with lower glutamine, glycine, and histidine levels as well as higher tryptophan levels. Higher glucose levels were associated with lower and HDL with higher glutamine, glycine and histidine concentrations. Higher LDL levels were associated with higher phenylalanine and acetate concentrations. High-sensitive C-reactive protein (hs-CRP) and PROCAM score were associated with lower glutamine, glycine and histidine as well higher tryptophan levels. Fast walking and VO_2 peak were positively associated with glutamine and histidine. Further associations are described in Table 3.

3.2. Metabolites and vascular health

Glutamine, glycine, histidine, tryptophan, phenylalanine, acetate and total lipoprotein-bound lipids together explained 20% of the PWV

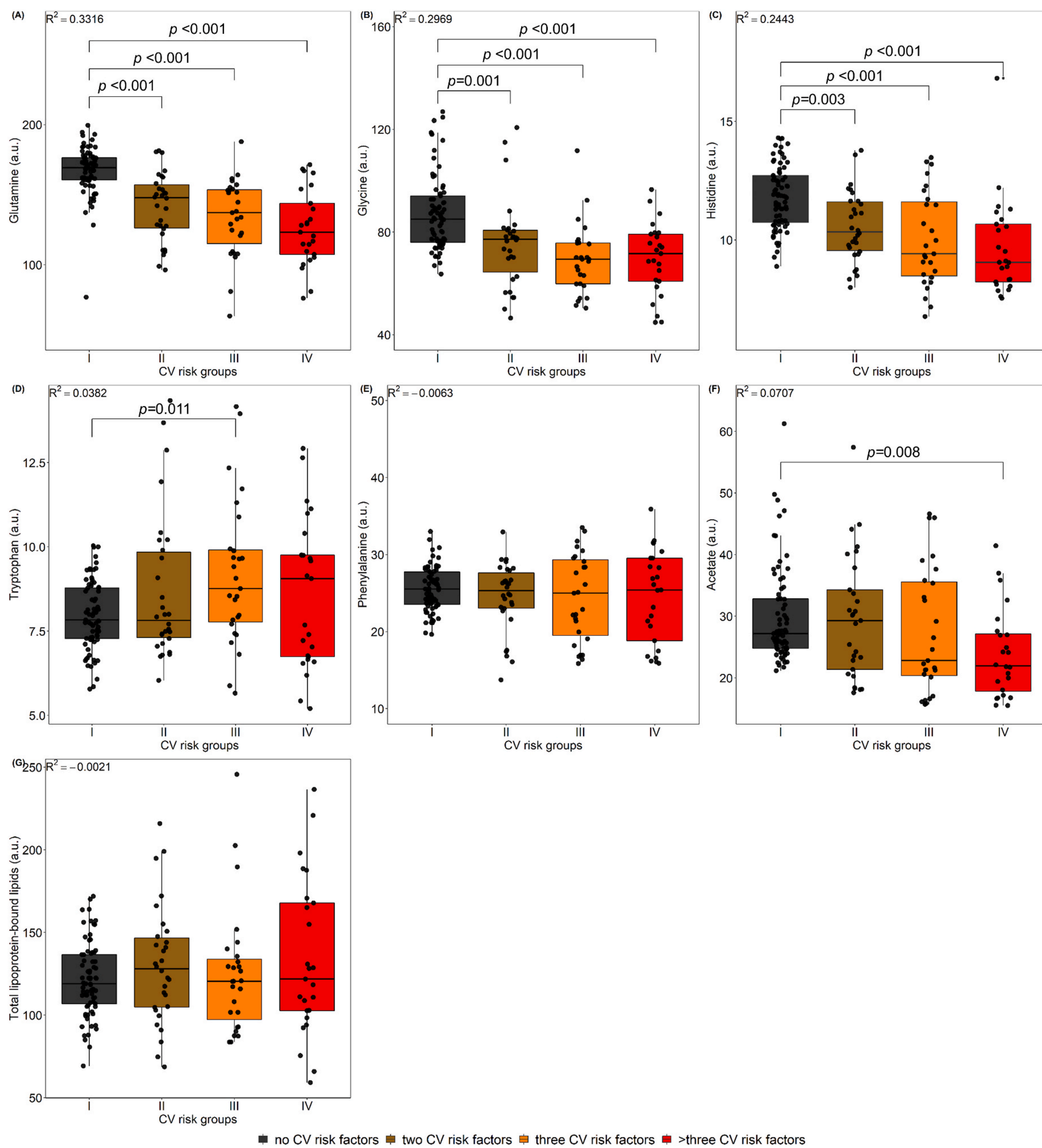

Fig. 2. Cardiovascular risk and metabolic profile.
Group differences (analysis of variance) of cardiovascular (CV) risk groups and metabolites as well as adjusted R^2 of linear regression models analysing potential associations of metabolites and CV risk in all 155 individuals.

Table 2
Adjusted R^2 of linear regression models of metabolites as dependent variable and cardiovascular risk factors as predictor.

predictor	Glutamine	Glycine	Histidine	Tryptophan	Phenylalanine	Acetate	Lipoprotein-bound lipids
BMI	0.097	0.058	0.085	0.016	0.004	0.018	0.005
Hypertension	0.080	0.102	0.053	0.010	0.036	0.008	0.027
Diabetes	0.095	0.067	0.132	0.003	0.021	0.033	0.018
Triglyceride	0.064	0.013	0.023	0.008	0.002	0.015	0.007
HDL	0.018	0.045	0.034	0.031	0.002	0.003	0.025
LDL	0.002	0.004	0.009	0.002	0.038	0.030	0.011
Smoking status	0.069	0.042	0.028	0.022	0.037	0.011	0.036
CRF	0.030	0.046	0.017	0.008	0.019	0.031	0.044

BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; CRF, cardiorespiratory fitness.

Table 3
Correlation coefficient and 95% confidence interval of correlations between metabolites and sample characteristics.

	Glutamine	Glycine	Histidine	Tryptophan	Phenylalanine	Acetate	Lipoprotein-bound lipids
Body mass	−0.50 (−0.614; −0.377)***	−0.44 (−0.559; −0.304)***	−0.50 (−0.609; −0.371)***	0.27 (0.119; 0.411)***	−0.07 (−0.227; −0.087)	−0.24 (−0.384; −0.087)**	−0.02 (−0.174; 0.141)
BMI	−0.53 (−0.631; −0.402)***	−0.36 (−0.494; −0.220)***	−0.46 (−0.572; −0.321)***	0.19 (0.037; 0.341)*	−0.03 (−0.184; 0.131)	−0.14 (−0.295; 0.013)	0.01 (−0.149; 0.167)
WC	−0.54 (−0.641; −0.416)***	−0.47 (−0.584; −0.337)***	−0.50 (−0.612; −0.376)***	0.21 (0.056; 0.357)**	−0.04 (−0.194; 0.120	−0.21 (−0.360; −0.059)**	−0.02 (−0.182; 0.133)
Fat mass	−0.46 (−0.579; −0.331)***	−0.25 (−0.388; −0.002)**	−0.36 (−0.488; −0.212)***	0.20 (0.042; 0.345)*	0.02 (−0.137; 0.178)	−0.08 (−0.239; 0.074)	−0.03 (−0.183; 0.132)
24 h. sys. BP	−0.22 (−0.365; −0.064)**	−0.24 (−0.388; −0.090)**	−0.07 (−0.227; 0.088)	0.03 (−0.131; 0.185)	0.09 (−0.066; 0.248)	−0.05 (−0.205; 0.111)	−0.13 (−0.284; 0.026)
24 h. dia. BP	−0.17 (−0.324; −0.017)*	−0.27 (−0.409; −0.115)***	−0.06 (−0.217; 0.098)	−0.07 (−0.224; 0.091)	0.06 (−0.102; 0.214)	−0.03 (−0.185; 0.131	−0.01 (−0.173; 0.144)
Glucose	−0.44 (−0.555; −0.299)***	−0.25 (−0.395; −0.099)**	−0.41 (−0.532; −0.268)***	−0.05 (−0.205; 0.109)	−0.00 (−0.159; 0.157)	−0.13 (−0.277; 0.033)	−0.07 (−0.222; 0.092)
Triglyceride	−0.30 (−0.437; −0.149)***	−0.06 (−0.22; 0.099)	−0.08 (−0.236; 0.077)	−0.07 (−0.230; 0.084)	0.23 (0.074; 0.373)**	−0.09 (−0.240; 0.073)	−0.10 (−0.256; 0.056)
HDL	0.28 (0.132; 0.422)***	0.32 (0.170; 0.454)***	0.29 (0.142; 0.431)***	−0.21 (−0.353; −0.051)**	−0.04 (−0.193; 0.122)	0.05 (−0.109; 0.205)	0.12 (−0.042; 0.269)
LDL	0.01 (−0.154; 0.166)	0.05 (−0.107; 0.212)	0.08 (−0.079; 0.238)	0.00 (−0.159; 0.160)	0.23 (0.073; 0.376)**	0.20 (0.038; 0.345)**	0.03 (−0.126; 0.193)
Hs-CRP	−0.37 (−0.502; −0.229)***	−0.16 (−0.314; −0.006)*	−0.25 (−0.393; −0.096)**	0.17 (0.017; 0.324)*	0.13 (−0.032; 0.279)	−0.11 (−0.261; 0.052)	−0.20 (−0.343; −0.039)*
PROCAM score	−0.34 (−0.480; −0.189)***	−0.25 (−0.401; −0.093)**	−0.30 (−0.438; −0.138)***	0.18 (0.019; 0.337)*	0.06 (−0.105; 0.222)	0.02 (−0.145; 0.183)	−0.02 (−0.185; 0.143)
Fast walking	0.26 (0.108; 0.407)**	0.12 (−0.043; 0.273)	0.21 (0.053; 0.359)**	−0.21 (−0.356; −0.049)*	0.06 (−0.102; 0.218)	−0.02 (−0.179; 0.142)	−0.04 (−0.198; 0.122)
$V0_2$peak	0.36 (0.217; 0.492)***	0.06 (−0.100; 0.214)	0.25 (0.099; 0.394)**	−0.15 (−0.303; 0.005)	−0.08 (−0.236; 0.077)	−0.06 (−0.212; 0.102)	0.14 (−0.019; 0.290)
CRAE	0.17 (0.006; 0.320)*	0.13 (−0.030; 0.287)	0.16 (−0.004; 0.311)	−0.12 (−0.276; 0.042)	0.06 (−0.098; 0.223)	0.15 (−0.009; 0.307)	−0.11 (−0.269; 0.050)
CRVE	−0.20 (−0.351; −0.041)*	−0.12 (−0.277; 0.041)	−0.15 (−0.302; 0.013)	0.04 (−0.124; 0.198)	−0.00 (−0.163; 0.160)	0.07 (−0.091; 0.230)	−0.06 (−0.215; 0.107)
AVR	0.42 (0.276; 0.543)***	0.28 (0.126; 0.424)***	0.35 (0.202; 0.485)***	−0.18 (−0.327; −0.014)*	0.07 (−0.091; 0.230)	0.09 (−0.069; 0.251)	−0.07 (−0.226; 0.095)
PWV	−0.27 (−0.413; −0.116)***	−0.27 (−0.416; −0.120)***	−0.32 (−0.458; −0.171)***	0.14 (−0.017; 0.296)	−0.09 (−0.243; 0.074)	−0.24 (−0.384; −0.082)**	−0.06 (−0.220; 0.099)

BMI, body mass index; WC, waist circumference; BP, blood pressure; HDL, high-density lipoprotein, LDL, low-density lipoprotein; Hs-CRP, high-sensitive C-reactive Protein; VO_2peak, peak oxygen uptake; CRAE, central retinal arteriolar equivalent; CRVE, central retinal venular equivalent; AVR, arteriolar-to-venular diameter ratio; PWV, pulse wave velocity; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

variation. In addition, lower levels of glutamine, glycine, and histidine were associated with higher PWV. All metabolites and total lipoprotein-bound lipids together explained 23% of the AVR, 9% of the CRVE, and 5% of the CRAE variation. Higher levels of glutamine, glycine, and histidine and lower levels of tryptophan were associated with a higher AVR (Table 3).

4. Discussion

As a main result of our study, classic CV risk factors were found to be associated with alterations of the metabolic profile. Classic CV risk factors explained up to 45% of the variation in metabolites. In addition, metabolites were shown to explain up to 20% of macro- and 23% of microvascular variation. The implications for translational and clinical medicine are discussed.

4.1. Amino acids, metabolic syndrome and inflammation

We showed that risk group I (low CV risk) was characterized by a homogenous metabolic profile, whereas the heterogeneity of the metabolic profile gradually increased with increasing CV risk factors. In addition, higher CV risk was associated with lower glutamine, glycine, and histidine as well as higher tryptophan levels. The variation of these amino acids was mainly explained by CV risk factors reflecting the metabolic syndrome such as BMI, blood pressure, serum glucose levels, and HDL. Amino acids are involved in several fundamental biological processes such as cell proliferation, energy balance, ammonia buffering, maintenance of the acid-base balance, and others [22]. Imbalances of amino acids in serum samples of high CV risk patients describe proteolytic processes, for instance elevated protease functions, leading to disturbance of several biological processes. Glutamine for example is a carbon source for the TCA cycle via conversion to alpha-ketoglutarate [23]. Low glutamine levels may lead to reduced cytosolic aspartate delivery with ensuing metabolic consequences [24]. Previous studies showed that an altered circulating metabolic profile may predict development of metabolic disorders [25,26]. Wang et al. demonstrated that a combination of three amino acids was able to predict development of type 2 diabetes 12 years later, which highlighted the use of circulating amino acids as early biomarkers for future diabetes progression [25]. Low levels of the amino acids glutamine, glycine, and histidine, as well as high levels of tryptophan, were associated with metabolic risk in our study. The strong association of amino acids and metabolic risk was the consequence of the high prevalence of metabolic risk factors in CV risk groups II to IV. The CV risk groups II to IV were characterized by a high prevalence of obesity (60%), hypertension (50%), diabetes (44%) as well as low HDL levels (14%), which are, by definition, key metabolic risk factors. HDL, especially delipidated apolipoprotein (apo) A, prevents endothelial cell death as a potential mechanism of how CV risk affects vascular health [27]. However, phenylalanine showed no statistically significant association with metabolic risk factors in our study. Floegel et al. demonstrated that serum phenylalanine was independently associated with increased risk of type 2 diabetes, as well as insulin secretion [26]. However, serum phenylalanine levels were equally distributed through all CV risk groups in our study cohort. Therefore, we would like to speculate that phenylalanine is a subclinical circulating biomarker to help predict development of type 2 diabetes at early stages

[25,26], but it seems to have limited potential to discriminate between healthy individuals and CV risk patients in our cohort. The underlying mechanism of how phenylalanine affects insulin resistance or impairs insulin secretion is still not fully understood and needs to be investigated in future studies. Amino acids are also involved in the mediation of inflammation processes [28,29]. Four out of five amino acids showed significant correlations with hs-CRP in our study. Lower levels of serum glutamine, glycine and histidine, as well as higher levels of tryptophan were correlated with higher inflammation. A recent review summarized the functions and signalling pathways of amino acids in systemic inflammation, which are mainly the suppression of oxidative stress and the inhibition of proinflammatory cytokines [30].

4.2. Amino acids, physical activity and fitness

Acute exercise sessions and long-term exercise interventions lead to changes in metabolite concentrations [31,32]. In addition, amino acid household has been associated with physical activity (PA), sedentary lifestyle and CRF [33,34]. Interestingly, we showed a positive correlation of glutamine and histidine with fast walking and CRF. Glutamine, as the most abundant amino acid in the human body, is involved in various fundamental biologic processes such as lipoprotein, fatty acid and tricarboxylic acid energy metabolism, glycolysis, fluid balance and inflammation. Low glutamine serum levels best reflect reduced stores in the human muscle [29]. Glutamine supplementation has been shown to increase muscle and decrease adipose tissue glucose uptake and to inhibit hepatic glucose production [35]. In addition, type 2 diabetic patients showed reduced CV risk after six weeks of glutamine supplementation [36]. Exercise and glutamine supplementation have been shown to be equally effective in reducing inflammatory processes and biomarkers of oxidative stress in rats [37]. Glutamine in particular seems to mediate some of the anti-inflammatory effects of exercise.

4.3. Amino acids and vascular health

Metabolites seem to have predictive value for CV disease risk [38]. Amino acids are, among other mechanisms, involved in processes regulating oxidative stress [39] and nitric oxide [40]. These processes play a central role in the pathogenesis of atherosclerosis [41]. Our study showed that metabolites explained 20% of macro- and 23% of microvascular variation. In addition, all amino acids showed significant correlations with the macro- or microvascular biomarker. Welsh et al. have previously shown that amino acids improved the risk classification for macro- and microvascular events in diabetic patients [13]. Previous studies highlighted the protective role of amino acids in endothelial cell (EC) senescence [42]. Glutamine seems to be an essential energy source for ECs [42]. Low glutamine concentrations in CV risk patients may be one reason for macro- and microvascular dysfunction, reflected by higher PWV and a lower AVR in our study. CV risk factors affect circulating amino acid concentrations, which may consequently contribute to vascular end organ damage. Circulating glutamine concentrations are, in large part, determined by the individual CV risk profile (45%). Tryptophan concentrations, on the other hand, are associated with the CV risk profile to a much lesser extent (10%). In addition, tryptophan showed only weak associations with microvascular phenotype. These findings point to a metabolic fingerprint that links specific metabolites with CV risk factors and vascular health. For example, we found high glutamine concentrations in the lowest risk group with a stepwise decrease in risk group II-IV. In addition, we showed strong associations of high glutamine levels with a beneficial macro- and microvascular phenotype (PWV and AVR).

4.4. Acetate, cardiovascular risk and vascular health

Acetate is a short-chain fatty acid involved in various metabolic processes [43,44]. Previous studies already reviewed several important pathways through which acetate is involved in metabolic health. Higher systemic acetate levels may positively affect insulin sensitivity, oxidative capacity, satiety, and improve adipose tissue function that leads to an antilipolytic and anti-inflammatory effect [43,44]. Interestingly, we found decreasing circulating acetate levels with increasing CV risk. CV risk factors explained the variation of acetate by 15%. In addition, acetate was negatively associated with body mass and WC even in our heterogeneous CV risk cohort. It has previously been demonstrated that acetate seems to play an important role in weight control trough different pathways [45]. It has been reported that serum acetate may positively correlate with LDL cholesterol in men [46], which seems to confirm our finding of a positive association of acetate with LDL levels. Studies investigating associations of acetate and vascular health are scarce. We found a negative association of acetate with PWV. Previous studies showed lower acetate concentrations in postmenopausal women with carotid artery calcification [47] and in myocardial ischemia patients without stenosis [48] compared to controls.

4.5. Total lipoprotein-bound lipids

Dyslipidaemia is known as a CV risk factor related to atherosclerotic cardiovascular disease [49]. Although the PCA and O-PLS-DA analysis showed statistically significant differences between our CV risk groups, these differences were not confirmed by univariate analysis. In addition, we found no evidence for an association of total lipoprotein-bound lipids with triglyceride, HDL or LDL. Even if all CV risk factors together explained 17% of total lipoprotein-bound lipids variation, triglyceride, HDL and LDL explained only up to 3% of the total lipoprotein-bound lipids variation. Our approach did not include the analysis of non-HDL, apoA or apoB, even though previous studies showed their potential to sensitively detect long-term risk of atherosclerotic cardiovascular disease [50,51]. We therefore recommend to perform deeper analyses of the entire lipoprotein profile in future studies, including the quantification of lipid distribution within lipoprotein sub-classes, to allow for further differentiation of CV risk [52].

4.6. Strength and limitations

This study aimed to investigate associations of CV risk factors with metabolites and vascular health. We have previously shown a potential epigenetic link of how *p66^Shc^* gene expression, a determinant of oxidative stress, might determine microvascular health [53,54]. However, it is still not well understood how CV risk factors mechanistically determine vascular phenotypes. The metabolites presented in this work appear to be potential mediators of the effects of classical CV risk factors on vascular end organ damage, however, longitudinal studies are essential to investigate the predictive value of metabolic profiling on CV disease development. Only a small fraction of lipids remained after methanol extraction that we combined with total lipoprotein-bound lipids. Unfortunately, it was not possible to extract lipoproteins or lipid subclasses based on these samples. Further studies need to analyse changes in lipoprotein profiles using NMR-based lipoprotein sub-class analysis. The CV risk factor distribution in our cohort was very heterogenic, which did not allow for subgroup analysis of patients diagnosed, for example, with diabetes or hypertension only. Even if we found strong evidence for an association of CV risk factors and metabolites, the group differences between risk groups II to IV were not statistically significant. This might be explained by the heterogeneity of the CV risk factor distribution and the small risk difference between the groups.

4.7. Conclusions

Metabolic profiling has the potential to improve CV risk stratification. It may help better understand some of the underlying metabolic mechanisms of atherosclerotic disease development. Our findings help justify future preventive strategies to target specific metabolites and

thereby reduce CV risk and associated vascular disease. This study demonstrated a higher heterogeneity of the metabolic profile with increasing CV risk and a strong association of CV risk factors with circulating metabolites. These circulating metabolites may mediate some of the detrimental effects of CV risk factors on macro- and microvascular health. More integrated longitudinal research is warranted to disentangle the complex interrelation of clinical CV risk, underlying metabolic profiles and vascular end organ damage. Metabolic profiling may have added value for CV risk stratification and potentially affect clinical decision making. Integration of metabolic profiling in CV risk assessment may potentially enable a more translational approach, from CV risk to metabolites to vascular end organ damage.

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CRediT authorship contribution statement

Lukas Streese: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Visualization. **Anna Maria Springer:** Conceptualization, Methodology, Validation, Formal analysis. **Arne Deiseroth:** Methodology, Validation, Investigation. **Justin Carrard:** Writing – review & editing. **Denis Infanger:** Formal analysis. **Christoph Schmaderer:** Writing – review & editing. **Arno Schmidt-Trucksäss:** Writing – review & editing, Resources. **Tobias Madl:** Conceptualization, Methodology, Validation, Formal analysis, Resources, Writing – review & editing, Funding acquisition. **Henner Hanssen:** Conceptualization, Methodology, Resources, Supervision, Writing – review & editing, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2021.07.005.

References

[1] C. Vlachopoulos, K. Aznaouridis, C. Stefanadis, Prediction of cardiovascular events and all-cause mortality with arterial stiffness: a systematic review and meta-analysis, J. Am. Coll. Cardiol. 55 (13) (2010) 1318–1327, https://doi.org/10.1016/j.jacc.2009.10.061.
[2] Y. Ben-Shlomo, M. Spears, C. Boustred, M. May, S.G. Anderson, E.J. Benjamin, et al., Aortic pulse wave velocity improves cardiovascular event prediction: an individual participant meta-analysis of prospective observational data from 17,635 subjects, J. Am. Coll. Cardiol. 63 (7) (2014) 636–646, https://doi.org/10.1016/j.jacc.2013.09.063.
[3] J. Flammer, K. Konieczka, R.M. Bruno, A. Virdis, A.J. Flammer, S. Taddei, The eye and the heart, Eur. Heart J. 34 (17) (2013) 1270–1278, https://doi.org/10.1093/eurheartj/eht023.
[4] M.K. Ikram, F.J. de Jong, M.J. Bos, J.R. Vingerling, A. Hofman, P.J. Koudstaal, et al., Retinal vessel diameters and risk of stroke: the Rotterdam Study, Neurology 66 (9) (2006) 1339–1343, https://doi.org/10.1212/01.wnl.0000210533.24338.ea.
[5] T.Y. Wong, R. Klein, A.R. Sharrett, B.B. Duncan, D.J. Couper, J.M. Tielsch, et al., Retinal arteriolar narrowing and risk of coronary heart disease in men and women. The Atherosclerosis Risk in Communities Study, JAMA 287 (9) (2002) 1153–1159.
[6] J.J. Wang, G. Liew, R. Klein, E. Rochtchina, M.D. Knudtson, B.E. Klein, et al., Retinal vessel diameter and cardiovascular mortality: pooled data analysis from two older populations, Eur. Heart J. 28 (16) (2007) 1984–1992, https://doi.org/10.1093/eurheartj/ehm221.
[7] S.B. Seidelmann, B. Claggett, P.E. Bravo, A. Gupta, H. Farhad, B.E. Klein, et al., Retinal vessel calibers in predicting long-term cardiovascular outcomes: the atherosclerosis risk in communities study, Circulation 134 (18) (2016) 1328–1338, https://doi.org/10.1161/CIRCULATIONAHA.116.023425.
[8] K. McGeechan, G. Liew, P. Macaskill, L. Irwig, R. Klein, B.E. Klein, et al., Prediction of incident stroke events based on retinal vessel caliber: a systematic review and individual-participant meta-analysis, Am. J. Epidemiol. 170 (11) (2009) 1323–1332, https://doi.org/10.1093/aje/kwp306.
[9] J. Ivanisevic, A. Thomas, Metabolomics as a tool to understand pathophysiological processes, Methods Mol. Biol. 1730 (2018) 3–28, https://doi.org/10.1007/978-1-4939-7592-1_1.
[10] J. Deelen, J. Kettunen, K. Fischer, A. van der Spek, S. Trompet, G. Kastenmuller, et al., A metabolic profile of all-cause mortality risk identified in an observational study of 44,168 individuals, Nat. Commun. 10 (1) (2019) 3346, https://doi.org/10.1038/s41467-019-11311-9.
[11] S.H. Shah, W.E. Kraus, C.B. Newgard, Metabolomic profiling for the identification of novel biomarkers and mechanisms related to common cardiovascular diseases: form and function, Circulation 126 (9) (2012) 1110–1120, https://doi.org/10.1161/CIRCULATIONAHA.111.060368.
[12] P. Wurtz, A.S. Havulinna, P. Soininen, T. Tynkkynen, D. Prieto-Merino, T. Tillin, et al., Metabolite profiling and cardiovascular event risk: a prospective study of 3 population-based cohorts, Circulation 131 (9) (2015) 774–785, https://doi.org/10.1161/CIRCULATIONAHA.114.013116.
[13] P. Welsh, N. Rankin, Q. Li, P.B. Mark, P. Wurtz, M. Ala-Korpela, et al., Circulating amino acids and the risk of macrovascular, microvascular and mortality outcomes in individuals with type 2 diabetes: results from the ADVANCE trial, Diabetologia 61 (7) (2018) 1581–1591, https://doi.org/10.1007/s00125-018-4619-x.
[14] S. Cheng, S.H. Shah, E.J. Corwin, O. Fiehn, R.L. Fitzgerald, R.E. Gerszten, et al., Potential impact and study considerations of metabolomics in cardiovascular health and disease: a scientific statement from the American heart association, Circ. Cardiovasc. Genet. 10 (2) (2017), https://doi.org/10.1161/HCG.0000000000000032.
[15] L. Streese, A. Deiseroth, J. Schafer, A. Schmidt-Trucksass, H. Hanssen, Exercise, arterial crosstalk-modulation, and inflammation in an aging population: the ExAMIN AGE study, Front. Physiol. 9 (2018), https://doi.org/10.3389/fphys.2018.00116. ARTN 116.
[16] World-Medical-Association, World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects, J. Am. Med. Assoc. 310 (20) (2013) 2191–2194, https://doi.org/10.1001/jama.2013.281053.
[17] G. Assmann, P. Cullen, H. Schulte, Simple scoring scheme for calculating the risk of acute coronary events based on the 10-year follow-up of the Prospective Cardiovascular Munster (PROCAM) study, Circulation 105 (3) (2002) 310–315, https://doi.org/10.1161/hc0302.102575.
[18] A. Vignoli, V. Ghini, G. Meoni, C. Licari, P.G. Takis, L. Tenori, et al., High-throughput metabolomics by 1D NMR, Angew Chem. Int. Ed. Engl. 58 (4) (2019) 968–994, https://doi.org/10.1002/anie.201804736.
[19] J. Chong, O. Soufan, C. Li, I. Caraus, S.Z. Li, G. Bourque, et al., MetaboAnalyst 4.0: towards more transparent and integrative metabolomics analysis, Nucleic Acids Res. 46 (W1) (2018) W486–W494, https://doi.org/10.1093/nar/gky310.
[20] J. Wang, Z. Li, J. Chen, H. Zhao, L. Luo, C. Chen, et al., Metabolomic identification of diagnostic plasma biomarkers in humans with chronic heart failure, Mol. Biosyst. 9 (11) (2013) 2618–2626, https://doi.org/10.1039/c3mb70227h.
[21] X.Y. Du, H.Z. You, Y.L. Li, Y. Wang, P. Hui, B.K. Qiao, et al., Relationships between circulating branched chain amino acid concentrations and risk of adverse cardiovascular events in patients with STEMI treated with PCI, Sci. Rep-Uk 8 (2018), https://doi.org/10.1038/s41598-018-34245-6. ARTN 15809.
[22] A.Y. Coqueiro, M.M. Rogero, J. Tirapegui, Glutamine as an anti-fatigue amino acid in sports nutrition, Nutrients 11 (4) (2019), https://doi.org/10.3390/nu11040863. ARTN 863.
[23] S.M. Fendt, E.L. Bell, M.A. Keibler, B.A. Olenchock, J.R. Mayers, T.M. Wasylenko, et al., Reductive glutamine metabolism is a function of the alpha-ketoglutarate to citrate ratio in cells, Nat. Commun. 4 (2013) 2236, https://doi.org/10.1038/ncomms3236.
[24] H.F. Alkan, K.E. Walter, A. Luengo, C.T. Madreiter-Sokolowski, S. Stryeck, A. N. Lau, et al., Cytosolic aspartate availability determines cell survival when glutamine is limiting, Cell Metabol. 28 (5) (2018) 706–720 e6, https://doi.org/10.1016/j.cmet.2018.07.021.
[25] T.J. Wang, M.G. Larson, R.S. Vasan, S. Cheng, E.P. Rhee, E. McCabe, et al., Metabolite profiles and the risk of developing diabetes, Nat. Med. 17 (4) (2011) 448–453, https://doi.org/10.1038/nm.2307.
[26] A. Floegel, N. Stefan, Z.H. Yu, K. Muhlenbruch, D. Drogan, H.G. Joost, et al., Identification of serum metabolites associated with risk of type 2 diabetes using a targeted metabolomic approach, Diabetes 62 (2) (2013) 639–648, https://doi.org/10.2337/db12-0495.
[27] I. Suc, I. Escargueil-Blanc, M. Troly, R. Salvayre, A. Negre-Salvayre, HDL and ApoA prevent cell death of endothelial cells induced by oxidized LDL, Arterioscler. Thromb. Vasc. Biol. 17 (10) (1997) 2158–2166, https://doi.org/10.1161/01.atv.17.10.2158.
[28] H. Nicastro, C.R. da Luz, D.F. Chaves, L.R. Bechara, V.A. Voltarelli, M.M. Rogero, et al., Does branched-chain amino acids supplementation modulate skeletal muscle remodeling through inflammation modulation? Possible mechanisms of action, J. Nutr. Metabol. (2012) 136937, https://doi.org/10.1155/2012/136937.

[29] D. Meynial-Denis, Glutamine metabolism in advanced age, Nutr. Rev. 74 (4) (2016) 225–236, https://doi.org/10.1093/nutrit/nuv052.

[30] F. He, C. Wu, P. Li, N. Li, D. Zhang, Q. Zhu, et al., Functions and signaling pathways of amino acids in intestinal inflammation, BioMed Res. Int. 2018 (2018) 9171905, https://doi.org/10.1155/2018/9171905.

[31] K. Contrepois, S. Wu, K.J. Moneghetti, D. Hornburg, S. Ahadi, M.S. Tsai, et al., Molecular choreography of acute exercise, Cell 181 (5) (2020) 1112–1130, https://doi.org/10.1016/j.cell.2020.04.043, e16.

[32] Y.C. Koay, K. Stanton, V. Kienzle, M. Li, J. Yang, D.S. Celermajer, et al., Effect of chronic exercise in healthy young male adults: a metabolomic analysis, Cardiovasc. Res. (2020), https://doi.org/10.1093/cvr/cvaa051.

[33] U.M. Kujala, V.P. Makinen, I. Heinonen, P. Soininen, A.J. Kangas, T.H. Leskinen, et al., Long-term leisure-time physical activity and serum metabolome, Circulation 127 (3) (2013) 340–348, https://doi.org/10.1161/CIRCULATIONAHA.112.105551.

[34] F. Beutner, C. Ritter, M. Scholz, A. Teren, L.M. Holdt, D. Teupser, et al., A metabolomic approach to identify the link between sports activity and atheroprotection, Eur. J. Prev. Cardiol. (2020), https://doi.org/10.1093/eurjpc/zwaa122.

[35] K.Y. Abboud, S.K. Reis, M.E. Martelli, O.P. Zordao, F. Tannihao, A.Z.Z. de Souza, et al., Oral glutamine supplementation reduces obesity, pro-inflammatory markers, and improves insulin sensitivity in DIO wistar rats and reduces waist circumference in overweight and obese humans, Nutrients 11 (3) (2019), https://doi.org/10.3390/nu11030536. ARTN 536.

[36] A. Mansour, M.R. Mohajeri-Tehrani, M. Qorbani, R. Heshmat, B. Larijani, S. Hosseini, Effect of glutamine supplementation on cardiovascular risk factors in patients with type 2 diabetes, Nutrition 31 (1) (2015) 119–126, https://doi.org/10.1016/j.nut.2014.05.014.

[37] R.D. Santos, G.D. Cardoso, L.D. Lima, M.L.D. Cavalcante, M.S. Silva, A.K. M. Cavalcante, et al., l-Glutamine and physical exercise prevent intestinal inflammation and oxidative stress without improving gastric dysmotility in rats with ulcerative colitis, Inflammation (2020), https://doi.org/10.1007/s10753-020-01361-3.

[38] I. Tzoulaki, R. Castagne, C.L. Boulange, I. Karaman, E. Chekmeneva, E. Evangelou, et al., Serum metabolic signatures of coronary and carotid atherosclerosis and subsequent cardiovascular disease, Eur. Heart J. 40 (34) (2019) 2883–2896, https://doi.org/10.1093/eurheartj/ehz235.

[39] Y. Martinez, X. Li, G. Liu, P. Bin, W. Yan, D. Mas, et al., The role of methionine on metabolism, oxidative stress, and diseases, Amino Acids 49 (12) (2017) 2091–2098, https://doi.org/10.1007/s00726-017-2494-2.

[40] R. Marion, M. Coeffier, S. Lemoulan, G. Gargala, P. Ducrotte, P. Dechelotte, L-Arginine modulates CXC chemokines in the human intestinal epithelial cell line HCT-8 by the NO pathway, Biochimie 87 (12) (2005) 1048–1055, https://doi.org/10.1016/j.biochi.2005.06.009.

[41] U. Forstermann, N. Xia, H. Li, Roles of vascular oxidative stress and nitric oxide in the pathogenesis of atherosclerosis, Circ. Res. 120 (4) (2017) 713–735, https://doi.org/10.1161/CIRCRESAHA.116.309326.

[42] H. Unterluggauer, S. Mazurek, B. Lener, E. Hutter, E. Eigenbrodt, W. Zwerschke, et al., Premature senescence of human endothelial cells induced by inhibition of glutaminase, Biogerontology 9 (4) (2008) 247–259, https://doi.org/10.1007/s10522-008-9134-x.

[43] M.A.G. Hernandez, E.E. Canfora, J.W.E. Jocken, E.E. Blaak, The short-chain fatty acid acetate in body weight control and insulin sensitivity, Nutrients 11 (8) (2019), https://doi.org/10.3390/nu11081943.

[44] S. Bose, V. Ramesh, J.W. Locasale, Acetate metabolism in physiology, cancer, and beyond, Trends Cell Biol. 29 (9) (2019) 695–703, https://doi.org/10.1016/j.tcb.2019.05.005.

[45] M. Trajkovski, C.B. Wollheim, Physiology: microbial signals to the brain control weight, Nature 534 (7606) (2016) 185–187, https://doi.org/10.1038/534185a.

[46] T.M. Wolever, J. Fernandes, A.V. Rao, Serum acetate:propionate ratio is related to serum cholesterol in men but not women, J. Nutr. 126 (11) (1996) 2790–2797, https://doi.org/10.1093/jn/126.11.2790.

[47] M. Varri, L. Niskanen, T.P. Tuomainen, R. Honkanen, H. Kroger, M.T. Tuppurainen, Metabolite profiling of osteoporosis and atherosclerosis in postmenopausal women: a cross-sectional study, Vasc. Health Risk Manag. 16 (2020) 515–524, https://doi.org/10.2147/VHRM.S279028.

[48] M. Deidda, C. Piras, C.C. Dessalvi, D. Congia, E. Locci, F. Ascedu, et al., Blood metabolomic fingerprint is distinct in healthy coronary and in stenosing or microvascular ischemic heart disease, J. Transl. Med. 15 (2017), https://doi.org/10.1186/s12967-017-1215-7. ARTN 112.

[49] F. Mach, C. Baigent, A.L. Catapano, K.C. Koskinas, M. Casula, L. Badimon, et al., ESC/EAS Guidelines for the management of dyslipidaemias: lipid modification to reduce cardiovascular risk, Eur. Heart J. 41 (1) (2019) 111–188, https://doi.org/10.1093/eurheartj/ehz455, 2020.

[50] F.J. Brunner, C. Waldeyer, F. Ojeda, V. Salomaa, F. Kee, S. Sans, et al., Application of non-HDL cholesterol for population-based cardiovascular risk stratification: results from the Multinational Cardiovascular Risk Consortium, Lancet 394 (2019) 2173–2183, https://doi.org/10.1016/S0140-6736(19)32519-X, 10215.

[51] G. Walldius, I. Jungner, The apoB/apoA-I ratio: a strong, new risk factor for cardiovascular disease and a target for lipid-lowering therapy–a review of the evidence, J. Intern. Med. 259 (5) (2006) 493–519, https://doi.org/10.1111/j.1365-2796.2006.01643.x.

[52] P. Soininen, A.J. Kangas, P. Wurtz, T. Suna, M. Ala-Korpela, Quantitative serum nuclear magnetic resonance metabolomics in cardiovascular epidemiology and genetics, Circ. Cardiovasc. Genet. 8 (1) (2015) 192–206, https://doi.org/10.1161/CIRCGENETICS.114.000216.

[53] L. Streese, A.W. Khan, A. Deiseroth, S. Hussain, R. Suades, A. Tiaden, et al., Physical activity may drive healthy microvascular ageing via downregulation of p66(Shc), Eur. J. Prev. Cardiol. 27 (2) (2020) 168–176, https://doi.org/10.1177/2047487319880367. Unsp. 2047487319880367.

[54] L. Streese, A.W. Khan, A. Deiseroth, S. Hussain, R. Suades, A. Tiaden, et al., High-intensity interval training modulates retinal microvascular phenotype and DNA methylation of p66Shc gene: a randomized controlled trial (EXAMIN AGE), Eur. Heart J. 41 (15) (2020) 1514–1519, https://doi.org/10.1093/eurheartj/ehz196.