Branched-chain and aromatic amino acids related to visceral adipose tissue impact metabolic health risk markers

Ximena Orozco-Ruiz[1], Andrea Anesi[2], Fulvio Mattivi[2,3], Monique M.B. Breteler [1,4].

1. Population Health Sciences, German Center for Neurodegenerative diseases (DZNE), Venusberg-Campus 1-Building 99, 53127 Bonn, Germany.
2. Department of Food Quality and Nutrition, Research and Innovation Centre, Fondazione Edmund Mach (FEM), Via E. Mach 1, 38010 San Michele all'Adige, Italy.
3. University of Trento, Department of Cellular, Computational and Integrative Biology (CIBIO), Via Sommarive 9, 38123 Povo, Italy.
4. Institute for Medical Biometry, Informatics and Epidemiology (IMBIE), Faculty of Medicine, University of Bonn, Venusberg-Campus 1-Building 11, 53127 Bonn, Germany.

Corresponding Author:

Monique M.B. Breteler, MD PhD

German Center for Neurodegenerative Diseases (DZNE), Bonn.

Venusberg Campus 1/ 99, 53127 Bonn

Tel: +49-22843302-929

Mail: monique.breteler@dzne.de

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Abstract

Context: Visceral (VAT) and subcutaneous adipose tissue (SAT) function as endocrine organs capable of influencing metabolic health across adiposity levels.

Objective: To investigate whether metabolites associated with VAT and SAT impact metabolic health through metabolite concentrations.

Methods: Analyses are based on 1790 participants from the population-based Rhineland Study. We assessed plasma levels of Methionine (Met), branched-chain amino acids (BCAA), aromatic amino acids (AAA), and their metabolic downstream metabolites with liquid chromatography-mass spectrometry. VAT and SAT volumes were assessed by magnetic resonance imaging (MRI). Metabolically healthy and unhealthy phenotypes were defined using Wildman criteria.

Results: Metabolically unhealthy participants had higher concentrations of BCAA than metabolically healthy participants ($p < 0.001$). In metabolically unhealthy participants, VAT volumes were significantly associated with levels of L-Isoleucine, L-Leucine, indole-3-lactic acid, and indole-3-propionic acid (in log standard deviation units: β=0.16, p=0.003; β=0.12, p=0.038; β=0.11, p=0.035 and β= -0.16, p=0.010, respectively). Higher concentrations of certain BCAA and AAA-downstream metabolites significantly increased the odds of cardiometabolic risk markers. The relation between VAT volume and cardiometabolic risk markers was mediated by BCAA (indirect effects 3.7 to 11%, p=0.02 to <0.0001), while the effect of VAT on systemic inflammation was mediated through higher kynurenine concentrations (indirect effect 6.4%, p<0.0001).

Conclusions: Larger volumes of VAT in metabolically unhealthy individuals are associated with altered concentrations of circulating BCAA and AAA-downstream metabolites, increasing the odds of cardiometabolic risk markers. This suggests that these metabolites are involved in the mechanisms that underlie the relationship of abdominal VAT with metabolic health.

Keywords: branched-chain amino acids, aromatic amino acids, metabolites, cardiometabolic risk markers, visceral adipose tissue, subcutaneous adipose tissue.

Introduction

Obesity is worldwide one of the major risk factors for cardiovascular diseases (CVD), type 2 diabetes (T2D), different types of cancer, and a high rate of mortality (1,2). However, there is a growing awareness that obesity is a heterogeneous condition, and that risk profiles for metabolic and cardiovascular disease vary widely among individuals with the same body mass index (BMI). Thus, risk stratification of individuals according to their metabotype, i.e. grouping according to similarities in metabolic profile, becomes crucial (3,4) Among individuals with high BMI, a subset can be considered metabolically healthy as they have a healthy metabolic profile characterized by high insulin sensitivity, favorable lipid profile, low pro-inflammatory cytokine levels, and normal blood pressure. Conversely, there are also individuals who are metabolically unhealthy despite a low BMI (5,6). The variation of metabolic health across obesity groups is mainly due to differences in abdominal fat distribution (7) such as visceral (VAT) and subcutaneous abdominal adipose tissues (SAT) (8). Larger SAT and VAT have been associated with future conversion to metabolically unhealthy from a healthy phenotype (9). One important mechanism by which VAT and SAT are involved in the progression from metabolically to metabolically unhealthy phenotype is by changes in the functionality of the adipocytes through their ability to expand (10). Hypertrophic adipocytes are characteristic of an unhealthier mechanism of adipocyte expansion. They cause a cascade of metabolic dysfunction by promoting insulin resistance, glucose intolerance and induce inflammation by secreting high levels of pro-inflammatory cytokines (11,12). Furthermore, the higher release of free fatty acids by hypertrophic adipocytes leads to hepatic lipid accumulation and hypertriglyceridemia (13). Nevertheless, the connection between abdominal fat and metabolically unhealthy phenotype is yet not totally understood and could be explained by other factors such as metabolomic biomarkers. Metabolomics has emerged as a powerful tool for assessing perturbations in metabolic pathways and for determining biomarkers that are associated with specific health conditions or diseases. Metabolomic biomarkers are a measure of exposure and susceptibility to specific outcomes and allow us to classify at-risk/diseased individuals (14). It has been shown that

there are differences in circulating levels of BCAA and AAA across metabolic phenotypes (15-17). Furthermore, BCAA and AAA have been associated with metabolic abnormalities and obesity in cross-sectional studies(18,19), and could predict the development of diabetes (20,21) and cardiovascular diseases (CVD) in longitudinal studies (22).

Evidence from animal and human adipose tissue studies suggests that the adipose tissue is an important determinant of BCAA and AAA oxidation and metabolism (23–25).

In particular, the route of tryptophan (Trp) catabolism through the kynurenine (Kyn) pathway (KP) degrades Trp into several metabolites with toxic and inflammatory effects. The KP can be upregulated in the adipose tissue by activating indoleamine 2,3-dioxygenase (IDO) (24,25),

a rate-limiting enzyme that breaks down Trp into downstream products such as Kyn, kynurenic acid (KYNA) and xanthurenic acid (XA) (26). Thus, increased activity of KP in the adipose tissue is reflected in the higher circulation of toxic Trp-derived metabolites. On the other hand, gene expression of enzymes involved in the catabolism of BCAA in the adipocytes (23,27,28)

reportedly decreases mainly in VAT compartments (23), which leads to a significant increase in circulating levels of BCAA in persons with high levels of VAT.

To our knowledge, few studies have investigated the relationship between abdominal adipose tissue and Met. Two animal studies showed that a diet restricted on Met was associated with a reduction of VAT accumulation and hepatic triglyceride synthesis. Furthermore, in the VAT adipocytes, lipogenesis and fatty acid oxidation increased, and there was an improvement in insulin sensitivity (29,30). Similar results were further replicated in humans with Metabolic Syndrome (MetS) (31). Additionally, some authors showed that the uptake of Met was diminished in the VAT of obese subjects, reflecting an increased release of Met in the circulation (32,33). Therefore, circulating metabolites could constitute the biological link between adiposity and metabolic diseases.

Thus, we aimed to understand whether known metabolites associated with VAT and SAT impact metabolic health and how they are involved in the link between abdominal VAT and SAT with cardiometabolic risk markers.

Materials and methods

Study population

We selected the first 2000 participants from the Rhineland Study, who participated between March 2016 to April 2019 and for whom blood samples and abdominal MRI data were available, for further metabolomic analysis as described below.

The Rhineland Study is an ongoing community-based cohort study in Bonn, Germany, that started in 2016. One of its central aims is to find biomarkers and multimodal biomarker profiles to identify individuals at risk for neurodegenerative and other age-related diseases. Participants in the Rhineland Study are recruited from two municipal districts in Bonn and are primarily Caucasians of European descent. Inclusion criteria are being aged 30 years or older and having sufficient command of the German language to provide written informed consent.

At baseline examination, participants completed an 8-hour in-depth multi-domain phenotypic assessment of anthropometry, physical activity and fitness, cardiovascular health, brain imaging, cognitive testing, neurologic functioning, ophthalmologic health and functioning, and other sensory systems. No financial incentives were offered for study participation (34). The study was approved by the Medical Faculty Ethics Committee of the University of Bonn and conducted following the Declaration of Helsinki. We obtained informed written consent from all the participants before they underwent any of the examinations.

Blood samples

Overnight fasting plasma samples were collected in 2 x 10 mL EDTA tubes from all participants between 7:00 and 9:30 a.m. and directly processed. The plasma was centrifuged within less than 10

minutes after blood withdrawal for 10 minutes at 2000 x g at 20 °C (brake of the centrifuge set on off to avoid platelet activation). Automated aliquoting (Hamilton Microlab Star) of the plasma was done within less than 35 minutes after centrifugation into 500 µL aliquots. All aliquots were directly cooled (10°C) during the process. The aliquots were placed into a chest freezer (-80°C) within less than 45 minutes after aliquoting.

Targeted metabolomics

For metabolomics analysis, frozen plasma samples were shipped on dry ice to Fondazione Edmund Mach (FEM), in Trento Italy. Metabolomics analyses were done in two batches, with a 7-months time difference between batches.

LC-MS/MS targeted analyses were performed on 2000 frozen plasma samples, all samples were thawed at 4°C. An aliquot of 50 µl was loaded on 96 well plates Ostro (Water) and 20 µl of an internal standard mix in methanol were added (trypthophan-d5, tyrosine- d4, methionine- d4, serotonin- d4, kynurenic acid-d5, 5-hydroxyindole-acetic acid- d5 and dopamine- d5 at 2.5 ppm; final concentration in the extracted and recovered sample: 0.5 ppm).

- UHPLC-ESI-Triple-quadrupole-MS analysis

The detection was performed on a Waters ® Xevo Triple Quadrupole –MS equipped with ESI source and coupled on-line with an Aquity UHPLC (Waters). The MS operated in positive and negative ion modes. Separations were performed on a Water UPLC HSST3 (150 x 2.1 mm I.D., 1.8 µm particle size, 100 A pore diameter) purchased from Waters. Mobile phase A was water containing 0.1% formic acid, mobile phase B was acetonitrile with 0.1% formic acid. The gradient started at 5%B and was maintained for 0.5 min; then %B was increased to 10% at 2.5 min, to 15% at 3.5 min, to 25% at 4.5 min, to 35% at 5.5 min, to 45% at 6.5 min, to 55% at 7 min and then to 100%B at 7.5 min. Final conditions were kept for 3 min and then the column was re-equilibrated for 4 min. The flow rate was 0.3 ml/min, the injection volume was 2 µl, the column oven was set at 40°C and the sample tray

temperature was 5°C. With this method, we are able to quantify 3 BCCAs, L-valine (Val), L-isoleucine (Ile), L-leucine (Leu), 13 aromatic amino acids and their metabolic downstream products; 5-hydroxyindole-3-acetic acid (5-HIAA), serotonin (5-HT), indole-3-acetic acid (3-IAA), indole-3-carboxaldehyde (I3A), indole-3-lactic acid (ILA), indole-3-propionic acid (IPA), kynurenic acid (KYNA), kynurenine (Kyn), xanthurenic acid (XA), L-tryptophan (Trp), L-phenylalanine (Phe), L-tyrosine (Tyr) and dopamine (DA), and L-methionine (Met). Further details of the methods are described elsewhere (35).

Biochemical and clinical measurements

Fasting insulin, *high-density lipoprotein* cholesterol (HDL-C), triglycerides, glucose, and high sensitive C-reactive protein (hsCRP) were measured on the day of blood withdrawal according to standard procedures at the *University Hospital Bonn* (UKB). HsCRP was measured by high-sensitivity assay (Dimension Vista® System, Siemens Healthcare Diagnostics GmbH).

We used the homeostatic model assessment to calculate insulin resistance (HOMA-IR), as glucose levels (mmol/L) x insulin levels (mU/L)/22.5.

Systolic and diastolic blood pressure were measured 3 times in sitting position, with 10 minutes intervals each, in a resting and quiet environment. The mean of the blood pressure was obtained from the mean of the last two measurements.

Metabolic health classification

Metabolic health was defined using Wildman et al. criteria (5), which include six cardiometabolic risk markers defined as follow: 1) elevated blood pressure: systolic/diastolic blood pressure ≥130/85 mm Hg or antihypertensive medication use; 2) elevated triglyceride level: fasting triglyceride level ≥150 mg/dL; 3) low HDL-C level: HDL-C level < 40 mg/dL in men or < 50 mg/dL in women or lipid-lowering medication use; 4) systemic inflammation: hsCRP level >0.1 mg/L; 5) elevated glucose level: fasting

glucose level ≥100 mg/dL or antidiabetic medication use; 6) insulin resistance: HOMA-IR > 5.13. Metabolically unhealthy phenotype was defined when participants had ≥ 2 of the above cardiometabolic risk markers, and metabolically healthy when < 2 cardiometabolic risk markers were present.

Abdominal fat segmentation

Abdominal MR image acquisition was performed using a two-point Dixon sequence at two different sites, both with identical 3T Siemens MAGNETOM Prisma MR scanners (Siemens Healthcare, Erlangen, Germany). Data were acquired during a single breath-hold in a supine position with arms at the sides.

Abdominal MRI-fat variables were extracted from the predicted segmentation maps of the Fat-SegNet pipeline, a fully automated deep learning pipeline that accurately segments VAT and SAT inside a consistent anatomically defined abdominal region (36).

The segmented area was defined from the lower bound of the twelfth thoracic vertebra to the lower bound of the fifth lumbar vertebra. We calculated the height of the region of interest segmented (height of ROI) measuring the segmented slices on the Z-axis.

Total energy and protein intake

We assessed dietary intake with a self-administered semi-quantitative food frequency questionnaire (FFQ) (37). To calculate protein and energy intakes, we used an algorithm developed by The Institute of Nutritional and Food Sciences at the University of Bonn, utilizing as reference the German Food Code and Nutrient Data Base (version 3.02).

Statistical analysis

We compared differences in the adjusted mean concentration of metabolites and clinical characteristics between metabolically healthy and metabolically unhealthy participants with analysis of covariance (ANCOVA) adjusting for age, sex, and, additionally, BMI.

For further analyses, all metabolite concentrations were log-transformed to obtain approximately normal distributions. We applied rank-based inverse normal transformed due to skewness of the metabolite residuals. Outliers in metabolites were identified as concentrations above or below 3 times the interquartile range (IQR) before the rank-based inverse normal transformation.

We used multivariable linear regression to assess the association of VAT and SAT with metabolite concentrations, independent of BMI. In every model, we considered single metabolites as the dependent variable, and VAT and SAT as the main independent variables, adjusting for BMI, age, sex, batch effect, and height of the ROI. In additional analyses, we further adjusted the linear regression models for dietary total energy and protein intake. To account for multiple comparisons, we adjusted *p*-values for multiple testing using the Benjamini-Hochberg method (38). We evaluated whether the association of VAT and SAT with metabolite concentrations differed between sexes by including sex-VAT and sex-SAT interaction terms in the models.

To analyze the association of metabolite concentration with presence of cardiometabolic risk markers we used logistic regression models. We first adjusted for age, sex, BMI, batch effect, and smoking. Subsequently, we additionally included VAT and SAT to evaluate whether the associations of metabolites with cardiometabolic risk markers were independent of abdominal adiposity. In additional analyses, we further adjusted the logistic regression models for dietary total energy and protein intake.

To investigate whether the effects of metabolites on cardiometabolic risk markers differed between sexes, we added sex-metabolite interaction terms to our models.

The effect sizes from the linear and logistic regression models (beta coefficients and odds ratio) can be interpreted as standardized effect sizes due to the inverse rank normalization (1-SD increased in

log standardized units of metabolites). All models were adjusted for age, sex, BMI, batch effect, smoking, VAT, SAT, and the height of the ROI.

Mediation analysis

To investigate whether the association of abdominal fat with cardiometabolic risk markers is mediated through circulating metabolites, we performed a causal mediation analysis. Since VAT rather than SAT is strongly associated with higher odds of cardiometabolic risk markers, we considered VAT as the main independent variable to calculate the direct and indirect effect. All models were adjusted for sex, age, BMI, batch effect, and SAT. To evaluate the indirect effect, which depicts how much of the effect of VAT on cardiometabolic risk markers is mediated through metabolite concentration, we applied the product method (39). We used bootstrapping to assess whether the mediation effect was statistically significant (different from zero) (40).

Results

From the 2000 study participants with metabolomics analyses, we excluded participants with extreme values in metabolite concentration (n=152) and cardiometabolic risk markers (n=44), as well as those participants without valid data on abdominal MRI-fat segmentation (n=14), leaving 1790 participants that were included in the analyses. Table 1 shows the descriptive characteristics of the participants stratified by metabolic health phenotypes. Independently of age and sex, metabolically unhealthy participants had significantly higher concentrations of Val, Leu, Iso, Tyr, Phe, Kyn, Kyn/Trp, KYNA, I3A, and lower concentrations of IPA. When we further adjusted the mean differences for BMI, only concentrations of Val, Iso, and Leu remained statistically significantly higher in metabolically unhealthy compared with metabolically healthy participants.

Figure 1 depicts the association of VAT and SAT volumes with metabolite concentration in metabolic health phenotypes, independently of age, sex and BMI. We observed significant associations of VAT with Iso, Leu, ILA and IPA (in log standard deviation units per L increase in VAT: β=0.16, p = 0.002;

β=0.12, *p* = 0.02; β=0.11, *p* =0.02; β= -0.16, *p* = 0.005, respectively), only in metabolically unhealthy participants. Findings were also similar after adjustment for total diet energy and protein intake (data not shown)

The association of circulating metabolites with the presence of cardiometabolic risk markers without and with adjustment for VAT and SAT are shown in Figure 2a and Figure 2b. We observed a considerable reduction of the strength of the associations of metabolites with cardiometabolic risk markers after accounting for abdominal fat and BMI in the models. However, levels of some of the BCAA metabolites, such as Iso and Leu, remained statistically significantly associated with an increased odds of hypertriglyceridemia (OR per 1 SD increase in concentration =1.39, 95%CI: 1.19 - 1.62; OR= 1.34, 95%CI: 1.15 – 1.56), low HDL-cholesterol (OR=1.25, 95%CI: 1.08 – 1.45; OR= 1.19, 95%CI: 1.03 – 1.37), glucose impairment (OR=1.32, 95%CI: 1.13 – 1.54; OR=1.23, 95%CI: 1.06 – 1.42) and insulin resistance (OR=1.95, 95%CI: 1.50 – 2.58; OR=1.74, 95%CI: 1.33 – 2.28).

After adjusting for BMI and abdominal fat, higher levels of Tyr and Phe were associated with a significantly increased odds of insulin resistance (OR=1.83, 95%CI: 1.42 – 2.36; OR= 1.42, 95%CI: 1.10 – 1.83), whereas only Trp breakdown products (Kyn, Kyn/Trp, and ILA) were significantly associated with higher odds of systemic inflammation. Higher levels of ILA were associated with an increased odds of hypertriglyceridemia (OR= 1.26, 95%CI: 1.07 – 1.49), insulin resistance (OR= 1.38, 95%CI: 1.04 – 1.82) and systemic inflammation (OR= 1.26, 95%CI: 1.10 – 1.44), but with lower odds of low HDL-cholesterol (OR= 0.81, 95%CI: 0.69 – 0.95). We further observed that higher IPA levels were associated with lower odds of glucose impairment (OR= 0.80, 95%CI: 0.69 – 0.92). Findings were also similar after adjustment for total diet energy and protein intake (data not shown)

We found no significant sex effects except for the relation between 5-HT and hypertriglyceridemia ($p_{\text{sex-interaction}}$<0.001), where per SD increase 5-HT concentration the odds of having hypertriglyceridemia decreased with 29% (*p* =0.002) in women, but increased by 24% (*p*=0.027) in men.

Table 2 shows the associations where we found a significant mediation effect of metabolite concentrations in the relationship between VAT and cardiometabolic risk markers. Iso and Leu were the main metabolites with significant mediation effects for hypertriglyceridemia, low HDL-C, glucose impairment, and insulin resistance. For systemic inflammation, Kyn was the only metabolite that showed a statistically significant mediation effect, with 6.4% of the effect of VAT volumes on the increased risk of systemic inflammation being mediated through increases in concentrations of Kyn levels.

Discussion

We found that levels of BCAA metabolites differ between metabolically unhealthy and metabolically healthy participants, regardless of BMI. Moreover, in metabolically unhealthy participants, VAT rather than SAT was implicated in altered metabolism of some of the BCAA and AAA metabolites. We further observed that independently of abdominal and general adiposity, higher circulating concentrations of BCAA and AAA-downstream metabolites were associated with a greater likelihood of the presence of cardiometabolic risk markers, especially of insulin resistance. Causal analysis revealed that several of these metabolites partly mediated the link between abdominal VAT and cardiometabolic risk markers.

Some previous studies in smaller samples have evaluated how metabolites differ across metabolic phenotypes in obese individuals, using different criteria of MetS (41,42) and metabolically health definition (15,43) to classify healthy and unhealthy status. One study including 78 women showed that metabolically unhealthy obese participants had higher concentrations of BCAA, Tyr, and Phe compared with obese metabolically healthy (41). Likewise, metabolically unhealthy overweight/obese subjects showed significantly higher Kyn levels and Kyn/Trp ratio in comparison with healthy individuals (42). Other studies, however, found no differences in BCAA, Phe, and Tyr levels between metabolically healthy and metabolically unhealthy obese subjects (15). Furthermore, those metabolites were similarly associated with the odds of metabolically healthy and unhealthy

phenotypes in obesity (43). Our study extended the prior work by demonstrating that BCAA metabolites levels are significantly higher in metabolically unhealthy individuals independently of adiposity levels in a large cohort study.

We found a significantly stronger effect of VAT than SAT on abnormal plasma metabolite levels. This is in line with previous studies, performed in 40 to maximally 491 healthy individuals, showing that VAT rather than SAT was associated with plasma BCAA and AAA levels (44-46). Moreover, it fits with findings from a longitudinal study of diet-induced weight loss, which reported that a decreased VAT mass was significantly associated with a reduction in BCAA levels independently of weight loss after 2-years of follow-up (47).

Some studies have investigated abdominal fat tissue-specific differences in the up/downregulation of the metabolism of BCAA and AAA (23-25). They concluded that mainly in VAT, the expression of catabolizing enzymes for BCAA and certain AAA is altered. Piro et al. (33) reported that pathological obese people had lower concentrations of BCAA in the VAT tissue than healthy participants, whereas increased production of BCAA catabolites. That suggests that an impaired BCAA catabolism in VAT boosts higher plasma circulation of these metabolites. Moreover, the functionality of adipose tissue can also differ across metabolic health phenotypes. Genes related to BCAA catabolism reportedly are more down-regulated in the abdominal adipose tissue of metabolically unhealthy obese compared to metabolically healthy obese individuals (15,48). Thus, in the metabolically healthy phenotype, the abdominal adipose tissue is characterized by the maintenance of mitochondrial function and absence of inflammation, while in the metabolically unhealthy phenotype the adipose tissue is more dysfunctional (48). Our results could complement this approach in a large population-based study since we found that only in metabolically unhealthy participants, larger VAT was associated with an increased level of several circulating metabolites.

We found that BCAA, AAA, and AAA-downstream metabolites were strongly associated with higher odds of cardiometabolic risk markers, independently of the well-known effects of VAT and SAT. The strongest effects were for insulin resistance, mainly by high concentrations of BCAA, Tyr, Trp, and XA

metabolites. BCAA and certain AAA-downstream metabolites have been largely associated with insulin resistance in some population studies (20-22) suggesting that high concentrations of these metabolites are strong markers of an early manifestation of T2D.

To further elucidate possible mechanisms, an animal study showed that BCAA leads to insulin resistance by activation of the mechanistic target of rapamycin (mTOR) and P70-S6 kinase 1 (S6K-1) in exposure to a high-fat diet (49), resulting in insulin resistance through the phosphorylation of insulin receptor substrate 1 (IRS-1) (50). On the other hand, in a human study, it was observed that elevated concentrations of BCAA could induce insulin resistance in human skeletal muscle by the direct inhibition of muscle glucose transport and/or phosphorylation with a subsequent reduction in rates of glycogen synthesis (51). Furthermore, the accumulation of toxic intermediates from the BCAA oxidation and impairment of mitochondria functionality may also be involved in the association between BCAA and insulin resistance (50). Moreover, few studies have investigated the mechanisms linking AAA with insulin resistance. One study showed that beta-cell function is affected by oral Tyr and its derived breakdown metabolites such as DA (52). Certain Trp downstream metabolites such as XA and KYNA have been associated with an impaired production, release, and biological activity of insulin. One intermediate pathway of Trp metabolism is the Kyn–nicotinamide adenine dinucleotide (NAD). Downregulation of the NAD pathway leads to the production of XA and KYNA and a decreased formation of NAD leads to inhibition of synthesis and secretion of insulin and the death of pancreatic beta cells (53).

In addition to insulin resistance, prior epidemiological studies have also evaluated a wider number of cardiometabolic risk markers as outcomes of impaired metabolite concentrations (54,55). For instance, circulating levels of BCAA and AAA metabolites were associated with dyslipidemia, high blood pressure (54) and with a higher odds ratio of T2D, MetS, and dyslipidemia after a four-year follow-up period (55). This fits our observations that higher circulating concentrations of BCAA, AAA, and AAA-downstream metabolites were not only associated with insulin resistance, but also with the presence of other cardiometabolic risk markers, albeit to a lesser extent.

We found no sex differences for the associations between metabolite concentrations and cardiometabolic risk markers, except for the association of 5-HT levels with the odds of having hypertriglyceridemia. Higher 5-HT levels were associated with a significantly lower likelihood of hypertriglyceridemia in women, and a significantly increased likelihood in men. Serotonin (5-HT) is a metabolite from the hydroxylation pathway of Trp catabolism associated with energy homeostasis, appetite regulation, and depressive symptoms. Furthermore, 5-HT also participates in the regulation of hepatic lipid balance (56) and induces lipolysis of stored triacylglycerol increasing plasma levels of free fatty acids and glycerol (57). To the best of our knowledge, there are no population-based studies showing sex differences on the association of 5-HT levels with hypertriglyceridemia. One possible explanation, however, could lie in the different genetic architecture of 5-HT between men and women which may impact the variation on susceptibility to different phenotypes (58). Nonetheless, research on the molecular mechanism by which 5-HT associates with cardiometabolic risk markers in men and women is scarce. Therefore, we can not completely exclude that our finding of a sex-dependent association of 5-HT with hypertriglyceridemia, could have been spurious.

We observed that although BCAA had an abdominal fat-independent effect on the odds of some of the cardiometabolic risk markers, Iso and Leu partially mediate the association of VAT with hypertriglyceridemia, low HDL-C, glucose impairment, and insulin resistance. These results suggest that BCAA are associated with metabolic health in two ways; acting as mediators between the connection of high VAT accumulation and cardiometabolic risk markers, and having an individual contribution for a higher odds of cardiometabolic risk markers. We also observed that Kyn was the only metabolite that partly mediated the association of VAT with systemic inflammation. Menni et al. (59) analysed the mediation effect of VAT in the association of BCAA with insulin resistance and showed that 19.4% to 46.6% of the variance of HOMA-IR explained by BCAA metabolites was through high VAT mass. Our results, however, support the hypothesis that altered metabolite concentrations are

a consequence of a disrupted metabolism in adipocytes, and biologically, they could mediate the relationship between VAT and cardiometabolic risk markers.

Several limitations of this study should be considered. First, we based our analysis on cross-sectional data, which does not allow us to draw causal conclusions on whether high VAT volumes are the cause of disruptions in metabolite concentrations and whether metabolites have a causal effect on higher odds of cardiometabolic risk markers. Second, we identified metabolites in plasma and did not have tissue-specific information to draw more precise inferences on the effects of VAT and SAT metabolism. As a strength of our study, we consider the large and homogenous study population, including men and women from a broad spectrum of ages. Second, the targeted metabolomic approach performed in our study allowed us to quantify a large number of known metabolites with high sensitivity and accuracy. In a sensitivity analysis, we found neither influence of total energy and total protein intake on the associations of VAT and SAT with metabolites concentration, nor in the association of metabolite concentration with the odds of cardiometabolic risk markers (data not shown).

This is the first large-scale study highlighting the importance to include the simultaneous analysis in human plasma, by liquid chromatography-tandem mass spectrometry, of the metabolites belonging to Trp, BCAA, and AAA pathways. Several of these, as depicted in Figure 1, emerged as potentially useful clinical markers to understand the link between the abdominal VAT and metabolic health risk markers. We also used a validated method to accurately quantify abdominal fat from MRI images that enable us to compare the metabolic activities of VAT and SAT volumes as different fat compartments. We considered as outcomes different cardiometabolic risk markers (prior state of disease) that allow identifying individuals before the onset of a disease. This study incites the further integration of genetic and lifestyle information to help to elucidate causal effects of metabolites on cardiometabolic risk markers and to understand the mechanism behind changes in VAT metabolism impact circulating metabolites.

Conclusion

In summary, we have shown that in metabolically unhealthy individuals, VAT is associated with an altered BCAA and AAA metabolism, as reflected in circulating concentrations. BCAA, AAA, and AAA-downstream metabolites are important biomarkers in metabolic health abnormalities, and they are also partial mediators in the connection between VAT and cardiometabolic risk markers. Thus, these metabolites may provide a better insight into the biological mechanisms that underlie the relationship of abdominal VAT with metabolic health.

Author Contributions: O.R.X contributed to the study concept and design, analysis and interpretation of the data, drafting and revising the manuscript. A.A implemented the metabolomic analysis, contributed to the interpretation of data, and critical revision of the manuscript for important intellectual content. M.F designed the plan of the experiment and supervised the metabolomic analyses, and contributed to the critical revision of the manuscript for important intellectual content. M.M.B.B. contributed to the study concept and design, interpretation of data, revision of the manuscript, funding and supervision. All the authors have accepted responsibility for the entire content of this submitted manuscript and approved the final version of the manuscript for submission.

Data availability: Some or all datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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Figure and legends

Figure 1. Association of VAT and SAT with metabolite concentrations stratified by metabolic health phenotypes.

Models were adjusted for age, sex, BMI, batch effect, height of the ROI, VAT and SAT simultaneously. Multiple testing was performed using the Benjamini and Hochberg method to adjust *p*-values. Abbreviations: Met, L-methionine; Val, L-valine; Leu, L-leucine; Iso, L-Isoleucine; Tyr, L-tyrosine; Phe, L-phenylalanine; DA, dopamine; XA, xanthurenic acid; Trp, L-tryptophan; Kyn, kynurenine; Kyn/Trp, kynurenine/tryptophan ratio; KYNA, kynurenic acid; IPA, indole-3-propionic acid; ILA, indole-3-lactic acid; I3A, indole-3-carboxaldehyde; 3-IAA, indole-3-acetic acid; 5-HT, serotonin; 5-HIAA, 5-hydroxyindole-3-acetic acid; VAT, visceral adipose tissue; SAT, subcutaneous adipose tissue.

Figure 2. Odds ratio of cardiometabolic risk markers per increase in metabolite concentration. **a)** Logistic regression model adjusted for age, sex, smoking status, batch effect, and BMI. **b)** Additionally, models were adjusted for VAT, SAT, and height of the ROI. The horizontal lines crossing the circle and diamond shapes represent the 95% confidence interval. Abbreviations: Met, L-methionine; Val, L-valine; Leu, L-leucine; Iso, L-Isoleucine; Tyr, L-tyrosine; Phe, L-phenylalanine; DA, dopamine; XA, xanthurenic acid; Trp, L-tryptophan; Kyn, kynurenine; Kyn/Trp, kynurenine/tryptophan ratio; KYNA, kynurenic acid; IPA, indole-3-propionic acid; ILA, indole-3-lactic acid; I3A, indole-3-carboxaldehyde; 3-IAA, indole-3-acetic acid; 5-HT, serotonin; 5-HIAA, 5-hydroxyindole-3-acetic acid. * *P*-value <0.05, ** *P*-value <0.01, *** *P*-value <0.001. Ω *P*-value for sex-interaction <0.001.

Table 1. Characteristics of the study population.

	Metabolically healthy n=1017	Metabolically unhealthy n=773	Adjusted for age and sex*		Adjusted for age, sex and BMI*	
			Mean difference [95% CI]	P-value	Mean difference [95% CI]	P-value
Women, n (%)	621 (58.0)	341 (44.1)	-0.7 [-0.9 – -0.6]	<0.001	-0.7 [-0.9 – -0.5]	<0.001
Age, years (SD)	50.1 (12.4)	60.1 (13.7)	10.3 [9.0 – 11.5]	<0.001	10.7 [9.3 – 12.0]	<0.001
BMI, kg/m^2 (SD)	24.0 (3.4)	27.5 (4.1)	3.6 [3.2 – 4.0]	<0.001	3.6 [3.2 – 4.0]	<0.001
VAT, L (SD)	1.0 (0.8)	2.3 (1.2)	1.0 [0.9 – 1.1]	<0.001	0.4 [0.4 – 0.5]	<0.001
SAT, L (SD)	2.6 (1.3)	3.7 (1.6)	1.3 [1.2 – 1.5]	<0.001	0.2 [0.1 – 0.3]	<0.001
Total energy intake, kcal/day (SD)	2507 (821)	2545 (859)	-18.9 [-99 – 61.1]	0.642	-7.2 [-95.4 – 81]	0.873
Protein intake, g (SD)	79.5 (24)	81.4 (26)	0.9 [-1.5 – 3.3]	0.461	0.8 [-1.8 – 3.4]	0.552
Met, Umol (SD)	11.5 (2.78)	11.5 (2.8)	-0.02 [-0.3 – 0.3]	0.87	-0.04 [-0.3 – 0.3]	0.79
Val, Umol (SD)	50.7 (21.3)	54.7 (22.9)	4.6 [2.4 – 6.8]	<0.001	2.9 [0.5 – 5.4]	0.02
Leu, Umol (SD)	56.9 (13.8)	62.3 (15.4)	4.3 [2.9 – 5.6]	<0.001	2.5 [1.03 – 3.9]	0.001
Iso, Umol (SD)	27.1 (7.2)	30.3 (8.2)	2.6 [1.9 – 3.3]	<0.001	1.7 [0.9 – 2.4]	<0.001
Tyr, Umol (SD)	32.4 (8.9)	36.2 (9.8)	2.6 [1.6 – 3.5]	<0.001	0.9 [-0.1 – 1.9]	0.09
Phe, Umol (SD)	32.5 (8.0)	34.3 (8.4)	1.1 [0.3 – 1.9]	0.01	0.1 [-0.7 – 1.0]	0.77
DA, Umol (SD)	0.02 (0.01)	0.02 (0.01)	-0.0 [-0.0 – 0.0]	0.58	-0.0 [-0.0 – 0.0]	0.27
XA, Umol (SD)	0.3 (0.07)	0.3 (0.07)	0.0 [-0.0 – 0.01]	0.16	0.0 [-0.01 - 0.01]	0.54
Trp, Umol (SD)	30.7 (7.5)	31.4 (7.5)	0.6 [0.1- – 1.4]	0.10	0.2 [-0.6 – 1.0]	0.63
Kyn, Umol (SD)	1.5 (0.6)	1.7 (0.6)	0.09 [0.03 – 0.2]	0.002	0.01 [-0.05 – 0.08]	0.71
Kyn/Trp, Umol (SD)	0.04 (0.01)	0.05 (0.02)	0.0 [0.0 – 0.0]	0.01	-0.0 [-0.0 – 0.0]	0.88

KYNA, Umol (SD)	0.03 (0.01)	0.03 (0.02)	0.002 [0.0006 – 0.003]	0.007	-0.0 [-0.0 – 0.0]	0.82
IPA, Umol (SD)	1.4 (0.9)	1.2 (0.8)	-0.2 [-0.3 - - 0.1]	<0.001	-0.9 [-0.2 – 0.01]	0.07
ILA, Umol (SD)	0.8 (0.3)	0.9 (0.4)	0.03 [-0.0 – 0.06]	0.08	0.0 [-0.03 – 0.04]	0.81
I3A, Umol (SD)	0.05 (0.02)	0.05 (0.02)	0.0 [0.0– 0.0]	0.01	0.0 [-0.0 – 0.0]	0.11
3-IAA, Umol (SD)	2.02 (1.0)	2.1 (1.2)	-0.03 [-0.1 – 0.08]	0.62	-0.03 [-0.1 – 0.09]	0.58
5-HT, Umol (SD)	0.1 (0.07)	0.1 (0.06)	-0.0 [-0.01 – 0.0]	0.27	0.0 [-0.01 – 0.01]	0.98
5-HIAA, Umol (SD)	0.02 (0.01)	0.02 (0.01)	-0.0 [-0.0 – 0.0]	0.12	-0.0 [-0.0 – 0.0]	0.12
Prevalence of cardiometabolic risk factors						
Hypertension, N (%)	248 (24.4)	656 (84.8)				
Hypertriglyceridemia, N (%)	25 (2.6)	294 (38.7)				
Low HDL-C, N (%)	35 (3.4)	290 (37.5)				
Glucose impairment, N (%)	34 (3.3)	279 (36.1)				
Insulin resistance, N (%)	0 (0)	103 (13.8)				
Systemic inflammation, N (%)	197 (20.5)	550 (72.5)				

Data are presented as mean (SD) or frequencies (%). Characteristics and mean metabolite concentrations were compared using an ANCOVA test. * When applicable.

Abbreviations: VAT, visceral adipose tissue; SAT, subcutaneous adipose tissue; Met, L-methionine; Val, L-valine; Leu, L-leucine; Iso, L-Isoleucine; Tyr, L-tyrosine; Phe, L-phenylalanine; DA, dopamine; XA, xanthurenic acid; Trp, L-tryptophan; Kyn, kynurenine; Kyn/Trp, kynurenine/tryptophan ratio; KYNA, kynurenic acid; IPA, indole-3-propionic acid; ILA, indole-3-lactic acid; I3A, indole-3-carboxaldehyde; 3-IAA, indole-3-acetic acid; 5-HT, serotonin; 5-HIAA, 5-hydroxyindole-3-acetic acid.

Table 2. Mediation effect of metabolites in the relation of VAT with cardiometabolic risk factors.

Cardiometabolic risk markers	Metabolite	Direct effect (95% CI)	Indirect effect (95% CI)	Proportion mediated, % (95% CI)	*P-value*
Hypertriglyceridemia	Iso	0.053 (0.04 – 0.06)	0.004 (0.002 – 0.01)	6.5 (3.1 – 11.0)	<0.001
	Leu	0.054 (0.05 – 0.06)	0.002 (0.0008 – 0.001)	4.03 (1.6 – 8.0)	<0.001
	ILA	0.055 (0.05 – 0.06)	0.009 (0.00007 – 0.002)	1.7 (0.3 – 4.0)	0.02
Low HDL-C	Iso	0.044 (0.03 – 0.05)	0.003 (0.001 – 0.01)	6.7 (2.0 – 14.0)	0.002
	Leu	0.045 (0.003 – 0.08)	0.002 (0.0001 – 0.004)	3.7 (0.2 – 9.0)	0.02
Systemic inflammation	Kyn	0.037 (0.02 – 0.04)	0.002 (0.0007 – 0.005)	6.4 (2.0 – 11.0)	<0.001
Glucose impairment	Iso	0.036 (0.02 – 0.05)	0.003 (0.002 – 0.01)	8.8 (3.2 – 18.0)	<0.001
	Leu	0.037 (0.02 – 0.05)	0.002 (0.0003 – 0.001)	4.6 (0.7 – 11.0)	0.02
	IPA	0.036 (0.02 – 0.05)	0.002 (0.0007 – 0.001)	6.3 (1.9 – 13.0)	0.004
Insulin resistance	Iso	0.009 (0.006 – 0.013)	0.001 (0.0005 – 0.002)	11.0 (5.8 – 18.0)	<0.001
	Leu	0.010 (0.008 – 0.013)	0.0007 (0.0002 – 0.001)	6.5 (2.5 – 12.0)	0.002

ILA	0.011 (0.007 – 0.015)	0.0003 (0.00002 – 0.001)	2.5 (0.2 – 6.0)	0.02
Tyr	0.010 (0.007 – 0.013)	0.0008 (0.0003 – 0.002)	7.8 (3.2 – 14.0)	<0.001
Phe	0.011 (0.007 – 0.01)	0.0003 (0.00002 – 0.001)	2.6 (0.4 – 6.0)	0.02

Models for mediation analysis were adjusted for age, sex, BMI, VAT, SAT, height of ROI, and batch effect.

Figure 1

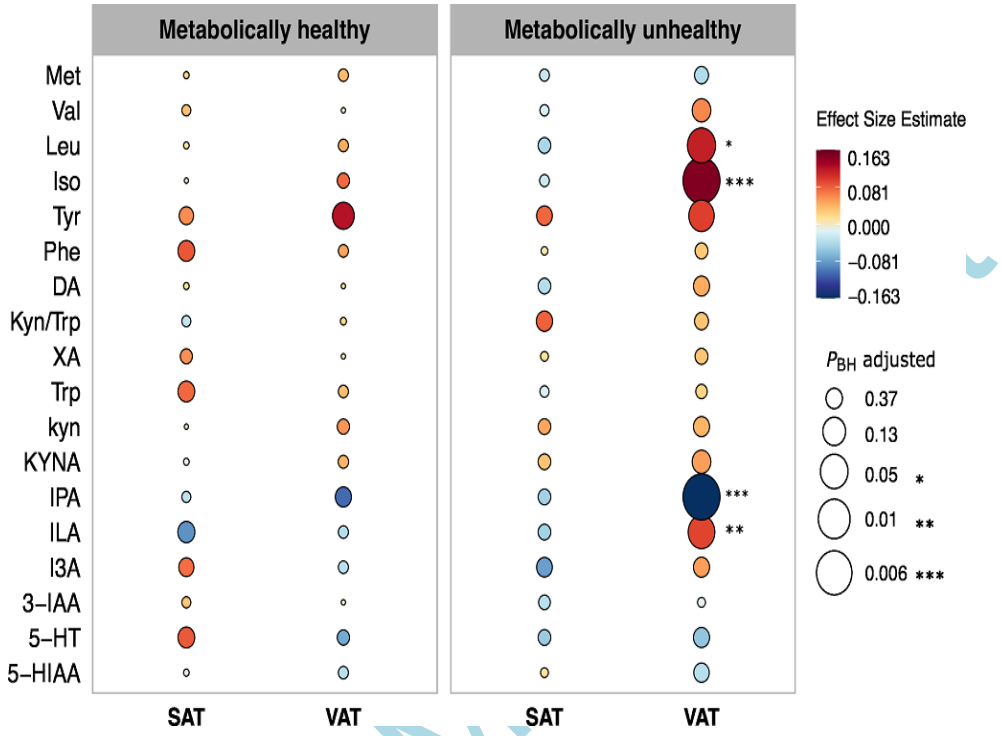

Figure 2