Serum metabolite profiles of habitual diet: evaluation by ^{1}H-nuclear magnetic resonance analysis

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ABSTRACT

Background: Objective and reliable methods to measure dietary exposure and prove associations and causation between diet and health are desirable.

Objective: The aim of this study was to investigate if ^{1}H-nuclear magnetic resonance (^{1}H-NMR) analysis of serum samples may be used as an objective method to discriminate vegan, vegetarian, and omnivore diets. Specifically, the aim was to identify a metabolite pattern that separated meat-eaters from non–meat-eaters and vegans from nonvegans.

Methods: Healthy volunteers (45 men and 75 women) complying with habitual vegan ($n = 43$), vegetarian ($n = 24$ + vegetarians adding fish $n = 13$), or omnivore ($n = 40$) diets were enrolled in the study. Data were collected on clinical phenotype, body composition, lifestyle including a food-frequency questionnaire (FFQ), and a 4-d weighed food diary. Serum samples were analyzed by routine clinical test and for metabolites by ^{1}H-NMR spectroscopy. NMR data were nonnormalized, UV-scaled, and analyzed with multivariate data analysis [principal component analysis, orthogonal projections to latent structures (OPLS) and OPLS with discriminant analysis]. In the multivariate analysis volunteers were assigned as meat-eaters (omnivores), non–meat-eaters (vegans and vegetarians), vegans, or nonvegans (lacto-ovo-vegetarians, vegetarians adding fish, and omnivores). Metabolites were identified by line-fitting of 1D ^{1}H-NMR spectra and the use of statistical total correlation spectroscopy.

Results: Although many metabolites differ in concentration between men and women as well as by age, body mass index, and body composition, it was possible to correctly classify 97.5% of the meat-eaters compared with non–meat-eaters and 92.5% of the vegans compared with nonvegans. The branched-chain amino acids, creatine, lysine, 2-aminobutyrate, glutamine, glycine, trimethylamine, and 1 unidentified metabolite were among the most important metabolites in the discriminating patterns in relation to intake of both meat and other animal products.

Conclusions: ^{1}H-NMR serum metabolomics appears to be a possible objective tool to identify and predict habitual intake of meat and other animal products in healthy subjects. These results should be confirmed in larger cohort studies or intervention trials. This trial was registered at clinicaltrials.gov as NCT02039609. *Am J Clin Nutr* 2019;110:53–62.

Keywords: habitual diet, vegan, vegetarian, omnivore, meat, metabolomics, ^{1}H-NMRs

Introduction

Today, environmental concern has sparked interest in vegetarianism or "flexitarianism," i.e., reducing the intake of meat, especially red meat, or choosing a vegetarian diet plus fish. Consequently, the "omnivore dietary group" today constitutes a wide range of meat and fish consumption. Some vegetarians substitute meat for full-fat dairy products such as cheese and eggs (lacto-ovo-vegetarians); others adhere to an almost vegan diet, exchanging dairy products with new substitutes based on soy, rice, or oats, whereas a vegan diet excludes all food of animal origin. These modern variations in actual food intake may explain the inconclusive results on health outcomes in studies comparing vegetarian and omnivore diets (1). In general, vegetarians have been more health conscious and many studies report lower BMI and improved blood lipids among vegetarians compared with omnivores (1), for reasons that may not be related to diet. Further, the mechanisms behind possible beneficial health effects of vegetarian diets are not fully understood. Effects from specific foods such as soy, whole grain, vegetable oils, and the exclusion of meat have been discussed, as well as the overall content of specific nutrients, fiber, and fat quality. More specific information on exact dietary intake in the era of multiple modern

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Supplemental Figure 1 is available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/ajcn/.

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Abbreviations used: BCAA, branched-chain amino acid; FFQ, food-frequency questionnaire; Hb, hemoglobin; OPLS-DA, orthogonal projections to latent structures with discriminant analysis; PCA, principal component analysis; TG, triglyceride; TMA, trimethylamine; ^{1}H-NMR, ^{1}H-nuclear magnetic resonance.

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diets will improve our understanding of why certain diets are healthy. Thus, objective methods for evaluating the intake of products of animal and nonanimal origin are important to further understand likely protective effects against noncommunicable diseases of vegetarian diets.

Metabolomics has emerged as a possible means to capture habitual diet (2). To date only a few studies have been performed to measure intake of meat or foods from animal sources in urine and serum samples with a metabolomics approach (3–6). ^{1}H-nuclear magnetic resonance (^{1}H-NMR) analysis in serum has some advantages over urine in that the pH does not fluctuate substantially, which complicates the peak identification in urine. Also, urine may be more or less diluted, i.e., vary in concentration, complicating comparison between samples. Factors that may influence pH and dilution are medication and coffee (6), which could lead to misclassification of individuals. This seems less likely to occur in serum samples. On the other hand, urine samples may contain a wider range of metabolites and 24-h collections can be employed instead of only fasting or spot serum samples. Previous results imply that the serum and urine metabolomes in response to a meal partially overlap, but are mainly complementary (7).

One study using mass spectrometry–based metabolomics to discriminate between omnivores, vegans, and vegetarians in serum has been published (3). The results are promising but should be repeated in studies with more optimal sample collection procedures and in fasting samples, because the last meal is known to influence the metabolome and thus possibly mask the "habitual" diet. Here we aimed for a high-quality sample collection procedure to capture the habitual metabolites. The primary outcome was to investigate if ^{1}H-NMR analysis of serum samples could be used as an objective method to discriminate between individuals adhering to a habitual vegan, vegetarian, or omnivore diet. Specifically, the aim was to find metabolite patterns that separated meat-eaters from non–meat-eaters and vegans from nonvegans, i.e., to evaluate if the NMR metabolomics approach can be used to evaluate a subject's intake of meat or other products of animal origin.

Methods

Subjects

Volunteers were recruited by advertisement for healthy individuals complying with a habitual vegan, (lacto-ovo-)vegetarian, or omnivore diet during April–May, 2013 and August–December, 2015. A fourth group—vegetarians adding fish or shellfish to their diet—was also included because this emerged as a rather common habitual diet. Before entering the study (NCT02039609), participants provided written informed consent. Subjects were considered suitable if aged between 18 and 65 y, healthy [i.e., having a normal blood test including hemoglobin (Hb), vitamin B-12, folate, serum electrolytes, creatinine, liver transaminases, bilirubin, alkaline phosphatase, C-reactive protein, plasma glucose, and thyroid status, with no regular use of medications (contraceptives were permitted)], and having a BMI (in kg/m^2) of 18–30. Screening included clinical phenotype, a short lifestyle questionnaire including a food-frequency questionnaire (FFQ) and 2 questions on physical activity, and a 4-d weighed food diary (the gold standard in nutritional assessment) for the days preceding sampling. The short FFQ developed for this study included 10 questions on food intake regarding soy or soy products, legumes, vegetables, fruit and berries, milk products, eggs and egg-based foods, fish and shellfish, poultry, red meat, and cookies and confectionery. Body composition was measured with bioimpedance analysis (ImpediMed Bioimp version 5.3.1.1). Subjects who were pregnant, lactating, or used nicotine products regularly were excluded. In addition, volunteers were not allowed to drink alcohol the night before sampling or consume food supplements 1 wk before sampling. No NMR-metabolomics studies on habitual diet had been published, to our knowledge, when planning the study and a sample size of >90 individuals was estimated to be sufficient because this was in line with other metabolomics studies.

The project was approved by the Regional Ethical Review Board in Gothenburg (reference number 561-12) and adhered to the Helsinki Declaration.

Sampling and sample preprocessing

Fasting serum samples were collected at 1 time point. Venous blood was drawn into a 5-mL BD vacutainer glass tube (BD Hemogard, BD Vacutainer), turned ∼5 times, allowed to clot at 4°C for 30 min, and centrifuged at 4°C at 2600 *g* for 10 min. The serum, once divided into aliquots, was stored at −20°C within 1 h and at −80°C within 2 h. Samples were stored at −80°C until analysis. Before ^{1}H-NMR analysis, serum samples were thawed for 60 min at 4°C, then 100 μL serum was mixed with 100 μL phosphate buffer (75 mM Na_2HPO_4, 20% D_2O, 0.2 mM imidazole, 4% NaN_3, 0.08% TSP-d_4, pH 7.4) in a deep well plate. Next, 180 μL sample mix was transferred to 3.0-mm NMR tubes (Bruker BioSpin, 96 sample racks for SampleJet) using a SamplePro liquid handling robot (Bruker BioSpin). Samples were kept at 6°C in the SampleJet sample changer until analysis.

NMR spectroscopy

^{1}H-NMR spectra were measured at 800 MHz using a Bruker Avance III HD spectrometer with a 3-mm TCI cryoprobe and a cooled (6°C) SampleJet for sample handling. All ^{1}H-NMR experiments were performed at 25°C. NMR data (1D perfect echo with excitation sculpting for water suppression) were recorded using the Bruker pulse sequence "zgespe." The spectral width was 20 ppm, the relaxation delay 3 s, the acquisition time 2.04 s, and a total of 128 scans were collected into 64k data points resulting in a measurement time for each sample of 12 min 4 s. All data sets were zero filled to 128k and an exponential line-broadening of 0.3 Hz was applied before Fourier transformation. All data processing was performed with TopSpin 3.2pl6 (Bruker BioSpin) and TSP-d_4 was used for referencing.

Chenomx NMR suite 8.31 (Chenomx Inc.) was used for annotation with the aid of the Human Metabolome Database (8) and an in-house implementation of the statistical total correlation spectroscopy (STOCSY) routine (9). Metabolic pathway information was retrieved from the Kyoto Encyclopedia of Genes and Genomes pathway database (10).

Data processing

^{1}H-NMR spectra were aligned using *ic*oshift (11) and manual integration of peaks was performed to a linear baseline on all spectra in parallel using an in-house MatLab (MathWorks) routine. In total 237 peaks were integrated within the chemical shift range of 0.721–8.362 ppm. No sections of the spectra were excluded. Data were nonnormalized and UV-scaled. Cross-validation groups were set to 7 (the default in the SIMCA software).

Dietary habits, i.e., vegan (consuming no food of animal origin), vegetarian (including dairy and eggs), vegetarian adding fish, or omnivore (consuming a mixed diet), were evaluated by general questions about diet and the FFQ. For data analysis 2 new dietary groups were formed: nonvegan, including omnivores, vegetarians, and vegetarians adding fish; and nonmeat, including vegans and vegetarians. Food-frequency data on red meat, poultry, fish and shellfish, eggs, and dairy were used to construct a "grade of omnivore eating." The original frequencies for dairy, eggs, fish, poultry, and red meat (never, less than a few times per month, 1–2 times/wk, >3 times/wk) were set to 0, 1, 2, and 3 points and added for each individual, resulting in a simple index of 0–15 points. Due to well-known gender differences in serum metabolites and a skewed distribution between men and women in the dietary groups, the larger group of women was also analyzed separately, to confirm that the separating metabolites were due to the diet and not to gender. The number of men was regarded as too few for separate multivariate modeling, so instead all data from men were used as a test set and projected onto the model created only with data from women.

Multivariate methods

A principal component analysis (PCA) model was used to explore clustering patterns of observations, trends in the data, and outliers. An orthogonal projections to latent structures (OPLS) model was used to evaluate the impact of known metadata on the metabolites and models. Separation of classes and variables related to separation in the data according to classification of diet (vegan compared with nonvegan, meat compared with nonmeat) was evaluated using OPLS with discriminant analysis (OPLS-DA). Receiver operating curve analysis was performed and the AUC was used as an estimate of the predictive accuracy of each dietary group in the OPLS-DA model. To select class-discriminating variables of interest for annotation, loadings ($pq \geq \pm 0.1$) and top-ranked variables in variable importance scores in the OPLS-DA model were assessed. All multivariate analyses were performed using SIMCA software version 15.0 (Umetrics AB).

Univariate methods

Statistical analyses were performed using SPSS version 25 (SPSS Inc.). Pearson's chi-square test and Fisher's exact test were used for comparing categorical data such as level of physical activity (low, medium, or high) between the groups. Comparisons of other characteristics and outcome variables were performed with 1-factor ANOVA with Tukey's post hoc test and Student's *t* test. Nonnormally distributed data were log transformed before statistical analysis. Data are presented as means ± SDs with significance set at $\alpha = 0.05$. Student's *t* test and logistic multivariable regression analysis were used to evaluate metabolites driving the separation in OPLS-DA models. The logistic regression models were adjusted for age, gender, BMI, and body fat mass percentage. To adjust for multiple testing a Bonferroni correction was applied; the 237 variables represent ~70 metabolites and with a margin we adjusted for 100 tests, i.e., *P* values < 0.0005 were regarded as significant.

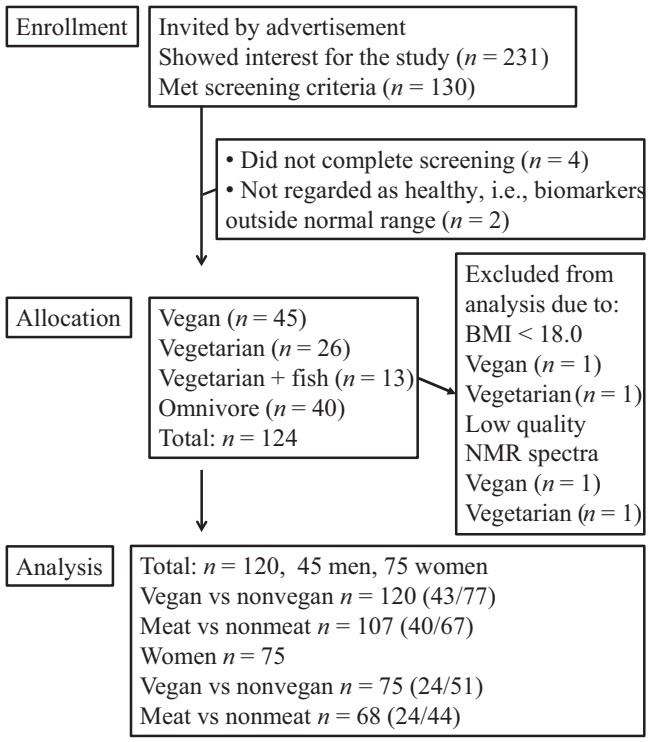

FIGURE 1 Consolidated Standards of Reporting Trials (CONSORT) diagram. NMR, nuclear magnetic resonance.

Results

Participant characteristics

In total, 231 individuals showed interest to participate and 130 met the screening criteria, out of whom 2 were regarded as not healthy, 2 were excluded because of BMI < 18.0, and 4 did not complete screening. Two NMR spectra displayed low quality and were excluded from analysis. Thus, 120 healthy volunteers, 45 men and 75 women, were included in the study (**Figure 1**). The number of women and men in each dietary group differed, although nonsignificantly (**Table 1**). Regular use of supplements was high: 52% and 95% among nonvegans and vegans, respectively (Table 1). Even so, vegans and vegetarians had lower serum vitamin B-12 concentrations than omnivores. Vegans and vegetarians had (as expected) lower serum creatinine but higher folate concentrations. Only 8 subjects had a BMI > 25 and none had a BMI > 30. Fat mass percentage was significantly higher in vegans and vegetarians than in omnivores, although their serum cholesterol and lipoprotein concentrations were significantly lower. Most participants were young (mean age 28–30 y) and reported a high level of physical activity. Omnivores

TABLE 1 Participant characteristics[1]

Participant characteristics	Omnivore/meat		Vegetarian adding fish		Vegetarian		Vegan		Nonvegan		Nonmeat	
n	40		13		24		43		77		67	
Sex, *n* men/women (% men)	16/24 (40)		6/7 (46)		4/20 (17)		19/24 (44)		26/51 (34)		23/44 (34)	
	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range
Omnivore index	15.2 ± 2.5	10–20	6.1 ± 1.2	4–8	3.6 ± 1.2	2–6	0.1 ± 0.4	0–2	10.0 ± 5.8	2–20	1.3 ± 1.2	0–6
Age, y	27.9 ± 7.7	19–53	29.9 ± 5.7	21–40	29.6 ± 8.1	19–57	29.3 ± 6.7	19–54	28.8 ± 7.5	19–57	29.4 ± 7.2	19–57
Weight, kg	66.3 ± 10.5	44.9–90.3	64.5 ± 9.9	48.0–78.1	62.4 ± 9.9	46.0–82.5	65.1 ± 10.5	41.3–89.8	64.7 ± 10.2	44.9–90.3	64.1 ± 10.3	41.3–89.8
BMI, kg/m^2	22.2 ± 1.8	19.4–28.2	20.7 ± 1.9	18.6–24.0	21.8 ± 2.6	18.0–28.9	21.6 ± 2.2	18.2–26.2	21.8 ± 2.1	18.0–28.9	21.7 ± 7.2	18.0–28.9
Fat mass, %[3]	17.0 ± 7.1	4.0–29.7	20.1 ± 4.7	13.7–30.9	23.8 ± 6.6[2]	8.3–32.6	21.3 ± 7.6[3]	5.3–36.5	19.6 ± 7.2	4.0–32.6	22.2 ± 7.3	5.3–36.5
Fat-free mass, kg	55.3 ± 11.3	39.1–76.4	51.8 ± 9.4	36.6–64.3	47.5 ± 9.2[2]	34.6–72.9	51.2 ± 9.4	30.9–77.6	52.3 ± 10.8	34.6–76.4	49.9 ± 9.5[2]	30.9–77.6
Systolic blood pressure, mm Hg	119 ± 9	100–140	114 ± 9	100–128	117 ± 11	98–135	119 ± 10	100–140	118 ± 10	98–140	118 ± 10	98–140
Diastolic blood pressure, mm Hg	73 ± 6	62–88	68 ± 6	62–80	69 ± 6	60–79	71 ± 8	60–94	71 ± 6	60–88	70 ± 7	60–94
Serum cholesterol, mmol/L	4.9 ± 1.0	3.4–7.5	4.2 ± 0.7	2.8–5.2	4.1 ± 0.8[2]	3.0–5.9	3.9 ± 0.7[2]	2.8–5.6	4.5 ± 1.0	2.8–7.5	4.0 ± 0.7[2]	2.8–5.9
Serum LDL, mmol/L	2.6 ± 0.9	1.4–4.9	2.1 ± 0.6	1.2–3.3	1.9 ± 0.7[2]	1.0–3.3	2.0 ± 0.6[2]	0.9–3.2	2.3 ± 0.9	1.0–4.9	1.9 ± 0.6[2]	0.9–3.3
Serum HDL, mmol/L	1.8 ± 0.5	0.8–2.9	1.8 ± 0.5	1.2–3.1	1.8 ± 0.3	1.3–2.6	1.6 ± 0.4[2]	0.9–2.4	1.8 ± 0.4[4]	0.8–3.1	1.6 ± 0.4[2]	0.9–2.6
Serum triglycerides, mmol/L	0.80 ± 0.28	0.43–2.00	0.71 ± 0.12	0.50–0.98	0.81 ± 0.35	0.34–1.70	0.84 ± 0.27	0.36–1.60	0.79 ± 0.29	0.34–2.00	0.83 ± 0.30	0.34–1.70
Hemoglobin, g/L	145 ± 13	127–175	138 ± 22	81–168	140 ± 14	112–172	141 ± 13	119–166	142 ± 15	81–175	141 ± 13	112–172
Creatinine, μmol/L	83 ± 11	61–111	72 ± 8[2]	57–86	73 ± 11[2]	57–104	73 ± 11[2]	53–97	78 ± 12[5]	57–111	73 ± 11[2]	53–104
Folate, nmol/L	21 ± 5	14–35	23 ± 9	11–42	25 ± 7[2]	13–39	27 ± 8[2]	10–45	22 ± 7[4]	11–42	26 ± 8[2]	10–45
Vitamin B-12, pmol/L	343 ± 109	170–710	303 ± 94	150–500	241 ± 99[2]	110–480	293 ± 161[3]	110–930	304 ± 111	110–710	274 ± 243[2]	110–930
Glucose, mmol/L	5.1 ± 0.4	4.1–6.2	5.2 ± 0.4	4.5–5.7	5.0 ± 0.4	4.2–5.9	5.0 ± 0.4	4.1–6.3	5.1 ± 0.4	4.1–6.2	5.0 ± 0.4	4.1–6.3
C-reactive protein, mg/L	0.9 ± 1.6	0–6	0.5 ± 1.3	0–4	0.5 ± 1.2	0–5	0.8 ± 2.5	0–15	0.7 ± 1.4	0–6	0.7 ± 2.1	0–15
Moderate physical activity <30 min/wk, %	0	—	0	—	4	—	2	—	1	—	3	—
Moderate physical activity >2.5 h/wk, %	68	—	31	—	58	—	61	—	65	—	60	—
Intense physical exercise <30 min/wk, %	8	—	39	—	17	—	30	—	16	—	24	—
Intense physical exercise >2 h/wk, %	63	—	31	—	50	—	47	—	53	—	48	—
Taking food supplements, %	53	—	62	—	50	—	95	—	52[6]	—	79[7]	—

[1] Values are means ± SDs and ranges unless otherwise indicated. Nonvegan includes omnivores, vegetarians, and vegetarians adding fish; nonmeat includes vegans and vegetarians. One-factor ANOVA and Tukey's post hoc test were performed between the 4 dietary groups. Nonnormally distributed data were log transformed before testing (vitamin B-12, BMI).
[2] Significantly different ($P < 0.02$) from omnivore.
[3] Significantly different ($P < 0.05$) from omnivore.
[4] Significantly different ($P < 0.02$) from vegan.
[5] Significantly different ($P < 0.05$) from vegan.
[6] Pearson's chi-square test $P < 0.02$ compared with vegan.
[7] Pearson's chi-square test $P < 0.02$ compared with omnivore.

reported a slightly, but not significantly, higher physical activity level than others; ~63% reported >2 h/wk of intense physical activity compared with 48% in the nonmeat group. The reasons for adhering to a vegan or vegetarian diet were related to ethical, environmental, and health aspects in all cases but 2, where cultural reasons were cited.

Four-day dietary records collected on the days preceding the blood sampling were used to assess intake of red meat, processed meat, poultry, fish and shellfish, eggs, and dairy products. All omnivores reported consuming meat in the FFQ and only 1 did not consume red meat according to the 4-d weighed dietary record. In addition, 75% reported consuming processed meat, 63% reported consuming poultry, and 50% reported consuming fish or shellfish once or more during the 4 d. All individuals reported consuming dairy products and all except 2 individuals consumed them on all 4 d. Among the vegetarians and vegetarians adding fish, ~60% reported consuming dairy products daily but 6 individuals reported not consuming dairy products at all. Five of these 6 individuals reported also not consuming any eggs during the 4 d. Eight of the 13 vegetarians adding fish reported consuming fish during the 4 d.

Dietary effects on serum metabolomics

In a PCA model including all dietary groups ($n = 120$), the fourth-largest variation in the data [5.3% of the explained variation (R^2X)] was related to habitual diet (**Figure 2**). The third-largest variation [6.6% of the explained variation (R^2X)] was related to gender (**Supplemental Figure 1**). Other known factors such as age, BMI, triglyceride (TG), HDL, and Hb did not show clustering patterns or trends in the PCA model. An OPLS model ($n = 120$) including age, length, weight, BMI, fat mass percentage, TG, HDL, glucose, Hb, and omnivore index is shown in **Figure 3**A, B. The permutation test was regarded as good for all included *y*-variables except for age. The model clearly separated by factors related to gender, i.e., it was driven by higher length, weight, and Hb for men and higher fat mass percentage for women in the first component [8.9% of the explained variation (R^2X)]. The third component [3.6% of the explained variation (R^2X)] was separated by fat mass percentage, TG, and glucose compared with HDL, i.e., factors related to health status. ANOVA testing of cross-validated predictive residuals resulted in length ($P = 3.4{*}10^{-8}$), weight ($P = 2.3{*}10^{-6}$), TG ($P = 8.3{*}10^{-4}$), HDL ($P = 0.025$), Hb ($P = 1.4{*}10^{-6}$), and omnivore index ($P = 2.6{*}10^{-14}$) being significant. BMI was nonsignificant, even when excluding related covariates.

OPLS-DA models were used to investigate discrimination between dietary groups and to identify variables responsible for class separation (**Figures 4** and **5**). Statistics for the final OPLS-DA models, including models with only women, are presented in **Table 2** and misclassifications for the main models in **Table 3**. It was possible to correctly classify 97.5% of the samples in the meat compared with nonmeat model and 92.5% of the samples in the vegan compared with nonvegan model (Table 3, Figures 4A and 5A).

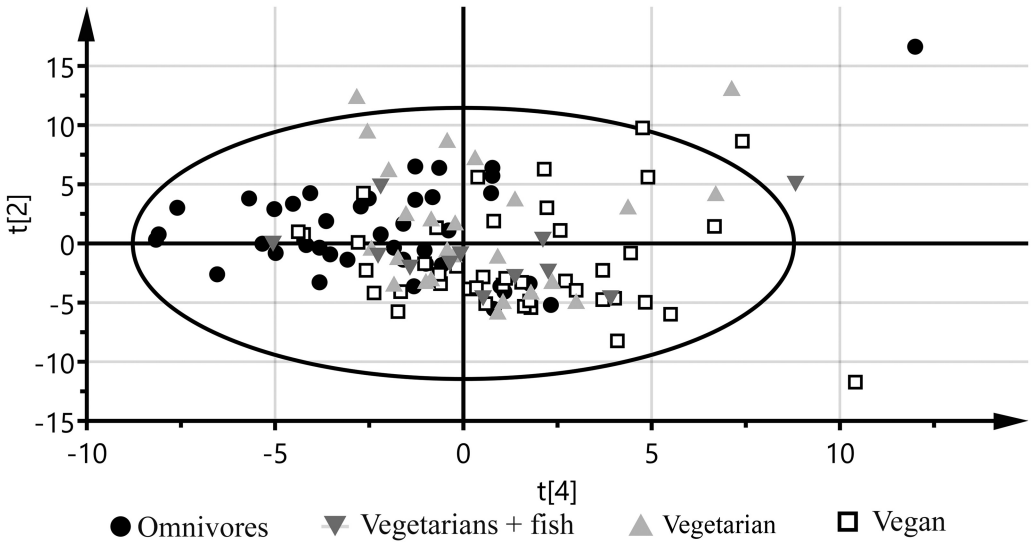

FIGURE 2 Principal component analysis model ($n = 120$) for component 4, showing the impact of habitual diet in the model.

The 3 misclassified nonmeat individuals in Figure 4A, B, in both the FFQ and the 4-d weighed dietary record, reported a relatively high intake of eggs and dairy compared with other individuals classified as nonmeat consumers. One meat-consuming individual was misclassified in the same model. This individual was an omnivore, but reported low meat intake in the FFQ and no meat or fish intake during the 4 d before sample collection.

Figures 4C and 5C display class separation between meat and nonmeat and between vegan and nonvegan consumers, respectively, in women alone. These models, built on only women participants' data, were useful in predicting the metabolic fingerprint of men; see Figures 4D and 5D. Classifying men in the female meat compared with the nonmeat model (Figure 4C) worked well with 1 exception (Figure 4D). Classifying men in the female vegan compared with the nonvegan model (Figure 5C) resulted in several misclassifications (Figure 5D). Classifying the subjects according to diet using only the last day before sampling from their 4-d dietary record did not improve the models (data not presented). Excluding the 4 omnivores who reported only

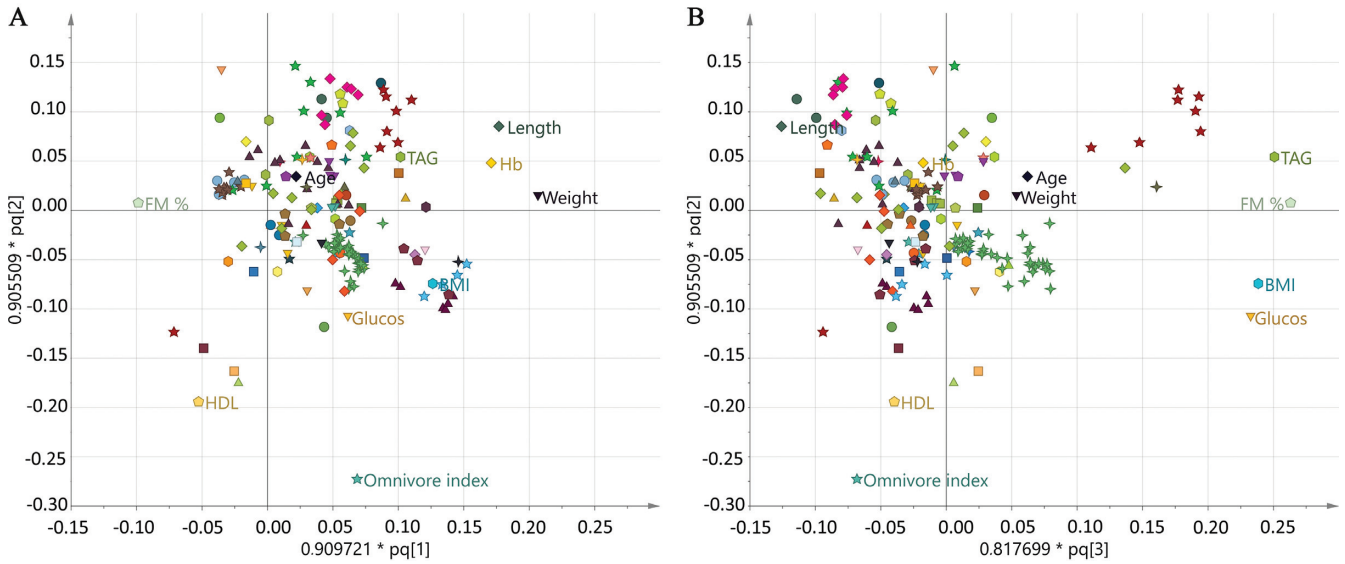

FIGURE 3 Orthogonal projections to latent structures model ($n = 120$) describing the relation between known metadata and metabolites. Included metadata are age, length, weight, BMI, FM percentage, TGs, HDL, glucose, Hb, and omnivore index. (A) The first component is shown separated by factors related to gender, i.e., driven by higher length, weight, and Hb for men and higher fat mass percentage for women. Identities for a selection of metabolites are as follows: light blue circles are citrate, brown 5-pointed stars are 3-hydroxybutyrate, pink diamonds are glutamine, green 5-pointed stars are glutamine + an unidentified metabolite, brown boxes are ornithine and tyrosine, green 4-pointed stars are glucose, dark red 5-pointed stars are unidentified lipids or free fatty acids, light blue 5-pointed stars are isoleucine, black inverted triangles are valine, and brown pentagons are leucine. (B) The third component is shown separated by factors related to health status such as fat mass percentage, TG, and glucose and on the other hand HDL. FM, fat mass; Hb, hemoglobin; TG, triglyceride.

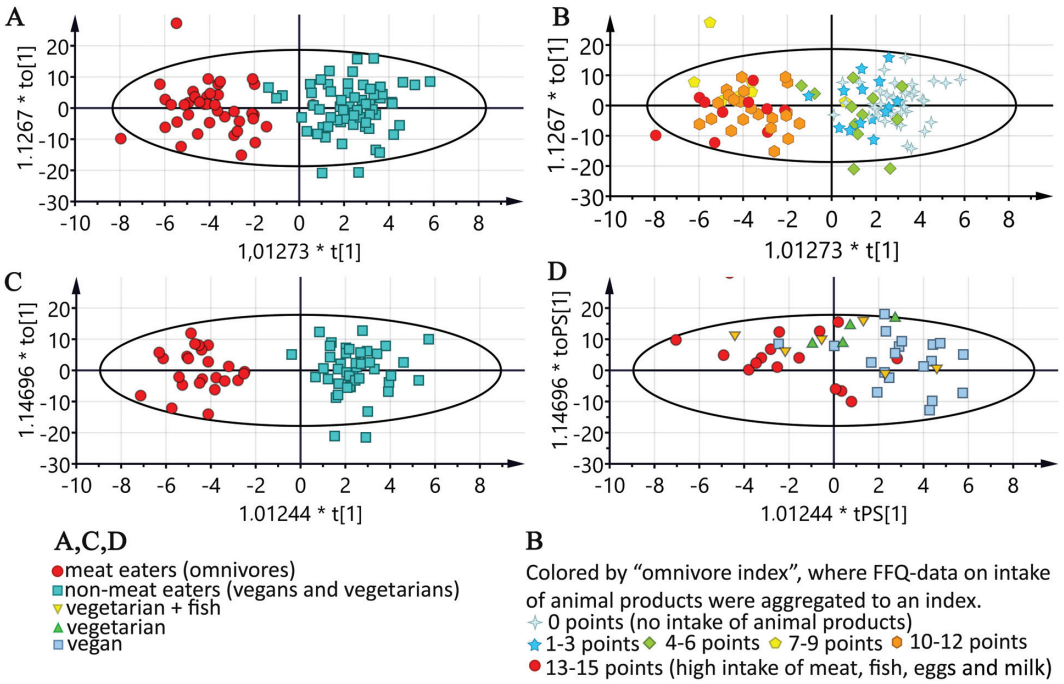

FIGURE 4 Meat-eaters compared with non–meat-eaters in orthogonal projections to latent structures with discriminant analysis models. (A) $n = 107$ (40/67), colored by group; (B) $n = 107$ (40/67), colored by omnivore index; (C) model built on only women's data, $n = 68$ (24/44), colored by group; (D) men predicted in the women's model in panel C, $n = 39$ (16/23), colored by habitual diet. FFQ, food-frequency questionnaire.

≤2 d consuming red meat, processed meat, and poultry during these 4 d improved the meat compared with the nonmeat model slightly ($n = 103$, cumulative R2X = 0.418, cumulative R2Y = 0.822, cumulative Q2 = 0.622). Excluding the vegetarians that could be classified as vegans from the 4-d dietary records did not improve the vegan compared with the nonvegan model ($n = 115$, cumulative R2X = 0.363, cumulative R2Y = 0.667, cumulative Q2 = 0.443).

Discriminating metabolites

Metabolites that contributed to the separation in OPLS-DA models are presented in **Table 4**. In NMR analysis, 1 metabolite often generates several peaks. Thus, several of the unidentified peaks may have arisen from the same metabolite or any of the identified ones.

A number of selected discriminant metabolites in the 2 main models (meat compared with nonmeat and vegan compared with nonvegan) were identical (Table 4). This was true for the branched-chain amino acids (BCAAs) isoleucine and valine, which were higher in meat-eaters and nonvegans than in non–meat-eaters and vegans. A peak that likely represents 3-hydroxyisobutyrate, an intermediate in valine metabolism, followed the same pattern. Lysine and creatine were also higher in meat-eaters and nonvegans. The meat compared with nonmeat model differed from the vegan compared with nonvegan model in that creatinine was among the discriminating metabolites and that glutamine and leucine discriminated only in the vegan compared with nonvegan model. Creatinine was significantly higher among meat-eaters than among non–meat-eaters, glutamine was higher among vegans than among nonvegans, and leucine was higher among nonvegans than among vegans. Glycine, glutamine (in a variable overlapping with an unknown metabolite), trimethylamine (TMA), and 2-aminobutyrate (a tentative identification not supported by 2D data) were higher among non–meat-eaters and vegans than among meat-eaters and nonvegans, respectively. In addition, 1 unidentified peak differed significantly in both models. When analyzing models including only women, the pattern was similar as for the mixed-gender models. However, leucine and proline/taurine also were discriminant for meat-eaters compared with non–meat-eaters in the women-only model. In this women-only model, comparing vegans and nonvegans, TMA and leucine/isoleucine were not discriminating but they did discriminate in the mixed-gender model. In both models including only women, there was 1 additional lipid peak that was higher in non–meat-eaters and vegans than among meat-eaters and nonvegans, respectively. There was also 1 additional unidentified peak for the vegan compared with nonvegan model.

Gender metabolic differences were investigated in a separate OPLS-DA model (Table 2). Comparing all men and women, BCAAs, creatinine, and TMA were discriminating and higher among men. Interestingly, creatine was also significant and in higher concentrations in women. Also glutamine, lysine, and several lipid peaks discriminated between men and women (data not shown).

When only including the discriminating metabolites in Table 4 in the OPLS-DA models, the ability to predict into the correct category was decreased (data not shown), indicating that a metabolomics approach including many metabolites improves the predictability compared with using a few selected ones.

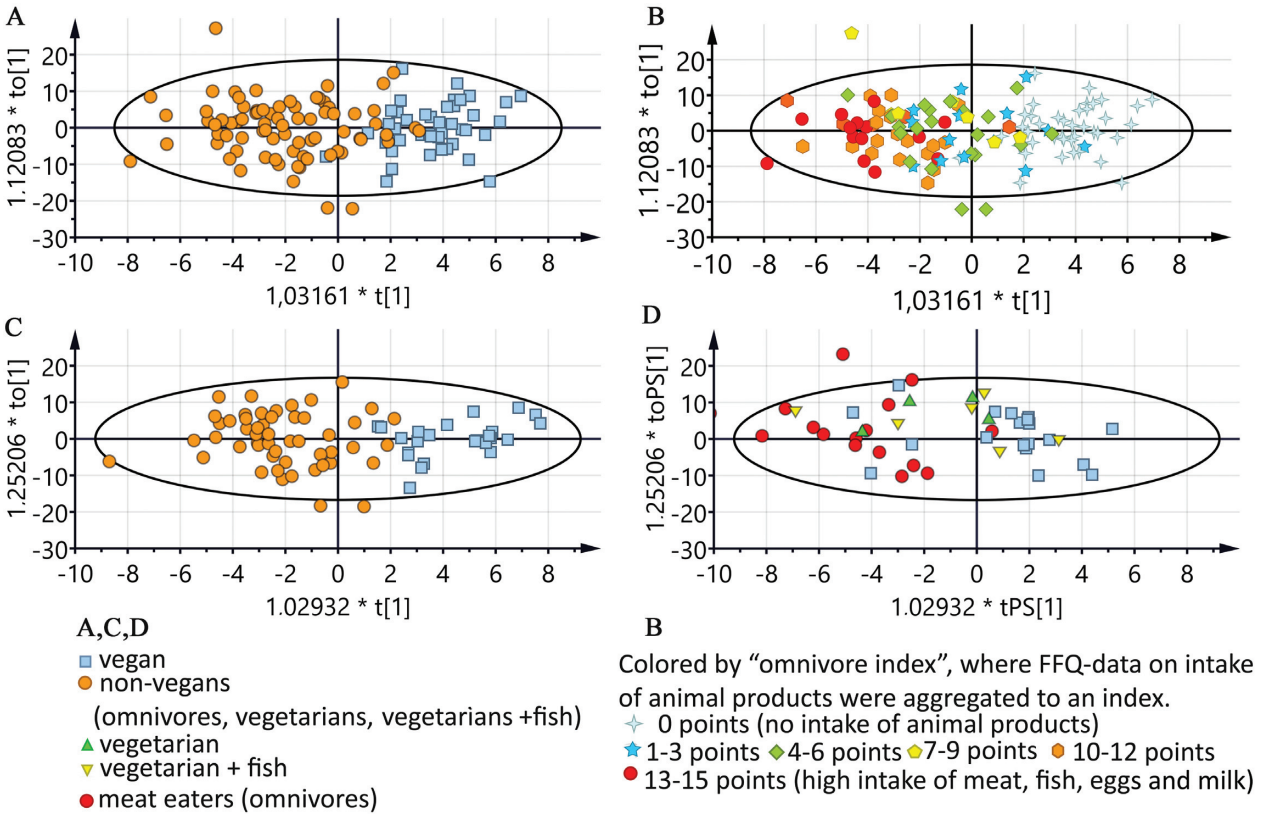

FIGURE 5 Vegan compared with nonvegan (omnivores, vegetarians, and vegetarians adding fish) eaters in orthogonal projections to latent structures with discriminant analysis models. (A) $n = 120$ (43/77), colored by group; (B) $n = 120$ (43/77), colored by omnivore index; (C) model built on only women's data, $n = 75$ (24/51), colored by group; (D) men predicted in the women's model in panel C, $n = 45$ (19/26), colored by habitual diet.

Discussion

This study shows that NMR metabolomics of fasting serum may assess habitual intake of foods of animal origin. Although the OPLS-DA models constitute the main results, the most discriminating metabolites are discussed in the following section to verify the models' biological plausibility.

Discriminating patterns between diets have previously been shown in both serum and urine (5, 6). Blood is highly controlled by homeostasis and comprises both endogenous and exogenous metabolites alongside inorganic salts and lipids, lipoproteins, and proteins, whereas urine is constituted of inorganic salts together with water-soluble waste products. Thus, we choose to discuss our findings only in relation to other findings in serum.

The most important metabolites that differed in concentration in omnivores, vegetarians, and vegans were BCAAs such

TABLE 2 Model statistics[1]

Model	LVs (*n*)	*n*	R2X	R2Y	Q2	CV-ANOVA (*P* value)	ROC AUC	Permutation test[2] (Q2)
PCA	7	120	0.578	—	0.398	—	—	—
OPLS	3 + 3 + 0	120	0.511	0.394	0.246	—	—	—
OPLS-DA meat vs. nonmeat all	1 + 3 + 0	107 (40/67)	0.411	0.805	0.583	1.1×10^{-15}	1.00/1.00	− 0.578
OPLS-DA meat vs. nonmeat women	1 + 3 + 0	68 (24/44)	0.408	0.889	0.576	1.1×10^{-08}	0.98/0.98	− 0.651
OPLS-DA vegan vs. nonvegan all	1 + 2 + 0	120 (43/77)	0.365	0.631	0.349	6.2×10^{-09}	0.98/0.98	− 0.433
OPLS-DA vegan vs. nonvegan women	1 + 2 + 0	75 (24/51)	0.362	0.704	0.330	9.5×10^{-05}	0.92/0.92	− 0.505
OPLS-DA men vs. women	1 + 2 + 0	120 (45/75)	0.360	0.708	0.559	4.2×10^{-18}	0.99/0.99	− 0.441

[1]Meat includes omnivores; nonvegan includes omnivores, vegetarians, and vegetarians adding fish; nonmeat includes vegans and vegetarians. PCA, Principal component analysis; OPLS, Orthogonal Projections to Latent Structures; CV-ANOVA, ANOVA testing of cross-validated predictive residuals; LV, latent variable; Q2, cumulative fraction of the sum of squares of Y predicted by the selected latent variables, estimated by cross-validation; ROC, receiver operating curve; R2X, cumulative fraction of the sum of squares of X explained by the selected latent variables; R2Y, cumulative fraction of the sum of squares of Y explained by the selected latent variables.

[2]The intercept between real and random models, degree of overfit.

TABLE 3 Classification of samples in the orthogonal projections to latent structures with discriminant analysis model

	Classification (*n*)			
True intake	Meat (*n* = 40)	Nonmeat (*n* = 67)	Vegan (*n* = 43)	Nonvegan (*n* = 77)
Meat	39 (98%)	1 (2%)	—	—
Nonmeat	2 (3%)	65 (97%)	—	—
Vegan	—	—	41 (95%)	2 (5%)
Nonvegan	—	—	8 (10%)	69 (90%)

as leucine, isoleucine, and valine: all were higher in meat-eaters and nonvegans than in non–meat-eaters/vegans. BCAA concentrations in serum depend on dietary intake (12). However, factors such as gender, fat-free mass, BMI, and changes in BMI after weight reduction have also been reported to influence BCAA concentrations (13–17). Because BCAAs are derived from protein degradation in muscle tissue in the fasted state, BCAA concentration in fasting serum correlates with fat-free mass. In fact, leucine oxidation is paralleled by plasma leucine concentrations in the fed state (18). BCAAs are in addition regulated by insulin and glucagon. BCAA concentrations increase with enhanced protein degradation but are also influenced by dietary intake. Thus, BCAAs are not reliable biomarkers for either muscle mass or dietary intake. However, our results imply that valine and isoleucine could be used as dietary markers if combined with other markers of meat or animal products.

In our subjects, creatine concentrations in fasting serum samples were higher in meat consumers than in vegans and vegetarians also after adjustments, which is in line with previous findings (19). We also confirm the dimorphisms for creatine being higher in women and creatinine being higher in men (15). The dietary source of creatine is muscle meat, including fish, and only small amounts are found in dairy products. Thus, vegetarians and vegans must synthesize creatine by de novo biosynthesis from glycine, methionine, and arginine (20). It has been shown that the muscle content of creatine is lower in muscle tissue in vegetarians than in omnivores, indicating that the dietary intake increases tissue content above the de novo synthesis (21), which our study confirms. In addition, the concentrations of other metabolites are influenced by dietary creatine intake; 5 d supplementation with creatine in healthy young men and women raised plasma concentrations of creatine, citrulline, valine, and lysine, and decreased glycine, glutamine, and taurine (22). This is similar to our findings with lower glycine and glutamine and higher valine content in serum in meat consumers than in non–meat consumers and in nonvegans than in vegans. In addition, our results are consistent with the findings in the metabolomics study by Schmidt et al. (3), which reported higher serum concentrations of glycine in vegans, but also other studies have reported an inverse relation between red meat intake and the glycine concentration in biofluids (5, 23). Glycine has been related to insulin resistance and oxidative stress by its essential role in gluconeogenesis and the formation of glutathione (24) and has been independently associated with decreased diabetes risk (23).

TMA was higher in non–meat-eaters. The amino acid choline can be converted to TMA, hence foods rich in choline, e.g., beef, chicken, egg, and soy, are expected to increase the concentration of TMA. However, significantly higher postprandial concentrations of TMA in urine have only been confirmed after seafood intake (25). About 80% of TMA N-oxide present in seafood is estimated to be converted to TMA by the gut microbiota (26). TMA conversion from choline and carnitine is estimated to be lower (26). Accordingly, higher TMA in omnivores, and especially in individuals with high fish consumption, was expected. However, this was not the case and might be explained by the microflora, as responsible for the conversion to TMA, or by the variable rate of conversion of TMA to TMA N-oxide that might differ in subjects with high or low intake of TMA precursors. A difference in gut microflora was discussed as one explanation for the finding that some individuals had more than 4 times higher TMA N-oxide concentration in plasma compared to others, when investigating choline uptake and conversion to TMA from eggs (27). To our knowledge there are no studies to show that TMA in serum is a good marker for habitual meat or even fish intake.

Several weaknesses of our study should be noted. In this study, men and women were not evenly distributed within dietary groups. The concentrations of many metabolites differ by gender; thus, the larger group of women was also analyzed separately to ensure that differences between groups were not due to this gender skewness. In addition, *P* values for metabolites driving the separation in OPLS-DA models were adjusted for age, gender, BMI, and body fat mass percentage in a logistic regression analysis (Table 4). The level of physical activity has also been shown to influence the metabolome (28, 29). Further, omnivores reported an overall higher level of physical activity (although not significantly) and this may have affected our metabolomics results. In addition, fat mass percentages and vitamin B-12, cholesterol, folate, and LDL concentrations were other factors that were not evenly distributed between groups and therefore may have affected the present results. Still, these factors likely were influenced by the diet and thus should be regarded as concomitant outcomes rather than as confounders. Finally, the study population comprised mainly young and healthy individuals with a high level of physical activity and this might limit the generalizability.

Our study has several important strengths. Fasting serum samples were used and these were rigorously handled following a strict protocol, resulting in high-quality NMR data. In the study by Schmidt et al. (3), nonfasting samples were collected at the participants' local general practitioners, resulting in significantly different times from last food or drink to collection and times from collection to processing. They reported that almost all amino acids were influenced by the sample collection procedure. This emphasizes the importance of sample handling in metabolomics research. Our subjective dietary data included both food-frequencies data and 4-d weighed dietary records. This aided us in interpreting the serum data and made it possible to explain misclassifications in the multivariate models (6).

To conclude, ^{1}H-NMR serum metabolomics appears to be a possible objective tool to identify and predict habitual intake of meat and other animal products in healthy subjects adhering to a vegan, vegetarian, or omnivore diet. Discriminating patterns reflecting the intake of meat and other products of animal origin were identified. However, a number of metabolites identified in increased concentration for the different diets

TABLE 4 Differences in metabolites between meat-eaters and non–meat-eaters and between vegans and nonvegans[1]

Metabolite	[1]H chemical shift region	Meat vs. nonmeat						Vegan vs. nonvegan						ANOVA all significance[2]
		All (n = 107) (40/67)			Women (n = 68) (24/44)			All (n = 120) (43/77)			Women (n = 75) (24/51)			
		M	NM	P	M	NM	P	NV	V	P	NV	V	P	
(2-aminobutyrate)	0.88	↑	↓	<0.00001[3,4]	↑	↓	0.0011	↑	↓	<0.00001[3]	↑	↓	0.00026[3,4]	V vs. all
(3-hydroxyisobutyrate)	0.98	↑	↓	0.000018[3,4]	↑	↓	0.00097	↑	↓	0.000025[3,4]	↑	↓	0.00059	V vs. O and Veg + F
Creatine	3.84	↑	↓	<0.00001[3]	↑	↓	<0.00001[3,4]	↑	↓	0.00021[3,4]	↑	↓	0.0018	O vs. all
Creatine + lysine	2.94	↑	↓	<0.00001[3]	↑	↓	<0.00001[3,4]	↑	↓	<0.00001[3]	↑	↓	0.00014[3]	O vs. all
Creatinine	3.96	↑	↓	0.000033[3,4]	↑	↓	0.000071[3,4]	—	—	—	↑	↓	0.0044	O vs. V and Veg
Glutamine	2.37	—	—	—	—	—	—	↓	↑	0.0065	—	—	—	—
Glutamine[5]	2.38	↓	↑	0.041	↓	↑	0.095	↓	↑	0.0056	↓	↑	0.02	V vs. O
Glutamine[5]	2.04	↓	↑	0.000013[3]	—	—	—	—	—	—	—	—	—	V vs. O and Veg
Glycine	3.46	↓	↑	<0.00001[3]	↓	↑	<0.00001[3]	↓	↑	0.00022[3,4]	↓	↑	0.0098	O vs. all
Isoleucine	0.83	↑	↓	0.000011[3,4]	↑	↓	0.0037	↑	↓	0.000015[3]	↑	↓	0.00049[3,4]	O vs. V
Leucine	0.87	—	—	<0.00001[3]	↑	↓	0.000082[3,4]	↑	↓	<0.00001[3]	↑	↓	0.000018[3]	O vs. V and Veg
Leucine + isoleucine	0.85	↑	↓	0.000042[3,4]	↑	↓	0.00045[3,4]	↑	↓	0.016	—	—	—	O vs. V and Veg
Lysine + arginine	1.79	↑	↓	0.017	↑	↓	0.000079[3,4]	↑	↓	<0.00001[3]	↑	↓	0.00044[3,4]	V vs. all
Proline + taurine	3.33	—	—	—	↑	↓	0.0003[3,4]	—	—	—	—	—	—	V vs. O and Veg + F
Trimethylamine	2.80	↓	↑	0.00015[3]	↓	↑	0.00045[3,4]	↓	↑	0.017	—	—	—	O vs. V and Veg
Valine	0.94	↑	↓	<0.00001[3]	↑	↓	0.00036[3,4]	↑	↓	<0.00001[3]	↑	↓	0.00062	O vs. V and Veg
Lipids	0.79	—	—	—	↓	↑	0.0058	—	—	—	↓	↑	0.0094	—
Unknown	3.26	—	—	—	—	—	—	—	—	—	↑	↓	0.0016	—
Unknown	0.99	↓	↑	0.00032[3,4]	↓	↑	0.0037	↓	↑	0.000083[3]	↓	↑	0.0007	V vs. O and Veg + F

[1]Chemical shift region for the peak used for t tests. Shown are discriminating metabolites that have a loading score pq > ± 0.1. P for Student's t test is presented for all discriminating metabolites. F, fish; M, meat (omnivores); NM, nonmeat (vegans and vegetarians); NV, nonvegan (omnivores, vegetarians, and vegetarians + fish); O, omnivore; V, vegan; Veg, vegetarian.

[2]ANOVA analysis between omnivores, vegetarians + fish, vegetarians, and vegans, with Tukey's post hoc test.

[3]Significant Student's t test after Bonferroni correction ($P < 0.0005$).

[4]Nonsignificant in a logistic regression model when adjusted for age, gender, BMI, and fat mass percentage, Bonferroni corrected ($P < 0.0005$).

[5]In a variable overlapping with an unidentified metabolite.

were also associated with gender, age, BMI, and body composition. Accordingly, metabolic patterns in relation to diet alone must be confirmed in intervention studies, controlling for individual factors that could potentially influence metabolite concentrations.

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