



# Stable isotope ratios of nitrogen and carbon as biomarkers of a vegan diet

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## Abstract

**Purpose** Dietary biomarkers can potentially overcome the limitations of self-reported dietary data. While in ecology and archaeology, stable isotope ratios of carbon and nitrogen are widely used as biomarkers, this is not the case in nutrition research. Since the abundance of the  $^{13}\text{C}$  and the  $^{15}\text{N}$  isotope differ in food sources from plant and animal origin, stable isotope ratios of carbon and nitrogen ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) may differ in human biological material. Here, we investigated the stable isotope ratios of nitrogen and carbon in serum and urine from vegans and omnivores.

**Method** Measurement of  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  in serum and 24 h urine was performed by Elemental Analyzer–Isotope Ratio Mass Spectrometer in the cross-sectional study “Risks and Benefits of a Vegan Diet”. The study included 36 vegans and 36 omnivores with a median age of 37.5 years (matched for age and sex), who adhered to their diet for at least 1 year.

**Results** Both  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  were significantly lower in both the serum and 24 h urine of vegans compared to omnivores.  $\delta^{15}\text{N}$  either in serum or urine had 100% specificity and sensitivity to discriminate between vegans and omnivores. Specificity of  $\delta^{13}\text{C}$  was also > 90%, while sensitivity was 93% in serum and 77% in urine.

**Conclusion**  $\delta^{15}\text{N}$  both in serum and urine was able to accurately identify vegans and thus appears to be a promising marker for dietary habits.

**Keywords**  $\delta^{15}\text{N}$  ·  $\delta^{13}\text{C}$  · Stable isotope ratios · Vegan diet · Biomarkers · Dietary intake data · Nitrogen isotopes

## Introduction

Dietary intake is widely recognized as one of the most important lifestyle factors that influence both human health and planetary health. Meat intake in particular has been linked to non-communicable diseases, and its production has implications for land and water use, as well as greenhouse gas production [1]. Diets that exclude meat, or more radically all animal products, have attracted increasing attention in the Western world.

Even though the importance of diet in relation to health outcomes has been identified, challenges concerning the validity and reliability of dietary intake data continue to undermine research in this field. Methods for assessing dietary intake typically involve self-report and rely on memory recall and objectivity. Dietary intake methods based on self-reported data are prone to be influenced by factors such as the social desirability of foods, lack of memory or lack of consciousness that a food item(s) have been consumed, and lack of ability to estimate portion sizes or amounts of foods

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consumed [2]. Thus, there is a need for a more objective assessment of dietary intake, and enormous progress has been made during the last decade concerning the use of food-specific biomarkers [3, 4].

Stable isotope ratios are among the biomarkers that have been investigated as indicators of meat and fish [5], and added sugar intake [6]. Stable isotopes are atoms of the same element that differ in the number of neutrons in the nucleus and thus differ in their atomic mass. In nature, each element occurs as a mixture of its isotopic forms, but metabolic rates in plants and animals are usually different for certain isotopes, resulting in small differences in the permille range. The isotope distribution in samples is usually expressed relative to the distribution of universal standard material, which is usually limestone (V-PDB) for carbon and nitrogen in air for nitrogen [7]. The ratio of naturally occurring stable isotopes of carbon ( $^{13}\text{C}/^{12}\text{C}$  ratio expressed as  $\delta^{13}\text{C}$ , sometimes also called CIR) and nitrogen ( $^{15}\text{N}/^{14}\text{N}$ ) ratio expressed as  $\delta^{15}\text{N}$ , also called NIR) have been used extensively in archaeological and ecological studies, and their use in dietary assessment studies is increasing [5, 7–10].

In biology, stable isotopes have been also used to characterize trophic positions in the food web. Due to greater retention of the heavier  $^{15}\text{N}$  isotope than the lighter  $^{14}\text{N}$  isotope in the production of nitrogenous waste, the nitrogen ratio of  $^{15}\text{N}$  to  $^{14}\text{N}$  ( $\delta^{15}\text{N}$ ) shows a stepwise enrichment from food producers to food consumers and is therefore indicative of relative trophic position [7, 11, 12]. Thus,  $\delta^{15}\text{N}$  can differentiate between trophic levels, as the relative abundance of the heavy nitrogen isotope, increases by approximately 2–4‰ per increasing trophic levels in the food web [7]. Although the trophic level or position is a well-known concept within biology, it has not been used in the field of human nutrition science. Based on the trophic model, humans who consume omnivorous diets would accordingly be seen as ‘higher predators’, while vegans would be on a lower trophic level as they only consume plant-based food. Vegetarians, who do not consume meat, but milk and dairy, eggs and honey, would be between vegans and omnivores.

While the  $\delta^{15}\text{N}$  ratio in a food web reflects the trophic position, differences in the  $\delta^{13}\text{C}$  are more dependent on the type of plants consumed. C3 plants (the majority of food plants such as wheat, rice, or beans) to a greater degree than C4 plants utilize the  $^{12}\text{C}$  rather than  $^{13}\text{C}$  in the photosynthesis when trapping/converting C from  $\text{CO}_2$  reflected by a lower  $\delta^{13}\text{C}$  value. C4 plants, among them sugar cane, corn and sorghum, have  $\delta^{13}\text{C}$  values approximately 12–13‰ higher than C3 plants. This difference can be used to measure the consumption of added sugar made from either sugar cane or corn while added sugar produced from sugar beet (C3 plant) would not show any difference in the  $\delta^{13}\text{C}$  [6, 13, 14]. However, carbon atoms in the diet are derived from all macronutrients and are thus more difficult to interpret.

Indeed, the feed of husbandry animals like pork is mainly based on corn, which would reflect the  $\delta^{13}\text{C}$  of C4 plants. Indeed,  $\delta^{13}\text{C}$  has recently been suggested as a marker for animal protein intake [15].

Stable isotope ratios as dietary biomarkers can be measured in different tissues or body fluids, including skin [16], urine [5, 17], fingernails [18], exhaled air [13, 19], hair [8, 20], blood [6] and serum [5, 21]. Stable isotope ratios in these biological specimens may reflect different time periods and varying nutrient turnover rates [7].

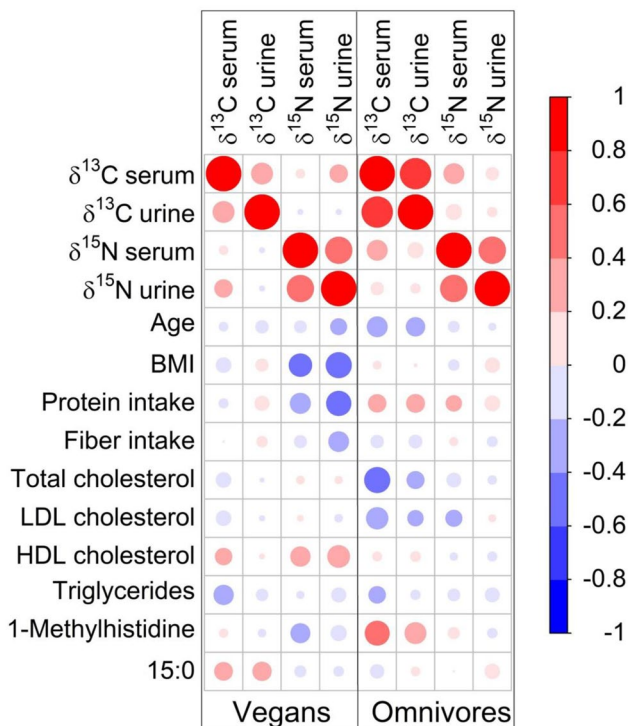
Here, we investigate in an exploratory manner whether the stable isotope ratios of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  in serum and 24 h urine can distinguish between healthy vegans and omnivores in a cross-sectional study. In addition, we investigate the discriminative power of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  in comparison to two other dietary biomarkers for dairy and meat intake, plasma pentadecanoic acid (15:0) and plasma 1-methylhistidine [3].

## Subjects and methods

This is a cross-sectional study investigating the nutritional status of vegans, compared to omnivores. The recruitment process for healthy volunteers, aged 30–60 years, is described elsewhere [22]. Briefly, the 36 vegans and 36 omnivores of the study “Risks and Benefits of a Vegan Diet” (RBVD) were recruited in Berlin (Germany) at the German Federal Institute for Risk Assessment (BfR) in the period from January to July 2017, matched for age and sex. The sample size for this study is based on the power calculation for the primary research question (bone health in vegans compared to omnivores) and due to the exploratory nature of the current analysis, a sample size calculation is not provided here [23]. The study was approved by the Ethics Committee of Charité—Universitätsmedizin Berlin (no. EA4/121/16). A flow chart of the study process is shown in Supplemental Fig. 1. Written informed consent was obtained from all participants during the first visit.

As an inclusion criterion, vegans should follow the vegan diet for at least a year, and omnivores should consume at least three times meat or two times meat and two times processed meat per week. Dietary intakes were recorded using three-day weighed food protocols. With the help of the German Nutrient Database (BLS, Bundeslebensmittelschlüssel) Version 3.02, the mean daily intake of food items, macronutrients and micronutrients was calculated. Information about age, educational attainment, and lifestyle factors were collected using tablet-based questionnaires. Height and body weight, waist circumference, and blood pressure were measured using standardized methods.

From all participants in the study, 60 mL of blood was obtained. Blood lipids and creatinine were measured in a certified routine laboratory (Labor 28 GmbH, Berlin,



**Fig. 1** Spearman correlation matrix for stable isotope ratios ( $\delta^{15}\text{N}$ ,  $\delta^{13}\text{C}$ ) in urine and serum with age, BMI, dietary intake and serum or plasma variables (lipids, 15:0 and 1-methylhistidine) in vegans ( $n=36$ ) and omnivores ( $n=36$ ).  $\delta^{15}\text{N}$  nitrogen stable isotope ratio,  $\delta^{13}\text{C}$  carbon stable isotope ratio, 15:0 pentadecanoic acid;

Germany) by standard methods on the same day of blood collection. 24 h urine was collected by the participants, and urine creatinine concentrations were determined also determined on the day of the visit to the study center. All other biochemical analyses were performed on samples stored at a temperature of  $-80\text{ }^{\circ}\text{C}$ . Urine was collected on the days prior to the visit to the study center and were done from Sunday to Thursday.

### Stable isotope ratio and biomarker assessment

Stable isotope ratios  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  were measured at the Stable Isotope Laboratory at the University of Oslo (UIO:CLIPT), using a method described by Kraft [24]. Briefly, serum (8  $\mu\text{L}$ ) and urine (15  $\mu\text{L}$ ) were pipetted into tin capsules and air dried. The  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  were measured simultaneously using an Elemental Analyzer (EA) IsoLink Isotope Ratio Mass Spectrometer (IRMS) System, consisting of a Flash EA and a DeltaV IRMS (Thermo Scientific, Germany). The  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values were normalized to the Vienna Pee Dee Belemnite (VPDB) and AIR scales, respectively, using two different internal reference materials incorporated into each analytical run: JGLUT (L-glutamic acid;  $\delta^{13}\text{C} = -13.43\text{ }‰$ ;  $\delta^{15}\text{N} = 4.34\text{ }‰$ ) and POPPGLY

(glycine;  $\delta^{13}\text{C} = -36.58\text{ }‰$ ;  $\delta^{15}\text{N} = 11.25\text{ }‰$ ) (both from Fisher Scientific). An additional quality control material, JALA (alanine, calibrated value  $\delta^{13}\text{C} = -20.62\text{ }‰$ ; measured value ( $n=40$ )  $-20.58 \pm 0.07\text{ }‰$ ; calibrated value:  $\delta^{15}\text{N} = 3.16\text{ }‰$ , measured value ( $n=31$ )  $-3.23 \pm 0.06\text{ }‰$ ) (Fisher Scientific) was incorporated into every run.  $\delta^{13}\text{C}$  of both reference materials and quality control sample were calibrated to the VPDB scale using LSVEC (lithium carbonate,  $\delta^{13}\text{C} = -46.6\text{ }‰$ ) and NBS-19 (calcium carbonate,  $\delta^{13}\text{C} = 1.95\text{ }‰$ ) (both obtained from the International Atomic Energy Agency, Austria). The  $\delta^{15}\text{N}$  values were calibrated to the AIR scale using USGS40 (L-glutamic acid,  $\delta^{15}\text{N} = 47.57\text{ }‰$ ) and USGS41 (L-glutamic acid,  $\delta^{15}\text{N} = 47.57\text{ }‰$ ) (both obtained from the United States Geological Survey). Analytical precision was based on repeated analyses of quality assurance material JALA (Fisher Scientific).

The fatty acid pentadecanoic acid (15:0) was measured as % of all fatty acids in plasma phospholipids at the German Institute of Human Nutrition Potsdam-Rehbrücke (Germany). The method for 15:0 measurement was described previously by Weitkuna [25]. In addition, we measured 1-methylhistidine (m1His) in plasma at Bevital AS (Bergen, Norway, <http://www.bevital.no>). 1-methylhistidine was quantified using an isotope-labeled internal standard to an existing assay utilizing liquid chromatography combined with tandem mass spectrometry, as previously described [26].

### Statistics

The study was powered by a primary research question about differences in bone health between vegans and omnivores. Data were analysed exploratory to answer the research questions.

Variables were reported using mean and standard deviation (SD) for normally distributed variables, median and interquartile range (IQR) for non-normally distributed variables, and relative percentages for categorical variables. Differences between vegans and omnivores were tested using a Chi-Square test for categorical variables and a Student's *T*-test (normally distributed) or Kruskal–Wallis test (non-normally distributed) for continuous variables. Normal distribution of variables was proven using the Shapiro–Wilk test, which indicated the non-normal distribution of  $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ , 15:0 and 1-methylhistidine. Spearman correlations were calculated to investigate potential correlations between isotopes and variables of interest. To study the discrimination performance of biomarkers ( $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ , 15:0 and 1-methylhistidine) regarding the dietary group (vegan vs. omnivorous diet), receiver operating characteristic curves (ROC) were plotted using the R package ROCit with a parametric binormal approach. The

ROC curve represents a plot of sensitivity versus false-positive rate (1-specificity) of logistic regression prediction models (Diet ~ Exposure). The area under the ROC curve (AUC) represents the probability that the prediction model assigns a true vegan as vegan compared to an omnivore. The AUC may range from 0.5 indicating no discrimination to 1.0 indicating perfect discrimination. Scatter plots were used to derive cut-offs for the discrimination analysis (sensitivity and specificity).

For statistical analyses of data, SAS software (version 9.4, SAS institute, Cary, N.C., USA) and R software (version 3.6.3) was used. Even though the analyses in this report are exploratory, a *p* value of 0.05 was regarded as significant [27].

## Results

### Cohort characteristics

In total, 72 healthy volunteers, 36 vegans and 36 omnivores (each 50% men) were included. Median age was 37.5 years (min–max: 30–57), and median duration of vegan diet was almost 5 years. Main characteristics of the study sample are presented in Table 1.

### Differences of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ in serum and urine

Table 2 shows the measured  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values in serum and 24 h urine samples in both vegans and omnivores. In both groups, stable isotope ratios for carbon

**Table 1** Characteristics of the study sample

	Vegans ( <i>n</i> = 36)	Omnivores ( <i>n</i> = 36)	<i>p</i> values
Men [ <i>n</i> (%)]	18 (50)	18 (50)	1.00
Age (years)	37.5 (32.5–44.0)	38.5 (32.0–46.0)	0.75
Body weight (kg)	70.1 ( $\pm$ 13.9)	73.6 ( $\pm$ 10.3)	0.24
BMI	22.9 ( $\pm$ 3.2)	24.0 ( $\pm$ 2.1)	0.08
Creatinine serum (mg/dL)	0.82 ( $\pm$ 0.15)	0.89 ( $\pm$ 0.15)	0.046
Duration of vegan diet (years)	4.8 (3.1–8.7)	n.a	
Education [ <i>n</i> (%)]			0.60
Lower	0 (0)	1 (2.8)	
Middle	11 (30.6)	11 (30.6)	
High	25 (69.5)	24 (66.7)	
Physical activity (h/week)	2.8 (0.9–3.8)	2.3 (1.2–4.1)	0.69
Smoking behavior [ <i>n</i> (%)]			0.3
Never-smoker	24 (66.7)	21 (58.3)	
Former smoker	8 (22.2)	6 (16.7)	
Smoker	4 (11.1)	9 (25.0)	
Dietary intake			
Total energy (kcal)	2270 (1800–2762)	2386 (2081–2737)	0.32
Protein (g/d)	72 (55–92)	86 (71–107)	0.02
Fat (g/d)	86 (64–111)	104 (88–143)	0.004
Carbohydrates (g/d)	259 (212–371)	230 (199–291)	0.12
Fibre (g/d)	46 (34–58)	24 (19–30)	< 0.0001
Food intake (g/d)			
Poultry	0 (0–0)	15 (0–61)	
Red meat	0 (0–0)	18 (0–85)	
Processed meat	0 (0–0)	32 (3–77)	
Fish	0 (0–0)	0 (0–39)	
Dairy	0 (0–0)	351 (185–481)	
Egg	0 (0–0)	17 (0–40)	

Variables expressed as percentage (*n*), mean ( $\pm$  SD) or median (IQR). Differences between the diet groups were assessed using chi-square tests for categorical variables by Chi-Square test, Student's *T*-test for normally distributed continuous variables and Kruskal–Wallis test for not normally distributed continuous variables. Education categories are low (no qualifications), middle (vocational training) or high (university or other higher education degree), smoking categories are never smokers, former smokers (smoking previously but stopped) and smoker (current smoker)

**Table 2** Dietary biomarkers in vegans and omnivores

	<i>N</i>	Median (IQR)	Min	Max	<i>p</i> value
$\delta^{15}\text{N}$ urine					
Vegans	36	2.74 (2.51; 3.18)	1.76	3.99	
Omnivores	36	4.69 (4.39; 5.06)	4.04	5.67	<0.0001
$\delta^{15}\text{N}$ serum					
Vegans	36	7.68 (7.48; 7.87)	6.61	8.43	
Omnivores	36	9.49 (9.32; 9.65)	9.04	10.31	<0.0001
$\delta^{13}\text{C}$ urine					
Vegans	36	-25.38 (-25.76; -25.20)	-26.64	-24.15	
Omnivores	36	-24.34 (-24.75; -23.88)	-25.56	-21.06	<0.0001
$\delta^{13}\text{C}$ serum					
Vegans	36	-24.35 (-24.54; -24.16)	-24.88	-23.05	
Omnivores	36	-22.76 (-23.08; -22.4)	-23.77	-22.11	<0.0001
15:0 (%)					
Vegans	36	0.15 (0.13; 0.16)	0.09	0.22	
Omnivores	36	0.26 (0.22; 0.30)	0.16	0.40	<0.0001
1-methylhistidine ( $\mu\text{mol/L}$ )					
Vegans	36	0.38 (0.31; 0.46)	0.10	0.69	
Omnivores	36	3.57 (1.72; 10.25)	0.40	37.2	<0.0001

Stable isotope ratios (‰) for carbon ( $\delta^{13}\text{C}$ ) and nitrogen ( $\delta^{15}\text{N}$ ) in serum and 24 h urine in and plasma concentrations of pentadecanoic acid (15:0) and 1-methylhistidine of vegans and omnivores. The stable isotope ratios have been obtained against standard material (Vienna Pee Dee Belemnite (VPDB) for carbon and air for nitrogen, as described in Methods) which explains the negative values for  $\delta^{13}\text{C}$ .  $\delta^{15}\text{N}$  nitrogen stable isotope ratio,  $\delta^{13}\text{C}$  carbon stable isotope ratio

and nitrogen, were lower in urine compared to serum. Compared to omnivores, vegans had lower levels of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  in urine and serum, respectively. The  $\delta^{15}\text{N}$  in vegans was approximately 2 ‰ lower in urine and approximately 1.5 ‰ lower in serum than in omnivores. For  $\delta^{13}\text{C}$ , the difference between vegans and omnivores was approximately -1 ‰ in urine and -1.5 ‰ in serum.

The biomarkers 15:0 (% of total fatty acids) and 1-methylhistidine in plasma were also highly significantly different between vegans and omnivores, with higher values in omnivores compared to vegans for both biomarkers.

### Correlations of biomarkers

There were no differences between men and women for either  $\delta^{15}\text{N}$  nor  $\delta^{13}\text{C}$ . In omnivores, there was a strong correlation of  $\delta^{13}\text{C}$  in serum with  $\delta^{13}\text{C}$  in urine ( $r = 0.78$ ), which was much weaker in vegans ( $r = 0.36$ ). Correlation of  $\delta^{15}\text{N}$  in serum with  $\delta^{15}\text{N}$  in urine was, however, similar in both omnivores ( $r = 0.59$ ) and vegans ( $r = 0.58$ ). The strength of the correlations of both  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  with other factors such as age, BMI, or lipids, differed in the omnivorous group compared with the vegan group (Fig. 1).

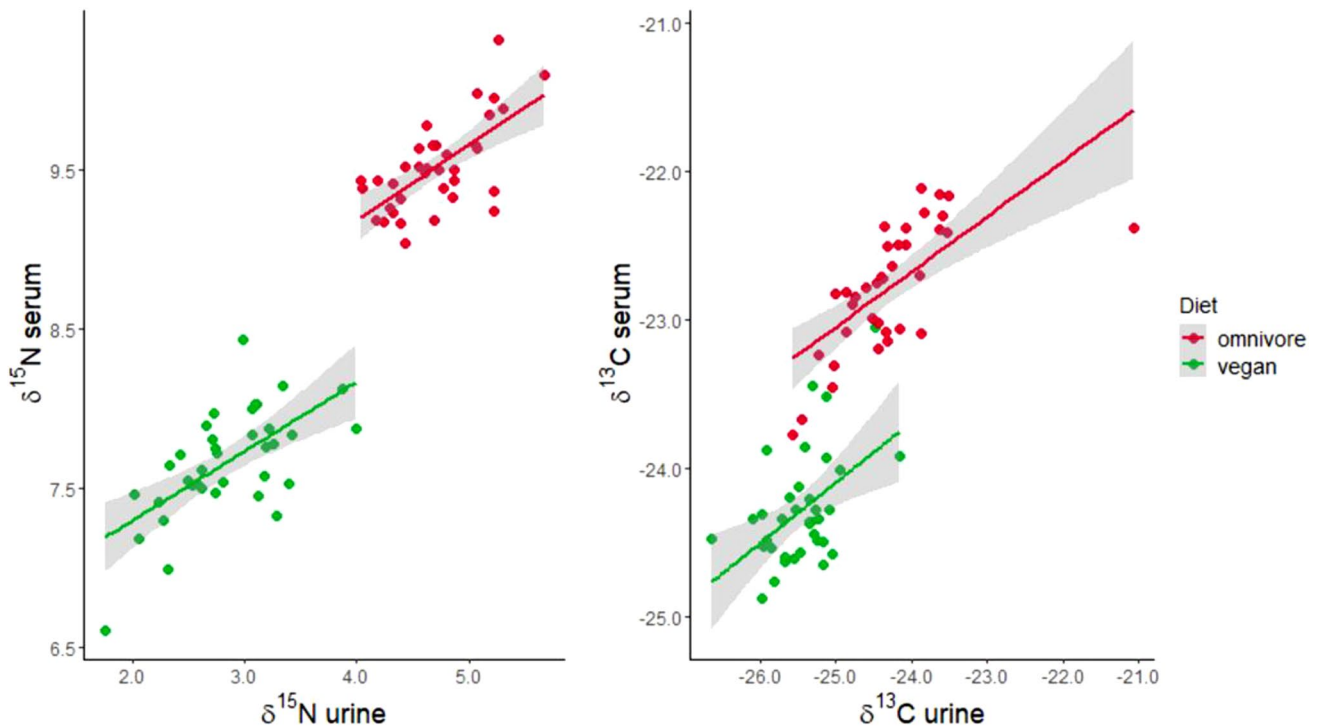
### Sensitivity and specificity analyses for biomarkers

The sensitivity and specificity of the SIR biomarkers, 15:0, and 1-methylhistidine to predict whether an individual practices a vegan or omnivorous diet was evaluated by ROC analyses. The  $\delta^{15}\text{N}$  in serum and urine had 100% sensitivity and specificity to discriminate between vegans and omnivores.  $\delta^{13}\text{C}$ , 15:0 and m1His also had high sensitivity and specificity. Results are shown in Figs. 2, and 3 and in Table 3.

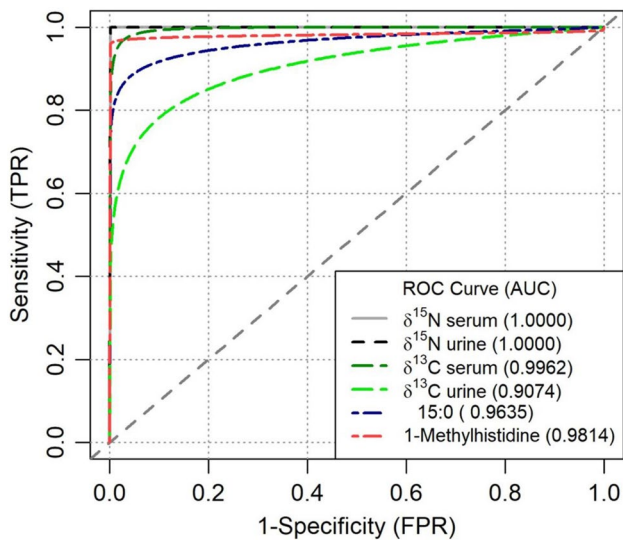
### Discussion

This cross-sectional study investigated biomarkers of vegan or omnivorous diet in plasma and 24 h urine. The main results are that  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  from vegans are much lower both in plasma and in 24 h urine, compared to omnivores with at least three meat consumption occasions per week. In particular,  $\delta^{15}\text{N}$  seems to be well suited to discriminate between participants following a vegan or an omnivorous diet. Further, in a ROC analysis,  $\delta^{15}\text{N}$  performed better than 1-methylhistidine or 15:0, which are discussed as specific biomarkers of meat or dairy intake, respectively.

Stable isotope ratios have been used for several years to characterize dietary habits in contemporary humans [8, 9],



**Fig. 2** Scatter plots of  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  in urine and serum, separated for the two dietary groups



**Fig. 3** Receiver characteristic curve for  $\delta^{15}\text{N}$ ,  $\delta^{13}\text{C}$ , 15:0, 1-methylhistidine to distinguish between vegans ( $n=36$ ) and omnivores ( $n=36$ ). (TPR = true positive rate, FPR = false positive rate)

including both vegan or vegetarian diets and different body tissues or fluids including hair [8, 9, 21], fingernails [18], whole blood [5], or serum [28].

Most of these studies reported lower  $\delta^{15}\text{N}$  in vegans compared to omnivores, even though the sample size in most

**Table 3** Estimated sensitivity and specificity of  $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ , 15:0 and 1-methylhistidine to distinguish between vegans and omnivores based on cut-offs

Parameter AND cut-offs	Vegan diet		Sensitivity	Specificity
	Yes	No		
<b><math>\delta^{13}\text{C}</math> serum (‰)</b>				
< - 23.5	34	2	94%	94%
> - 23.5	2	34		
<b><math>\delta^{13}\text{C}</math> urine (‰)</b>				
< - 24.5	34	12	94%	66%
> - 24.5	2	24		
<b><math>\delta^{15}\text{N}</math> serum (‰)</b>				
< 8.5	36	0	100%	100%
> 8.5‰	0	36		
<b><math>\delta^{15}\text{N}</math> urine (‰)</b>				
< 4.0	36	0	100%	100%
> 4.0	0	36		
<b>15:0 (%)</b>				
< 0.2	34	6	94%	83%
> 0.2	2	30		
<b>1-methylhistidine (µmol/L)</b>				
< 1	36	8	100%	78%
> 1	0	28		

studies was low and not exceeding 16 persons, and the time period since these persons followed a vegan diet was only occasionally provided [8, 9, 18, 21]. In our study, adherence to a vegan diet for at least 1 year was an inclusion criterion. Further, diets in the omnivore groups are often not characterized in detail [8, 18, 25, 28], unlike our omnivorous participants who reported consumption of meat or meat products at least three times per week [22]. Most studies did not report differences between vegans and omnivores for  $\delta^{13}\text{C}$  (9, 18, 21). Three studies compared also stable isotope ratios of vegans and vegetarians and reported either no differences between these (21, 28) or lower ratios in vegans for  $\delta^{15}\text{N}$  only (8).

Different tissues have been used in nutritional stable isotope ratio research, including hair [9, 21, 29, 30] and urine [5, 17], which can both be obtained non-invasively. Indeed, stable isotope ratios in different tissues may be different. Nash et al. measured both  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  in plasma, red blood cells and hair, and reported lower  $\delta^{15}\text{N}$  in erythrocytes compared to plasma and hair, while  $\delta^{13}\text{C}$  was higher in hair compared to plasma and RBC [29]. Very similar findings were reported for hair, plasma and erythrocytes [30]. Kuhnle reported lower  $\delta^{15}\text{N}$  values in urine compared to whole blood for  $\delta^{15}\text{N}$ , while differences for  $\delta^{13}\text{C}$  were less evident [5]. Hülsemann reported variation in the  $\delta^{15}\text{N}$  of urinary urea in 69 samples obtained from 8 omnivorous participants over a period of 48 to 104 h, the mean  $\delta^{15}\text{N}$  was  $4.4 \pm 0.6\text{‰}$  [17]. We are not aware of studies that compared spot urine and 24 h urine, and how far short-term changes in hydration and diuresis affect SIRs in urine.

Generally, it is understood that the overall  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  in body fluids and tissues should reflect the stable isotope ratios observed in food sources. However, there are not many contemporary analyses of stable isotope ratios in foods. According to Hülsemann, cereals and legumes have lower  $\delta^{15}\text{N}$  values compared to meat and dairy (examples from their analyses: whole grain bread 2.3‰, soya Bolognese 2.4‰, yoghurt 6.0‰, beef roulade 7.4‰) [31].

Thus, lower  $\delta^{15}\text{N}$  in vegans may reflect the consumption of plant-based protein sources instead of animal protein with higher  $\delta^{15}\text{N}$ . We observed lower urinary  $\delta^{15}\text{N}$  than plasma  $\delta^{15}\text{N}$  (both in vegans and omnivores) which may reflect to a stronger extent the preferred excretion of  $^{14}\text{N}$  [17]. While  $^{14}\text{N}$  is preferably excreted in urine,  $^{15}\text{N}$  is retained and leads to higher  $\delta^{15}\text{N}$  in serum. There are also some other noteworthy dietary differences between vegans and omnivores. Vegans reported lower total protein intake, although on average, it still exceeded 1 g per kg body weight and lower fat and higher carbohydrate intake (Table 1). The lower protein intake may also lead to differences in urinary nitrogen excretion, however, as we did not measure urinary nitrogen, this remains speculative.

In comparison to the  $\delta^{15}\text{N}$  ratio, specificity, and sensitivity of the  $\delta^{13}\text{C}$  ratio to distinguish between vegans and omnivores were lower, but comparable to two other biomarkers of animal food intake. The fatty acid, 15:0, has been suggested as a biomarker of dairy intake and has shown a good correlation with reported dairy intakes in epidemiologic studies [32], although it has not been widely used. Recently it has been shown that odd-chain fatty acids (15:0 and 17:0) can also be synthesized internally from propionic acid, derived from dietary fiber, which would limit their use as a biomarker of dairy intake, although this seemed to be more an issue for 17:0 [25]. 1-methylhistidine has recently been proposed as a biomarker of cod and salmon intake in a randomized controlled trial [33] and as a biomarker of animal protein intake in clinical [34] and epidemiologic studies [35]. Significant differences in 1-methylhistidine urinary concentrations has also been described in vegans and non-vegans in the Adventist Health Study 2 [36]. Indeed, highly significant differences were observed for both markers upon a comparison of the two groups in the present study. Yet, both markers showed lower sensitivity and specificity than  $\delta^{15}\text{N}$ . To our knowledge, the present study is the first to have measured and evaluated these biomarkers in combination.

Although the cross-sectional nature and sample size of our study precludes more advanced statistical analyses, the findings are nonetheless promising and should inform future work investigating reliable biomarkers of dietary intake and patterns. The results warrant therefore confirmation in studies with a more advanced study design. Of note, we relied on self-reported dietary habits when grouping the participants into a vegan or omnivorous diet. However, the risk of misclassification in this study seems to be low as participants filled in 3 days of dietary records and the call for participation was specifically addressing vegan diet and omnivorous diet, including the duration of a vegan diet and the requirement of 3 or more meat consumption occasions per week.

In conclusion, the RBVD study included strict definitions of a vegan and omnivorous diet, implemented different nutritional status measurements, and provided the opportunity for stable isotope ratios and dietary biomarkers such as 15:0 percentage or 1-methylhistidine concentration to be investigated together for the first time. Using these biomarkers in combination may be promising and help to master the challenge to distinguish between for instance vegetarian and flexitarian diets, even if the absolute differences in stable isotopes between vegans and omnivores were small. Further research should focus on the added value of these combinations of biomarkers to monitor dietary changes, and whether stable isotope ratios alone or in combination with other biomarkers provide greater sensitivity, specificity, and ultimately reliability and reproducibility when distinguishing between omnivores, vegetarians, and vegans.

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**Author contributions** Statement of authors' contribution to manuscript: CW, KA and BM designed the cross-sectional study (project conception, development of overall research plan, study oversight). KB, AM and JD conducted the research (hands-on conduct of the experiments and data collection), SD performed the statistical analysis, JD and CW wrote the paper and JD had the primary responsibility for final content. All authors have read and approved the final manuscript. Data described in the manuscript will not be made available because of provisions of the data protection regulations for participants.

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## Declarations

**Conflict of interest** None of the authors declares a conflict of interest.

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