The Influence of Dietary Habits and Meat Consumption on Plasma 3-Methylhistidine—A Potential Marker for Muscle Protein Turnover

*Bastian Kochlik, Christiana Gerbracht, Tilman Grune, and Daniela Weber**

Scope: 3-Methylhistidine (3-MH) as a potential biomarker for muscle protein turnover is influenced by meat intake but data on the impact of meat on plasma 3-MH are scarce. We determined the association of plasma 3-MH, 1-methylhistidine (1-MH), and creatinine with dietary habits and assessed the impact of a single white meat intervention during a meat-free period.

Methods and results: Plasma 3-MH, 1-MH, and creatinine concentrations of healthy young omnivores ($n = 19$) and vegetarians ($n = 16$) were analyzed together with data on anthropometry, body composition, grip strength, and nutrition. After baseline measurements omnivores adhered to a meat-free diet for 6 days and received a defined administration of chicken breast on day four. At baseline, omnivores had higher plasma 3-MH and 1-MH concentrations than vegetarians. White meat administration led to a slight increase in plasma 3-MH in omnivores. The elevated 3-MH concentrations significantly declined within 24 h after white meat intake.

Conclusion: 1-MH concentrations in plasma seem to be suitable to display (white) meat consumption and its influence on 3-MH plasma concentration. 3-MH in plasma may be used as a biomarker for muscle protein turnover if subjects have not consumed meat in the previous 24 h.

1. Introduction

3-Methylhistidine (3-MH; *N*-tau-methylhistidine) seems to be a suitable biomarker to identify elevated muscle degradation or an unfavorable muscle protein turnover as shown in muscle wasting disease and elderly populations.[1–5] 3-MH is synthesized only in the muscle by the methylation of one histidine residue in actin and in varying amounts in myosin depending on the type of muscle.[6–8] 3-MH is released into the circulation during muscle degradation and then excreted quantitatively in the urine without being metabolized.[9,10] Thus, muscle protein degradation is the only endogenous source of 3-MH in human plasma. In healthy adults (20–70 years) the content of 3-MH in muscle and the 3-MH-to-creatinine excretion (3-MH/Crea) ratio remain constant.[2,4] Thus, we suggest that 3-MH might be a helpful biomarker in the assessment of muscle protein turnover, which is important in the diagnosis of frailty and sarcopenia. Up to date the diagnosis of sarcopenia and frailty is mainly performed by physical tests and imaging parameters.[11,12] However, both approaches have some drawbacks, including patients' compliance, availability in the clinical routine, and costs. Therefore, an objective and specific biomarker for the assessment of muscle protein turnover is needed. However, plasma 3-MH might be influenced by food intake. Meat, fish, and their products are the only exogenous sources of 3-MH and hence are able to influence plasma concentrations of 3-MH. The current recommendation to measure endogenously released 3-MH in plasma is to adhere to a meat-free diet for at least 3 days before blood sampling.[2] This is problematic when measuring samples of elderly patients, in the clinical routine and in large scale human studies.

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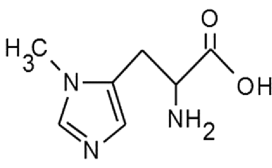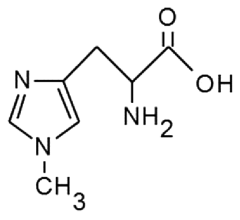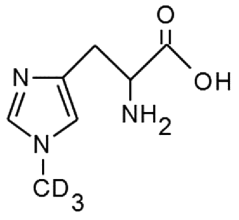Munich-Neuherberg, Germany
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Table 1. Structure and nomenclature of methylhistidines.

Structure			
Biochemical nomenclature[a]	1-Methylhistidine N-pi-methylhistidine	3-Methylhistidine N-tau-methylhistidine	3-Methyl-D$_3$-histidine N-tau-methyl-D$_3$-histidine
IUPAC nomenclature	(2S)-2-amino-3-(3-methylimidazol-4-yl) propanoic acid	(2S)-2-amino-3-(1-methylimidazol-4-yl) propanoic acid	

[a] Nomenclature used in this study.

Another form of methylhistidine, namely 1-methylhistidine (1-MH; N-pi-methylhistidine), exists besides 3-MH. 1-MH is mainly synthesized in muscle of animals but not in humans and occurs as part of the dipeptide anserine (β-alanyl-N1-methyl-histidine).[13–16] Therefore, it has been hypothesized that urinary 1-MH may function as a specific marker for meat consumption[17,18] and may indicate exogenous 3-MH.

In the literature there is some inconsistency about the nomenclature of methylhistidine metabolites in terms of numbering the methyl group of the imidazole ring of histidine.[16,19–21] In the present study, we prefer the biochemical names N-tau-methylhistidine and N-pi-methylhistidine which refer to 3-methylhistidine and 1-methylhistidine, respectively (**Table 1**), and which is in contrast to the IUPAC (International Union of Pure and Applied Chemistry) nomenclature but allows better comparison to previous literature in this field.

Creatinine, its urinary excretion and its plasma concentrations are associated with muscle metabolism[22–24] and smaller interindividual variations of methylhistidines (MH) have been shown when adjusted to creatinine.[25,26]

The aim of this pilot study was to investigate 1) the associations of plasma 1-MH, 3-MH, and creatinine with dietary habits in young omnivores and vegetarians, and 2) the impact of a single white meat intervention on plasma 1-MH and 3-MH during a meat-free period in omnivores. Further, we aimed to display exogenous 3-MH and to reexamine the recommendation of avoiding all meat for 3 days before blood sampling.

2. Experimental Section

This study was conducted with each participant's understanding and informed written consent. Ethical clearance was given by the ethics committee of the University of Potsdam, Potsdam, Germany (permission number 36/2016). This study was conducted in accordance with the Declaration of Helsinki (1964).

2.1. Study Population and Study Design

Healthy participants (absence of chronic or acute diseases) were recruited for the study by bulletin advertisements at the University of Potsdam and the German Institute of Human Nutrition. The participants were divided according to their nutritional habits into a vegetarian and an omnivorous group.

Inclusion criteria for both groups were: age between 20–30 years and a BMI of 20–30 kg m^{-2}. An additional inclusion criterion for the vegetarian group was ovo-lacto vegetarian eating behavior. Exclusion criteria for both groups were: pregnancy, lactating period, chronic medication (except oral contraceptive), and chronic diseases. For the vegetarian group the additional exclusion criteria were consuming meat, fish, and such products.

The study scheme is shown in **Figure 1**. After baseline, the omnivore group followed an ovo-lacto vegetarian diet for 6 days, that is, no meat, fish, and such products were consumed (Figure 1B). On day three (d3) a blood sample was taken. After blood sampling on day four (d4), the participants received a single bolus of 160 g cooked chicken breast together with a mixed salad and bread. This amount of 160 g chicken meat contains about 35.52 g protein (22.2% protein) resulting in 126.5 μmol 3-MH (3.56 μmol 3-MH g^{-1} protein).[27] An additional blood sample was taken 3 h after white meat intervention (d4.1). Further blood collections were performed on day five (d5) and day six (d6) during the meat-free period. Then the participants were allowed to resume their normal eating habits for 7 days followed by a final blood sampling on day 13 (d13).
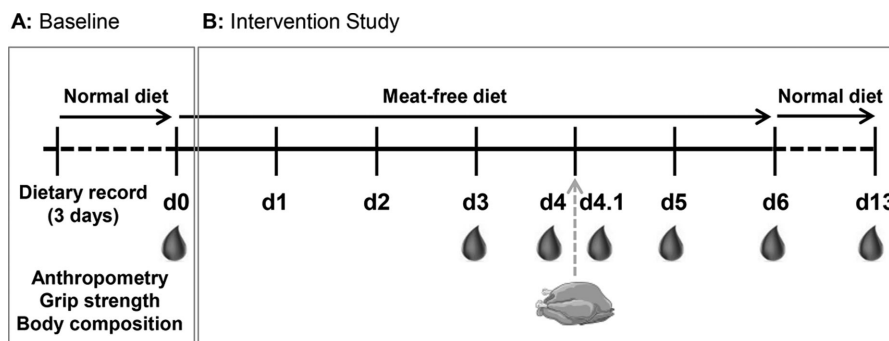
All anthropometric measurements and blood samplings were performed by trained and qualified study nurses. Plasma preparation and biomarker analysis were performed by trained and qualified scientists.

2.2. Nutritional Data

Before baseline measurement, the participants were instructed to eat according to their usual dietary habits and fill out a 3-day dietary record. The intake of energy, protein, and meat was evaluated using PRODI Software Version 6.5 (Nutri-Science GmbH, Hausach, Germany).

2.3. Measurements of Anthropometry and Body Composition

Anthropometrics and body composition were assessed at baseline by using a SECA Stadiometer 274, a nonelastic tape measure and a SECA mBCA 515 with integrated scale for the

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Figure 1. Study Design. A) Baseline examination and blood sampling in omnivores ($n = 19$) and vegetarians ($n = 16$). B) Time course of dietary conditions and time points of blood samplings and white meat intervention for the omnivores.

bioelectrical impedance analysis (BIA; Seca GmbH & Co. KG, Hamburg, Germany). Physical activity level (PAL) was self-reported by participants during the BIA measurement. PAL was categorized according to participants' lifestyle and profession: ≤1.2 (inactive), 1.4, 1.6, 1.8, and ≥2.0 (highly active).

2.4. Measurement of Grip Strength

Grip strength was measured at baseline by using a Jamar Plus Digital Hand Dynamometer (Patterson Medical, Sammons Preston Bolingbrook, IL, USA) and carried out according to Roberts et al.[28]

2.5. Blood Samples

Blood samples (4 mL) were collected by venipuncture into EDTA vacutainers (Sarstedt, Numbrecht, Germany). Blood samples were kept at 4 °C until centrifugation (EBA 200, Hettich Zentrifugen, Tuttlingen, Germany) for 10 min at 2700 g and room temperature to separate the plasma. Plasma samples were stored at −80 °C until analysis and thawed immediately before preparation for analysis.

2.6. Analysis of Plasma Methylhistidines

The simultaneous measurement of 3-MH and 1-MH (μmol L^{-1}) in plasma was carried out by ultra-performance liquid-chromatography tandem mass spectrometry (UPLC-MS/MS) using an Acquity Ultra Performance LC system coupled to a Waters Quattro Premier XE mass spectrometer (both Waters Corporation, Milford, MA, USA) and the Waters MassLynx Software (Version 4.1). UPLC conditions recommended by the column manufacturer were modified as described below in section 2.6.2.

2.6.1. Materials

Standards used were 1-MH and 3-MH (both Sigma Aldrich, Taufkirchen, Germany) and deuterated 3-methylhistidine (d$_3$-3-MH; Cambridge Isotope Laboratories Inc., Andover, MA, USA) as internal standard.

Reagents used were water, methanol, acetonitrile (all LC-MS grade; Merck, Darmstadt, Germany), ammonium formate (for MS; Sigma Aldrich, Taufkirchen, Germany), and formic acid (eluent additive for MS; Honeywell, Offenbach, Germany).

1-MH and 3-MH stock solutions were prepared in water and then mixed to a standard mixture followed by dilutions in various concentrations (0.24 μM–120 μM) with water and treated like plasma samples for quantification.

2.6.2. Sample Preparation and UPLC-MS/MS Analysis

Internal standard (2.4 μM; 25 μL) was added to plasma samples (25 μL), vortex mixed, and incubated for 1 h at 7 °C; then 150 μL methanol was added to precipitate proteins, vortex mixed, and incubated for 2 h at 7 °C; samples were centrifuged for 10 min at 4 °C and 16 500 × g (Biofuge primo R; Heraeus, Thermo Fisher Scientific, Germany). The supernatant was consequently centrifuged for 10 min at 4 °C and 16 500 × g; then the supernatant was diluted with an equal volume of water; transferred to an autosampler vial; kept at 15 °C in the autosampler and 4 μL of the sample was injected into the UPLC-MS/MS system. Analyte separation was carried out on an Intrada Amino Acid column (3 μm, 50 × 3 mm; Imtakt USA, Portland, OR, USA) at a column temperature of 37 °C. Eluents A (0.25% formic acid in ACN v/v) and B (0.25% formic acid in 100 mM ammonium formate v/v) were used at a flow rate of 0.8 mL min^{-1}. Analytes were eluted at 7.0 min (3-MH and d$_3$-3-MH) and 7.3 min (1-MH) from the column with a stepwise gradient (initial: 95% A; min 1–3: 95–85% A; min 3–8: 85–20% A; min 8–9: 0% A; min 9–10: 95% A). Electrospray ionization operated in positive mode (ESI+), and MS/MS settings were as follows—capillary voltage: 0.75 kV, cone voltage: 20 V, cone gas flow: 100 L h^{-1}, source temperature: 120 °C, desolvation temperature: 400 °C, desolvation gas flow: 850 L h^{-1}. Three mass transitions with optimal collision energies (CE) each were defined for MRM analysis of 1-MH, 3-MH, and d$_3$-3-MH. Mass transitions of the quantifier ion of 1-MH (m/z 170 > 96, CE: 20 V), 3-MH (m/z 170 > 124, CE: 16 V), and d$_3$-3-MH (m/z 173 > 127, CE: 16 V) were used for quantification. Mass transitions of the qualifier ions were as follow: 1-MH (m/z 170 > 126, CE: 18 V; m/z 170 > 109, CE: 16 V), 3-MH (m/z 170 > 109, CE: 18 V; m/z 170 > 96, CE: 30 V) and d$_3$-3-MH (m/z 173 > 112, CE: 18 V; m/z 173 >

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99, CE: 30 V). Quantification of 1-MH in plasma was performed by external standard calibration and quantification of 3-MH in plasma was performed by standard isotope-dilution approach.

In total, 148 plasma samples were randomly prepared in batches of 20 samples and 6-point standard calibration (0.015–7.5 μM 1-MH and 3-MH including 0.15 μM IS) was used for quantification. Two quality control samples (pooled plasma samples; $n = 5$ measurements) were measured between batches.

2.6.3. Method Validation

Linearity was assessed by triplicate measurement of six concentration levels (0.015–7.5 μM) of a standard mixture of 1-MH, 3-MH, and d_3-3-MH dissolved in water or spiked to pooled plasma.[29] The same set of samples was used to determine the *matrix effect* on 1-MH, 3-MH, and d_3-3-MH by comparing the slopes obtained from calibration curves in water with those in plasma.[29,30] *Recoveries* were calculated by spiking six plasma samples with standard mixtures of 1-MH, 3-MH, and d_3-3-MH with two different concentrations (0.075 μM and 1.5 μM) before and after sample extraction. Subsequently, peak areas of the analytes in the pre-spiked samples were compared with peak areas in the post-spiked samples. Further, on three different days a set of six plasma samples was prepared (see Section 2.6.2) in duplicate, measured in triplicate, and quantities were calculated to determine *inter-assay* and *intra-assay variations* for 1-MH and 3-MH in plasma.[31] For quantification three 6-point calibration mixtures (0.015–7.5 μM for 1-MH and 3-MH including 0.15 μM IS) were prepared and measured in triplicate. For 3-MH, peak areas were normalized to peak areas of the corresponding IS, plotted against the applied concentration, and the resulting calibration curve used to quantify sample concentrations. 1-MH quantification was performed by external standard calibration due to a lack of internal standard. The same sets of calibration mixtures were used to estimate the *theoretical limit of detection (tLOD)*. The *limit of quantification (LOQ)* was defined as the lowest concentration of the calibration curve.[29,32] *tLOD* was estimated using the formula:

$$tLOD = t_\alpha \times S_{\bar{x}}, \tag{1}$$

with t_α according to Student's *t*-test using $n - 1$ measurements and $S_{\bar{x}}$ as an estimate of the true standard deviation of the distribution of sample means.[32]

Stability of processed samples and calibration mixtures was determined by measuring concentrations of the same set of samples and calibration mixtures kept for 24 or 48 h at −20 °C. Autosampler *stability* was assessed by measuring concentrations of six samples and by a set of calibrators kept for 36 h in the autosampler (at 15 °C).

2.7. Analysis of Plasma Creatinine

Creatinine (μmol L^{-1}) in plasma was measured by using the ABX Pentra Creatinine CP kit in a Pentra 400 (ABX Diagnostics, Montpellier, France) device. The analysis was based on the enzymatic

Table 2. Characteristics of the study population.

	Omnivores	Vegetarians	*P*
n (% female)	19 (84.2)	16 (87.5)	—
Age [years]	26.3 ± 2.4	26.4 ± 2.9	n.s.
Assessment before baseline measurement			
Energy intake [kcal d^{-1}]	1973 ± 563	1986 ± 484	n.s.
Protein intake [g d^{-1}]	75.1 ± 33.0	62.9 ± 20.7	n.s.
Meat intake [g d^{-1}]	106.5 ± 79.7	0 ± 0	—
Meat intake [g] (at d1)	93.9 ± 126.1	0 ± 0	—
At baseline			
BMI [kg m^{-2}]	22.0 ± 2.10	21.9 ± 2.10	n.s.
FMI [kg m^{-2}]	5.38 ± 1.75	6.25 ± 1.58	n.s.
FFMI [kg m^{-2}]	16.6 ± 2.00	15.6 ± 1.26	n.s.
SMM [kg]	21.4 ± 5.39	20.6 ± 3.94	n.s.
PAL	1.64 ± 0.08	1.65 ± 0.09	n.s.
Grip strength [kg]	31.7 ± 9.27	30.8 ± 8.08	n.s.

Values are given as (means ± SD) or (n [%]). FMI, fat-mass index; FFMI, fat-free mass index; SMM, skeletal muscle mass; PAL, physical activity level; n.s., not significant.

Jaffé method and was carried out according to the manufacturer's instructions.[33,34]

2.8. Statistical Analysis

For all analyses, $p < 0.05$ was considered statistically significant. Student's *t*-test was used to compare intergroup differences and one-way ANOVA (post hoc Tukey test) was used to compare intragroup differences. Normal distribution of data was checked groupwise for each time point by Kolmogorov–Smirnov test. Two outliers of 1-MH from time points d3 and d4.1 were excluded. One sample is missing in the omnivore group on d3. This results in data from $n = 19$ omnivores at d0, d4, d4.1, d5, d6, and d13 and $n = 18$ omnivores at d3 for 3-MH and creatinine. For 1-MH, data from $n = 19$ omnivores at d0, d4, d5, d6, and d13, $n = 18$ omnivores at d4.1 and $n = 17$ omnivores at d3 were analyzed. Finally, data of $n = 16$ vegetarians at baseline was assessed for 3-MH, 1-MH, and creatinine. IBM SPSS Statistics Version 20 (Release 20.0.0) was used for statistical analyses.

3. Results

3.1. Baseline and Intervention Study

In total, 35 participants were enrolled into the study and grouped according to their eating behavior into an omnivorous ($n = 19$) or a vegetarian ($n = 16$) group. Characteristics of both groups are shown in **Table 2**. Both groups were similar regarding age, anthropometric data, body composition, grip strength, energy, and protein intake and PALs.

At baseline, plasma 3-MH, 1-MH, and creatinine concentrations as well as the 1-MH/Crea ratio were higher in omnivores than in vegetarians ($p < 0.05$; **Table 3** and **Figure 2**).

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Table 3. Influence of the dietary habit on plasma methylhistidine concentrations and creatinine ratios in omnivores and vegetarians at baseline.

	Omnivores	Vegetarians	*P*
3-MH [μM]	3.03 ± 0.85	2.46 ± 0.59	0.032
1-MH [μM]	4.86 ± 7.11	0.54 ± 0.09	0.016
Creatinine [μM]	80.7 ± 10.9	71.1 ± 9.7	0.010
3-MH/Crea ratio	0.037 ± 0.008	0.035 ± 0.007	n.s.
1-MH/Crea ratio	0.055 ± 0.079	0.008 ± 0.001	0.013

Plasma concentrations of 3-MH, 1-MH, creatinine, and corresponding MH/Crea ratios of omnivores and vegetarians at baseline. Values are given as (means $\pm$ SD). n.s., not significant.

Additionally, we observed a positive correlation between 1-MH and meat intake ($r = 0.483$; $p = 0.036$) in omnivores as well as between 3-MH and both skeletal muscle mass ($r = 0.648$; $p = 0.007$) and grip strength ($r = 0.504$; $p = 0.046$) in vegetarians.

Plasma 3-MH and 1-MH concentrations and both MH/Crea ratios declined to a basal level within 3–4 days on a meat-free diet in omnivores ($p < 0.05$; **Figure** 3A,B). Plasma concentrations and MH/Crea ratios in omnivores were similar between d3 and d4 on a meat-free diet and thus may represent endogenously released 3-MH and 1-MH in plasma. Further, 3-MH and 1-MH plasma concentrations and MH/Crea ratios in omnivores on d4 were similar to those in vegetarians at baseline (Figure 3C–F). Plasma creatinine was significantly higher in omnivores on d4 than in vegetarians at baseline ($p = 0.038$; data not shown).

An increase in plasma 3-MH ($p < 0.001$), 1-MH ($p < 0.001$), and both MH/Crea ratios ($p < 0.001$) was found 3 h after white meat intervention (d4.1; **Figure** 4A). The elevated 3-MH concentrations and 3-MH/Crea ratios in plasma declined significantly within 24 h after this intervention (d5). The increased 1-MH concentrations and 1-MH/Crea ratios in plasma fell to basal levels after 48 h (d6). Further, we observed that plasma 3-MH and

3-MH/Crea on d0, d5, and d13 were similar (Figure 4C,E). Regarding plasma 1-MH and 1-MH/Crea we found higher values on d5 compared to d0 and d13 ($p < 0.05$; Figure 4D,F). The relative amount of endogenously released methylhistidines at baseline, d5, and d13 were 79, 82, and 80 for 3-MH (**Table 4**) and 12, 6, and 14 for 1-MH, respectively (data not shown).

3.2. Method Validation

Calibration curves of a standard mixture of 1-MH, 3-MH, and d$_3$-3-MH in water and plasma extracts showed *linearity* in the range of 0.015–7.5 μM ($r^2 > 0.999$; Table 2). The *tLOD* was estimated to be 6.0 nM and 5.3 nM for 1-MH and 3-MH, respectively. The *LOQ*, defined as the lowest concentration of the calibration curve, was shown to be 15 nM for both analytes. The *matrix effect*, calculated by comparing the slopes obtained from calibration curves in water with those in plasma, was between 102.62% and 111.12% for 3-MH and 1-MH, respectively. *Recovery* values, showing analyte signal loss during sample preparation, varied between 85.95% (1-MH) and 90.52% (3-MH) for low-concentration spiked samples and 107.83% (3-MH) and 112.06% (1-MH) for high-concentration spiked samples. *Intra-assay* and *inter-assay variation coefficients* of 1-MH and 3-MH concentrations in plasma were <10% for both analytes. *Stability* of 1-MH and 3-MH concentrations in plasma extracts varied between 82.98%–101.29% (36 h autosampler at 15 °C), 99.75–107.77% (24 h at −20 °C), and 85.27–104.08% (48 h at −20 °C).

4. Discussion

Our results show that plasma 3-MH concentrations and 3-MH/Crea ratios only slightly increase in omnivores after a defined single white meat intervention. The elevated plasma 3-MH concentrations and 3-MH/Crea ratios significantly
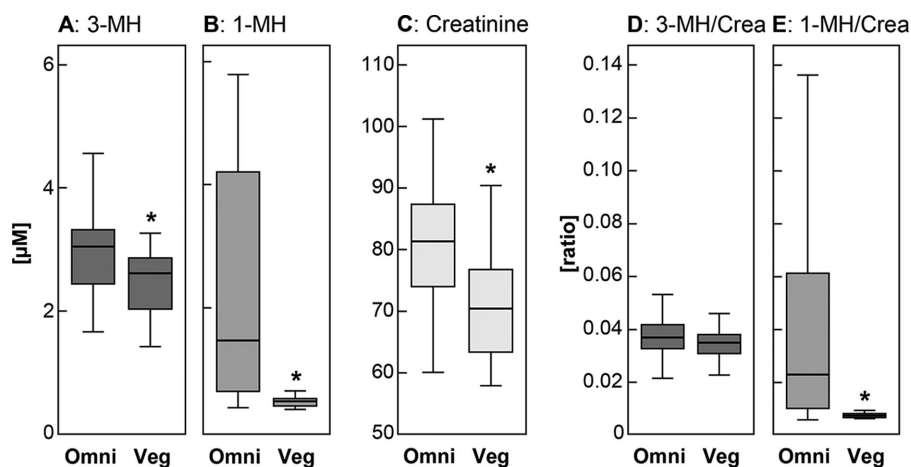

Figure 2. Influence of dietary habits on plasma methylhistidine concentrations and creatinine ratios in omnivores and vegetarians at baseline. A,B) Plasma 3-MH and 1-MH (one extreme value is not shown in the figure but included in the statistical analysis). C) Plasma concentrations of creatinine. D,E) Ratios of 3-MH/Crea and 1-MH/Crea (one extreme value is not shown in the figure but included in the statistical analysis). Values are given as (means $\pm$ SD); horizontal lines represent the mean, with the box representing the 25th and 75th percentiles, the whiskers the 5th and 95th percentiles. *$p < 0.05$. Omni, omnivores/omnivore group; Veg, vegetarians/vegetarian group.
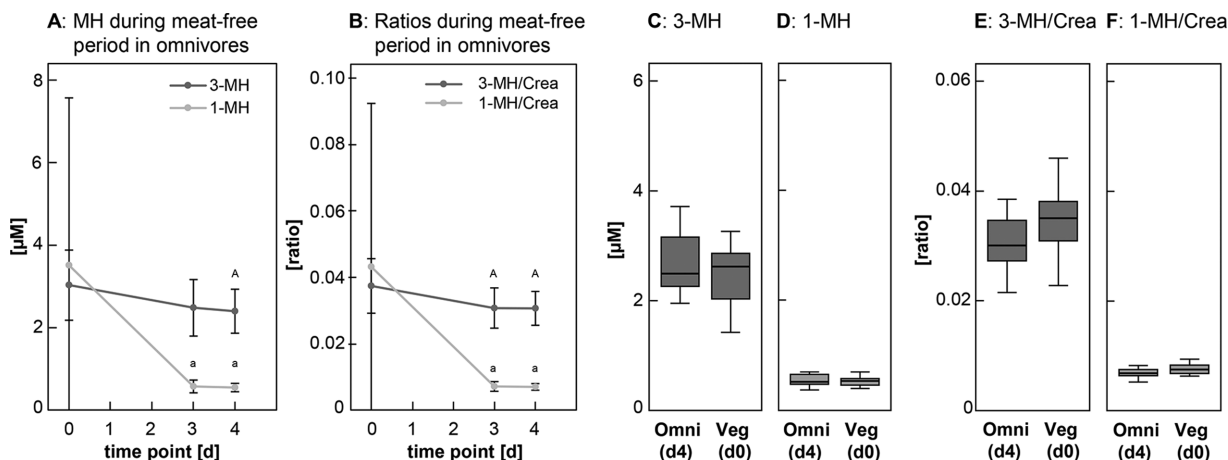
Figure 3. Influence of a meat-free diet on plasma methylhistidine concentrations and creatinine ratios. A) Plasma concentrations of 3-MH and 1-MH (one extreme value at d0 is not shown in the figure but included in the statistical analysis); 3-MH (A = d0, B = d3, C = d4); 1-MH (a = d0, b = d3, c = d4). B) Ratios of 3-MH/Crea and 1-MH/Crea (one extreme value at d0 is not shown in the figure but included in the statistical analysis); 3-MH/Crea (A = d0, B = d3, C = d4); 1-MH/Crea (a = d0, b = d3, c = d4). C,D) Plasma concentrations of 3-MH and 1-MH in omnivores on d4 and in vegetarians at baseline. E,F) Ratios of 3-MH/Crea and 1-MH/Crea in omnivores on d4 and in vegetarians at baseline. Values are given as (means ± SD); horizontal lines represent the mean, with the box representing the 25th and 75th percentiles, the whiskers the 5th and 95th percentiles. A+B: A, a: $p < 0.05$. Omni, omnivores/omnivore group; Veg, vegetarians/vegetarian group.
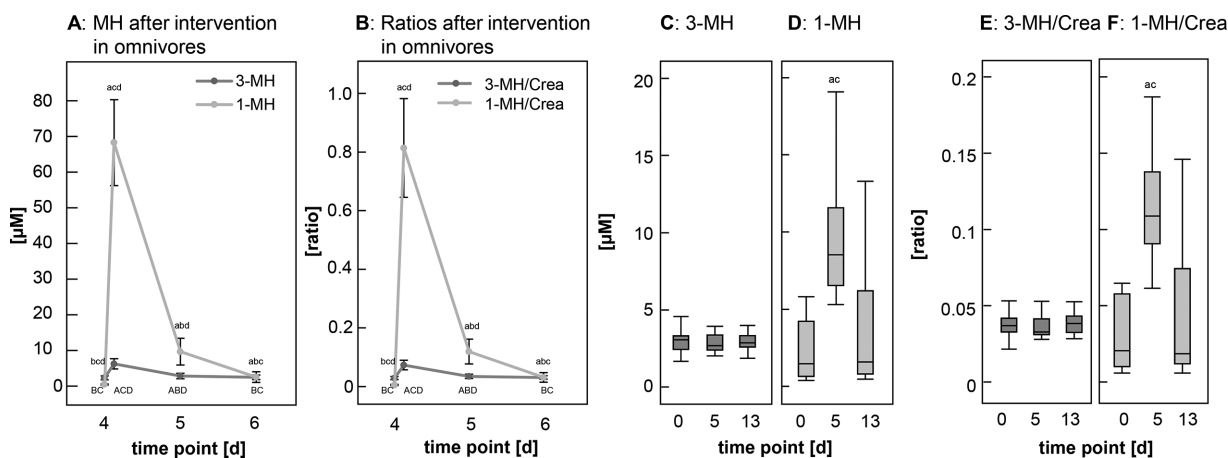
Figure 4. Influence of a single white meat administration and a general meat-containing diet on plasma methylhistidine concentrations and creatinine ratios in omnivores. A) Plasma concentrations of 3-MH and 1-MH; 3-MH (A = d4, B = d4.1, C = d5, D = d6); 1-MH (a = d4, b = d4.1, c = d5, d = d6). B) Ratios of 3-MH/Crea and 1-MH/Crea; 3-MH/Crea (A = d4, B = d4.1, C = d5, D = d6); 1-MH/Crea (a = d4, b = d4.1, c = d5, d = d6). C,D) Plasma concentrations of 3-MH and 1-MH at baseline, d5 and d13; 3-MH (A = d0, B = d5, C = d13); 1-MH (a = d0, b = d5, c = d13). E,F) Ratios of 3-MH/Crea and 1-MH/Crea at baseline, d5, and d13; 3-MH/Crea (A = d0, B = d5, C = d13); 1-MH/Crea (a = d0, b = d5, c = d13). Values are given as (means ± SD); horizontal lines represent the mean, with the box representing the 25th and 75th percentiles, the whiskers the 5th and 95th percentiles. A+B) repeated measures ANOVA with Bonferroni post hoc test: BC, ACD, ABD, bcd, acd, abd, abc: $p < 0.05$; D+F) ac: $p < 0.05$.

Table 4. Endogenous and exogenous 3-MH in plasma of omnivores.

Time point	d0	d4	d5	d13
3-MH$_{total}$ [% (μM)]	100 (3.03 ± 0.85)	100 (2.39 ± 0.53)	100 (2.92 ± 0.73)	100 (3.00 ± 0.71)
3-MH$_{endogenous}$ [% (μM)]	**79** (2.39)	**100** (2.39)	**82** (2.39)	**80** (2.39)
3-MH$_{exogenous}$ [%(μM)]	21 (0.63)	0 (0.00)	18 (0.53)	20 (0.60)

The total absolute concentration of 3-MH at d4 represents endogenous 3-MH, which is used to calculate the exogenous 3-MH amounts on d0, d5, and d13 (μM 3-MH$_{exogenous}$ = μM 3-MH$_{total}$ − μM 3-MH$_{endogenous}$). Total absolute 3-MH concentrations on d0, d4, d5, and d13 are set to 100% and the relative amounts of endogenous and exogenous 3-MH were calculated. Values are given as (% [mean ± SD]) or (% [mean]).

Table 5. Method validation parameters.

Parameter	1-MH	3-MH
Linearity [μM][a]	0.015–7.5 ($r^2 = 0.9993$)	0.015–7.5 ($r^2 = 0.9999$)
tLOD [nM][b]	6.0	5.3
LOQ [nM][c]	15	15
Matrix effect [%][d]	111.12 ± 2.8	102.62 ± 2.8
Intra-assay variation [%][e]	8.5 ± 4.8	4.0 ± 2.2
Inter-assay variation [%][e]	7.5 ± 10.9	6.6 ± 12.7

[a] Standard calibration mixtures measured in triplicate in water and plasma; [b] Repeated injection of LOQ concentration of standard calibration mixtures on three different days followed by using peak areas (1-MH) or analyte-to-IS-peak area ratios (3-MH) and Equation (1) to calculate tLOD; [c] Defined as the lowest concentration of the calibration curve; [d] Comparison of slopes obtained from standard curves in water and plasma; [e] % CV of concentrations of six plasma samples prepared in duplicate and measured in triplicate on three different days.

declined within 24 h. We assume that about 80% of plasma 3-MH is of endogenous origin after an overnight fast of omnivores. Further, we suppose that plasma 1-MH is able to reflect an exogenous uptake of 3-MH in plasma. Furthermore, we found that plasma 1-MH concentrations and 1-MH/Crea ratios are strongly influenced by white meat consumption. Moreover, we observed plasma 3-MH and 1-MH concentrations and both MH/Crea ratios to be higher in omnivores than in vegetarians at baseline.

The higher plasma 3-MH and 1-MH levels in omnivores at baseline reflect the general meat consumption of omnivores which is confirmed by a positive correlation of 1-MH and meat intake. Additionally, we observed a significant positive correlation between 3-MH and both skeletal muscle mass and grip strength in vegetarians showing the association of 3-MH with muscle status.

Methylhistidines are assessed primarily in the urine; however, the sampling of 24 h urine can be prone to errors especially in an elderly study population.[14,26] Therefore, our aim was to measure 3-MH, 1-MH, and creatinine in plasma samples which is more practical, more objective, and not biased by patients' compliance. However, for the interpretation of 3-MH, information on the subjects' previous meat consumption is required since the influence of meat intake on urinary 3-MH excretion is well described.[14,15,17,18,22,27,35] Contrarily, the impact of meat consumption on plasma 3-MH has hardly been investigated. The purpose of our study was therefore to generate novel data on plasma levels of 3-MH, 1-MH, and creatinine regarding dietary influences.

Our findings of elevated plasma 3-MH due to an omnivorous diet and a white meat administration during a meat-free period are in accordance with previous examinations in urine and plasma. Urinary 3-MH excretion was significantly higher in subjects on omnivorous diets and increased due to interventions with different types and amounts of meat during meat-free periods.[18,22,27] On the other hand, urinary 3-MH excretion did not increase after the administration of 100 g of different types of meat.[15] This may be due to lower amounts of meat in comparison to the present study. A dose-dependent increase of 3-MH has previously been shown after the intake of different meats (100 g) or capsules (20 mg, 60 mg, or 120 mg of 3-MH).[36] Additionally,

the half-life of 3-MH in plasma was found to be about 12 h.[36] In contrast, another study observed a half-life of only about 130 min after an intravenous injection of ^{14}C-labeled 3-MH.[10]

These findings of meat consumption on 3-MH urinary excretion and plasma levels led to the recommendation not to eat any meat 3 days before sample collection. We found that increased plasma 3-MH and creatinine ratios significantly declined within 24 h after white meat administration. Additionally, we found similar plasma 3-MH and 3-MH/Crea ratios in fasted omnivores and 24 h after white meat administration. Further, plasma 3-MH at d0, d5, and d13 represent about 80% of endogenously released 3-MH in omnivores. We suggest that avoiding meat consumption 3 days before blood sampling may not be necessary. Furthermore, we recommend that participants avoid meat consumption for at least 24 h before blood sampling.

To adhere to strict dietary recommendations for 3 days or to give correct statements on previous meat intake may be difficult, especially in elderly patients. Therefore, we additionally measured 1-MH in plasma to reexamine this recommendation and to assess whether samples can be excluded on the basis of 1-MH values indicating that subjects consumed meat in the previous 24 h. 1-MH is independent of the human muscle metabolism and it is more sensitive to meat consumption, especially to white meat, than 3-MH.[14,17,18] 1-MH has been described as suitable urinary biomarker for meat consumption. We suggest that plasma 1-MH can also be used as a marker for exogenous 3-MH in plasma. Previously, Myint et al. were able to distinguish between omnivores and vegetarians based on urinary 1-MH excretion.[17] Cross et al. found a dose-dependent increase in 1-MH excretion in omnivores on a red meat diet and elevated excretion levels on an omnivorous compared to a lacto-vegetarian diet.[18] Further studies showed that the impact of different meat sources on urinary 1-MH was strong and that an omnivorous diet led to higher urinary 1-MH than 3-MH excretion.[14,15,17] In accordance with these findings, our results show the same impact of dietary habits and white meat consumption on plasma 1-MH. Hence, by the measurement of plasma 1-MH, reflecting general and white meat consumption, data from participants may be excluded or adjusted when assessing muscle protein turnover.

The strong influence of the chicken meat intervention on plasma 1-MH compared to a small influence on 3-MH in this study may have occurred due to varying contents of methylhistidines in different types of meat. White meat, that is, chicken, contains high amounts of 1-MH and anserine, whereas the amount of 3-MH is rather low.[13–16]

Interestingly, we found a low basal level of 1-MH in plasma although neither 1-MH nor anserine is synthesized in human muscle. One explanation could be that anserine is present in non-muscle tissues[16] and thus anserine degradation may lead to low 1-MH plasma concentrations.

Creatinine excretion and serum concentrations have previously been discussed as useful markers for muscle mass,[22–24,37] despite being influenced by meat and protein intake.[25,38] Urinary creatinine was shown to correlate positively with urinary 3-MH excretion ($r > 0.7$).[22,25] Similarly, we found a positive correlation between plasma creatinine and plasma 3-MH in omnivores ($r = 0.622$; $p = 0.004$) and vegetarians ($r = 0.642$; $p = 0.007$). Additionally, plasma creatinine was significantly correlated with skeletal muscle mass ($r = 0.577$; $p = 0.010$) and grip strength ($r = 0.482$;

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$p = 0.037$) in omnivores and with skeletal muscle mass ($r = 0.607$; $p = 0.013$) in vegetarians. Interestingly, we observed neither an influence of chicken intake on creatinine nor a correlation of creatinine with meat and protein intake confirming the dependence of plasma creatinine on muscle metabolism.

Previously, urinary MH/Crea ratios have been described to have smaller interindividual variations[26] and this led to the recommendation to prefer the MH/Crea ratios in the assessment of muscle protein breakdown in heterogeneous populations.[25] In our study, the 1-MH/Crea ratio is significantly higher in omnivores than in vegetarians and both ratios are significantly increased after the administration of chicken to omnivores. Increased 3-MH/Crea ratios declined significantly and returned to values in the same range as before the intervention (d4: 0.0306 ± 0.0051 μM vs d5: 0.0362 ± 0.0075 μM) 24 h after white meat intervention. The same was true for 1-MH/Crea. These results lead to the suggestions that plasma 1-MH/Crea ratios can detect short-term white meat consumption and that plasma 3-MH/Crea ratios may depict reliable data concerning muscle protein turnover in participants who did not consume meat in the previous 24 h.

One limitation of our study is that we did not correct for exercise and nutrition during the intervention study. Thus, we cannot exclude our results being biased since there may be an influence of exercise and protein intake on muscle metabolism[39,40] leading to a varying release of 3-MH into the blood and resulting in altered urinary 3-MH and 3-MH/Crea.[39,41,42] Future studies should include participants' physical activity and data on nutrition to evaluate the influence of these factors. Alternatively, further studies may include avoiding exercise and may implement a standardized diet in the study design. In our study, we normalized methylhistidine concentrations to creatinine levels to minimize the influence of muscle mass. Another limitation is that we did not draw blood samples in smaller intervals after the white meat administration to provide more details concerning the bioavailability of methylhistidines from white meat. Our study investigated only young and healthy subjects and thus a real association between plasma 3-MH and muscle degradation as occurring in elderly or in a muscle wasting condition cannot be assessed here. Future studies in elderly populations diagnosed with muscle wasting diseases, sarcopenia or frailty and also regarding dietary influences will be needed to validate the suitability of plasma 3-MH as a biomarker for muscle protein turnover.

In conclusion, here we provide novel data on the impact of dietary habits and white meat intake on plasma levels of 3-MH and 1-MH as well as on MH/Crea ratios in young and healthy omnivores and vegetarians. Our results confirm that white meat intake only has a weak impact on plasma 3-MH but a strong impact on plasma1-MH in omnivores. Hence, we suggest that plasma 1-MH and the 1-MH/Crea can be used both as biomarkers for meat consumption by signaling short-term meat consumption, and to display exogenous 3-MH in plasma. Furthermore, our data suggest that plasma 3-MH and 3-MH/Crea may be used as biomarkers for muscle protein turnover when subjects are on a meat-free diet, but this must be further validated in another study population. Finally, we suggest that blood sampling for 3-MH measurements should at least be performed in subjects which did not consume meat in the previous 24 h.

Abbreviations

1-MH, 1-methylhistidine; 3-MH, 3-methylhistidine; Crea, creatinine; FA, formic acid

Conflict of Interest

The authors declare no conflict of interest.

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