Specificity, Dose Dependency, and Kinetics of Markers of Chicken and Beef Intake Using Targeted Quantitative LC-MS/MS: A Human Intervention Trial

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Scope: Common methods for food intake assessment are error-prone. Estimating food intake via metabolite biomarkers in blood/urine is challenged by inter-individual variation. Here, meat intake markers based on criteria defined within the FoodBAll consortium, including dose dependency, specificity, kinetics, and their ability to predict meat dose, are evaluated. **Methods and results:** In two randomized human interventions, meat at different doses are consumed. Plasma concentrations of 100 analytes, including previously proposed meat intake markers, are determined at different time points up to 24 h after meat ingestion using targeted liquid chromatography–tandem mass spectrometry. Plasma concentrations of π-methylhistidine (π-M-His) correlated best with the chicken meat amount consumed even after 24 h ($R^2 = 0.96$). Both, anserine and π-M-His show first-order elimination kinetics, irrespective of meat dose ($t_{1/2}$ is 1.4 and 5.9 h, respectively). Surprisingly, π-M-His best predicted the amount of beef consumed, albeit at lower concentrations. Furthermore, trimethylamine-N-oxide (TMAO) increases only after beef, while dimethylglycine only after chicken consumption. The lack of baseline concentrations for π-M-His and anserine is likely the strength of these compounds to predict meat dose.
Conclusion: Quantitative assessment of meat intake within 24 h is most accurate with π-M-His, whereas TMAO and dimethylglycine best discriminate between chicken and beef.

1. Introduction

Dietary habits are crucial determinants for the development of numerous chronic diseases. In particular, Western-style diets are associated with increased risk for metabolic syndrome, type 2 diabetes as well as cardiovascular diseases and different forms of cancers. For example, the consumption of red meat has been identified as a predominant risk factor for colorectal, pancreatic, and gastric cancers[1–3] as well as for type 2 diabetes[4,5] and for overall mortality.[6] In contrast, white meat is suggested to have beneficial effects on health and to prevent sarcopenia in elderly.[7]

Traditional methods for the assessment of food intake use food-frequency questionnaires, food diaries, and 24-h dietary recalls.[6,8] These methods are based on self-reporting and are known to be error-prone in particular due to underreporting.[9] Incorrect assessment of dietary intake weakens the observed relations between dietary intake and disease risk. To better estimate diet–health interrelations, it is thus crucial to better follow nutritional intake.

To overcome the inaccuracy of traditional assessment methods, various attempts have been undertaken to find suitable nutrition markers in body fluids like blood and urine. The

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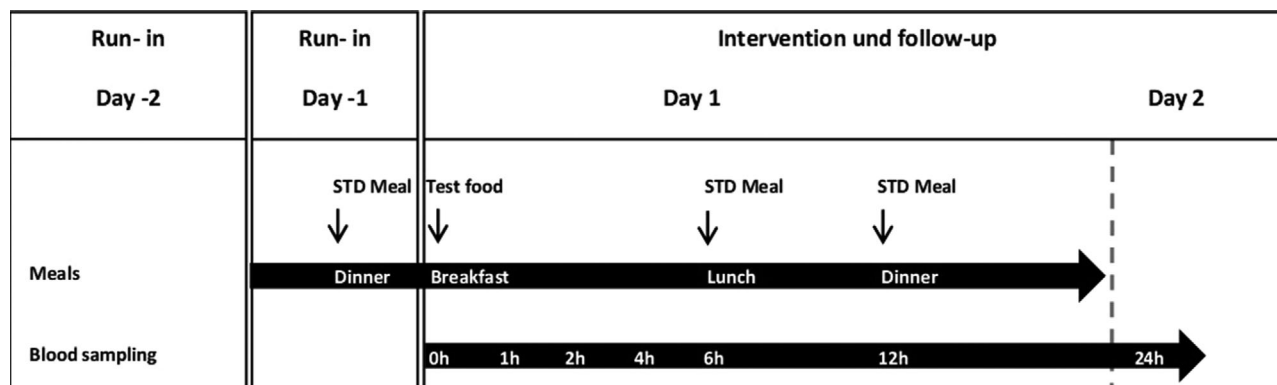Technical University of Munich
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Figure 1. Study design of the randomized controlled meat intervention trial. STD, standardized.

developments in metabolomics techniques to measure large numbers of chemical compounds and metabolites allow to explore the biomarker properties of novel metabolite classes, at the same time validating classically reported metabolites.[10] Nonanimal food sources, mostly plants, produce numerous xenobiotic compounds as a result of a wide array of plant-specific enzymes, making these compounds potentially interesting to define the intake of these foods. The search for markers of meat intake is more challenging, as almost all components in meat are also endogenously present in human plasma or urine and vary largely in concentrations under different physiological conditions such as fasting or physical activity.[11] The classically described chicken meat markers anserine (beta-alanyl-π-methylhistidine) and the anserine-derived amino acid π-methylhistidine (π-M-His) were described as highly specific for intake of poultry meat.[12,13] This is owing to the high carnosine methyltransferase activity in birds, which is lacking in humans. As a result, these animals have high levels of anserine, while it is absent in humans.[14] Other proposed meat intake markers include metabolites of the creatine energy supply system and intermediates of carnitine metabolism.[15–17] Also, urinary levels of guanidinoacetic acid (GAA), a precursor of creatine, were proposed in an attempt to estimate chicken meat intake, and revealed a good agreement of reported and calculated intakes of chicken meat.[18] A recent study performed in vitro colonic digestion of red and white meat preparations and proposed a number of new red-meat specific metabolite markers including intermediates of kynurenine metabolism.[16] Furthermore, the recent work by Cuparencu et al. compared the intake of chicken, pork, beef, and a control protein source in an untargeted metabolomics approach and reported a set of compounds derived from collagen degradation, amino acid metabolism as well as flavor compounds, which were validated for plausibility, robustness, time-response, and prediction performance.[19]

Here, we searched for novel compounds and additionally evaluated reported meat biomarkers in literature. We performed a human intervention trial focusing on various defined doses of meat (either chicken or beef) in a crossover design. Targeted quantitative LC-MS/MS was used to analyze plasma concentrations of 100 metabolites from different classes (amino acids, amines, dipeptides, and acylcarnitines). We looked at dose–response relationships, compared the plasma appearance after chicken and beef intake, and explored the kinetics of appearance

and disappearance of markers in plasma. Furthermore, we tested how well plasma analyte concentrations can predict a consumed amount of meat after different time points.

2. Experimental Section

2.1. Study Participants

The study protocol for testing foods and validation biomarkers was approved by the ethical committee of the Faculty of Medicine of the Technical University of Munich in Germany (approval no. 51/16S). The study was registered in the German Clinical Trial Register (DRKS00010133). Written informed consent was obtained from all participants before inclusion.

In total, 18 lean women and men (BMI 23.0 ± 3.8 kg m^{-2}) aged 22 to 37 years were recruited on a voluntary basis between February 2015 and July 2018 at the campus of the Technical University of Munich in Freising, Germany. The participants' eligibility was assessed and exclusion criteria were checked: BMI < 18.5 kg m^{-2}, smokers, gastro-intestinal diseases resulting in malabsorption and digestion, disease impairing metabolism and excretion, chronic illness, acute infection, history of chronic or infectious disease, intake of antibiotics in the previous 6 months, regular intake of medication, pregnancy or lactation, known allergies or intolerances to tested foods.

2.2. Study Design

Figure 1 summarizes the study design of the randomized controlled trial. Participants were invited to three separate appointments and received either 100 g sous-vide cooked meat (chicken breast/beef) or 200 g sous-vide cooked meat (chicken breast/beef) with 125 g rice or as a control food in a random order. Concerning the run-in period, participants of the study were asked to eat only vegetarian food. One day before intervention, participants received a standardized meal (STD Meal) containing 125 g rice, 30 g margarine, and 1.5 g salt. After an overnight fast, the first intervention day started with sampling of blood. Afterward, participants received either the test food or the STD Meal (see above). Regarding blood collection, sampling time points were before breakfast (= 0 h) and 1, 2, 6, 11, and

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24 h after breakfast (Figure 1). During the first 6 h, participants were asked to drink 250 mL of water every hour. Afterward, participants were allowed to drink water ad libitum. On the intervention day, participants received the STD Meal after 6 and 11 h. To ensure compliance during the intervention phase, participants received all foods in the Core Facility for Human Studies of the ZIEL—Institute for Food and Health in Freising, Germany.

2.3. Phenotyping

Anthropometric parameters (height, weight, waist-to-hip ratio) were measured in a highly standardized manner between 8 and 9 a.m. following an overnight fast. BMI was calculated by dividing body weight in kilogram by height in meter squared (kg m⁻²). Body weight and composition were measured using the TANITA Body Composition Analyzer Type BC-418 MA III (Amsterdam, The Netherlands).

2.4. Blood Sampling

Blood samples were collected in the fasting state. Liver enzymes (aspartate transaminase [AST], alanine transaminase [ALT], γ-glutamyltransferase [γ-GT]), creatinine, uric acid, bilirubin (total), and thyroid-stimulating hormone (TSH) were analyzed in serum by SynLab (Munich, Germany). Additionally, plasma (EDTA KE monovettes, Nümbrecht, Sarstedt) was collected and centrifuged at $1800 \times g$ for 10 min at 4 °C. Serum (Sarstedt monovettes) was collected, left for 20 min to allow clotting, and was finally centrifuged ($2500 \times g$ for 10 min at 4 °C). Plasma and serum were aliquoted and stored at −80 °C for later measurement of selected biochemical parameters.

2.5. Targeted Metabolite Profiling via LC-MS/MS

Quantitative analysis of amino acid and acylcarnitine concentrations was performed using LC-MS/MS based on the methods described earlier.[20,21] A complete list of all analytes in the measurement, including HPLC retention times, mass transitions, and MS settings, can be found in Table S1, Supporting information. Calibration data, accuracies, and precisions are found in Tables S2–S4, Supporting information, respectively. Briefly, 10 µL plasma was dissolved in 500 µL ice-cold methanol containing isotope-labeled internal standards (see Table S1, Supporting information). Samples were centrifuged (10 min, 4 °C, $3600 \times g$) and supernatants were dried using liquid nitrogen. Amino acids and acylcarnitines were derivatized to their butyl esters as described by Gucciardi et al. Briefly, a mixture of 95% n-butanol and 5% acetylchloride (v/v) was added to the samples. Samples were subsequently incubated at 60 °C for 15 min while shaken at 600 rpm (Eppendorf Thermomixer Comfort; Eppendorf, Hamburg, Germany). The samples were dried and reconstituted in a 300 µL mixture of methanol/water/formic acid (70/30/0.1% v/v).

The analysis was performed on a triple quadrupole QTRAP 5500 LC-MS/MS system operating in positive ESI mode (Sciex, Framingham, MA, USA) equipped with a 1200 series binary pump (Agilent, Santa Clara, CA) and coupled to an HTC pal autosampler (CTC Analytics, Zwingen, Switzerland). Chromatographic separation was achieved using a Zorbax Eclipse XDB-C18 column (length 150 mm, internal diameter 3.0 mm, particle size 3.5 µm; Agilent). Eluent A consisted of 0.1% formic acid, 2.5 mм ammonium acetate, and 0.01% heptafluorobutyric acid in water. Eluent B consisted of 10% Isopropanol, 0.1% formic acid, 2.5 mм ammonium acetate, and 0.01% heptafluorobutyric acid in acetonitrile. Amino acids and acylcarnitines were measured in two separate runs. The gradient elution programs for both runs are listed in Table S5, Supporting information. The measurement was performed in scheduled multiple reaction monitoring (sMRM). For absolute quantification of amino acids, a 10-point calibration of amino acid concentrations in different ranges depending on the physiological concentrations of the respective analyte was applied. Acylcarnitine concentrations were calculated based on analyte-to-internal standard area ratios and respective concentrations of internal standards. Data analysis was done using Analyst 1.7 software (Sciex).

2.6. Statistical Analysis

Data analysis was performed using the R software environment[22] and its base packages. For every analyte and every dose, the area under the curve per individual was calculated using the trapezoidal rule as a measure for the response to the meat consumption, resulting in 36 values for every analyte (3 doses × 12 individuals). These values were correlated with the corresponding amounts of meat consumed (12 × 0 g, 12 × 100 g, 12 × 200 g). The resulting Pearson correlation coefficient was used as a measure for the overall dose–response relationship for every single analyte.

To find analytes with a significantly different appearance in plasma after the consumption of chicken compared to the intake of beef, we compared the mean AUCs for the 200 g doses of both meat types. AUC values were non-normally distributed and variances were not homogenous (normality testing was done using the R package *MVN*), therefore a nonparametric test was applied (Kolgomorov–Smirnov, two-sided) to test the significance of the deviation in average AUC between chicken and beef intake.

For kinetic analysis, plasma levels were plotted in logarithmic scale. Peak concentrations in plasma were determined and the least square methods were used to determine elimination constants and half-lifes. In a second step, the elimination constants were utilized for fitting of the Bateman function.[23]

Finally, for chicken intake, prediction models at individual time points were calculated using linear regression (using the *lm()* package in R). This was done in a two-step process. In the first step, for every individual analyte in the analysis, linear regression was applied to determine the relationship between the analyte as dependent variable of the chicken dose (being the independent variable). Per single analyte, this relationship was then used to predict the actual dose. The individual dose predictions were correlated with the actual dose, determining Pearson correlations. In the second step, the 20 best correlating analytes were selected and used in multiple linear regression followed by stepwise variable selection in both directions, finding the model with
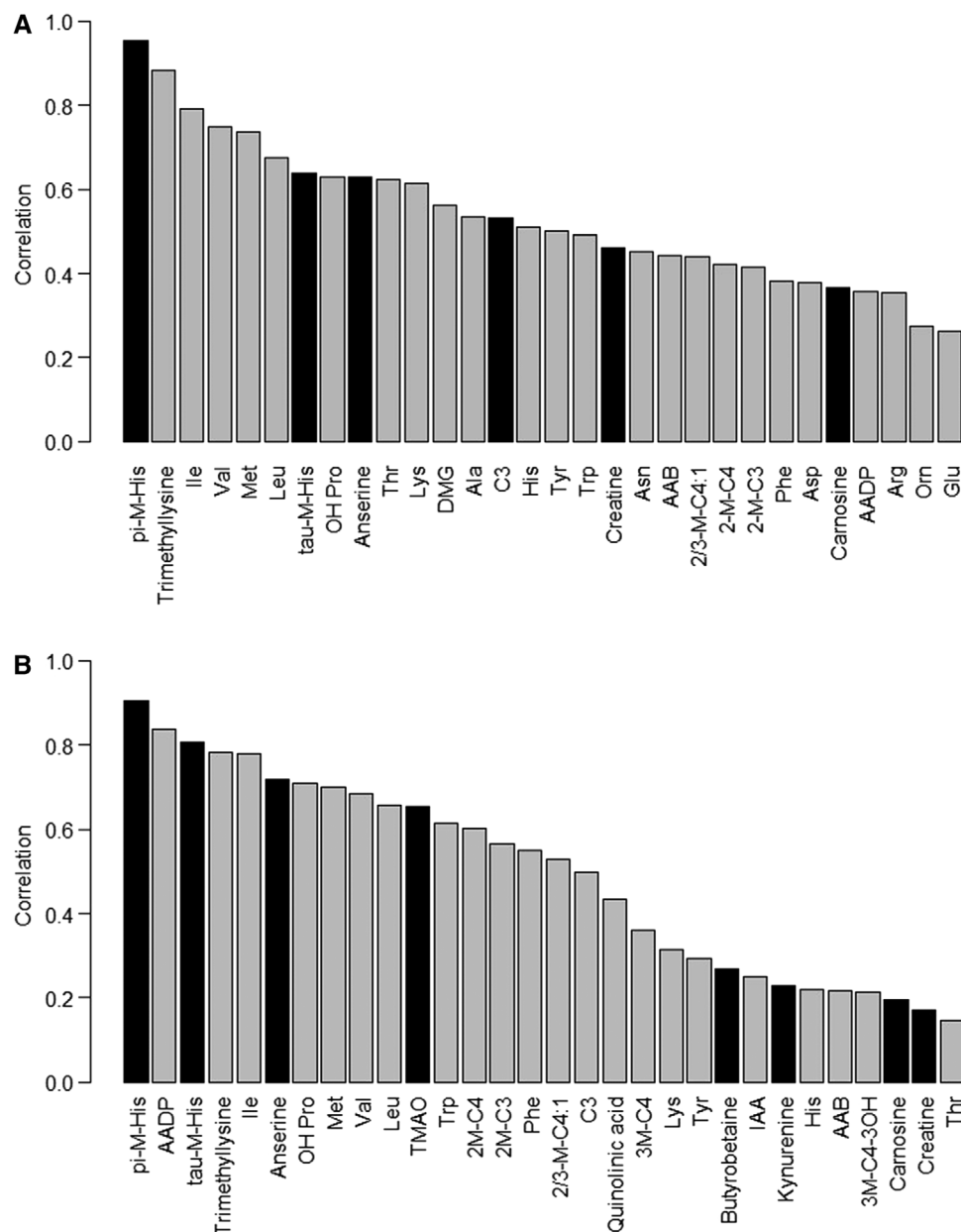
Figure 2. Bar plot ranking the top 30 analytes with the best correlation between meat dose and plasma response (area under the concentration curve in plasma) for A) chicken meat and B) beef.

the smallest Akaike information criterion (AIC). These models were then applied to predict the actual intake of chicken meat and the Pearson correlation of actual and predicted meat consumed was determined at each individual time point.

3. Results

3.1. Metabolites Displaying the Best Dose–Response Relationship in Plasma

To determine which analytes display the closest relationship between the amount of meat consumed and the analyte appearance in plasma, we calculated per dose the area under the plasma curve for each analyte. We then used correlation analysis and ranked the analytes according to the strength of the correlation between ingested dose of meat and the AUC response in plasma. **Figure 2** shows a ranking of the 30 analytes with the highest correlation between meat intake and plasma concentration after intake of chicken A) and beef B). Analytes that were previously suggested as putative meat markers are marked in black.

For both, intake of chicken and beef, π-M-His showed the strongest correlation between the dose ingested and the plasma response. Furthermore, τ-methylhistidin (τ-M-His), anserine, carnosine, and creatine were among the 30 highest correlating compounds in both chicken and beef arms. Additionally, for beef,

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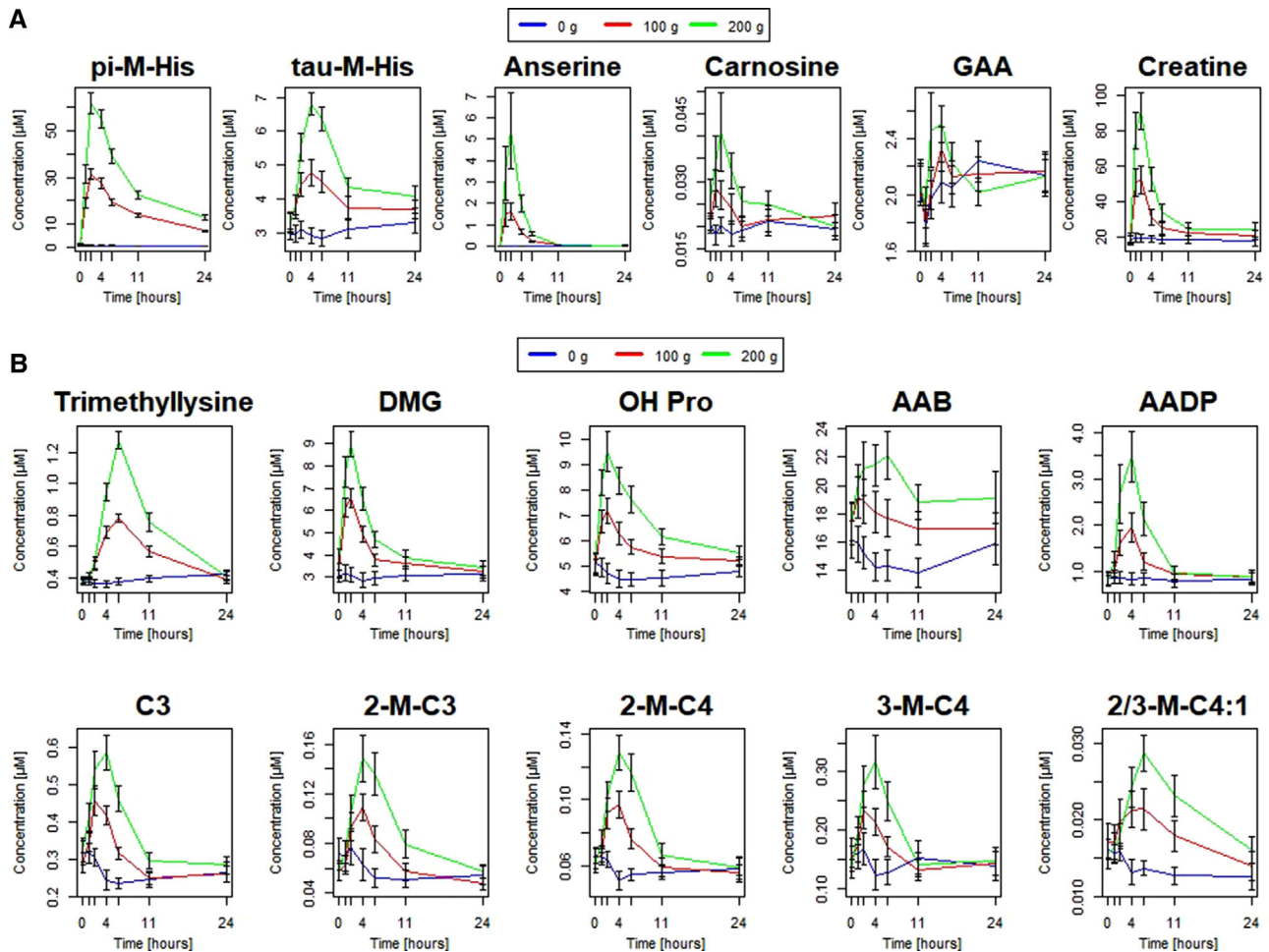Molecular Nutrition
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Figure 3. Course of metabolite concentrations after the ingestion of 0, 100, or 200 g chicken meat with A) displaying markers previously proposed and B) depicting other amino acid intermediates and acylcarnitines with strong dose–response relationships. Error bars display SEM values.

trimethylamine-*N*-oxide (TMAO), appeared with high a correlation coefficient larger than 0.7.

Besides the markers reported in literature, trimethyllysine was revealed as the second best entity in chicken and the fourth best in case of beef. In the beef trial, AADP ranked second, while for chicken, dimethylglycine (DMG) strongly correlated with the ingested dose. Furthermore, for both chicken and beef arms, strong dose-responses were found for essential amino acids and in addition for hydroxyproline (OH-Pro), BCAA-derived acylcarnitines.

Figure 3 shows the plasma response of selected analytes after intake of chicken meat (responses of all 30 analytes can be found in Figure S1, Supporting information). Shown are markers (Figure 3A) previously proposed as well as a variety of new entities, mainly amino acid intermediates that displayed strong dose–response relationships (Figure 3B). π-M-His, anserine, carnosine, and creatine concentrations reached peak values in plasma at 2 h, while τ-M-His peaked after 4 h. A dose-response could not be observed for GAA. Furthermore, we could identify DMG, trimethylglycine, hydroxyproline (OH-Pro), AADP, and AAB as well as acylcarnitines derived from BCAA breakdown with strong dose-responses in plasma.

As a more general phenomenon, we observed that metabolites from amino acid degradation had a retarded appearance compared to the precursor amino acid. Thus, metabolites more downstream in metabolic pathways appeared later in plasma than the compounds derived directly from the ingested food. This was most clearly seen for intermediates of Ile breakdown (Figure S5, Supporting information). Ile concentrations peaked in plasma 2 h after chicken meat intake, while its breakdown product 2-methylbutyrylcarnitine and the subsequent breakdown product 2-methylcrotonylcarnitine peaked 4 and 6 h after intake of meat, respectively.

At baseline, π-M-His and anserine were the only two analytes that were completely absent and remained absent after the ingestion of rice serving as a control. For the proteinogenic amino acids, a clear depression of plasma baseline values was observed after rice intake (Figure S1, Supporting information). This is likely a consequence of insulin release and its effects in inhibiting proteolysis and increasing amino acid uptake into insulin-sensitive tissues.[24] Accordingly, this decrease was not observed for τ-M-His and other non-proteinogenic analytes, like trimethyllysine and AADP. Even after 11 h
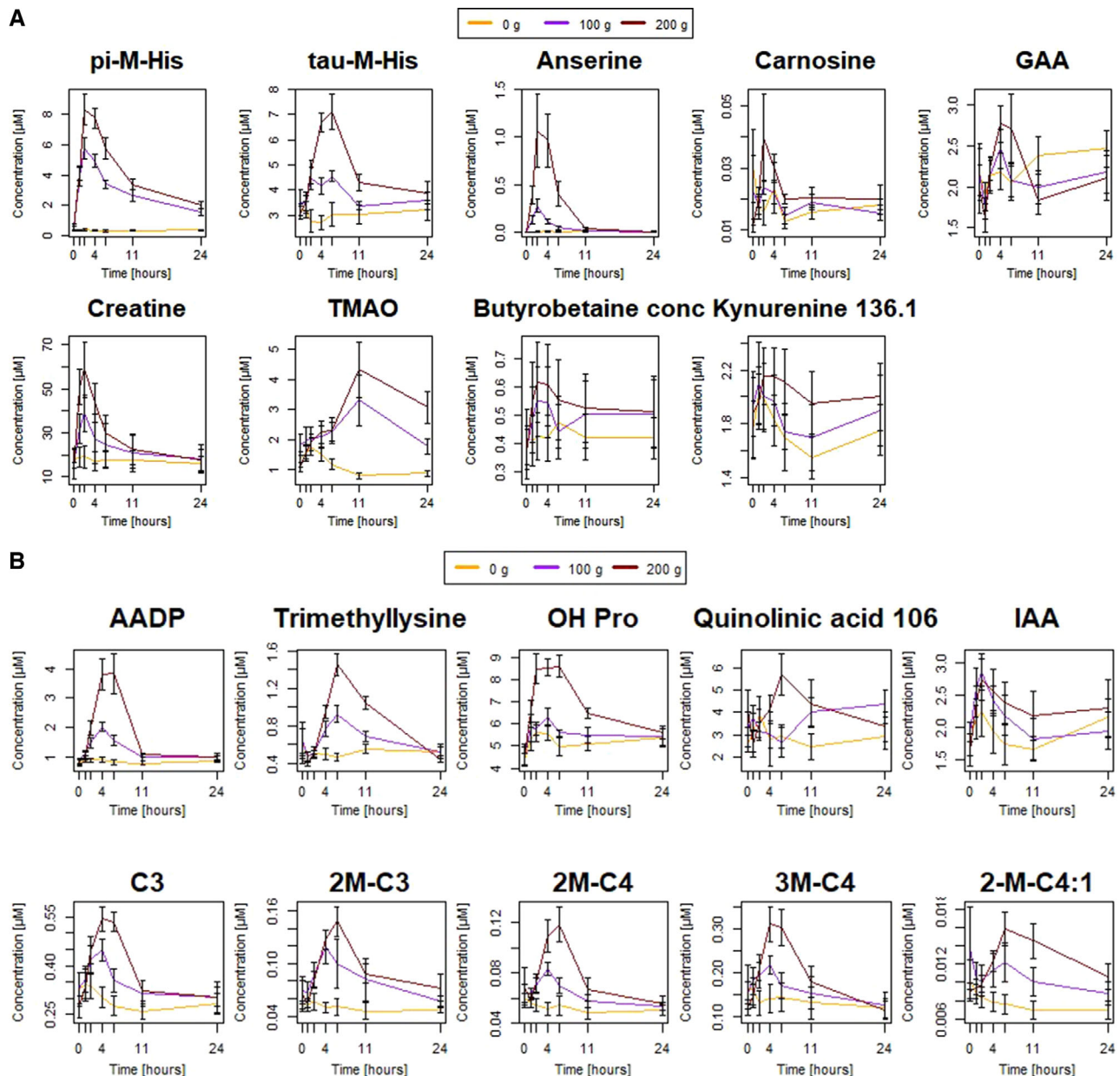
Figure 4. Course of metabolite concentrations after the ingestion of 0, 100, or 200 g beef with A) displaying markers previously proposed and B) depicting other amino acid intermediates and acylcarnitines with strong dose–response relationships. Error bars display SEM values.

following meat intake trimethyllysine, π-M-His, OH-Pro, and 2-methylcrotonylcarnitine (2-M-C4:1) levels in plasma showed clear differences between the different meat doses. After 24 h, a clear difference in plasma levels between the different meat doses could only be observed for π-M-His, which was significant between rice intake and either dose of chicken intake, as well as between the 100 and 200 g chicken dose.

Figure 4 shows the plasma response of selected analytes after intake of beef and Figure S2, Supporting information, summarizes the responses of all 30 analytes. π-M-His and anserine displayed similar plasma courses as observed in the chicken arms but concentrations were generally 4- to 6-times lower. A clear dose-response was also observed for τ-M-His, while the

effect for creatine was less clear. GAA failed to show a dose-response in plasma. Other beef-derived compounds were TMAO and butyrobetaine as well as the Trp-intermediate kynurenine. Here the clearest dose-response was observed for TMAO, with maximum plasma concentrations around 11 h after intake. In general, all analytes showed a later plasma peak as compared to chicken intake: most proteinogenic amino acids peaked at 4 h after beef intake compared to 2 h after chicken consumption (see Figures S1 and S2, Supporting information). Also, τ-M-His, and amino acid intermediates like OH-Pro, AADP, and the BCAA-derived carnitines peaked at 6 h after beef as compared to 4 h after chicken intake. Finally, TMAO produced in liver from TMA as an intermediate of carnitine, choline, tri- and

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Table 1. Analytes with the strongest differences in the plasma response (AUC) after intake of either 200 g chicken or 200 g beef.

Metabolite	AUC (200 g chicken)	AUC (200 g beef)	Significance (Kolgomorov–Smirnov, two-sided)
π-M-His	654.33	100.14	$4.6*10^{-4}$
Dimethylglycine	112.10	66.03	0.00275
Anserine	18.26	4.64	0.0293
TMAO	33.25	69.23	0.014

dimethylglycine degradation showed the latest plasma response all analytes with a peak reached after 11 h. Its dose-dependency is still detectable in plasma 24 h after beef intake.

3.2. Which Metabolites Discriminate Best between Chicken and Beef?

First, we noted, that nearly all markers considered as discriminative were present after both interventions, although to a different extent.

To find the analytes that best discriminate between chicken and beef intake in our experimental setting, we compared the plasma responses after consumption of 200 g chicken and 200 g beef and searched for the strongest differences in the corresponding plasma AUCs. Most significant differences in the plasma AUCs between chicken and beef intake were found for π-M-His, anserine, DMG, and TMAO, with p-values of $4.6*10^{-4}$, 0.0293, 0.0027, and 0.0137, respectively (see **Table 1**). For TMAO, despite the large average difference in the AUC between the consumed meat sources, the large variation between individuals reduced the

discriminative power for this analyte. In contrast, the high significance levels for π-M-His and anserine resulted mainly from their absence in fasting plasma and thus a lack of inter-individual variation.

Figure 5A shows the course of plasma levels for those analytes with the strongest differences in the response to the consumption of 200 g chicken breast versus 200 g beef. For anserine, π-M-His, and DMG, although an increase in concentrations could be seen for both test meals, the increase was much more prominent after chicken intake. In contrast to that, TMAO was the only analyte which only increased after beef consumption and remained above baseline even after 24 h.

Other analytes with a strong relationship between meat dose and plasma response showed similar plasma changes in both types of test meals (Figure 5B).

3.3. Plasma Kinetics of Selected Metabolites

For the analytes with the strongest dose–response relationships we explored their kinetics in plasma. Figure S6, Supporting Information, shows the plasma disappearance of selected analytes as logarithmic concentrations over time after chicken consumption (Figure S6A, Supporting information) and after beef intake (Figure S6B, Supporting information). For beef, owing to the retarded appearance of analytes in plasma, the 2- or 4-h measurement points were still during the absorption phase for a number of analytes. Elimination was not estimated for these analytes. Also, the limited rise in carnosine concentrations, in combination with a fast elimination, did not allow the determination of half-life. For the remaining analytes in Figure S6, Supporting information, we observed that logarithmic concentrations decreased linearly. This linear character argues for first-order kinetics in elimination. Notably, linearity holds true up to 12 h. Thereafter (at the 24-h
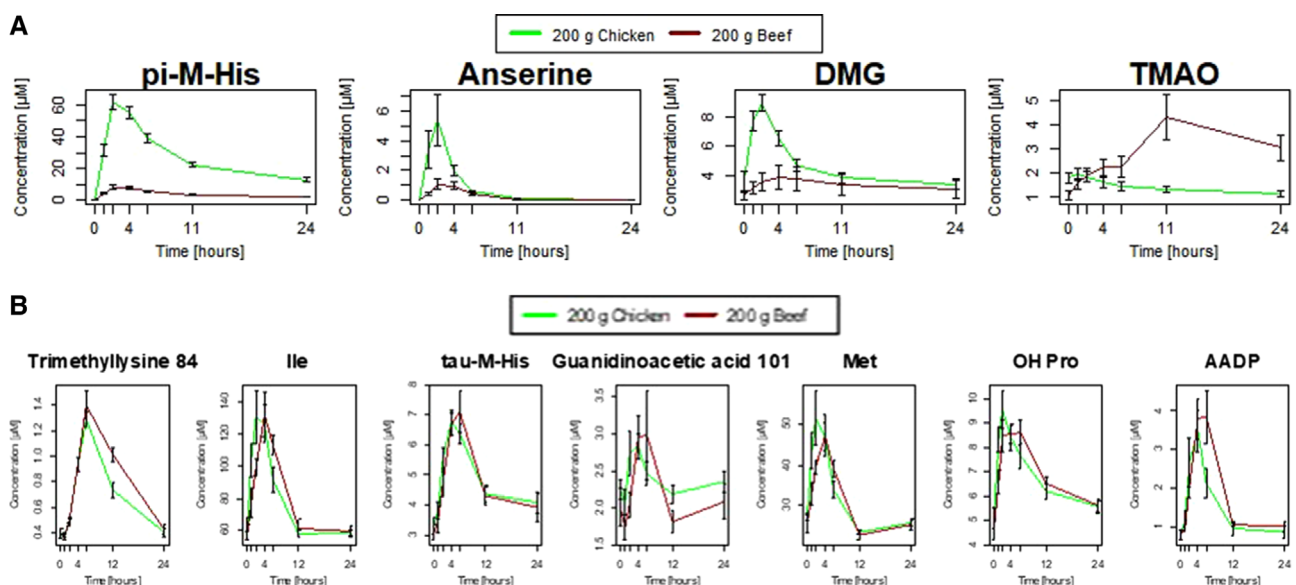

Figure 5. A) Appearance of analytes in plasma with the largest differences in the response comparing intake of either 200 g chicken or 200 g beef. Shown are the plasma metabolite responses after the intake of 200 g of chicken (green) and 200 g of beef (brown). Error bars display SEM values. B) Appearance of analytes in plasma with strong dose–response relationships with comparable changes after chicken and beef intake. Shown are the plasma metabolite responses after the intake of 200 g of chicken (green) and 200 g of beef (brown). Error bars display SEM values.

Table 2. Time range of elimination for the selected analytes with elimination constants and half-life values after either chicken or beef intake.

Metabolite	200 g chicken			200 g beef		
	Linear elimination [h]	K_{Elim}	$t_{1/2}$ (Elim)	Linear elimination [h]	K_{Elim}	$t_{1/2}$ (Elim)
π-M-His	2–11	0.118	5.89	4–11	0.116	5.95
Anserine	2h–11	0.506	1.37	4–11	0.466	1.49
Ile	2–11	0.096	7.19	4–11	0.112	6.21
Val	2–11	0.043	16.06	4–11	0.056	12.28
Leu	2–11	0.079	8.82	4–11	0.097	7.16
Asp	2–6	0.091	7.63	n.d.	n.d.	n.d.
τ-M-His	4–11	0.066	10.53	n.d.	n.d.	n.d.
C3	4–11	0.096	7.21	4–11	0.080	8.72
Creatine	2–6	0.247	2.81	2–6	0.167	4.16
AADP	4–11	0.179	3.86	4–11	0.195	3.56

n.d., not determined; $t_{1/2}$ (Elim) in hours.

time point), elimination rate seems to decrease, as the fitted line underestimates the actual concentrations measured in plasma. For the proteinogenic amino acids, this is because baseline levels are reached at the 24-h time point (see Figures 3 and 4). However, this cannot be the complete explanation as π-M-His, which was completely absent at baseline, is also underestimated at 24 h. A possible factor that might cause a deviation from linear excretion is a variation in the glomerular filtration rate. The period between the 12- and 24-h time points includes the night and it is known that glomerular filtration rate is reduced during nighttime.[25] Elimination of anserine and creatine was clearly faster than those of the proteinogenic amino acids. **Table 2** lists the individual elimination constants and half-life values of the analytes as well as the time range in which elimination was linear. Since we observed a first-order elimination for π-M-His and anserine, we next applied the Bateman function of invasion and elimination[23] to these analytes and fitted the plasma response after chicken intake. The fit according to Bateman also underestimated concentrations of π-M-His at the 24-h time point (Figure S3, Supporting information).

3.4. Can Kinetic Parameters Predict the Amount of Meat Consumed?

To determine how well the consumed meat dose can be predicted by plasma analyte concentrations, we performed linear regression analysis, using the dataset both as test and training data. The analysis was performed for individual sampling time points to explore early and late predictors and analytes were first individually used for prediction of meat dose. They were then ranked based on the correlation between predicted and actual meat dose consumed We then increased the predictive model by adding single analytes. Figure S4, Supporting Information, shows that, when using only the best predictor variable, already a dose prediction is achieved with a coefficient of correlation of 0.9.

To prevent overfitting, we next applied stepwise variable selection to determine which combination of metabolites best estimates the consumed meat dose. The regression models are listed in **Table 3**. For all time points, π-M-His was found to be part of the prediction model. Furthermore, the models for early time points contained amino acids, while the models for the later time points consised mainly of amino acid breakdown products and acylcarnitines. We used the prediction model based on 24 h-plasma concentrations and estimated the actual meat dose consumed (**Figure 6**). For the 100 g dose, our estimation is within a 20% inaccuracy for 8 out of 12 subjects (for three subjects, the dose was overestimated at 124%, 128%, and 163% and for one subject, the dose was underestimated at 74%). For the 200 g dose this is the case for 10 out of 12 subjects (for one subject, the dose was overestimated at 128% and for one subject, the dose was underestimated at 78%).

4. Discussion

A number of metabolites have been postulated to function as biomarkers for meat consumption. A recent study compared chicken, beef, and pork intake as well as a nonmeat protein source and evaluated the metabolite response in urine by applying untargeted metabolomics.[19] A set of compounds was acquired with high selectivity for the meat type. These compounds were then used by the authors to set up classification models for the estimation of the type of meat consumed. We here extend this study by looking at dose–response relationships for previously suggested biomarkers and also novel compounds. We used a targeted quantitative metabolite profiling to assess the response of the compounds in plasma upon consumption of chicken and beef. The metabolite profiling included dipeptides, amino acids, amino-acid derivatives, and acylcarnitines to evaluate known meat markers and candidate meat markers from amino acid metabolism. Compounds were evaluated based on dose-dependency, specificity, and selectivity to meat type. Moreover, we determined the kinetics of plasma appearance and disappearance of various markers and used regression models based on selected marker metabolites for estimation of the amount of meat consumed from plasma concentrations.

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Table 3. Regression models for the prediction of chicken meat intake.

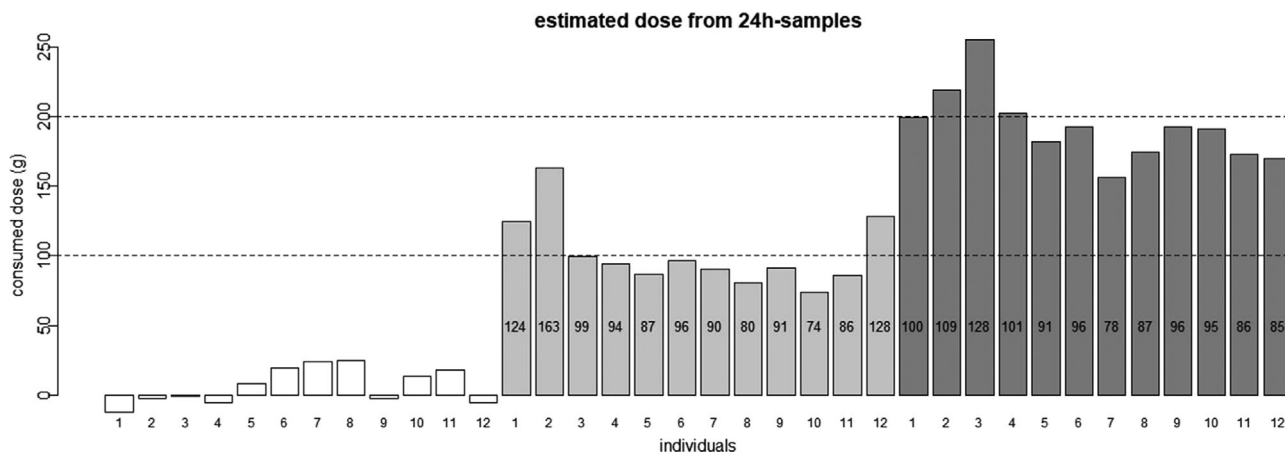|	Analytes in the regression model	Correlation (pred. vs actual dose)
Prediction after 4 h	π-M-His + trimethyllysine + Ile + Val + His	0.981
Prediction after 6 h	trimethyllysine + π-M-His + anserine + 2-M-C4 + Leu + 2-M-C3 + Thr + Lys + 3-M-C4	0.983
Prediction after 12 h	π-M-His + Thr + C4-OH b + C4:1 + DMG + Ala + C14:1 + C16:1 + C4-OH a	0.976
Prediction after 24 h	π-M-His + τ-M-His + kynurenine + C5-OH + Asp + C4:1 + Tyr + creatine	0.963

Figure 6. Prediction of meat dose consumed from blood samples collected 24 h after consumption. White, light gray, and dark gray columns show the prediction in samples from an actual consumption of 0, 100, and 200 g of chicken meat, respectively. The numbers in the gray bars show the percentage of over- or underestimation as compared to the actual consumed dose.

4.1. π-M-His and Anserine Best Reflect Consumed Meat Dose

Our findings show that π-M-His and anserine best reflect the amount of chicken meat consumed. This is in line with previous results proposing these two compounds as specific meat markers.[12,19] Interestingly, these compounds also reflect best the dose of beef consumed in our study, although at much lower plasma levels. It has been shown that bovine species also contain anserine although much less than birds.[14] The superior quality of metabolites like π-M-His and anserine as markers of meat consumption is obviously owing to their absence in human plasma in a fasting state. This would otherwise cause additional variation in the plasma levels by inter-individual differences in fasting levels, blurring the estimation of dietary intake. Anserine appears to be the main source of π-M-His released by peptidases without further intracellular metabolism and almost complete excretion via urine.[26] π-M-His is not used in proteinogenesis, due to the lack of complementing tRNA. This specific metabolic fate of anserine/π-M-His may explain the good fit of the Bateman function (Figure S1, Supporting information) to the corresponding plasma levels. The elimination of π-M-His from plasma occurred with a half-life of ≈ 5.9 h and was independent of dose and meat type and even after 24 h π-M-His concentrations in plasma were still detectable and still revealed differences between the meat doses consumed. Plasma concentrations of anserine are in a lower range and the half-life of anserine is much shorter. The fast disappearance of anserine is likely owing to the high hydrolytic activity serum carnosinase, degrading anserine to beta-alanine and π-M-His.[27]

4.2. τ-M-His, Trimethyllysine, AADP, Hydroxyproline, and Essential Amino Acids Increase Dose-Dependently but Show No Specificity

It is obvious that the intake of meat causes dramatic changes in plasma amino acid levels and changes in various amino acid breakdown products. Although we observed that concentrations of most of these compounds increase to a similar extent after chicken or beef intake, in case of beef the peak plasma concentrations were reached slightly later then after chicken intake. The cause for this delay remains elusive. One possibility might be that the fat content, generally described to be higher in beef tenderloin as compared to chicken breast,[28] causes a delay in gastric emptying and consequently delays absorption of digestion products.[29] This hypothesis, however, needs further studies, as we have no data on the actual fat content of the foods in this study. Furthermore, while proteinogenic amino acids reach their maximum concentration at 2 and 4 h after intake of chicken and beef, respectively, amino acid breakdown products and amino acid-derived acylcarnitines generally peak later between 4 and 6 h after intake. In the rice-based control meal—low in protein—proteinogenic amino acids show a decline in plasma levels by inhibition of proteolysis and increased uptake into tissues mediated by the increase in plasma insulin. This decline of basal plasma levels was not observed for non-proteinogenic amino acid intermediates like alpha-amino adipic acid and trimethyllysine, suggesting that insulin mainly affects protein turnover into and from single amino acids, but affects amino acid breakdown to a lesser extent. These amino acid

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intermediates might therefore be interesting candidates for the assessment of protein intake, as they have generally low baseline levels, that are less variable compared to the proteinogenic amino acids.

4.3. TMAO and Compounds Linked to Carnitine Metabolism Increase to a Larger Extent after Beef Intake

TMAO was the only analyte that increased after beef intake but not after chicken intake. It is a breakdown product of choline, lecithin, and carnitine. L-carnitine levels were previously shown to be higher in beef as compared to chicken and higher in red compared to white chicken muscle, relating L-carnitine concentrations in muscle to mitochondrial oxygen metabolism and myofiber type.[30] Besides red meat, TMAO was shown to increase after intake of dairy products and salt-water fish.[17] Thus, TMAO lacks the specificity as a food intake marker for beef, but it might be of interest for understanding health effects described for red meat. With respect to the increased risk of colonic cancer and type 2 diabetes associated with red meat intake, TMAO was suggested as a therapeutic target for insulin resistance and cancer.[31] In comparison to most other analytes, TMAO peaks very late in plasma ($\approx$12 h) where it discriminates best the intake of both meat types. This late plasma peak is likely the result of a more complex and longer-lasting process of TMAO formation from its precursors involving the concerted action of hepatic enzymes and the gut microbiome.[32,33] This may also cause the larger inter-individual variation that we observed. In line with this, the carnitine metabolites γ-butyrobetaine and trimethyllysine also increased to a larger extent after the consumption of beef in comparison to chicken meat.

Amongst the acylcarnitines, only those derived from amino acid degradation and most prominently propionylcarnitine, revealed dose-dependent changes while medium- and long-chain species—derived from fatty acid breakdown—did not. This is in line with previous studies and is an obvious reflection of protein load when ingesting meat.[17,34] Consequently, consumption of other protein sources like dairy products also cause an increase in the concentrations of these acylcarnitines and they are thus not specifically marking the intake of meat.[35]

In essence, we thus could not find any compound that is specific at its own to function as food intake marker for beef. To achieve accurate estimations of the amount of red meat consumed, novel compound classes clearly need to be investigated. Recently, Cuparencu et al.[19] found a number of putative red meat intake markers, mainly hydroxyproline-containing di- and tri-peptides that were not part of our analysis. These might be a good starting point for further study of dose-dependent red meat markers to improve estimation of red meat dose.

4.4. Dimethylglycine Discriminates Chicken and Beef Intake at Early Time Points and May Originate from Broiler Feed

DMG was increased to a much larger extent after the intake of chicken meat when compared to beef. As a metabolite of choline, it is present in all plants and animals and it seems not specific

for the chicken metabolome. It might therefore perhaps be surprising to find large differences in plasma appearance of DMG among different meat types. A possible explanation might be that DMG was present in the broiler feed. DMG was found to improve nutrient digestibility and to reduce broiler ascites syndrome.[36] The presence of DMG as a broiler feed additive for the chickens from which the meat was derived cannot be excluded in this study.

4.5. Relevance for Diet Intake Assessment

Marker metabolites that associate with the intake of specific food items should provide better measures of diet than classical questionnaire methods. In addition, they may provide a mechanistic understanding for the empirical associations found between good or bad health and intake of particular food items or categories. The latter is exemplified by metabolites like TMAO[32] and dityrosine, that were shown to promote atherosclerosis and to relate to oxidative stress,[16,37] respectively, thus linking red meat intake mechanistically to adverse health effects.

We assessed the quality of described meat intake markers for selectivity and specificity and whether they can quantitatively predict the amount of meat consumed. It should be pointed out that our comparative study of chicken and beef intake does not allow a confirmation of the specificity of meat intake markers. It rather aimed to evaluate whether suggested specificities for markers truly hold when comparing a red and white meat source. Obviously, a large number of compounds investigated here are present in other protein sources besides meat, like dairy products and plant-based protein sources. According to the recent classification by Maruvada et al., they are referred to as food component intake biomarkers (FCIBs).[38] To assure the specificity of food markers, a thorough understanding as to why compounds are specific to a certain food source. Also, food composition databases need significant expansion to be able to make conclusions about specificity. The two compounds for which the literature provides a plausibility on specificity for poultry meat are π-M-His and anserine. We used these two compounds and combined them with nonspecific but dose-dependent plasma metabolites to generate linear regression models for different time windows after intake. Most relevant for the prediction at any given time point was π-M-His, while anserine and trimethyllysine were particularly relevant at early time points. GAA, previously suggested as a urinary marker for chicken intake,[18] appeared here not relevant for the prediction based on plasma samples and GAA levels increased as well after beef intake. For the 24-h model, that seems particularly interesting as the time point furthest away from the actual consumption, we could reach a correlation between predicted and actual dose consumed of 0.96. At the level of an individual, this translates into an over- or under-estimation of intake of maximal 20%. Such a prediction of meat dose, as performed here, can of course only be achieved when a single meal is consumed and the approach is dramatically compromised when multiple meals over a 24 h period are consumed as under real-life conditions. However, these new approaches by using metabolite profiling for food intake assessment are still in its infancy and need more conceptual approaches.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

P.G. and B.B. contributed equally to this work. P.G., B.B., and T.S. wrote the manuscript. T.S. and H.D. conceived the experiments. All authors critically revised the manuscript. Y.M.L. and B.B. performed the experiments. P.G. performed the analytics.

[1] D. S. Chan, R. Lau, D. Aune, R. Vieira, D. C. Greenwood, E. Kampman, T. Norat, *PLoS One* **2011**, *6*, e20456.

[2] C. A. Gonzalez, P. Jakszyn, G. Pera, A. Agudo, S. Bingham, D. Palli, P. Ferrari, H. Boeing, G. del Giudice, M. Plebani, F. Carneiro, G. Nesi, F. Berrino, C. Sacerdote, R. Tumino, S. Panico, G. Berglund, H. Simán, O. Nyrén, G. Hallmans, C. Martinez, M. Dorronsoro, A. Barricarte, C. Navarro, J. R. Quirós, N. Allen, T. J. Key, N. E. Day, J. Linseisen, G. Nagel, et al., *JNCI: J. Natl. Cancer Inst.* **2006**, *98*, 345.

[3] S. C. Larsson, A. Wolk, *Br. J. Cancer* **2012**, *106*, 603.

[4] U. Ericson, E. Sonestedt, B. Gullberg, S. Hellstrand, G. Hindy, E. Wirfält, M. Orho-Melander, *Br. J. Nutr.* **2013**, *109*, 1143.

[5] A. Steinbrecher, E. Erber, A. Grandinetti, L. N. Kolonel, G. Maskarinec, *Public Health Nutr.* **2011**, *14*, 568.

[6] R., Sinha, A. J. Cross, B. I. Graubard, M. F. Leitzmann, A. Schatzkin, *Arch. Intern. Med.* **2009**, *169*, 562.

[7] M. Rondanelli, M. Faliva, F. Monteferrario, G. Peroni, G. Peroni, E. Repaci, F. Allieri, S. Perna, *Biomed. Res. Int.* **2015**, *2015*, 524948.

[8] K. J. Joshipura, F. B. Hu, J. E. Manson, M. J. Stampfer, E. B. Rimm, F. E. Speizer, G. Colditz, A. Ascherio, B. Rosner, D. Spiegelman, W. C. Willett, *Ann. Intern. Med.* **2001**, *134*, 1106.

[9] K. Poslusna, J. Ruprich, J. H. de Vries, M. Jakubikova, P. van't Veer, *Br. J. Nutr.* **2009**, *101*, S73.

[10] M. M. Ulaszewska, C. H. Weinert, A. Trimigno, R. Portmann, C. Andres Lacueva, R. Badertscher, L. Brennan, C. Brunius, A. Bub, F. Capozzi, M. Cialiè Rosso, C. E. Cordero, H. Daniel, S. Durand, B. Egert, P. G. Ferrario, E. J. M. Feskens, P. Franceschi, M. Garcia-Aloy, F. Giacomoni, P. Giesbertz, R. González-Domínguez, K. Hanhineva, L. Y. Hemeryck, J. Kopka, S. E. Kulling, R. Llorach, C. Manach, F. Mattivi, C. Migné, et al., *Mol. Nutr. Food Res.* **2019**, *63*, 1800384.

[11] S. Krug, G. Kastenmuller, F. Stuckler, M. J. Rist, T. Skurk, M. Sailer, J. Raffler, W. Römisch-Margl, J. Adamski, C. Prehn, T. Frank, K. H. Engel, T. Hofmann, B. Luy, R. Zimmermann, F. Moritz, P. Schmitt-Kopplin, J. Krumsiek, W. Kremer, F. Huber, U. Oeh, F. J. Theis, W. Szymczak, H. Hauner, K. Suhre, H. Daniel, *FASEB J.* **2012**, *26*, 2607.

[12] J. Sjolin, G. Hjort, G. Friman, L. Hambraeus, *Metabolism* **1987**, *36*, 1175.

[13] K. Bachmann, R. Galeazzi, A. Millet, A. G. Burger, *Metabolism* **1984**, *33*, 107.

[14] A. A. Boldyrev, G. Aldini, W. Derave, *Physiol. Rev.* **2013**, *93*, 1803.

[15] C. Stella, B. Beckwith-Hall, O. Cloarec, E. Holmes, J. C. Lindon, J. Powell, F. van der Ouderaa, S. Bingham, A. J. Cross, J. K. Nicholson, *J. Proteome Res.* **2006**, *5*, 2780.

[16] C. Rombouts, L. Y. Hemeryck, T. Van Hecke, S. De Smet, W. H. De Vos, L. Vanhaecke, *Sci. Rep.* **2017**, *7*, 42514.

[17] W. Cheung, P. Keski-Rahkonen, N. Assi, P. Ferrari, H. Freisling, S. Rinaldi, N. Slimani, R. Zamora-Ros, M. Rundle, G. Frost, H. Gibbons, E. Carr, L. Brennan, A. J. Cross, V. Pala, S. Panico, C. Sacerdote, D. Palli, R. Tumino, T. Kühn, R. Kaaks, H. Boeing, A. Floegel, F. Mancini, M. C. Boutron-Ruault, L. Baglietto, A. Trichopoulou, A. Naska, P. Orfanos, A. Scalbert, *Am. J. Clin. Nutr.* **2017**, *105*, 600.

[18] X. Yin, H. Gibbons, M. Rundle, G. Frost, B. A. McNulty, A. P. Nugent, J. Walton, A. Flynn, M. J. Gibney, L. Brennan, *J. Nutr.* **2017**, *147*, 1850.

[19] C. Cuparencu, A. Rinnan, L. O. Dragsted, *Mol. Nutr. Food Res.* **2019**, *63*, https://doi.org/10.1186/s12263-019-0656-4.

[20] U. Harder, B. Koletzko, W. Peissner, *J. Chromatogr. B* **2011**, *879*, 495.

[21] P. Giesbertz, J. Ecker, A. Haag, B. Spanier, H. Daniel, *J. Lipid Res.* **2015**, *56*, 2029.

[22] R Development Core Team, *R: A Language and Environment for Statistical Computing*, R Foundation for Statistical Computing, Vienna **2009**.

[23] H. Bateman, *Proc. Cambridge Phil. Soc.* **1910**, *15*, 423.

[24] T. Skurk, I. Rubio-Aliaga, A. Stamfort, H. Hauner, H. Daniel, *Metabolomics* **2011**, *7*, 388.

[25] M. G. Koopman, G. C. M. Koomen, R. T. Krediet, L. Arisz, *Kidney Int.* **1989**, *35*, 470.

[26] J. H. Butt, B. Fleshler, *Exp. Biol. Med.* **1965**, *118*, 722.

[27] K. J. Yeum, M. Orioli, L. Regazzoni, M. Carini, H. Rasmussen, R. M. Russell, G. Aldini, *Amino Acids* **2010**, *38*, 847.

[28] J. F. Hocquette, F. Gondret, E. Baeza, F. Medale, C. Jurie, D. W. Pethick, *Animal* **2010**, *4*, 303.

[29] D. Gentilcore, R. Chaikomin, K. L. Jones, A. Russo, C. Feinle-Bisset, J. M. Wishart, C. K. Rayner, M. Horowitz, *J. Clin. Endocrinol. Metab.* **2006**, *91*, 2062.

[30] K. Shimada, Y. Sakuma, J. Wakamatsu, M. Fukushima, M. Sekikawa, K. Kuchida, M. Mikami, *Meat Sci.* **2004**, *68*, 357.

[31] J. Oellgaard, S. A. Winther, T. S. Hansen, P. Rossing, B. J. von Scholten, *Curr. Pharm. Des.* **2017**, *23*, 3699.

[32] R. A. Koeth, Z. Wang, B. S. Levison, J. A. Buffa, E. Org, B. T. Sheehy, E. B. Britt, X. Fu, Y. Wu, L. Li, J. D Smith, J. A. DiDonato, J. Chen, H. Li, G. D Wu, J. D Lewis, M. Warrier, J. Mark Brown, R. M. Krauss, W H W. Tang, F. D. Bushman, A. J. Lusis, S. L. Hazen, *Nat. Med.* **2013**, *19*, 576.

[33] Z. Wang, B. S. Levison, J. E. Hazen, L. Donahue, X. M. Li, S. L. Hazen, *Anal. Biochem.* **2014**, *455*, 35.

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[34] M. Rousseau, F. Guenard, V. Garneau, B. Allam-Ndoul, S. Lemieux, L. Pérusse, M. C. Vohl, *Nutrients* **2019**, *11*, 173.

[35] A. S. Hoefle, A. M. Bangert, A. Stamfort, K. Gedrich, M. J. Rist, Y. M. Lee, T. Skurk, H. Daniel, *J. Nutr.* **2015**, *145*, 467.

[36] I. D. Kalmar, M. W. A. Verstegen, D. Vanrompay, K. Maenner, J. Zentek, C. Iben, R. Leitgeb, A. Schiavone, L. Prola, G. P. J. Janssens, *Livestock Sci.* **2014**, *164*, 81.

[37] M. Estevez, *Meat Sci.* **2011**, *89*, 259.

[38] P. Maruvada, J. W. Lampe, D. S. Wishart, D. Barupal, D. N. Chester, D. Dodd, Y. Djoumbou-Feunang, P. C. Dorrestein, L. O. Dragsted, J. Draper, L. C. Duffy, J. T. Dwyer, N. J. Emenaker, O. Fiehn, R. E. Gerszten, F. B Hu, R. W. Karp, D. M. Klurfeld, M. R. Laughlin, A. R. Little, C. J. Lynch, S. C. Moore, H. L. Nicastro, D. M. O'Brien, J. M. Ordovás, S. K. Osganian, M. Playdon, R. Prentice, D. Raftery, N. Reisdorph, et al., *Adv. Nutr.* **2019**, https://doi.org/10.1093/advances/nmz075.