

Estimation of chicken intake using metabolomics derived markers

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Abbreviations used: NANS, National Adult Nutrition Survey; OPLS-DA, orthogonal partial least-squares discriminant analysis; PCA, principal components analysis; PLS-DA, partial least-squares discriminant analysis; PhIP, 2-amino-1-methyl-6-phenylimidazo [4, 5-b] pyridine; ROC, receiver operating characteristic; 1-methylhistidine, 1-Meth-His; 3-methylhistidine, 3-Meth-His.

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Abstract

Background: Improved assessment of meat intake using metabolomics derived markers can provide objective data and could be helpful in clarifying proposed associations between meat intake and health.

Objective: The objective was to identify novel markers of chicken intake using a metabolomics approach, and use markers to determine intake in an independent cohort.

Methods:

Ten participants (age, 62 y; BMI, 28.25 Kg/m²) in NutriTech Food Intake Study (NCT01684917) consumed increased amounts of chicken from 88 to 290 g/day over three weeks. Urine and blood samples were analyzed by NMR and MS, respectively. Multivariate data analysis was performed to identify markers associated with chicken intake. A calibration curve was built based on dose response association using NutriTech data. Bland and Altman analysis evaluated the agreement between reported and calculated chicken intake in National Adult Nutrition Survey (NANS) cohort.

Results: Multivariate data analysis of postprandial and fasting urine samples collected in NutriTech revealed good discrimination between high (290 g/day) and low (88 g/day) chicken intakes. Urinary metabolite profiles showed differences in metabolite levels between low and high chicken intakes. Examining metabolite profiles revealed guanidoacetate significantly increased from 1.47 to 3.66 mmol/L following increasing chicken intake from 88 to 290 g/day ($P < 0.01$). Using a calibration curve developed from NutriTech study, chicken intake was calculated in NANS, where chicken consumers had higher guanidoacetate excretion (0.70 mmol/L) than non-consumers (0.47 mmol/L) ($P < 0.01$). Bland and Altman analysis revealed good agreement between reported and calculated intakes with a bias of -30.2g/day. Plasma metabolite analysis demonstrated that 3-methylhistidine (3-Meth-His) was a more suitable indicator of chicken intake compared with 1-methylhistidine (1-Meth-His).

26 **Conclusions:** Guanidoacetate was successfully identified and confirmed as a marker of
27 chicken intake, and importantly its measurement in fasting urine samples could be used to
28 determine chicken intake in a free-living population.

29

30 **Keywords:** metabolomics, dietary markers, guanidoacetate, estimated chicken intake, 3-
31 methylhistidine

Introduction

Meat is widely consumed globally and is an important contributor to dietary protein. Meat intake has the potential to influence a number of nutrition and health outcomes (1). Over the past several decades, the associations between meat consumption and disease incidence and mortality have been evaluated in a number of epidemiological studies (2, 3). A number of studies reported that meat consumption interacts with various ongoing metabolic processes to increase or decrease the incidence of diseases such as diabetes, obesity, heart disease, and cancer (4-8). From these and other studies the type of meat appears to be important with some studies reporting beneficial effects of white meat such as chicken while other reports indicate that red meat or processed meat intake is associated with an increased risk of rectal cancer or colon cancer (6, 9, 10). Therefore, it has now become imperative to obtain reliable and accurate dietary assessment for the different types of meat in order to examine the associations between meat intake and disease risk.

Traditional dietary assessment methods include FFQs, 24 h dietary recalls, and weighed food diaries (11). These methods are based on self-reporting and can be subject to errors including recall bias, difficulty in assessing portion sizes and energy underreporting (12, 13). Dietary measurement errors can result in misreporting dietary intake and also attenuate the associations between food intake and disease risk in epidemiological studies (14). Therefore, there is an increased interest in developing new approaches for objective measures of dietary intake. One such approach is the use of dietary markers to provide a more objective measurement of intake (15). To date, many studies have identified putative dietary markers of exposure for many foods (15-18), including citrus fruit (19), coffee (20), red meat(21).

Metabolomics has played a key role in the discovery of dietary markers. This comprehensive analysis of small molecule metabolites in biofluids (including urine and blood) represents an ideal method for the discovery of dietary markers (16, 22, 23). In recent years, there have

been a number of studies examining dietary markers for red meat intake using metabolomics approaches. For example, creatinine, creatine, carnitine, carnosine, taurine, 1-methylhistidine (1-Meth-His) and 3-methylhistidine (3-Meth-His) have been put forward as putative markers of red meat intake (21, 24). With respect to white meat consumption, fewer studies have been reported. Nonetheless the following metabolites have emerged as potential markers: pyroglutamine, 2-amino-1-methyl-6-phenylimidazo [4, 5-b] pyridine (PhIP). However, there is a lack of validation of these markers of chicken intake (16, 25), and no study has demonstrated that these markers can accurately determine chicken intake. To this end the objective of the present study was to use a metabolomics approach to identify and confirm markers related specifically to chicken consumption and to determine chicken intake in an independent cohort.

Subjects and methods

NutriTech study design

The NutriTech food intake study was designed to detect markers of different food group intake using an untargeted metabolic profiling approach in a human nutrition intervention study. Ethical approval was received from London Brent Ethics Committee (reference number: 12/LO/0139). Participants attended the NIHR/Wellcome Trust Imperial Clinical Research Facility for three days over three consecutive weeks. Eligibility criteria included healthy males and females of all ethnicities, aged between 18 and 65 y with a BMI of 18.5-35 kg/m² and free from any chronic medical condition. Participants (n=50) were randomized into one of five different treatment groups including red meat, fish, chicken, processed meat, and vegetarian groups, and each group had 10 participants. The detailed NutriTech study participant flow chart can be seen in **Supplemental Figure 1**. In the present study we focus on the chicken group. In total, 5 men and 5 women with a mean age (\pm SEM) of 62 ± 1 y and

a mean BMI (\pm SEM) of 28.25 ± 1.25 Kg/m² were randomly assigned to the chicken group (see **Table 1**).

Participants were given set meals for breakfast (8am), lunch (12am) and evening meals (7pm) for three days (day 1, 2, and 3) during a week and this was repeated for three weeks (week 1, 2, and 3). The period between each week was minimum 3 days. In each group, the test food intake increased from week 1 to week 3 (see chicken intake in **Supplemental Table 1**). Leftovers were measured and recorded where appropriate.

Biological samples were collected over the course of the three days during each week. On day 1 no samples were collected. On day 2 a 24 h urine sample was collected and during this period all voids were collected in a single container which was kept chilled throughout. At the end of collection the urine was inverted 5 times and 50 mL was removed and processed. On day 3, after the 8 am void, participants were only allowed to urinate at 0 h (void immediately before the midday meal at 11.55 am), 2 h (spot sample 2 hours after the midday meal) and 6 h (spot sample 6 hours after the midday meal). They were asked not to urinate outside these designated times, and these spot urine samples were collected and kept on ice until processed, respectively. Blood samples were also collected at 0 h, 2 h, and 6 h; 4 mL of blood was collected in an EDTA tube and 10 mL of blood was collected in a lithium heparin tube. On day 4, the fasting morning urine and blood samples were collected. The schematic representation of biofluid collection is shown in **Supplemental Figure 2**. All urine samples were processed within 30 minutes of collection, centrifuged at 1800 x g for 10 min at 4 °C, and 1 mL aliquots were stored at -80 °C for NMR analysis. All blood samples were inverted 8 times, centrifuged at 1800 x g for 10 mins at 4 °C, and 500 μ L aliquots were stored at -80 °C until subsequent analysis.

NANS study design

The confirmation study was performed using data from NANS. NANS investigated habitual food and nutrient consumption, lifestyle, health indicators and attitudes to food and health in a representative sample of 1500 adults aged between 18 and 90 years in the Republic of Ireland during 2008-2010 (26). Ethical approval was obtained from the University College Cork Clinical Research Ethics Committee of the Cork Teaching Hospitals and the recruitment began in May 2008. Dietary intake was measured by using a 4-d semi-weighed food record (27). Participants were asked to record detailed information on the amount and type of all foods, drinks and nutritional supplements consumed over four consecutive days in a food diary. A fasting first-void urine sample was collected. Urine samples from 565 NANS participants, randomly selected from the main NANS database ensuring equal numbers of men and women across the age range, were analyzed by ^1H NMR. For the purpose of this study, the chicken consumers and non-consumers were selected from 565 NANS participants, and their fasting urinary spectra were used to identify and quantify the dietary markers.

Urine sample analysis- ^1H NMR spectroscopy

Urine samples were prepared by addition of 250 μL phosphate buffer (0.2 mol $\text{KH}_2\text{PO}_4/\text{L}$, 0.8 mol $\text{K}_2\text{HPO}_4/\text{L}$) to 500 μL urine. After centrifugation at $5360 \times g$ for 5 min at 4°C , 10 μL sodium trimethylsilyl [2,2,3,3- $^2\text{H}_4$] proprionate (TSP) and 50 μL deuterium oxide (D_2O) were added to 540 μL supernatant. Urine spectra were acquired on a 600-MHz Varian NMR spectrometer by using the first increment of a nuclear overhauser enhancement spectroscopy pulse sequence at 25°C . Spectra were acquired with 16,384 data points and 128 scans. Water suppression was achieved during the relaxation delay (2.5 s) and the mixing time (100 ms). All ^1H NMR urine spectra were referenced to TSP at 0.0 parts per million (ppm) and processed manually with the Chenomx NMR Suite (version 7.5) by using a line broadening of 0.2 Hz, followed by phase correction and baseline correction. Data were normalized to the

sum of the spectral integral. Metabolites were identified and quantified by Chenomx NMR Suite.

To confirm the metabolite assignment, a 50 μ L solution of pure compound (0.01 mol/L) was added to a urine sample. The ^1H NMR spectra were acquired prior to and after the addition of the pure compound.

^1H NMR spectroscopy of Chicken flesh homogenate

Metabolite extracts from cooked chicken breast were analyzed by ^1H NMR. Approximately 10 g of breast muscle was chopped and transferred into a 250 mL beaker. Following this, 120 mL deionized water was added into the beaker and homogenized using a homogenizer (Yellowline by IKA* DI 25 Basic Homogenizer). The resulting suspension was transferred into a 500 mL beaker and deionized water was added to a total volume of 450 mL. The solution was then stirred for 90 min and sonicated for 5 min. Deionized water was added to reach a final volume of 500 mL. Thirty milliliters of the suspension was centrifuged at 2700 x g at room temperature for 30 min. An aliquot of supernatant was filtered through a 0.22 μ m membrane filter. The 500 μ L filtrated chicken extract was combined with 250 μ L phosphate buffer, and then was centrifuged at 5360 x g for 5 min at 4 $^{\circ}\text{C}$. Following this, 10 μ L TSP and 50 μ L D_2O were added to 540 μ L supernatant. The final solution was transferred into an NMR tube. NMR spectra were acquired as described above.

Plasma sample analysis-AbsoluteIDQ® p180 Kit

Plasma samples were sent for analysis to BIOCRATES Life Sciences AG (Innsbruck, Austria), where the AbsoluteIDQ® p180 Kit was used for targeted plasma metabolite quantification. This kit is a 96-well plate format consisting of isotope-labelled and chemically homologous internal standards which are used for metabolite quantification. The kit

measurement consists of two parts: a HPLC separation step and a flow injection analysis step both followed by MS analysis. MS analysis were performed by a 4000 QTRAP® tandem mass spectrometry instrument coupled to an Agilent 1200-Series HPLC. Mass detection and compound identification were performed by multiple reaction monitoring. The identified metabolites covered amino acids, biogenic amines, acylcarnitines, phosphatidylcholines, phosphatidylcholines, lysophosphatidylcholines, sphingolipids, and hexoses. As part of the NutriTech project this assay was extended to include 6 additional metabolites including 1-Meth-His, 3-Meth-His, TMAO, anserine, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). The quantitative data analysis was performed with BIOCRATES software MetIDQTM enabling isotopic correction and basic statistical analysis. Concentrations of all analyzed metabolites are reported in $\mu\text{mol/L}$.

Statistical analysis

Biochemical data was analyzed using IBM SPSS Statistics 20.0. Data are presented as mean \pm SEM. Repeated measures ANOVA was performed to identify metabolites exhibiting significant differences across the weeks. A p value < 0.05 was considered to indicate significance.

Multivariate data analysis was carried out with Simca-P software (version 13.0.3; Umetrics) within the intervention study. Data sets were scaled using Pareto scaling. Principal components analysis (PCA), an unsupervised technique, reduced the dataset to a small number of principal components (28), and was also applied to explore any trends and outliers in the data. The differences between NMR spectral data were further explored by using partial least-squares discriminant analysis (PLS-DA). Subsequently, orthogonal PLS-DA (OPLS-DA) was performed, and the S-line plot was used to identify features that discriminated between groups.

A receiver operating characteristic (ROC) curve was constructed by using IBM SPSS Statistics 20.0. The ROC curve was used to determine whether the dietary marker could discriminate between chicken and red meat groups in the discovery study and assess the classification performance of the marker. The classification performance of dietary markers was assessed by the AUC. The shortest distance from the optimal point (0, 1) to the intersect of the ROC curve was used to measure the optimal cutoff for sensitivity and specificity calculation (29).

Bland and Altman analysis was performed by GraphPad Prism 7.02, which was used to evaluate agreement between the two measurements. The agreement between the two methods is calculated by constructing limits of agreement. These statistical limits are calculated using the mean and the standard deviation of the differences between the two measurements(30). The x-axis is an average of the two measurements and the y-axis is a difference of the two measurements. In this study, Bland and Altman analysis was performed to evaluate agreement between calculated chicken intake and reported chicken intake in NANS cohort. Calculated chicken intake was determined based on a calibration curve built using data from day 4 fasting urine samples in NutriTech study. This calibration curve was built to relate chicken intake and guanidoacetate excretion using data from day 4 fasting urine samples in NutriTech study. Reported chicken intake was based on a 4-d semi-weighed food record in NANS cohort. Results were plotted with y axis as the value of the difference between reported and calculated chicken intake and the x axis as the mean of reported and calculated chicken intake. The 95% limits of agreement (1.96SD) were presented for visual judgement of how well the two measurements agree.

Results

Postprandial urinary metabolic profile changes following the consumption of chicken based meals

In the present study individuals (n=10) consumed increasing amounts of chicken over three consecutive weeks in a controlled environment. The characteristics of participants are described in **Table 1**. Urine collected at post chicken consumption (time points 2 and 6 h) was analyzed using multivariate data analysis. The initial PCA of postprandial urine samples collected at 2 h and 6 h post consumption in day 3 showed no outliers in samples (see **Figure 1A, D**). Furthermore a good separation between low and high chicken consumption was observed. Robust PLS-DA models were built to enable better discrimination between week 1 and week 3 (see **Figure 1B, E**). The urinary metabolite profiles (S-line plot in **Figure 1C, F**) revealed differences in metabolite levels between low chicken intake in week 1 and high chicken intake in week 3. Further examination revealed that one of the spectral regions correlated with chicken intake was identified as guanidoacetate. Spectra regions of guanidoacetate acquired prior to and after the addition of the pure compound were overlaid and the assignment was confirmed (see **Supplemental Figure 3**).

Fasting urinary metabolic profile changes following the consumption of chicken based meals

PCA and PLS-DA models of fasting urine samples at day 4 were built to identify differences in spectral regions between week 1 and week 3 (see **Figure 2A, B**). The initial PCA of the ^1H NMR urine showed one outlying sample. The NMR spectrum of this outlying sample was inspected and revealed peaks associated with medication use. This sample was removed from subsequent analysis and PCA was repeated. According to the PCA and PLS-DA models, a good separation was observed when comparing fasting urine samples from week 3 with that

from week 1. Further examination of the discriminating metabolite profiles also revealed that guanidoacetate excretion strongly correlated with chicken intake (S-line; see **Figure 2C**).

Ability of guanidoacetate levels to distinguish between chicken and red meat intakes

Comparison of fasting urine samples obtained following chicken and red meat consumption revealed good separation between the meat types. Robust PLS-DA model was obtained and the spectra regions of guanidoacetate showed higher intensity in urinary profiles from the chicken group compared to that of the red meat group (see **Supplemental Figure 4**). Examining the guanidoacetate levels across the weeks demonstrated that the excretion of guanidoacetate significantly increased with increasing chicken intake ($P < 0.01$), and showed a strong dose response association. For example, in week 1 when participants consumed 88 g/day chicken, the excretion of guanidoacetate was 1.47 mmol/L; in week 2, the excretion of guanidoacetate increased to 2.48 mmol/L after participant consuming 187g/day chicken; in week 3, the excretion of guanidoacetate rose up to 3.66 mmol/L after participant consuming 290 g/day chicken. However, levels of urinary guanidoacetate in the red meat group remained constant during the three weeks (see **Figure 3**).

ROC curve analysis was performed to assess the ability of guanidoacetate to discriminate between chicken and red meat intakes. The excellent AUC value (0.99) was supported by a high of specificity and sensitivity of 90 % and 98 %, respectively (see **Supplemental Figure 5**).

Quantification of guanidoacetate in chicken breast

To investigate the origin of urinary guanidoacetate following chicken intake, we measured guanidoacetate in cooked chicken breast. Chicken breast extracts were analyzed by ^1H NMR and revealed the presence of guanidoacetate (see **Supplemental Figure 6**). The

concentrations of guanidoacetate in different types of chicken breast including normal, free range, and organic chicken are reported in **Supplemental Table 2**.

Confirmation of guanidoacetate as a marker of chicken intake using NANS data

To confirm these findings, guanidoacetate was quantified in urine samples from NANS. Participants who consumed chicken were selected from NANS, and 50 subjects with the highest chicken consumption (71-245 g/d) were selected and classified as consumers. Furthermore, another 50 subjects having no chicken intake were classified as non-consumers. Examining the guanidoacetate levels in the fasting urine samples demonstrated that the excretion of guanidoacetate significantly increased in consumers compared with non-consumers ($P < 0.01$) (**Table 2**).

Using guanidoacetate to calculate chicken intake

Using the linear calibration curve ($Y = 0.01X + 0.50$, $R^2 = 0.99$; Y = guanidoacetate content, mmol/L; X = chicken intake, g/day) developed from the NutriTech study, chicken intake was calculated for 565 NANS participants. The Bland and Altman plot to assess the difference between reported and calculated chicken intake against the average of reported and calculated chicken intake is shown in **Figure 4**. In this analysis, the 95% limits of agreement of measurement differences ranged from -124.9 to 64.4 g/day, and the mean difference (bias) between reported chicken intake and calculated chicken intake was -30.2 g/day (95% confidence intervals from -34.2 to -26.2 g/day). There were 31 participants (5 % of the observations) that fell outside 95% limits of agreements (the dotted lines), and most of these participants were predicted to have low chicken intake compared to the self-reported data. Bland and Altman plot also shows an increase in variability, shown by an increase in the scatter of the differences, as the magnitude of the measurement increased. However, overall

visual inspection of the plot revealed good agreement between reported and calculated chicken intake in NANS cohort.

Plasma measurements of 3-Meth-His increased with increasing chicken intake

The initial analysis of plasma data revealed interesting data for methylhistidine metabolites. In each intervention week, excretion of 3-Meth-His was rapid and peaked at 2 h post consumption, and on day 4 almost declined to similar levels with day 3 time points 0 h (see **Figure 5A**). Compared with 3-Meth-His, excretion of 1-Meth-His did not show many changes at different time points, except in week 3 (see **Figure 5B**). Day 4 fasting plasma samples were analyzed following increased chicken intake (see **Figure 5C, D**), 3-Meth-His and 1-Meth-His both significantly increased following the increment of chicken intake ($P < 0.01$). However, the response of 3-Meth-His was much higher and displayed a strong dose response association.

Discussion

In the present study, we used a metabolomics approach to identify a novel marker of chicken intake in a controlled intervention study, and subsequently confirmed this candidate marker in a free-living population. The urinary marker showed a strong dose response with chicken intake. Importantly using a calibration curve we were able to calculate chicken intake in an independent free-living cohort. Targeted plasma metabolite analysis demonstrated that 3-Meth-His was a more suitable indicator of chicken intake compared to 1-Meth-His.

There are some interesting metabolites were reported to associate with chicken intake in previous studies. For example, four major metabolites of PhIP metabolites, N^2 -OH-PhIP- N^2 -glucuronide, PhIP- N^2 -glucuronide, 4'-PhIP-sulfate and N^2 -OH-PhIP- N^3 -glucuronide were reported to be high in the urine samples following chicken meal intake (25). Similar results

can also be found in a study by Kulp et al. (2004) (31). However, PhIP are produced in meat during cooking at high temperatures and humans can be exposed to PhIP through the consumption of various cooked muscle meats, notably beef, pork and chicken (32, 33). Therefore the metabolites of PhIP are not specific for chicken consumption, and also are not suggested as markers. In contrast to those metabolites, the urinary guanidoacetate is a specific marker of chicken intake. The specificity of this marker was confirmed in NutriTech participants consuming red meat. Furthermore, a dose response association where the marker increased with increasing intake was also demonstrated. These qualities make guanidoacetate an attractive marker.

Guanidoacetate, referred to glycoamine or guanidinoacetic acid, is a natural and immediate precursor for creatine in the vertebrate body of animals (34, 35). It was first identified as a natural compound in humans about 80 years ago (36), and the reported concentration is 41.8 (10.6-97.3 $\mu\text{mol}/\text{mmol}$ creatinine) in normal human urine determined by NMR (37). Guanidoacetate is formed from the amino acids glycine and arginine mainly in the kidney and pancreas, and transformed to creatine after transportation to the liver (36). A recent study reported that guanidoacetate was associated with dietary intake: Schmedes et al. (2016) performed a randomized crossover intervention study, where participants were randomly assigned to the lean-seafood diet group with lunch and dinner and the other half of participants to a non-seafood containing chicken, lean beef, turkey, pork, egg, milk, and milk products. They analyzed the urinary metabolites and found a higher level of guanidoacetate excretion following the non-seafood intervention (38). Considering that this intervention group contained chicken as a protein source we believe that our data supports the origin of guanidoacetate being chicken.

Supplemental guanidoacetate is often used as feed additive for chickens and pigs (34, 39). Some studies investigated the effects of guanidoacetate on the performance, meat quality and

energy metabolism of broilers, and found supplemental guanidoacetate improved weight gain or breast meat yield (34, 35). In the study of Michiels et al. (2011), 308 broilers were assigned to 1 of 4 diets: negative control, all-vegetable corn-so-bean-based; negative control supplemented with either 0.6 or 1.2 g of guanidoacetate per kg of feed; and positive control (60, 30, and 30 g/kg of fish meal in the starter, grower, and finisher diets, respectively). They found the final weight of guanidoacetate-fed broilers was higher than that of the negative control birds. Dietary supplementation with guanidoacetate resulted in a higher percentage of breast meat in the carcass compared with that from birds of the negative control diet (35). Lemme et al. (2007) suggested that the optimal guanidoacetate supplementation level was between 0.06 % to 0.12 % depending on performance parameters (34). In our study we confirmed that guanidoacetate was present in chicken breast and propose that this is the main source of urinary guanidoacetate. The range observed in chicken breasts is unlikely to translate to major urinary differences depending on the type of chicken. In addition to urinary guanidoacetate, plasma 3-Meth-His was identified as a suitable marker of chicken intake. Previous studies have reported associations between methylhistidine and meat intake. For example, Cross et al. (2011) found urinary excretion of 1-Meth-His and 3--Meth-His elevated with increasing red meat intake in highly controlled, crossover studies, and recommended them as potential markers of meat intake (21). Several studies also demonstrated that the levels of urinary 1-Meth-His and 3-Meth-His were excellent parameters for discriminating between vegetarian and omnivorous subjects (40, 41). Myint et al. (2000) demonstrated that 1-Meth-His excretion differed greatly and significantly between vegetarians and omnivores, and found urinary 1-Meth-His had good correlations with meat consumption including red meat, chicken, and fish (40). However, to the best of our knowledge, the specificity for different types of meat has not been measured. For our targeted plasma metabolites analysis, 1-Meth-His and 3-Meth-His significantly increased following increasing dietary chicken

intake. However, only 3-Meth-His displayed a strong dose response indicating its superior quality as a marker of chicken intake.

The putative dietary markers discovered in a controlled intervention study cannot always be translated to free-living subjects. Some factors such as genetic variability, lifestyle, physiology and diet can have an influence on the marker measures of dietary intake. The confirmation of putative markers in an independent free-living study is essential for their acceptance. In NANS cohort, chicken consumers had significantly higher guanidoacetate excretion than non-consumers. Bland and Altman analysis demonstrated the calculated chicken intake based on urinary marker levels agreed with self-reported chicken intake from 4-d food record. The disagreement observed between two measurements became greater with very high intake, which may be caused by self-reporting issues such as over-reporting. Importantly the bias between the two measurements was low with respect to a chicken breast portion (150g according to 'Food Standards Agency: Food Portion Sizes' handbook). Therefore the marker performed as well as the 4-d food record in this NANS cohort. The use of the developed calibration curve to calculate chicken intake is an important development for the field of dietary markers.

The present study has many strengths. Firstly this study demonstrated a dose response for guanidoacetate, enabling determination of chicken intake from the concentration measurements. Secondly, the multivariate data analysis and ROC curve between red meat and chicken groups indicated that urinary excretion of guanidoacetate was specific for chicken intake. The determination of chicken intake in an independent cohort offers potential for this marker as an objective measure of intake. However, as chicken is one of the most commonly consumed meats across the world, further validation and testing in other populations may be desired.

In conclusion, the current study demonstrates that urinary guanidoacetate and plasma 3-Meth-
His are markers of chicken intake. With respect to guanidoacetate, our study demonstrates
that we can accurately determine dietary intake using a urinary measurement of the marker.
This opens the possibility for the marker to aid dietary assessment in future studies and marks
a significant development for metabolomics derived dietary markers.

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TABLE 1 Population characteristics in NutriTech and NANS study

	NutriTech study ¹		NANS study ²	
	Chicken	Red meat	Group 1	Group 2
n	10	10	565	100
Sex , n	5 (F), 5 (M)	5 (F), 5 (M)	281(F), 284(M)	45(F), 57(M)
Age, y	62 ± 1	58 ± 1	47 ± 1	47 ± 2
BMI, Kg/m ²	28.25 ± 1.25	30.95 ± 1.00	29.91 ± 0.59	26.24 ± 0.38

Values are presented as mean ± SEM.

¹ NutriTech study, an intervention study was used to discover dietary markers.

² NANS study, an independent free-living cohort was used to confirm dietary markers (18);

Group 1: 565 NANS participants randomly selected from the main NANS database were used to examine the agreement between calculated and reported chicken intakes; Group 2: 100 participants including 50 chicken consumers and 50 non-consumers were selected from 565 NANS participants, and were used to compare the difference of urinary markers between chicken consumers and non-consumers.

TABLE 2 Differences in quantified urinary guanidoacetate between consumers and non-consumers of chicken intake in **the** NANS cohort

	Consumers ¹	Non-consumers ²	<i>P</i> ³
	mmol/L	mmol/L	
Guanidoacetate	0.70 ± 0.06	0.47 ± 0.04	<i>P</i> < 0.01

Values are presented as mean ± SEM, n=100 (50 fish consumers, 50 non-consumers).

¹ Consumers (n=50) chosen from NANS consumed high chicken daily (97-245 g/d).

² Non-consumers (n=50) chosen from NANS did not consume chicken (0 g/d).

³ Based on Independent-Samples T-test between chicken consumers and non-consumers.

Figures

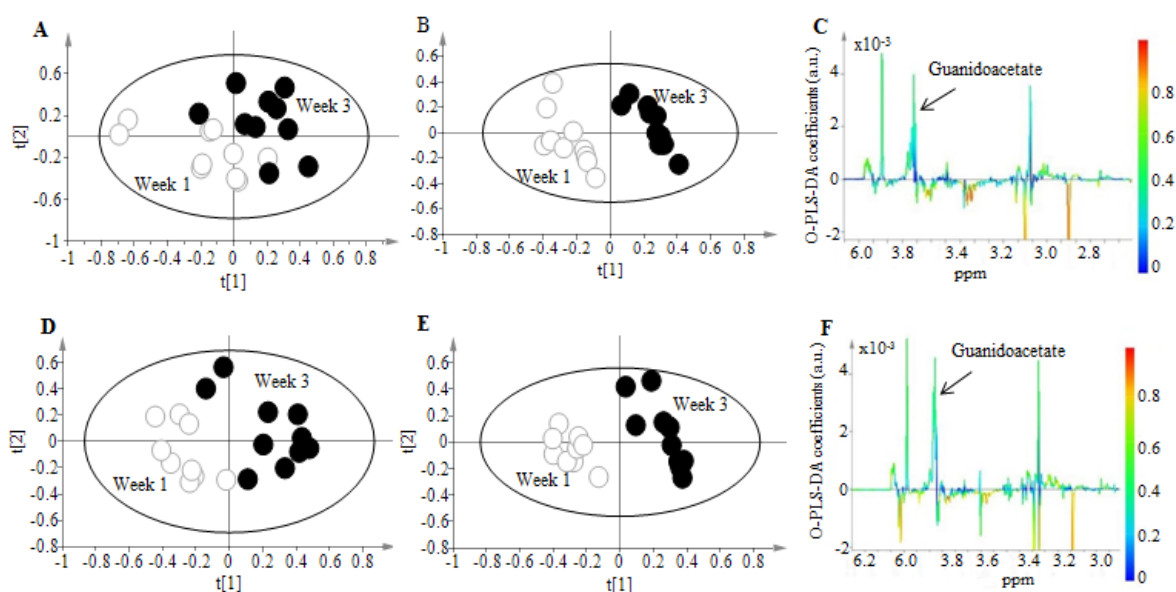


FIGURE 1 Identification of a putative marker in postprandial urine samples collected from NutriTech Food Intake Study. Week 1, low chicken intake (88g/day); Week 3, high chicken intake (290g/day). **A-B:** PCA scores plot (R²X=0.47, Q²=0.11) and PLS-DA scores plot (R²Y=0.95, Q²=0.69) of ¹H NMR urine samples collected on day 3, 2 h post consumption in week 3 (●) and week 1 (○); **D-E:** PCA scores plot (R²X=0.46, Q²=0.12) and PLS-DA scores plot (R²Y=0.95, Q²=0.78) of ¹H NMR urine samples collected on day 3, 6 h post consumption in week 3 (●) and week 1 (○); **C-F:** S-line plot of week 3 compared with week 1 on day 3 time point 2 h and 6 h post consumption, respectively. The spectral region in the positive section (top half) is indicative of the week 3 while the negative half (bottom half) is indicative of week 1. A putative marker for chicken consumption was identified as guanidoacetate.

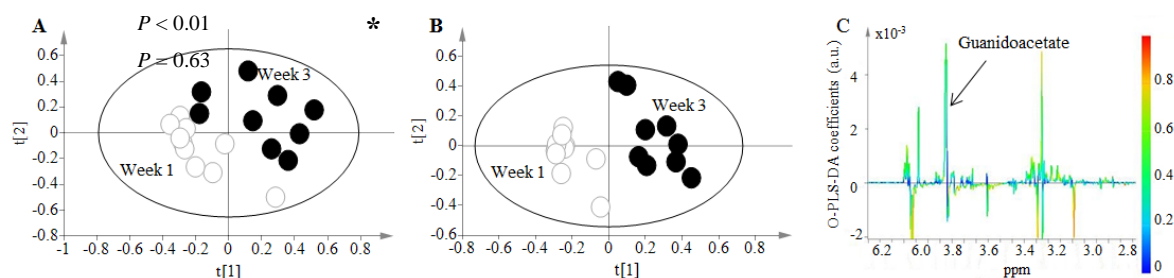


FIGURE 2 Identification of a putative marker in fasting urine samples collected from NutriTech Food Intake Study. Week 1, low chicken intake (88g/day); Week 3, high chicken intake (290g/day). **A-B**: PCA scores plot ($R^2X=0.47$, $Q^2=0.15$) and PLS-DA scores plot ($R^2Y=0.92$, $Q^2=0.67$) of ^1H NMR urine samples of week 3 (●) compared with week 1 (○) on day 4; **C**: S-line plot of week 3 compared with week 1. The spectral region in the positive section (top half) is indicative of the week 3 while the negative half (bottom half) is indicative of week 1. A putative marker for chicken consumption was identified as guanidoacetate.

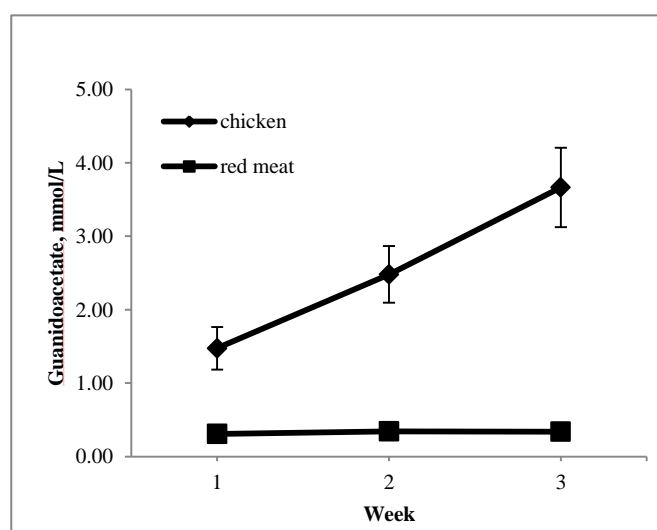


FIGURE 3 Urinary guanidoacetate concentrations (Mean \pm SEM) from NutriTech chicken and red meat groups on day 4 fasting urine samples during three consecutive weeks. In week 1, participants consumed 88 g/day chicken; in week 2, participants consumed 187 g/day

chicken; in week 3, participants consumed 290 g/day chicken. Repeated measures ANOVA was performed, and * means urinary guanidoacetate significantly increased from week 1 to week 3 in chicken group ($P < 0.01$).

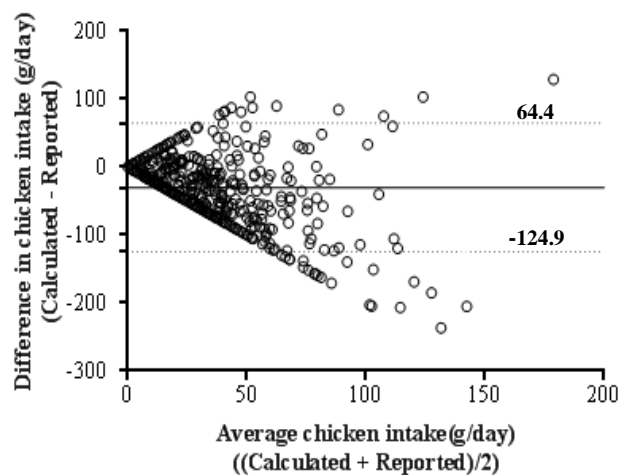
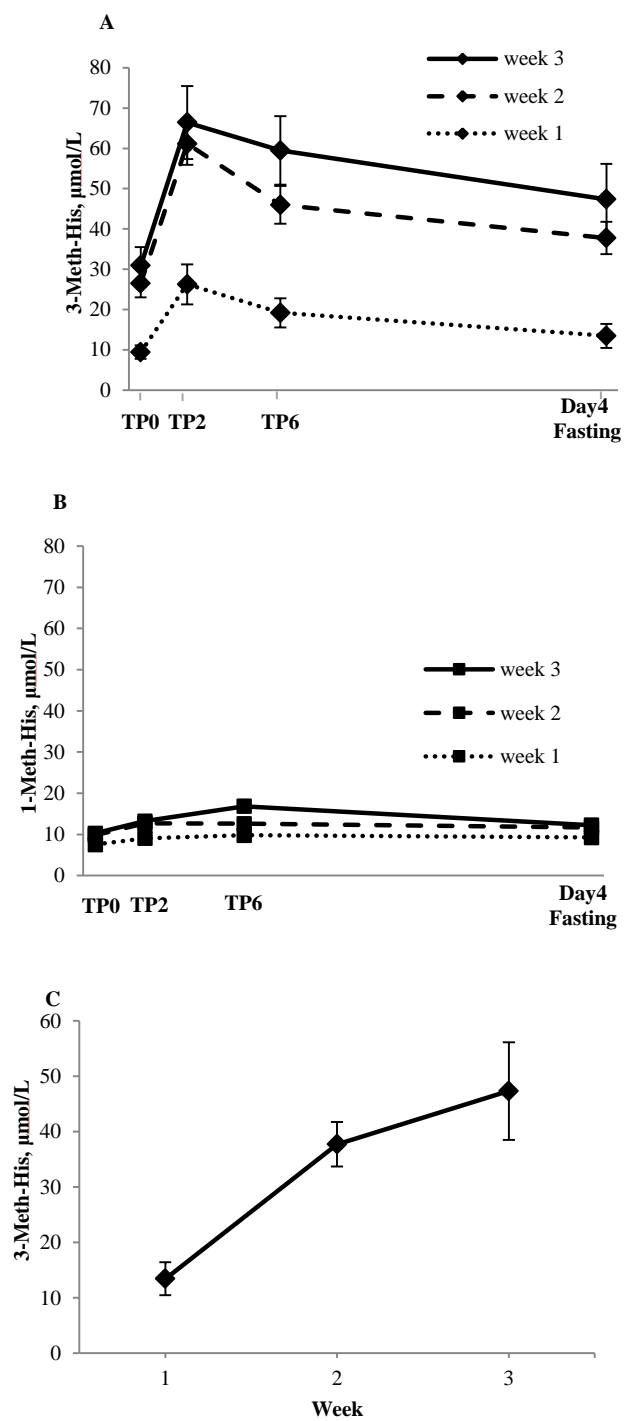


FIGURE 4 Bland and Altman plot of the difference between calculated and reported chicken intake vs the average of calculated and reported chicken intake in NANS study. The solid line (x-parallel line) represents the mean difference (bias) and the dotted line represents the 95% limits of agreement. ‘Calculated’ indicates the calculated chicken intake based on urinary guanidoacetate concentrations. ‘Reported’ indicates chicken intake recorded using a 4-d semi-weighed food record.



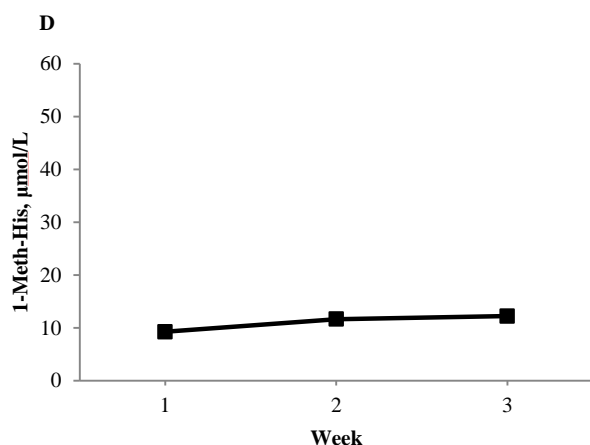


FIGURE 5 Plasma concentrations of 1-Meth -His and 3-Meth-His (mean \pm SEM, n=10) in NutriTech chicken group during three consecutive weeks. **A:** plasma excretion kinetics of 3-Meth-His; **B:** plasma excretion kinetics of 1-Meth-His; **C:** fasting plasma 3-Meth-His concentration changes across three weeks; **D:** fasting plasma 1-Meth-His concentration changes across three weeks. Repeated measures ANOVA was performed, and * means 1-Meth-His and 3-Meth-His significantly increased from week 1 to week 3 in plasma samples ($P < 0.01$). In week 1, participants consumed 88 g/day chicken; in week 2, participants consumed 187 g/day chicken; in week 3, participants consumed 290 g/day chicken.

Online Supporting Material

Supplemental Table 1 Chicken intake (g/day) in three consecutive weeks

Participants	Week 1	Week 2	Week 3
Female	82±3.0	174±1.5	253±35.3
Male	94±3.9	200±5.7	327±24.8
Average	88±3.0	187±5.1	290±23.8

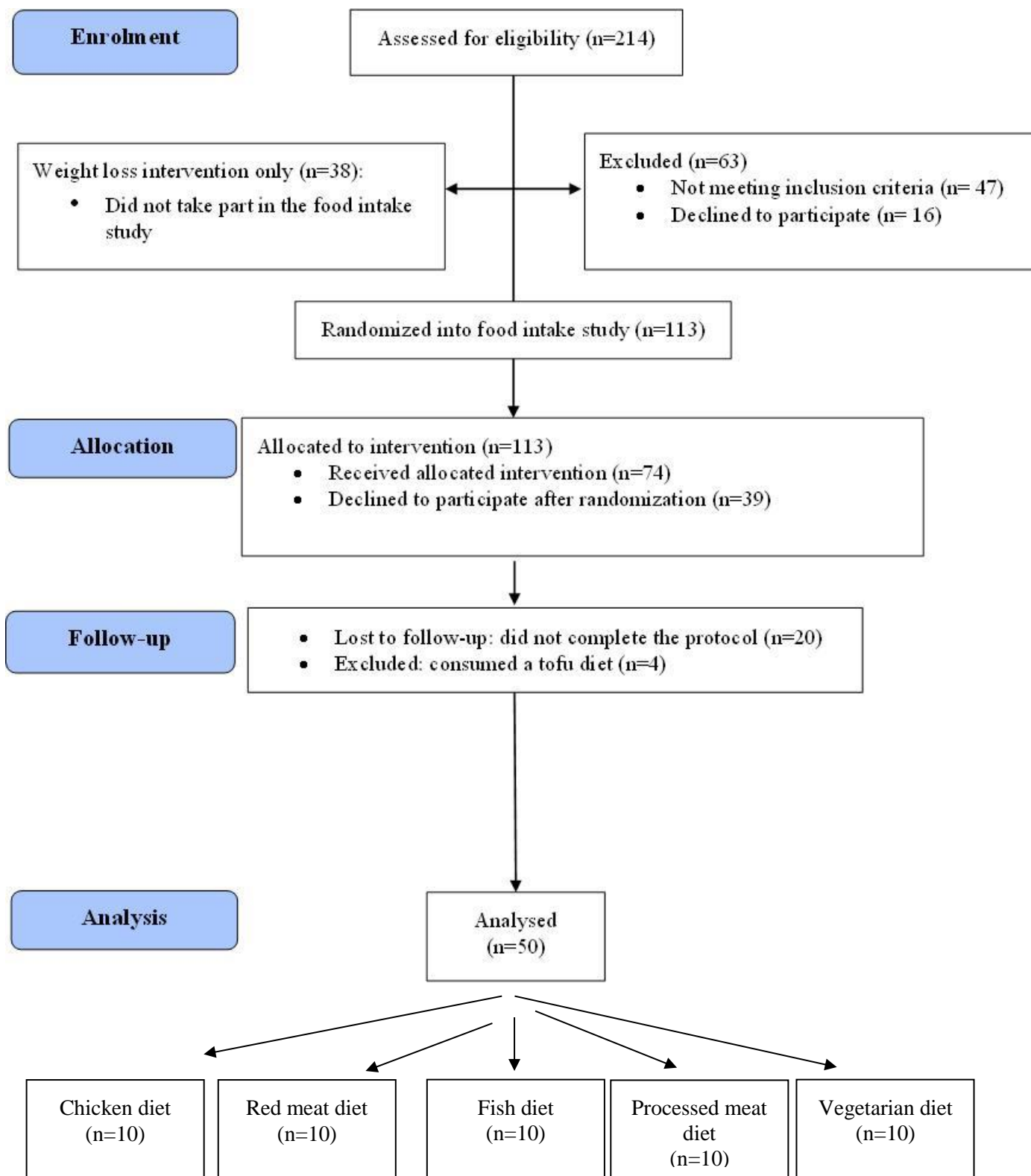
Values are presented as mean ± SEM, n=10 (5 men).

Supplemental Table 2 Concentrations of guanidoacetate extracted from different types of cooked chicken breast

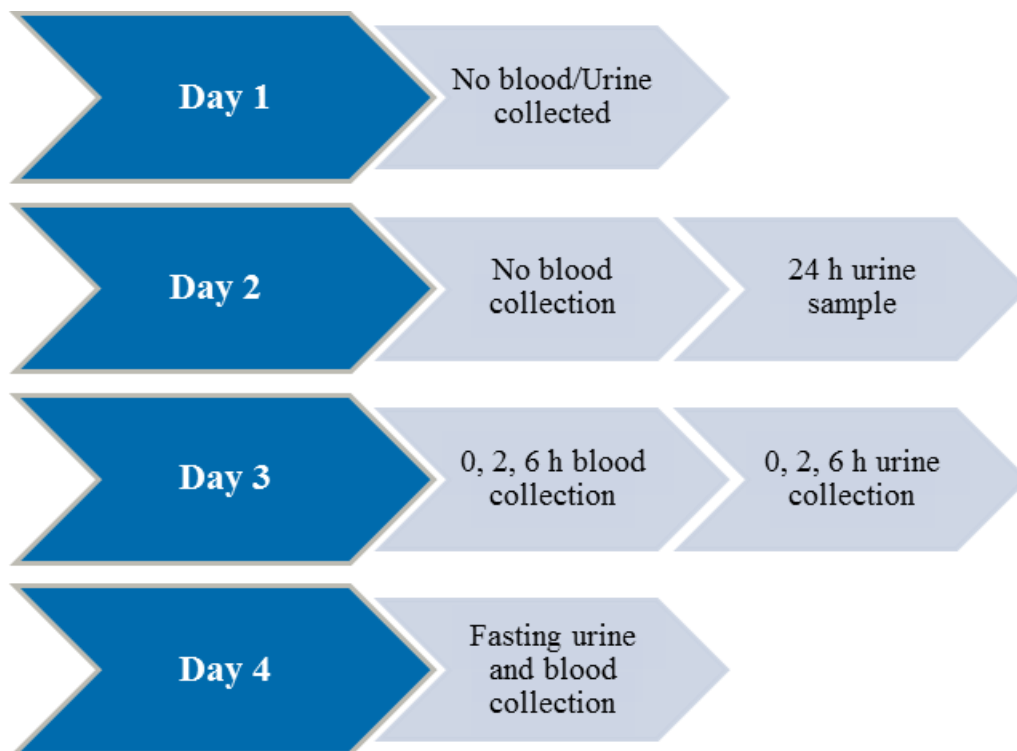
Type of chicken	Concentration	Range
	mg/g	mg/g
Normal	3.45±0.08	3.41-3.60
Free range	10.25±0.62	9.38-11.45
Organic	7.33±0.16	7.04-7.60
NutriTech ¹	3.19±0.11	3.02-3.41

Values are presented as mean ± SEM, and range of measurements.

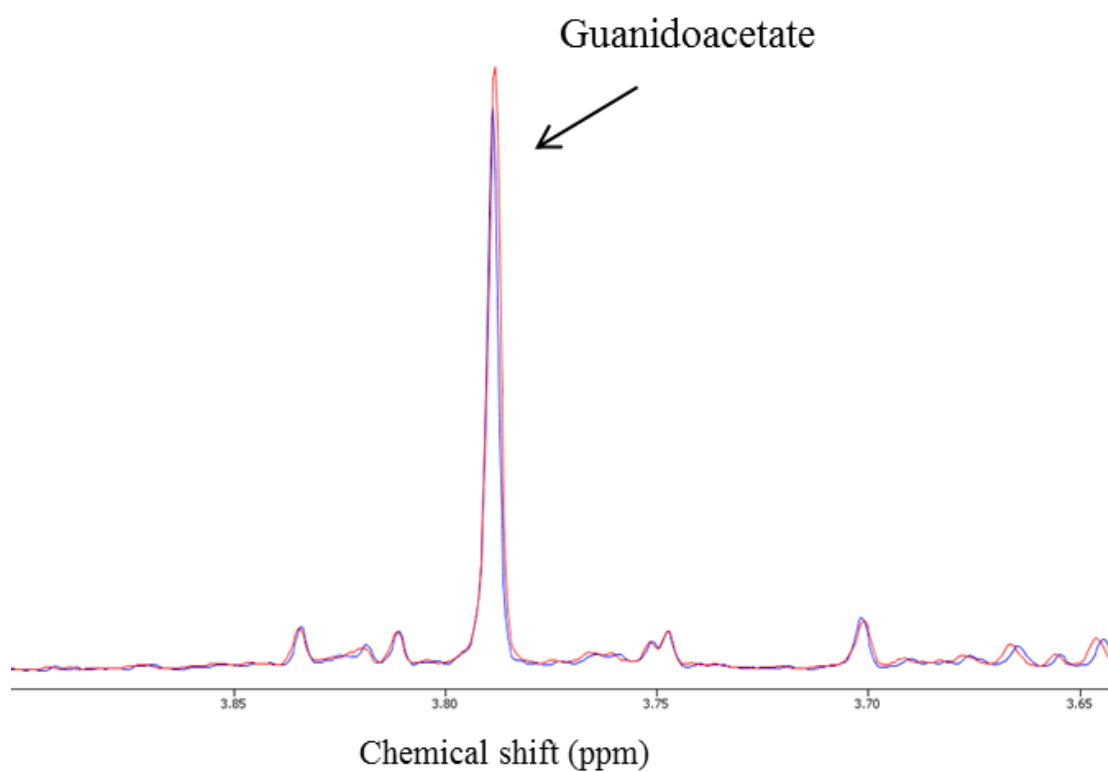
¹ NutriTech means chicken meal in the Nutritech project.



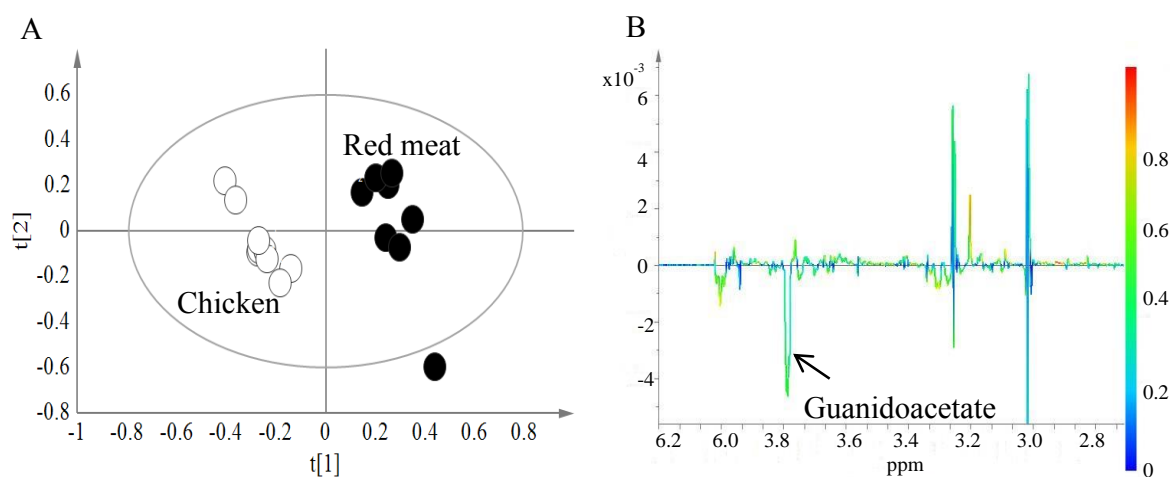
Supplemental Figure 1 NutriTech study participant flow chart



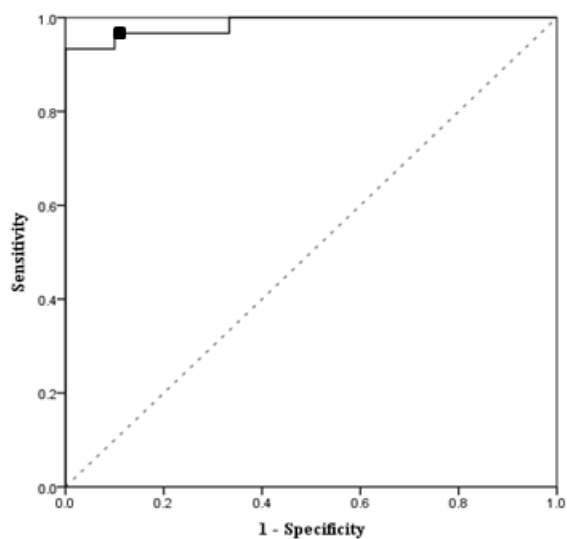
Supplemental Figure 2 Schematic representation of biofluid collection in the NutriTech food intake study. 24 h urine collection began at the first fasting void collected on day 2 until the first fasting collected void on day 3. 0 is prior to consumption, 2 h is 2h post consumption and 6 h is 6 h post consumption.



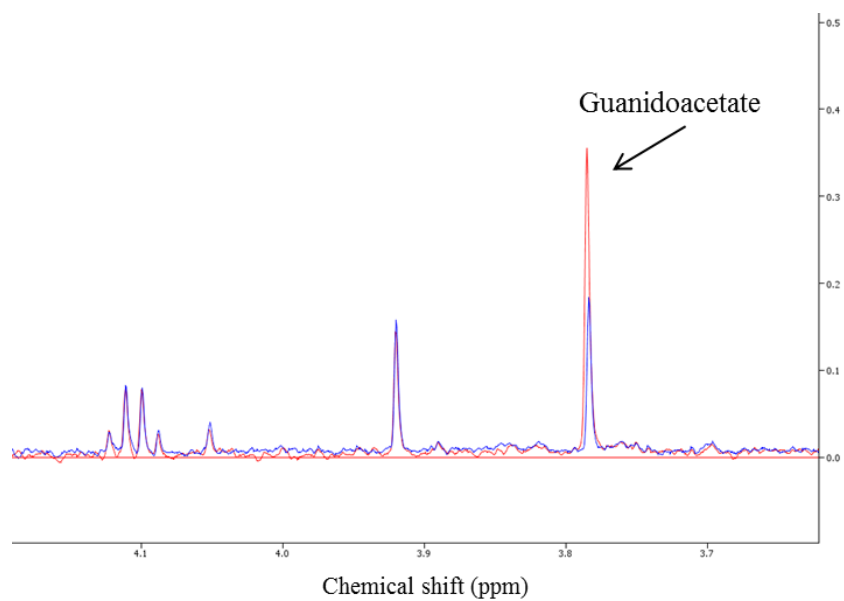
Supplemental Figure 3 ^1H NMR spectrum of a urine sample to confirm the guanidoacetate is correctly assigned. Blue, urine sample; Red, urine sample spiked with standard of guanidoacetate. The concentration of standard is 0.01 mol/L.



Supplemental Figure 4 Multivariate data analysis between chicken and red meat groups using day 4 fasting urine samples in week 3. **A:** PLS-DA scores plot, $R^2Y=0.99$ $Q^2=0.85$; **B:** S-line plot. The spectral region in the positive section (top half) is indicative of red meat group while the negative half (bottom half) is indicative of chicken group. Guanidoacetate was identified in chicken group.



Supplemental Figure 5 ROC curve to assess the classification ability of urinary guanidoacetate between red meat and chicken groups in NutriTech study. The optimal operating point (■) represented a specificity and sensitivity of 0.90 and 0.98, respectively. AUC was 0.99.



Supplemental Figure 6 ^1H NMR spectrum of chicken breast extracts to confirm the existence of the marker in the chicken breast sample. Blue, chicken breast sample; Red, chicken breast sample spiked with standard of guanidoacetate. The concentration of standard is 0.01 mol/L.