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Estimation of chicken intake using metabolomics derived markers

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Online supplemental material including Table 1-2 and Figure 1-6 has been submitted.

A running title is “Markers of chicken intake”.

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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1 **Abstract**

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

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

7 **Methods:**

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

15 **Results:** Multivariate data analysis of postprandial and fasting urine samples collected in
16 NutriTech revealed good discrimination between high (290 g/day) and low (88 g/day)
17 chicken intakes. Urinary metabolite profiles showed differences in metabolite levels between
18 low and high chicken intakes. Examining metabolite profiles revealed guanidoacetate
19 significantly increased from 1.47 to 3.66 mmol/L following increasing chicken intake from
20 88 to 290 g/day ($P < 0.01$). Using a calibration curve developed from NutriTech study,
21 chicken intake was calculated in NANS, where chicken consumers had higher guanidoacetate
22 excretion (0.70 mmol/L) than non-consumers (0.47 mmol/L) ($P < 0.01$). Bland and Altman
23 analysis revealed good agreement between reported and calculated intakes with a bias of -
24 30.2g/day. Plasma metabolite analysis demonstrated that 3-methylhistidine (3-Meth-His) was
25 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

32 **Introduction**

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

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

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

57 been a number of studies examining dietary markers for red meat intake using metabolomics
58 approaches. For example, creatinine, creatine, carnitine, carnosine, taurine, 1-methylhistidine
59 (1-Meth-His) and 3-methylhistidine (3-Meth-His) have been put forward as putative markers
60 of red meat intake (21, 24). With respect to white meat consumption, fewer studies have been
61 reported. Nonetheless the following metabolites have emerged as potential markers:
62 pyroglutamine, 2-amino-1-methyl-6-phenylimidazo [4, 5-b] pyridine (PhIP). However, there
63 is a lack of validation of these markers of chicken intake (16, 25), and no study has
64 demonstrated that these markers can accurately determine chicken intake. To this end the
65 objective of the present study was to use a metabolomics approach to identify and confirm
66 markers related specifically to chicken consumption and to determine chicken intake in an
67 independent cohort.

68

69 **Subjects and methods**

70 **NutriTech study design**

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

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

84 Participants were given set meals for breakfast (8am), lunch (12am) and evening meals (7pm)
85 for three days (day 1, 2, and 3) during a week and this was repeated for three weeks (week 1,
86 2, and 3). The period between each week was minimum 3 days. In each group, the test food
87 intake increased from week 1 to week 3 (see chicken intake in **Supplemental Table 1**).
88 Leftovers were measured and recorded where appropriate.

89 Biological samples were collected over the course of the three days during each week. On
90 day 1 no samples were collected. On day 2 a 24 h urine sample was collected and during this
91 period all voids were collected in a single container which was kept chilled throughout. At
92 the end of collection the urine was inverted 5 times and 50 mL was removed and processed.
93 On day 3, after the 8 am void, participants were only allowed to urinate at 0 h (void
94 immediately before the midday meal at 11.55 am), 2 h (spot sample 2 hours after the midday
95 meal) and 6 h (spot sample 6 hours after the midday meal). They were asked not to urinate
96 outside these designated times, and these spot urine samples were collected and kept on ice
97 until processed, respectively. Blood samples were also collected at 0 h, 2 h, and 6 h; 4 mL of
98 blood was collected in an EDTA tube and 10 mL of blood was collected in a lithium heparin
99 tube. On day 4, the fasting morning urine and blood samples were collected. The schematic
100 representation of biofluid collection is shown in **Supplemental Figure 2**. All urine samples
101 were processed within 30 minutes of collection, centrifuged at 1800 x g for 10 min at 4 °C,
102 and 1 mL aliquots were stored at -80 °C for NMR analysis. All blood samples were inverted
103 8 times, centrifuged at 1800 x g for 10 mins at 4 °C, and 500 μ L aliquots were stored at –
104 80 °C until subsequent analysis.

105

106 **NANS study design**

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

120

121 **Urine sample analysis- ^1H NMR spectroscopy**

122 Urine samples were prepared by addition of 250 μL phosphate buffer (0.2 mol $\text{KH}_2\text{PO}_4/\text{L}$,
123 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
124 sodium trimethylsilyl [2,2,3,3- $^2\text{H}_4$] proprionate (TSP) and 50 μL deuterium oxide (D_2O) were
125 added to 540 μL supernatant. Urine spectra were acquired on a 600-MHz Varian NMR
126 spectrometer by using the first increment of a nuclear overhauser enhancement spectroscopy
127 pulse sequence at 25°C . Spectra were acquired with 16,384 data points and 128 scans. Water
128 suppression was achieved during the relaxation delay (2.5 s) and the mixing time (100 ms).
129 All ^1H NMR urine spectra were referenced to TSP at 0.0 parts per million (ppm) and
130 processed manually with the Chenomx NMR Suite (version 7.5) by using a line broadening
131 of 0.2 Hz, followed by phase correction and baseline correction. Data were normalized to the

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

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

137

138 **^1H NMR spectroscopy of Chicken flesh homogenate**

139 Metabolite extracts from cooked chicken breast were analyzed by ^1H NMR. Approximately
140 10 g of breast muscle was chopped and transferred into a 250 mL beaker. Following this, 120
141 mL deionized water was added into the beaker and homogenized using a homogenizer
142 (Yellowline by IKA* DI 25 Basic Homogenizer). The resulting suspension was transferred
143 into a 500 mL beaker and deionized water was added to a total volume of 450 mL. The
144 solution was then stirred for 90 min and sonicated for 5 min. Deionized water was added to
145 reach a final volume of 500 mL. Thirty milliliters of the suspension was centrifuged at 2700 x
146 g at room temperature for 30 min. An aliquot of supernatant was filtered through a 0.22 μ m
147 membrane filter. The 500 μ L filtrated chicken extract was combined with 250 μ L phosphate
148 buffer, and then was centrifuged at 5360 x g for 5 min at 4 $^\circ\text{C}$. Following this, 10 μ L TSP and
149 50 μ L D_2O were added to 540 μ L supernatant. The final solution was transferred into an
150 NMR tube. NMR spectra were acquired as described above.

151

152 **Plasma sample analysis-AbsoluteIDQ[®] p180 Kit**

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

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

168

169 **Statistical analysis**

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

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

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

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

204

205 **Results**

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

208 In the present study individuals (n=10) consumed increasing amounts of chicken over three
209 consecutive weeks in a controlled environment. The characteristics of participants are
210 described in **Table 1**. Urine collected at post chicken consumption (time points 2 and 6 h)
211 was analyzed using multivariate data analysis. The initial PCA of postprandial urine samples
212 collected at 2 h and 6 h post consumption in day 3 showed no outliers in samples (see **Figure**
213 **1A, D**). Furthermore a good separation between low and high chicken consumption was
214 observed. Robust PLS-DA models were built to enable better discrimination between week 1
215 and week 3 (see **Figure 1B, E**). The urinary metabolite profiles (S-line plot in **Figure 1C, F**)
216 revealed differences in metabolite levels between low chicken intake in week 1 and high
217 chicken intake in week 3. Further examination revealed that one of the spectral regions
218 correlated with chicken intake was identified as guanidoacetate. Spectra regions of
219 guanidoacetate acquired prior to and after the addition of the pure compound were overlaid
220 and the assignment was confirmed (see **Supplemental Figure 3**).

221

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

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

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

232

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

234 Comparison of fasting urine samples obtained following chicken and red meat consumption
235 revealed good separation between the meat types. Robust PLS-DA model was obtained and
236 the spectra regions of guanidoacetate showed higher intensity in urinary profiles from the
237 chicken group compared to that of the red meat group (see **Supplemental Figure 4**).
238 Examining the guanidoacetate levels across the weeks demonstrated that the excretion of
239 guanidoacetate significantly increased with increasing chicken intake ($P < 0.01$), and showed
240 a strong dose response association. For example, in week 1 when participants consumed 88
241 g/day chicken, the excretion of guanidoacetate was 1.47 mmol/L; in week 2, the excretion of
242 guanidoacetate increased to 2.48 mmol/L after participant consuming 187g/day chicken; in
243 week 3, the excretion of guanidoacetate rose up to 3.66 mmol/L after participant consuming
244 290 g/day chicken. However, levels of urinary guanidoacetate in the red meat group remained
245 constant during the three weeks (see **Figure 3**).

246 ROC curve analysis was performed to assess the ability of guanidoacetate to discriminate
247 between chicken and red meat intakes. The excellent AUC value (0.99) was supported by a
248 high of specificity and sensitivity of 90 % and 98 %, respectively (see **Supplemental Figure**
249 **5**).

250

251 **Quantification of guanidoacetate in chicken breast**

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

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

257

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

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

266

267 **Using guanidoacetate to calculate chicken intake**

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

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

282

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

284 The initial analysis of plasma data revealed interesting data for methylhistidine metabolites.
285 In each intervention week, excretion of 3-Meth-His was rapid and peaked at 2 h post
286 consumption, and on day 4 almost declined to similar levels with day 3 time points 0 h (see
287 **Figure 5A**). Compared with 3-Meth-His, excretion of 1-Meth-His did not show many
288 changes at different time points, except in week 3 (see **Figure 5B**). Day 4 fasting plasma
289 samples were analyzed following increased chicken intake (see **Figure 5C, D**), 3-Meth-His
290 and 1-Meth-His both significantly increased following the increment of chicken intake ($P <$
291 0.01). However, the response of 3-Meth-His was much higher and displayed a strong dose
292 response association.

293

294 **Discussion**

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

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

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

314 Guanidoacetate, referred to glycoamine or guanidinoacetic acid, is a natural and immediate
315 precursor for creatine in the vertebrate body of animals (34, 35). It was first identified as a
316 natural compound in humans about 80 years ago (36), and the reported concentration is 41.8
317 (10.6-97.3 $\mu\text{mol}/\text{mmol}$ creatinine) in normal human urine determined by NMR (37).
318 Guanidoacetate is formed from the amino acids glycine and arginine mainly in the kidney and
319 pancreas, and transformed to creatine after transportation to the liver (36). A recent study
320 reported that guanidoacetate was associated with dietary intake: Schmedes et al. (2016)
321 performed a randomized crossover intervention study, where participants were randomly
322 assigned to the lean-seafood diet group with lunch and dinner and the other half of
323 participants to a non-seafood containing chicken, lean beef, turkey, pork, egg, milk, and milk
324 products. They analyzed the urinary metabolites and found a higher level of guanidoacetate
325 excretion following the non-seafood intervention (38). Considering that this intervention
326 group contained chicken as a protein source we believe that our data supports the origin of
327 guanidoacetate being chicken.

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

330 energy metabolism of broilers, and found supplemental guanidoacetate improved weight gain
331 or breast meat yield (34, 35). In the study of Michiels et al. (2011), 308 broilers were
332 assigned to 1 of 4 diets: negative control, all-vegetable corn-so-bean-based; negative control
333 supplemented with either 0.6 or 1.2 g of guanidoacetate per kg of feed; and positive control
334 (60, 30, and 30 g/kg of fish meal in the starter, grower, and finisher diets, respectively). They
335 found the final weight of guanidoacetate-fed broilers was higher than that of the negative
336 control birds. Dietary supplementation with guanidoacetate resulted in a higher percentage of
337 breast meat in the carcass compared with that from birds of the negative control diet (35).
338 Lemme et al. (2007) suggested that the optimal guanidoacetate supplementation level was
339 between 0.06 % to 0.12 % depending on performance parameters (34). In our study we
340 confirmed that guanidoacetate was present in chicken breast and propose that this is the main
341 source of urinary guanidoacetate. The range observed in chicken breasts is unlikely to
342 translate to major urinary differences depending on the type of chicken. In addition to urinary
343 guanidoacetate, plasma 3-Meth-His was identified as a suitable marker of chicken intake.
344 Previous studies have reported associations between methylhistidine and meat intake. For
345 example, Cross et al. (2011) found urinary excretion of 1-Meth-His and 3--Meth-His elevated
346 with increasing red meat intake in highly controlled, crossover studies, and recommended
347 them as potential markers of meat intake (21). Several studies also demonstrated that the
348 levels of urinary 1-Meth-His and 3-Meth-His were excellent parameters for discriminating
349 between vegetarian and omnivorous subjects (40, 41). Myint et al. (2000) demonstrated that
350 1-Meth-His excretion differed greatly and significantly between vegetarians and omnivores,
351 and found urinary 1-Meth-His had good correlations with meat consumption including red
352 meat, chicken, and fish (40). However, to the best of our knowledge, the specificity for
353 different types of meat has not been measured. For our targeted plasma metabolites analysis,
354 1-Meth-His and 3-Meth-His significantly increased following increasing dietary chicken

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

357 The putative dietary markers discovered in a controlled intervention study cannot always be
358 translated to free-living subjects. Some factors such as genetic variability, lifestyle,
359 physiology and diet can have an influence on the marker measures of dietary intake. The
360 confirmation of putative markers in an independent free-living study is essential for their
361 acceptance. In NANS cohort, chicken consumers had significantly higher guanidoacetate
362 excretion than non-consumers. Bland and Altman analysis demonstrated the calculated
363 chicken intake based on urinary marker levels agreed with self-reported chicken intake from
364 4-d food record. The disagreement observed between two measurements became greater with
365 very high intake, which may be caused by self-reporting issues such as over-reporting.
366 Importantly the bias between the two measurements was low with respect to a chicken breast
367 portion (150g according to 'Food Standards Agency: Food Portion Sizes' handbook).
368 Therefore the marker performed as well as the 4-d food record in this NANS cohort. The use
369 of the developed calibration curve to calculate chicken intake is an important development
370 for the field of dietary markers.

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

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

384

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386 X.Y. and H.G. conducted the research and analyzed data; X.Y. and L.B. analyzed data and
387 wrote the manuscript. G.F. and M.R. provided essential materials in NutriTech project;
388 B.A.M., A.P.N., M.J.G., A.F., and J.W. provided essential materials in NANS. All authors
389 read and approved the final manuscript.

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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

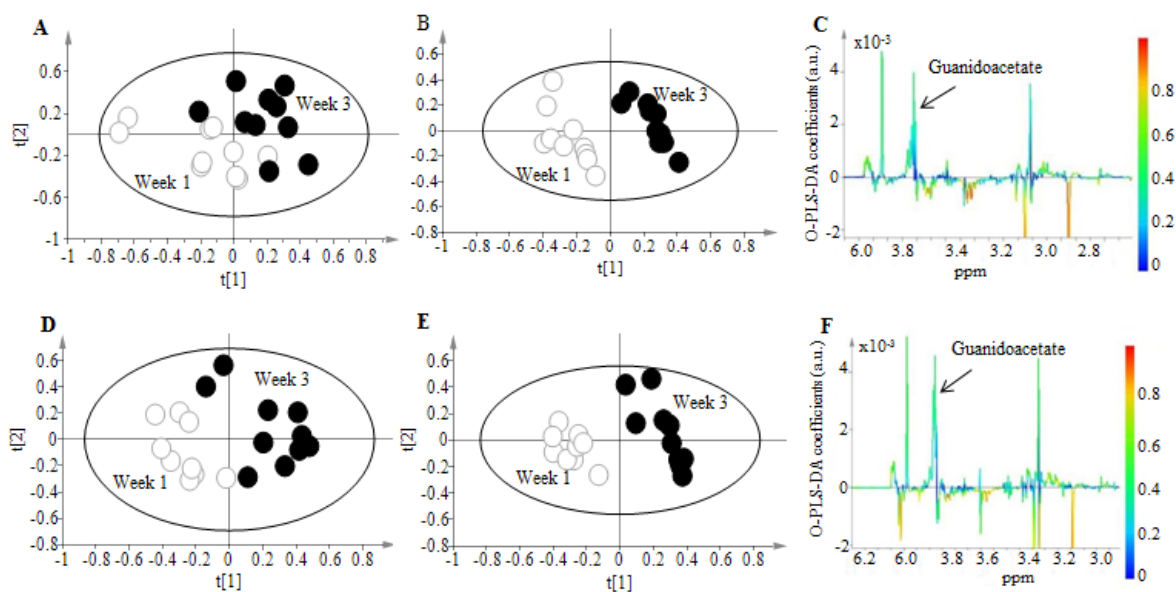


FIGURE 1 Identification of a putative marker in postprandial urine samples collected from NutriTech Food Intake Study. Week 1, low chicken intake (88g/day); Week 3, high chicken intake (290g/day). **A-B**: PCA scores plot ($R^2X=0.47$, $Q^2=0.11$) and PLS-DA scores plot ($R^2Y=0.95$, $Q^2=0.69$) of ^1H NMR urine samples collected on day 3, 2 h post consumption in week 3 (●) and week 1 (o); **D-E**: PCA scores plot ($R^2X=0.46$, $Q^2=0.12$) and PLS-DA scores plot ($R^2Y=0.95$, $Q^2=0.78$) of ^1H NMR urine samples collected on day 3, 6 h post consumption in week 3 (●) and week 1 (o); **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.

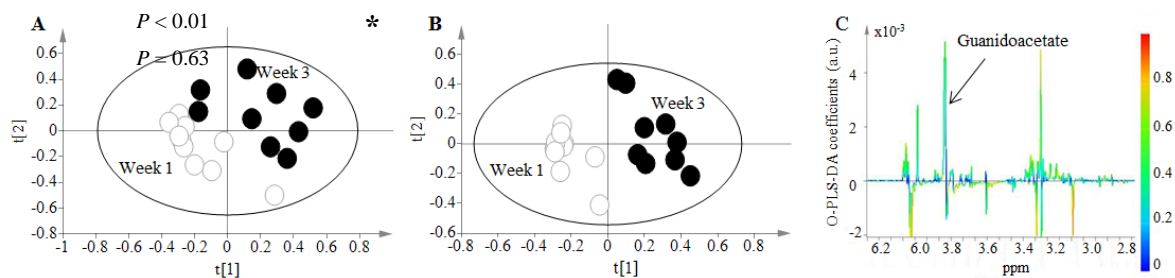


FIGURE 2 Identification of a putative marker in fasting urine samples collected from NutriTech Food Intake Study. Week 1, low chicken intake (88g/day); Week 3, high chicken intake (290g/day). **A-B**: PCA scores plot ($R^2X=0.47$, $Q^2=0.15$) and PLS-DA scores plot ($R^2Y=0.92$, $Q^2=0.67$) of ^1H NMR 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.

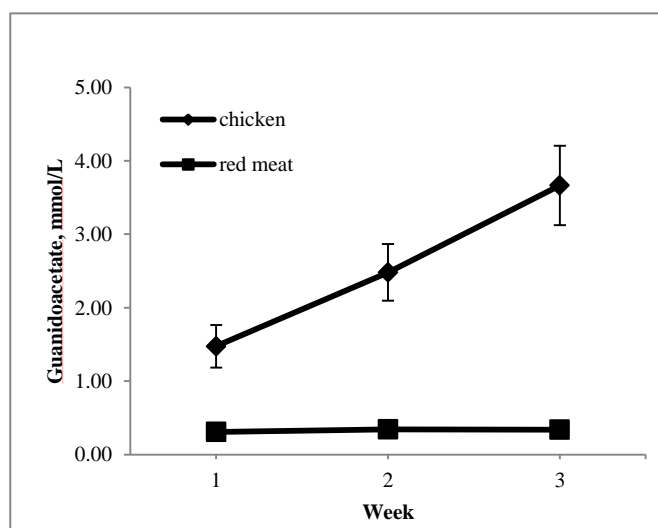


FIGURE 3 Urinary guanidoacetate concentrations (Mean \pm SEM) from NutriTech chicken and red meat groups on day 4 fasting urine samples during three consecutive weeks. In week 1, participants consumed 88 g/day chicken; in week 2, participants consumed 187 g/day

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 ($P < 0.01$).

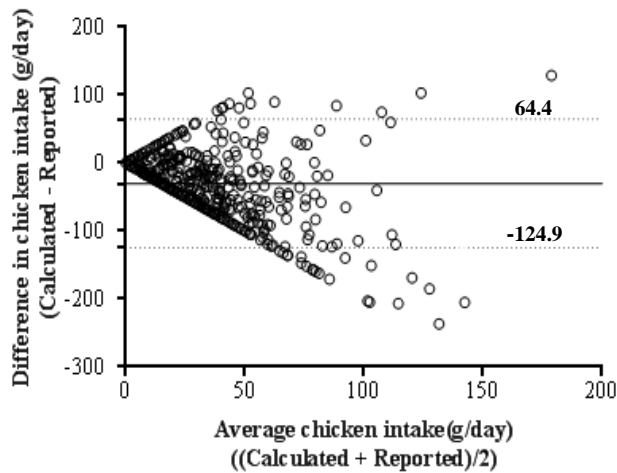
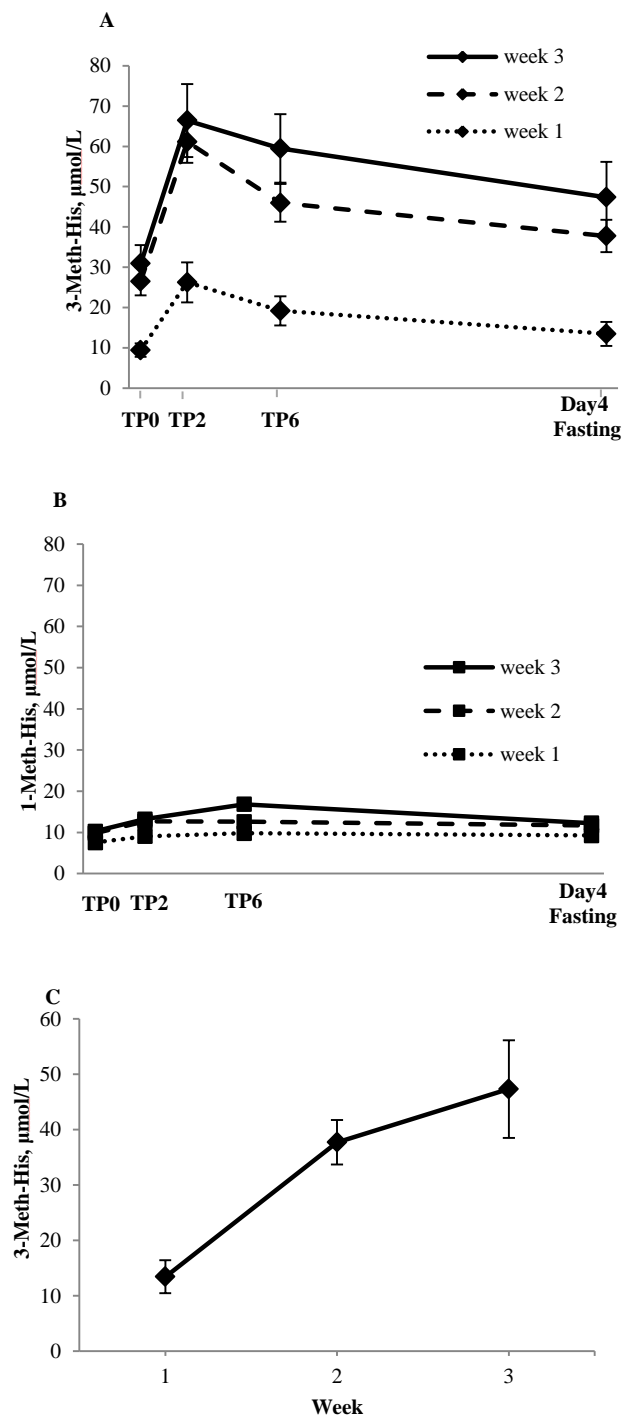


FIGURE 4 Bland and Altman plot of the difference between calculated and reported chicken intake vs the average of calculated and reported chicken intake in NANS study. The solid line (x-parallel line) represents the mean difference (bias) and the dotted line represents the 95% limits of agreement. ‘Calculated’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



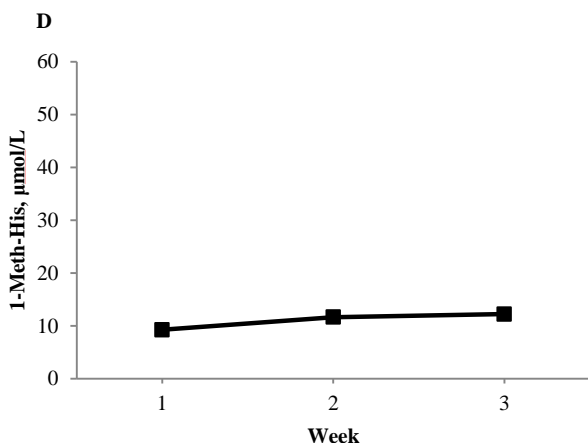


FIGURE 5 Plasma concentrations of 1-Meth -His and 3-Meth-His (mean \pm SEM, n=10) in NutriTech chicken group during three consecutive weeks. **A:** plasma excretion kinetics of 3-Meth-His; **B:** plasma excretion kinetics of 1-Meth-His; **C:** fasting plasma 3-Meth-His concentration changes across three 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

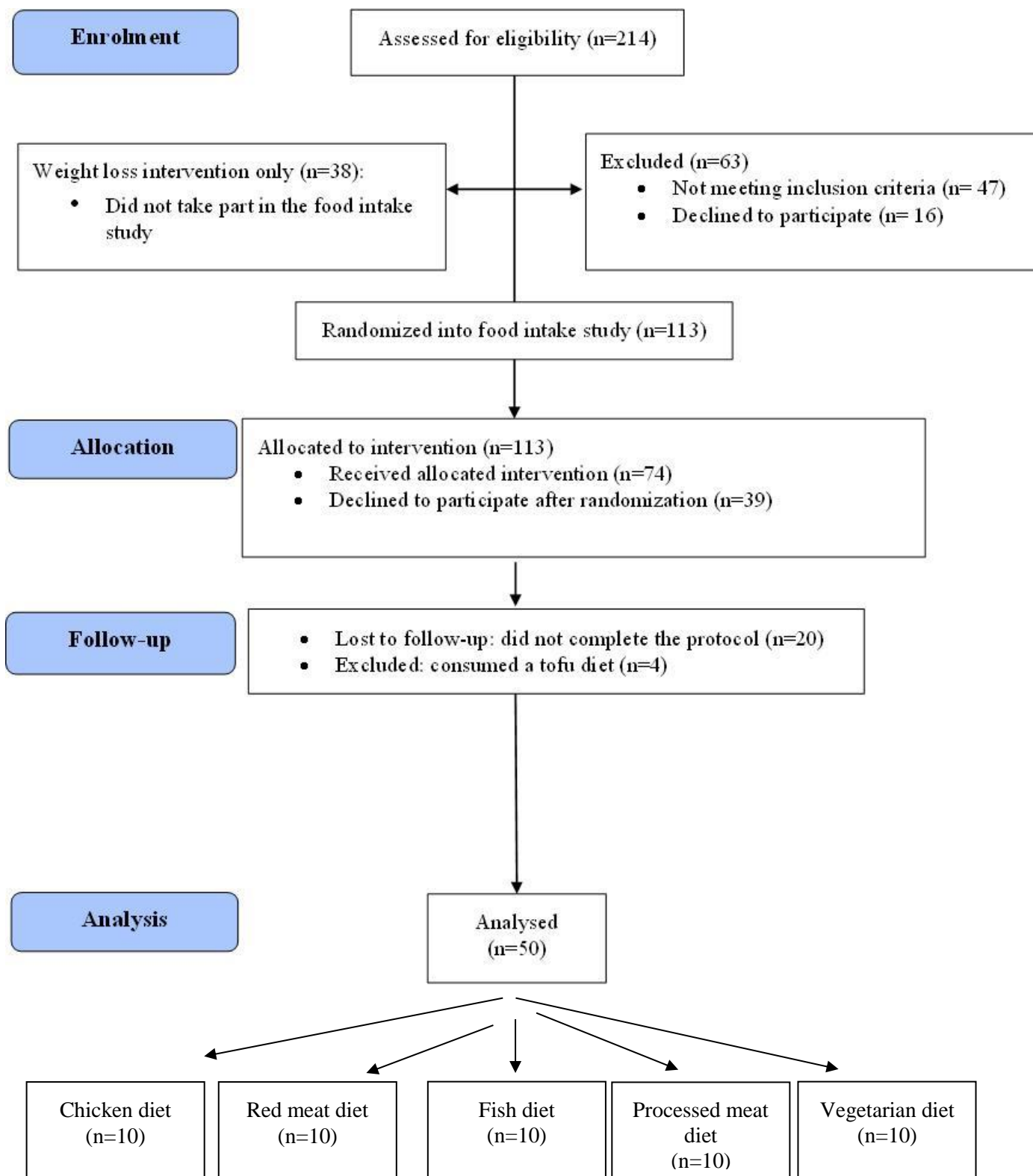
Values are presented as mean ± SEM, n=10 (5 men).

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

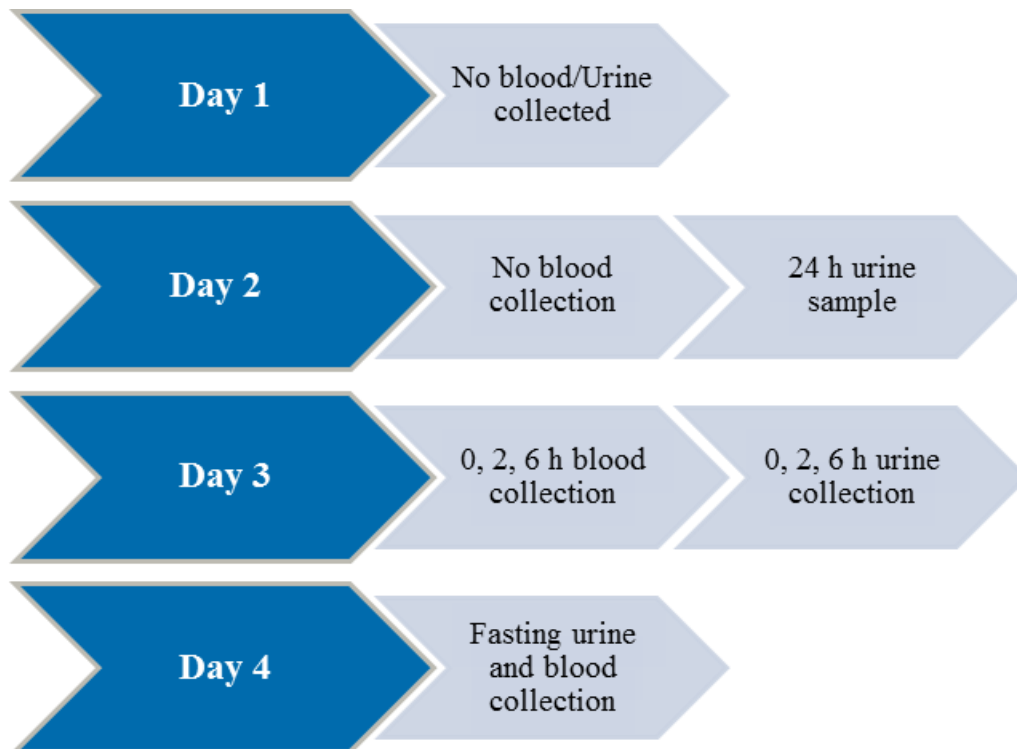
Type of chicken	Concentration	Range
	mg/g	mg/g
Normal	3.45±0.08	3.41-3.60
Free range	10.25±0.62	9.38-11.45
Organic	7.33±0.16	7.04-7.60
NutriTech ¹	3.19±0.11	3.02-3.41

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

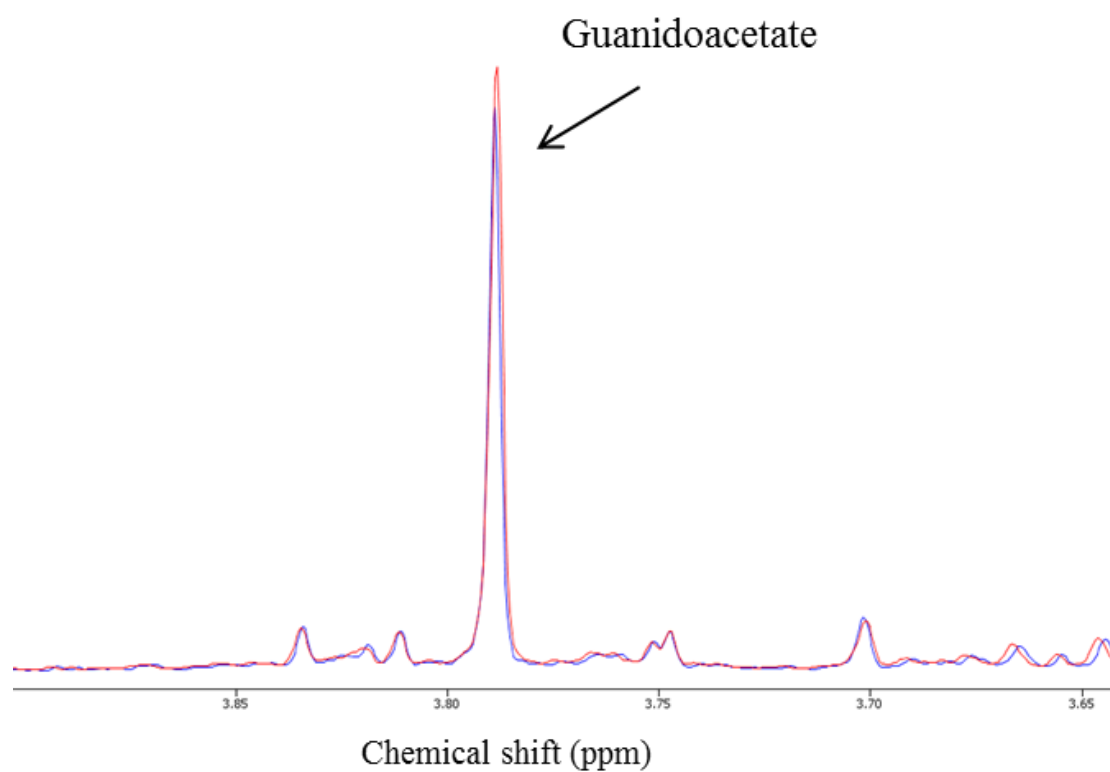
¹ NutriTech means chicken meal in the Nutritech project.



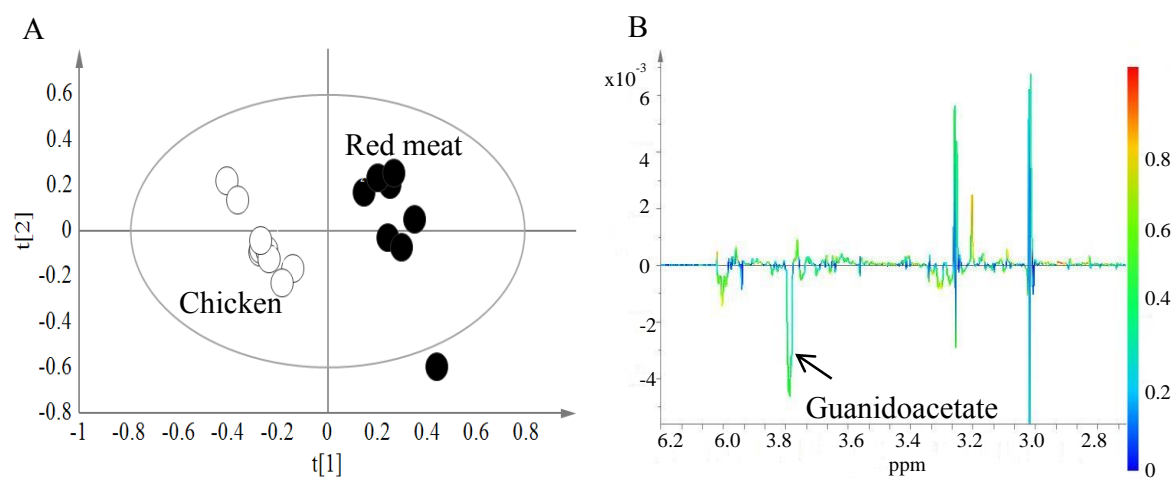
Supplemental Figure 1 NutriTech study participant flow chart



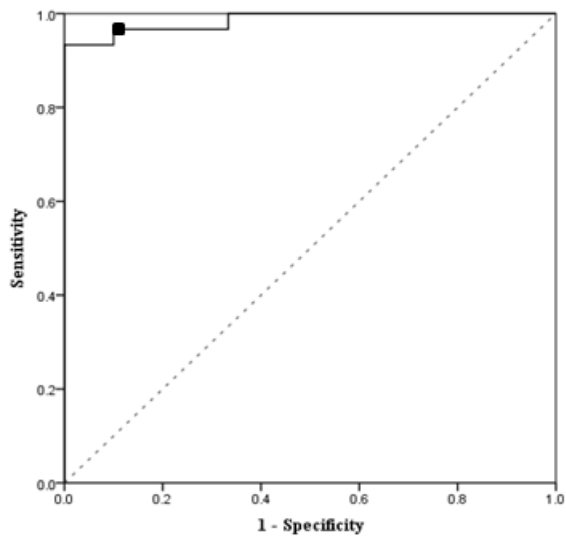
Supplemental Figure 2 Schematic representation of biofluid collection in the NutriTech food intake study. 24 h urine collection began at the first fasting void collected on day 2 until the first fasting collected void on day 3. 0 is prior to consumption, 2 h is 2h post consumption and 6 h is 6 h post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



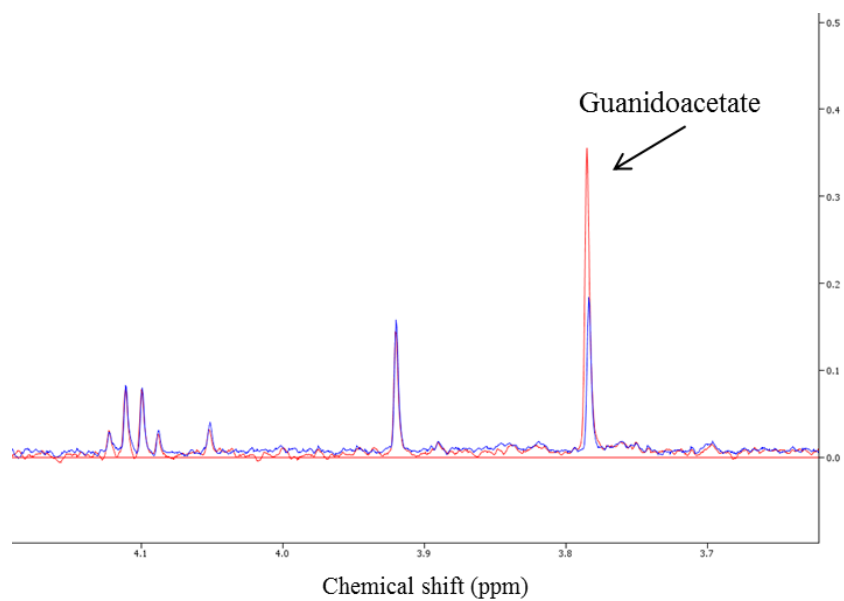
Supplemental Figure 3 ^1H NMR spectrum of a urine sample to confirm the guanidoacetate is correctly assigned. Blue, urine sample; Red, urine sample spiked with standard of guanidoacetate. The concentration of standard is 0.01 mol/L.



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.