

A METABOLOMIC ANALYSIS OF THE ACUTE RESPONSE TO MEALS THAT CONTAIN PORK OR CHICKEN 3B-103

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Pork

By

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Executive Summary

Amino acid (AA) status is determined by factors including nutrition, metabolic rate, and interactions between the metabolism of AA, carbohydrates and lipids. Analysis of the plasma AA profile, together with markers of glucose and lipid metabolism will shed light on metabolic regulation. The objectives of this study were to investigate the acute responses to consumption of meals containing either pork (PM) or chicken (CM), and to identify relationships between AA and markers of glycaemic and lipaemic control. A secondary aim was to explore AA predictors of plasma zinc concentrations.

Ten healthy adults participated in a post-prandial study on 2 separate occasions. In a randomised cross-over design, participants consumed PM or CM. The concentrations of 21 AA, glucose, insulin, triglycerides, non-esterified fatty acids (NEFA) and zinc were determined over 5h post-prandially.

The meal composition did not influence glucose, insulin, triglyceride, NEFA or zinc concentrations. Plasma histidine was higher following the consumption of PM ($P=0.014$), with consistently higher changes observed after 60 min ($P<0.001$). Greater percent increases were noted at limited time points for valine and leucine+isoleucine in those who consumed CM compared to PM. In linear regression some AA emerged as predictors of the metabolic responses irrespective of the meal that was consumed.

The present study demonstrates that a single meal of pork or chicken produces a differential profile of AA in the post-prandial state. The sustained increase in histidine following the consumption of a pork meal is consistent with the reported effects of lean pork on cardiometabolic risk factors.

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Running title: Post prandial metabolic responses to pork and chicken meals

Key words: Amino acids, glucose, insulin, non-esterified fatty acids, triglycerides, zinc

1. Introduction

Amino acid (AA) status is determined by a many factors including dietary adequacy and metabolic rate, and interactions that occur between the metabolism of AA, carbohydrates and lipids. Analysis of the plasma AA profile, together with markers of glucose and lipid metabolism will shed light on the overall network of metabolic regulation and the relationships among the measurable plasma metabolites that can be monitored.

A number of AAs have been implicated in glucose and lipid metabolism. The post-prandial insulin response can be increased substantially by the ingestion of a mixture of AA and/or proteins [1,2], and AA such as tryptophan are associated with appetite control and behavior [3]. The existing literature that reports on high protein diets has shown favourable metabolic responses, including weight loss [4], and improved cardiometabolic risk factors [5] and energy expenditure following the consumption of pork as the main source of protein [6].

Dietary proteins from different sources have differential effects on cholesterol metabolism [7], possibly due to their AA composition. For instance, animals fed a high dietary ratio of lysine to arginine (Lys:Arg) are reported to have hyperlipidaemia and a propensity to atherosclerosis as compared to animals fed a lower Lys:Arg ratio [8] and the addition of Arg ameliorates this effect [9]. Similarly the consumption of Arg was found to improve endothelial function acutely in patients with cardiovascular disease [10,11].

Proteins from animal sources deliver bioavailable inorganic nutrients such as iron and zinc [12,13]. We have shown that the consumption of pork meat by young women maintains hemoglobin levels to the same extent as low-dose iron supplementation [14], and analysis of a sample of the US adult population showed that fresh pork contributed 21% of the total intake of zinc [15]. The relationship between circulating AA and mineral concentrations is largely unexplored and warrants further investigation.

Metabolomic techniques could help identify AA that are associated with metabolic responses following the consumption of meals that contain different sources of protein. We propose to investigate the acute metabolic responses in humans following the consumption of meals that contain either pork (PM) or chicken (CM) meat as the dietary sources of protein, and to identify relationships between AA and markers of glycaemic and lipaemic control in healthy individuals. A secondary aim was to explore potential AA predictors of plasma zinc concentrations.

2. Methodology

Ten adults were recruited through advertising and leaflet distribution on the University of Sydney campus. Volunteers were screened by using a short questionnaire and selected based on age (18-35y). Exclusion criteria included vegetarians, individuals with philosophical or religious reasons for not consuming pork, those who were pregnant, lactating, or reported a major illness (e.g. gastrointestinal disease), body mass index >30 kg/m², and those consuming nutritional supplements or medication. All volunteers were requested to maintain their habitual diet and exercise regimens during the study. The University of Sydney Human Ethics Review Committee approved the study (protocol # 15143) and all subjects gave written informed consent prior to their participation.

Study design

Healthy male and female volunteers participated in a post-prandial study on 2 separate occasions, 2-3 weeks apart. In a randomised cross-over design, participants consumed a meal that contained meat obtained from pork (PM) or chicken (CM) as the main source of protein, prepared in a sweet and sour sauce, and served with rice (50g, cooked weight). The meals were isocaloric (1164 and 1181 kJ per serving, PM and CM, respectively), consisting of similar quantities of dietary protein (31 and 27 g), fat (5 and 7 g) and carbohydrate (27 g in both meals). The meals were formulated and prepared by a food service dietitian (Master Catering Services Pty Ltd, Lidcombe, NSW).

Subjects were asked to refrain from exercise or drinking alcoholic beverages on the day preceding the study. The subjects reported to a metabolic unit in a fasting (12 h) state, where they rested in a seated position before blood samples were obtained. An indwelling cannula (EBOS Healthcare, Kingsgrove NSW) was inserted into the antecubital vein or a dorsal hand vein, and maintained patent by infusion with sodium chloride (EBOS Healthcare, Kingsgrove NSW). Blood samples were collected into lithium heparin vacutainer tubes (Becton Dickinson, North Ryde, NSW), at timed intervals. After the baseline (0 h) blood sample was collected, the participants were provided with either PM or CM and were requested to consume the entire meal within 15 min. Additional blood samples were collected at 0.5, 1, 1.5, 2, 2.5, 3, 4 and 5 h post-prandially. Plasma was recovered immediately by centrifugation (1000 × g, 15 min, 4 °C) and stored at -80 °C until subsequent analysis. The subjects remained mainly sedentary during the test period but were allowed to walk within a restricted area of the metabolic unit, read, or use their computers. No food was provided following the meal but water was readily available.

Biochemical and anthropometric measurements

The subjects' height was measured to the nearest 0.1cm (Harpenden Stadiometer, Holtain Ltd, Crymch, UK) and they were weighed, in light clothing and no shoes, to the nearest 0.1kg on an electronic scale (Tanita, Corporation, Kewdale, USA). Weight and height were used to calculate BMI.

Plasma glucose concentrations were analysed using a glucose hexokinase assay (Roche Diagnostic Systems, Sydney, NSW) adapted for use on an auto-analyser (Roche/Hitachi 912®; Boehringer Mannheim GmbH, Mannheim, Germany). Plasma insulin concentrations were measured by a competitive radioimmunoassay (RIA, Coat-a-Count, Diagnostic Products, Los Angeles, CA, USA). Plasma triglycerides were analysed on a Cobas 8000 analyser (Roche Diagnostics, Basel, Switzerland) using enzymatic reagents supplied by Roche. Analysis of non-esterified fatty acids (NEFA) was performed on an auto-analyser (Konelab 20 XT, Vantaa, Finland), using enzymatic reagents supplied by Wako (Osaka, Japan). Plasma zinc concentrations were determined using inductively coupled plasma mass spectrometry (ICPMS, Agilent 7500ce, Santa Clara, CA). Samples were diluted (1:40 v/v) in ammonium EDTA using rhodium as an internal standard and measured against a matrix-matched standard curve [16].

Amino acids

Alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, ornithine, proline, phenylalanine, serine, threonine, tryptophan, tyrosine and valine, all with purity ≥98% were purchased from Sigma-Aldrich (Castle Hill, NSW). All analytical procedures were based on the method of Wei et al [17]. Briefly, samples were thawed gradually by placing them sequentially at -20 °C for 2 h, 4 °C for 1 h, and room temperature for 30 min. Plasma was incubated in extraction buffer (80 %

(v/v) ethanol, 0.1% (v/v) formic acid), at 4°C for 2 h, then centrifuged. The supernatant was centrifuged further in a vacuum centrifuge (Eppendorf, Hamburg, Germany). The dry extract was then stored at -20°C until required.

The HPLC mobile phase (A) was 0.1% (v/v) acetic acid and mobile phase (B) was acetonitrile. Each cycle was 9.5 min, and started with 0% B at 0.1 mL/min for 1 min, followed by a linear increase to 100% B in 0.5 min and held for 4 min at 0.2 mL/min, finalised by a decrease to 0% B and held for 4 min at 0.1 mL/min. Aliquots (100 µL) of each sample were dispensed into HPLC plates (Agilent, Santa Clara, CA) with a plastic cover to avoid evaporation. Metabolites were injected in technical triplicates onto a Luna Phenyl-Hexyl column (1.0 x 50 mm, 5 µm particle size, fitted with a guard cartridge; Phenomenex Australia Pty Ltd) using a Agilent 1200 HPLC system (Agilent, Santa Clara, CA). The column was coupled to a 5500 QTRAP quadrupole ion trap mass spectrometer which was fitted with a TurboVTM ion source (AB Sciex, Foster City, CA). The polarity for the ionisation mode was set at positive and the ion spray potential was set at 5000 V. The ion source gas 1 and 2 were set at 40 psi. The curtain and collision gases (ultra high purity nitrogen) were set at 20 psi and medium, respectively. The source temperature was at 400°C. The de-clustering potential, collision energy and product ions were optimized manually. The dwell time for all AA was 50 ms.

The LC-MS/MS data were analysed using MultiQuant 2.0 software. The relative quantity of the metabolite was determined by integrating the area under the curve. All chromatograms were checked manually for peak shape and retention time. Gaussian smooth width was set at 3 points and noise percentage at 40. Mean value of the triplicate samples was calculated after ensuring the CV was <5%.

Statistical analyses

Paired t-tests were used to determine whether the average post-prandial responses over 5h differed between CM and PM. As the shapes of the PM and CM curves were qualitatively similar, an average value was intended as a summary measure. Stepwise regression was used to identify whether any of the AA at baseline or at peak values were able to predict the response to any of the metabolic variables. Differential effects of CM and PM on plasma glucose, insulin, triglycerides, NEFA and zinc concentrations were tested by a general linear model with meal as fixed factor, subject as random factor and any quantitative variables identified by the regression as covariates. Statistical analyses were carried out using IBM SPSS v20 (<http://www-01.ibm.com/software/analytics/spss/>). A value of P < 0.05 was taken to designate statistical significance.

3. Outcomes

Ten subjects (4 male, 6 female) completed both metabolic profile test days. Their mean age was 24.9 y (range 18-32) and their BMI was 20.8 (range 18.9-24.0) kg/m². The participants' characteristics are shown in Table 1. There were no significant differences between any of the metabolic parameters at baseline during the 2 test days.

Table 1 - The participants' metabolic profiles¹ prior to consuming each test meal

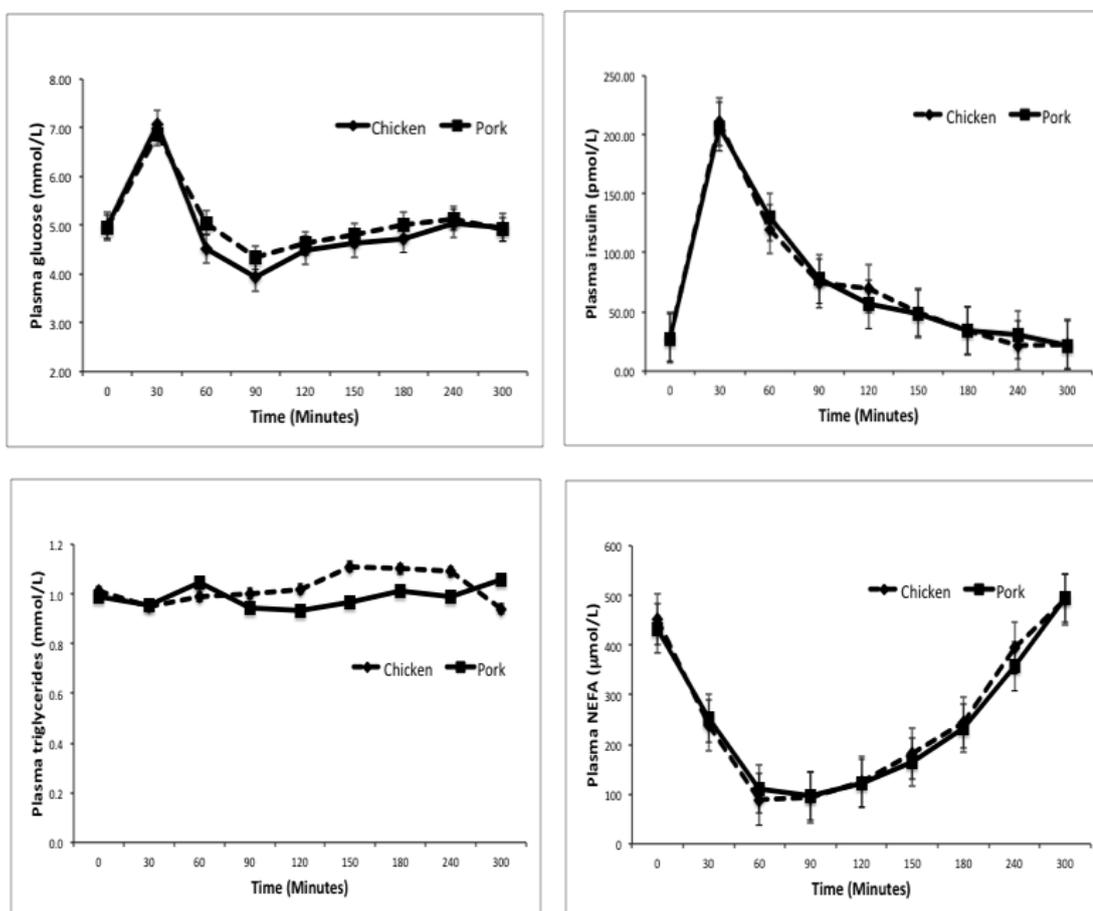
	PM ²	CM ³
Plasma glucose (mmol/L)	5.0 ± 0.2	5.0 ± 0.3
Plasma insulin (pmol/L)	27.5 ± 19.5	28.8 ± 14.1
Plasma triglycerides (mmol/L)	1.0 ± 0.3	1.0 ± 0.5

	PM ²	CM ³
Plasma NEFA (µmol/L)	433 ± 197	452 ± 201
Plasma zinc (µmol /L)	12 ± 3	11 ± 2

¹Values are shown as mean ± SD, n=10. ²Pork meal. ³Chicken meal

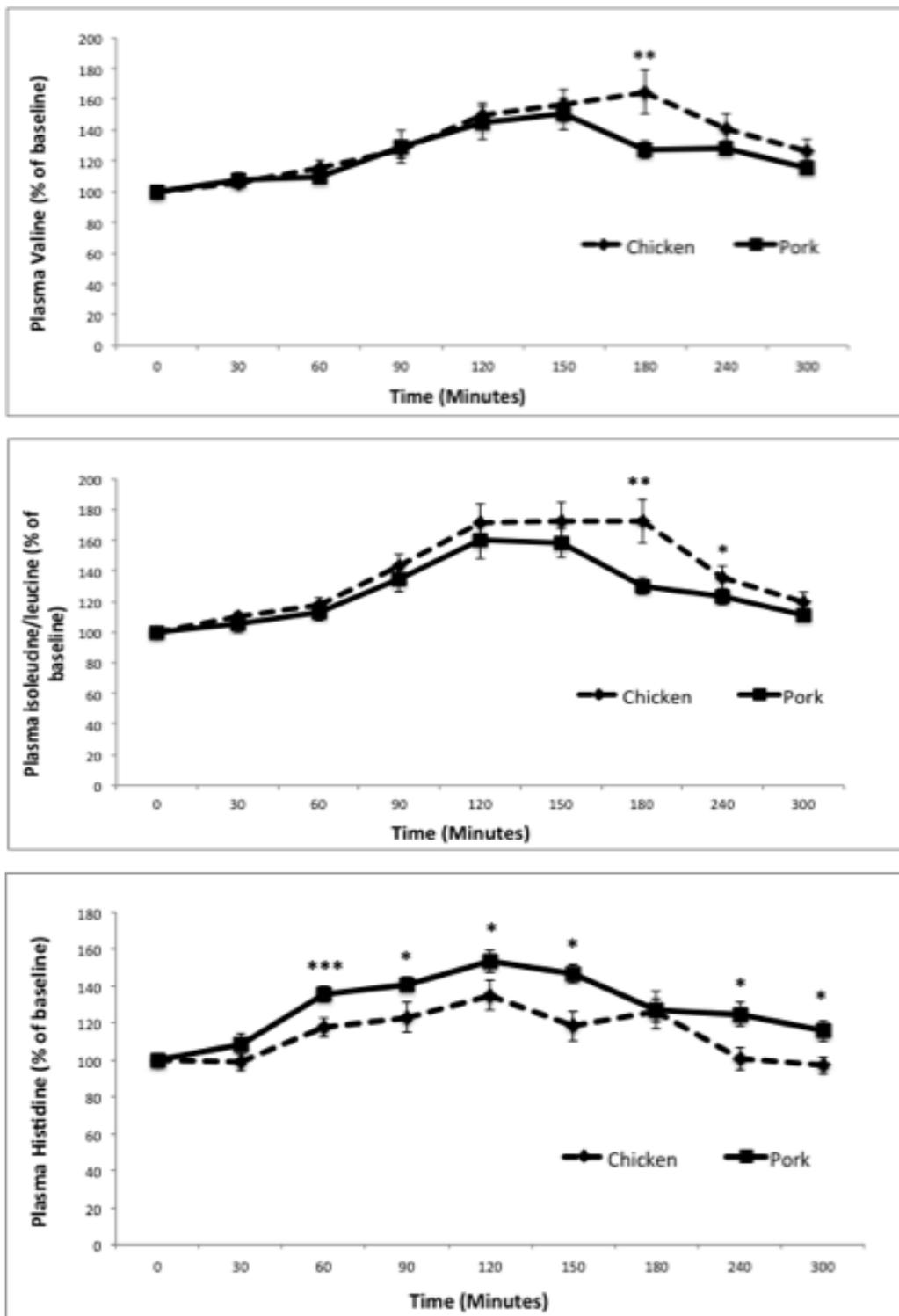
The meal composition did not influence the post-prandial plasma glucose, insulin, triglyceride, NEFA or zinc concentrations. Following the consumption of either CM or PM, significant increases were observed for plasma glucose (P<0.001) and insulin (P<0.001) concentrations, reaching peak values at 30 min. Plasma NEFA concentrations decreased significantly (P<0.001) to a nadir between 30-90 min then rose gradually to baseline values. Plasma triglyceride and zinc concentrations showed no significant change from baseline values (Figure 1).

Figure 1 - Post-prandial concentrations of plasma glucose, insulin, triglycerides and non-esterified fatty acids, following the consumption of meals that contain chicken or pork.



The response of AA was affected by the test meal (Figure 2). The mean concentrations of plasma valine (Val) and leucine + isoleucine (Leu+Iso; calculated over 5 h) were significantly higher following the consumption of CM (P=0.050 and P= 0.017, respectively) as compared to PM. Significantly (P<0.01) greater percent increases were noted at 180 min post-prandially for Val and Leu+Iso in those who consumed CM compared to PM. In contrast, the mean concentration of plasma histidine (His) was significantly higher post-prandially (P=0.014) in those who consumed PM, with consistently higher changes from baseline observed after 60 min (P<0.001). No other statistically significant differences were noted in plasma AA following the consumption of CM or PM.

Figure 2. Post-prandial response of selected plasma AA following the consumption of meals that contain chicken or pork



We investigated the relationships between AA and other metabolic biomarkers, with the responses measured at 30 min for glucose, insulin, triglycerides and zinc; and the minimum value of NEFA for each person, which usually occurred between 30-90 min post-prandially. AA values were investigated at 30 min and also as an average value over 5h. The main predictive relationships are shown in Table 2. Plasma glucose concentrations were predicted significantly by alanine (Ala; $P < 0.001$), Lys ($P = 0.006$) and His ($P = 0.029$), and a regression with these three variables had $r^2 = 0.74$. When these relationships were examined using a general linear model with meal as fixed factor, subject as random factor and the three AA

as covariates, only Ala was significant ($p=0.001$). None of the AA was identified as significant variables for insulin concentrations.

Table 2 - Plasma AA predictors of plasma glucose, insulin, triglycerides, NEFA and zinc concentrations¹

Concentration in plasma at 30 minutes post-prandially	Predictor amino acid ³	
	Regression analysis	General linear model
Glucose	<i>Ala^a, Lys^b, His^c</i>	<i>Ala^a</i>
Insulin	NS	NS
Triglycerides	<i>Orn^d, Tyr^a</i>	NS
NEFA ²	<i>Val^e</i>	NS
Zinc	<i>Arg^f</i>	NS

¹Values recorded at 30 minutes were frequently the peak value.

²The minimum value for each person was taken, usually occurred at 30, 60 or 90 min post-prandially.

³Predictor AA values were significant for the AA listed, measured at 30 min (glucose, triglycerides, zinc) or as an average response over 5 h (non-esterified fatty acids).

^{a-f} Superscripts indicate statistical significance ^a $P<0.001$, ^b $P<0.006$, ^c $P<0.029$, ^d $P<0.002$, ^e $P<0.033$, ^f $P<0.021$.

For plasma triglyceride concentrations, significant predictors were ornithine (Orn; $P=0.002$) and tyrosine (Tyr; $P<0.001$), with $r^2=0.49$. When investigated using a general linear model as described above, neither of these AA was significant. NEFA were predicted by the average Val over 5h ($r^2=0.23$, $p=0.033$). Arg was related to plasma zinc concentrations during the post-prandial period ($r^2=0.26$, $p=0.021$).

4. Discussion

In this controlled acute study in healthy subjects, we investigated the effects of consuming meals that contained either pork or chicken as the primary source of protein on plasma AA, glucose, insulin, triglycerides, NEFA, and zinc concentrations. For plasma AA, His was consistently and significantly higher following the consumption of PM, while Val and Leu+Iso were significantly higher following the consumption of CM. For the other measured variables, CM or PM consumption had no statistically significant effect. A number of plasma AA emerged as predictors of the metabolic response irrespective of the type of protein that was consumed.

The plasma AA response is believed to reflect differences in the rates of protein digestion, and differences between proteins were illustrated by milk proteins: the whey fraction is rapidly digested while casein has a relatively slower rate due to curd formation in the stomach. Differences in the rates of digestion have prompted the concept of “fast” and “slow” proteins, which was proposed based on the dynamics of the plasma AA responses [18]. In the present study plasma AA concentrations increased to a peak at 30 min post-prandially, with the majority of the analysed AA showing a similar profile of response following CM or PM. Similar responses have been observed following the consumption of beef and chicken [19], however AA from meals that contained fish [19], egg-white and cottage cheese [20] took longer to reach peak values, possibly due to differences in the rates of gastric emptying or protein hydrolysis, suggesting a lower rate of digestion [21,22].

The protein matrix also affects the rates of AA appearance in plasma. It has been reported [23,24] that the intake of liquid meals produced more rapid changes in the concentrations of plasma AA as compared to their intact counterparts. Factors that may alter the profile of AA include the presence of other components in

mixed meals, particularly those that are derived from vegetables and cereals that are consumed commonly with meat-containing meals e.g. cell wall material that may interfere with AA absorption [25]. There were no differences in the palatability of the test meals in the present study which were formulated using identical ingredients except the protein source, and were low in fat and dietary fibre.

One of the main differences in AA that emerged between PM and CM was the significantly higher levels of His following the consumption of PM. His levels were higher consistently over the 5h post-prandial period. Serum His levels are reported to be lower in obese women, and are negatively associated with inflammation and oxidative stress [26]. In a supplementation trial, His significantly decreased markers of insulin resistance, waist circumference, fat mass, serum NEFA, serum inflammatory cytokines and oxidative stress [27]. In the present study His was significantly associated with glucose concentrations in regression analysis, however this relationship was not evident in the general linear model. The lack of strong association of His and glycaemic control in the present study could be due to the subjects having a BMI and glucose concentrations in the normal range. His also resulted in an increased serum adiponectin [27], a hormone that is inversely correlated with body fat percentage in adults [28]. His intake is inversely associated with energy intake in young women [29] and in rats suppresses food intake and increases the expression of uncoupling protein in brown adipose tissue, leading to increased fat oxidation [30].

In a post-prandial study in humans, Charlton et al [31] determined the effects of pork, beef or chicken meals on satiety and hormones that are associated with appetite. There were no significant differences in measures of satiety after consumption of the test meals at breakfast, however a significantly higher concentration of peptide YY (PYY) and a tendency for an increased cholecystokinin (CCK) were observed in those who consumed the pork meal as compared to the chicken meal [31]. Thus it is possible that increasing the intake of pork, elicits a combination of increased His concentrations and satiety hormones leading to improved glycaemic control, reduces BMI and fat mass. The latter changes in cardio-metabolic risk factors have been reported in studies that used pork as the main source of protein in weight reducing diets [5,6].

Significantly higher levels of the branched chain AA (Val, Leu+Iso) were observed following the consumption of CM as compared to PM. The differences were observed at 180 min (Val, Leu+Iso) and 240 min (Leu+Iso) post-prandially, giving an overall average higher response following the consumption of CM. In cohort studies plasma branched chain AA concentrations were predictive of future development of type 2 diabetes mellitus [32,33]. Significant inverse correlations have been reported between Iso and Val concentrations, and insulin sensitivity [34]. Elevated plasma concentrations of branched chain AA are proposed as an early indicator of insulin resistance, however the evidence that they promote insulin resistance in humans is not clear [34].

It has been shown previously that proteins and individual AA could stimulate insulin secretion [1,2]. None of the AA that were analysed in the present study were correlated with the insulin response and only Ala remained a significant predictor of glucose concentrations when analysed in a general linear model. Few studies have investigated Ala: in obese subjects Ala consumption is associated with lower glucose and higher insulin concentrations at baseline [35]. The lack of any relationship with glucose and insulin is consistent with previous observations [1] that AA do not significantly affect the glucose response in normal young people although more subtle responses may be seen under conditions of a euglycaemic clamp [36]. In addition, the majority of studies that investigate the role of AA on glycaemic control are carried out with meals that contain little or no

carbohydrate, or consist of AA in isolation. Thus the lack of a relationship between AA and insulin in the present study could be due to the young age of the participants and absence of any underlying metabolic disease. It is possible also that the carbohydrate portion of the test meal prevented any specific effects of AA on glucose and insulin responses to be observed.

The identity of factors that enhance zinc absorption remains unclear. Increasing intake of dietary protein are associated with enhanced absorption [13] and it is believed that AA that are released during digestion may play an important role in maintaining zinc soluble in the intestine and therefore more readily absorbed [37]. In rats His enhances the absorption of zinc at a maximal rate at near physiological pH [38]. In the present study Arg was associated by linear regression to plasma zinc concentrations and this association is consistent with the proposed role of AA. The lack of effect of Arg and other AA in the general linear model is likely to be influenced by the minimal changes that occurred in plasma zinc concentrations.

A number of limitations need to be acknowledged: the inclusion of a small number of male and female subjects with normal glycaemic control may have limited our ability to observe the effect of pork and chicken meals on metabolic responses, and any potential gender differences that may exist. The analytical procedures did not allow for the adequate separation of Leu and Iso and thus these AA are presented as a composite. In some instances the plasma AA were close to the lower limits of detection and may have introduced an increased standard deviation in the reported mean values. Other limitations are the lack of data on the dietary AA composition of the test meals, and absence of a proximate analysis of the meal composition that was consumed.

In summary, the present study demonstrates that a single meal that contains pork or chicken produces a differential profile of AA in the post-prandial state. The results in young healthy subjects show that meals containing pork result in higher His and lower branched chain AA than meals that contain chicken. While the effects of branched chain AA in plasma are controversial, good evidence suggest that the sustained increase in His following the consumption of a pork meal is consistent with the reported favourable effects of lean pork on cardiometabolic risk factors.

5. Implications

1. We have generated pilot data that compare the AA profiles of pork and chicken meals post-prandially. The generation of these data employed state-of-the-art technology and represent a functional resource for nutrition research and training.
2. In this pilot study *His* emerged clearly as being significantly higher following the pork meal as compared to chicken. There is good evidence in the literature that *His* contributes to an improved metabolic profile and may be responsible partly for the published health benefits of pork, e.g. weight loss.
3. The *His* response will provide opportunities for innovative product development, e.g. selection of muscle compartments or other parts of the animal carcass that have high *His* concentrations.
4. Further research is needed to confirm the change in *His*. If confirmed this information may lead to new evidence-based statements regarding the health benefits of pork.
5. The trial should be repeated and extended to include a greater number of subjects and measurements of other potential benefits of *His*, such as measures of inflammatory markers and oxidative stress.

6. Branched chain AA were higher following the consumption of chicken meat. The response of these AA may be beneficial under some circumstances, e.g. in individuals with altered protein requirements such as the elderly, athletes or renal patients. However in obesity and diabetes the role of these AA is controversial. It is not clear if post-prandial responses to a meal necessarily lead to any benefit or adverse health effects. This warrants further investigation in individuals who are obese or diabetic.
7. Our methodology offers the opportunity to investigate further the relationships between AA from pork and the absorption of inorganic nutrients such as iron, zinc and selenium.

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