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Differential effects of L- and D-phenylalanine on pancreatic and gastrointestinal hormone release in humans: A randomized crossover study

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Abstract

Aim: To investigate the effects of L-phenylalanine on gastroenteropancreatic hormone release, glucose levels, subjective appetite and energy intake in humans, and to determine whether these effects were stereoisomer-specific by comparing them with D-phenylalanine.

Materials and methods: A dose-finding, non-randomized, unblinded, crossover study was conducted during October–December 2017 at the NIHR Imperial Clinical Research Facility in five participants, in which the tolerability of escalating doses of oral L-phenylalanine was assessed (0, 3, 6 and 10 g). Also, an acute, randomized, double-blind, placebo-controlled crossover study was conducted during January–May 2018 at the NIHR Imperial Clinical Research Facility in 11 participants, in which the effects of oral 10 g L-phenylalanine relative to D-phenylalanine and placebo on gastroenteropancreatic hormone (insulin, glucagon, glucose-dependent insulintropic polypeptide [GIP], peptide tyrosine tyrosine [PYY], glucagon-like peptide-1) and glucose concentrations, visual analogue scales for subjective appetite and energy intake at an *ad libitum* meal served 70 minutes post-ingestion, were investigated.

Results: L-phenylalanine was well-tolerated and increased insulin and glucagon concentrations prior to meal ingestion at several time points relative to placebo and D-phenylalanine ($P < .05$). L-phenylalanine also increased GIP concentrations relative to D-phenylalanine ($P = .0420$) and placebo ($P = .0249$) 70 minutes following ingestion. L-phenylalanine reduced postprandial glucose area under the curve (AUC)_{70–150mins} relative to placebo ($P = .0317$) but did not affect subjective appetite or energy intake ($P > .05$). D-phenylalanine increased postprandial PYY AUC_{70–150mins} concentrations relative to placebo ($P = .0002$).

Conclusions: Ingestion of L-phenylalanine, but not D-phenylalanine, increases insulin, glucagon and GIP concentrations without appearing to have a marked effect on appetite.

KEYWORDS

appetite, calcium-sensing receptor, glycaemia, humans, L-phenylalanine

1 | INTRODUCTION

High-protein diets (HPDs) improve long-term glycaemic control (as measured by HbA1c) in people with type 2 diabetes¹ and body composition in adults.^{2,3} Accordingly, HPDs may benefit individuals with type 2 diabetes (T2D) and/or obesity,^{4,5} but are difficult to adhere to.⁶ Understanding how HPDs mediate their beneficial effects may facilitate development of agents to treat or prevent T2D and obesity.

Protein stimulates the release of gastroenteropancreatic hormones involved in glycaemic control and appetite regulation,^{7,8} including insulin, glucagon, cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP) and peptide tyrosine tyrosine (PYY). These hormonal changes are believed to be partly responsible for the HPD-induced improvements in glycaemic control and body composition.

Following ingestion, protein is hydrolysed within the gastrointestinal tract into amino acids, which can be sensed via cellular mechanisms, including promiscuous amino-acid sensing G-protein-coupled receptors such as the calcium-sensing receptor (CaSR).⁹ The CaSR is widely expressed in tissues not associated with calcium metabolism,¹⁰ including pancreatic beta cells,¹¹ gastrointestinal enteroendocrine cells¹² and vagal afferents,^{13,14} suggesting physiological roles in glucose and energy homeostasis and gastroenteropancreatic hormone release. With regards to nutrient detection, the CaSR is allosterically modulated by amino acids, showing a stereoselective response to their L-isomers.¹⁵ Evidence suggests that the magnitude of change in both calcium mobilization and CaSR sensitivity varies depending on the L-amino acid utilized, with L-phenylalanine regarded as the most potent amino acid allosteric enhancer of the CaSR.¹⁵

Human interventions show that acute ingestion of ~10 g L-phenylalanine reduces the postprandial glucose area under the curve (AUC) by ~65%,¹⁶ decreases energy intake at a meal by ~2000 kJ and stimulates CCK release.¹⁷ Non-significant trends on PYY and GLP-1 levels have also been reported.¹⁸ Previous research suggests the glycaemic and appetite-suppressing effects of L-phenylalanine in animals are mediated by the CaSR, activation of which stimulates the release of GLP-1, GIP and PYY.^{19,20} Relative to humans, these animal models use comparatively high doses of L-phenylalanine (~3–12 mmol/kg, equivalent to ~35–140 g of L-phenylalanine for a 70 kg human). However, comparing preclinical in vivo doses with human doses is difficult because of interspecies differences in body weight, size and metabolism that alter pharmacokinetics and pharmacodynamics,²¹ and is particularly challenging when administering an agent believed to act within the gut lumen, where local exposure of the nutrient-sensing mechanism to the agent would be expected to matter more than systemic dose. It is therefore important to investigate whether these effects occur at doses feasible for human intervention.

Identifying whether effects on hormone release are specific to the L-phenylalanine stereoisomer would provide evidence that they are

mediated by a specific biological mechanism, and would be in accord with a role for the CaSR in energy and glucose homeostasis in humans. This can be achieved by the inclusion of the synthetic isomer D-phenylalanine as a comparator, which exhibits weak modulatory activity of the CaSR¹⁵ but provides an isonitrogenous, isosmotic and isocaloric control. Previous research shows that 10 g L- but not D-phenylalanine induces anorectic effects and CCK release, implying a role for the CaSR or another stereoisomer-specific mechanism in this response.¹⁷ However, the comparative release of other gastroenteropancreatic hormones following D- and L-phenylalanine administration is unknown. Additionally, for L-phenylalanine to have possible therapeutic utility, it is helpful to establish whether it has wider, and possibly detrimental, metabolic effects beyond those expected on downstream pathways such as tyrosine synthesis.

The present study therefore aimed to investigate the effects of L-phenylalanine on gastroenteropancreatic hormone release, glucose levels, subjective appetite and energy intake in humans, and to determine whether these effects were stereoisomer-specific by comparing them with D-phenylalanine. Nuclear magnetic resonance analyses were also used to investigate possible wider effects on metabolism.

2 | MATERIALS AND METHODS

2.1 | Ethics

This study was granted ethical approval (London - West London & GTAC Research Ethics Committee, UK; reference number: 16/LO/1040) and conducted in accordance with the Declaration of Helsinki. All participants provided written informed consent prior to study commencement.

2.2 | Study design

Two studies were conducted: (a) a dose-finding study to determine the tolerability of L-phenylalanine and (b) an acute study to investigate and compare the effects of L- and D-phenylalanine on gastroenteropancreatic hormone release, glycaemia, subjective appetite and energy intake.

L-phenylalanine and D-phenylalanine powders were purchased from Euro OTC Pharma and encapsulated in opaque hypromellose capsules (Blackburn Distributions, UK) using a capsule machine (The Capsule Machine, Capsule Connection, USA).

2.2.1 | Dose-finding study

The dose-finding study had a five-visit, unblinded, ascending dose design. The five visits involved participants consuming increasing doses of L-phenylalanine at sequential visits: (a) 0, (b) 0, (c) 3, (d) 6 and

(e) 10 g. The inclusion of two 0 g visits facilitated participant familiarization with the study protocol. The maximum dose (10 g) was based on the highest single dose used by prior studies investigating oral L-phenylalanine.^{16,17,22–24} All participants received doses in the same order with study visits separated by a minimum of 1 week.

2.2.2 | Acute study

The acute study had a four-visit, double-blind, placebo-controlled crossover design. The first visit for all participants was an acclimatization visit, during which participants consumed the placebo treatment to enable their familiarization with the study protocol. Participants were then randomly allocated to a sequence comprising the following treatments: (a) placebo, (b) 10 g D-phenylalanine or (c) 10 g L-phenylalanine. Study visits were separated by a minimum of 1 week.

Randomization Treatment sequences were generated using randomisation.com and allocated to participants using sealed opaque envelopes upon commencement of the first visit.

Blinding Treatments were blinded by an external staff member; participants and investigators were unaware of treatment. Visual analogue scales (VAS) were scored, energy intake calculated and assay performed while blinded. Treatment allocation was then revealed to permit statistical analysis.

2.3 | Recruitment

Healthy males or females aged 18–60 years with a body mass index (BMI) of 18.5–30.0 kg/m² were recruited through the Imperial Clinical Research Facility healthy volunteer database. Participant exclusion criteria are described in Appendix S1.

2.4 | Sample size

The dose-finding study was a descriptive pilot study and thus a formal power calculation was not used. Pancreatic hormones were measured in the dose-finding study; we were particularly interested in the effects of L-phenylalanine on glucagon, which appeared to reflect the effects of a high-protein meal on glucagon release.²⁵ Sample size for the acute study was therefore calculated based on glucagon data from the dose-finding study (Figure S1D). It was estimated that nine participants would provide an 80% chance of detecting a statistically significant difference with *P* less than .05. Consequently, we aimed to recruit 12 participants to account for an estimated dropout rate of 30%.

2.5 | Study visits

2.5.1 | Dose-finding study

Participants arrived at the Imperial Clinical Research Facility at about 08:30 AM following an ~12-hour overnight fast. Participants were

instructed to purchase and consume the same ready meal of their choice before 08:00 PM the evening prior to each study visit. Participants were also instructed to refrain from alcohol consumption and strenuous exercise for the 24 hours preceding the study. Upon arrival, an intravenous cannula was inserted into the antecubital vein of the participant to allow serial blood sampling. Following the collection of baseline measurements, participants ingested either (a) 0, (b) 0, (c) 3, (d) 6 or (e) 10 g L-phenylalanine at *T* = 0 (in 0, 0, 5, 9 and 13 capsules, respectively; size 00). Blood samples and 100 mm VAS were collected at 15-minute intervals (*T* = 0, 15, 30, 45, 60, 75, 90, 105, 120, 135 and 150) for 150 minutes following ingestion (Figure S2).

2.5.2 | Acute study

The study design of the acute study was identical to the dose-finding study except that following baseline measurements, participants ingested either (a) placebo, (b) 10 g D-phenylalanine or (c) 10 g L-phenylalanine at *T* = 0 (all in 13 capsules; size 00); participants were provided an *ad libitum* test meal 70 minutes following treatment ingestion (*T* = 70), resulting in blood samples and VAS also being taken at this time point (replacing *T* = 75; Figure S2). A 70-minute premeal interval was selected on data from the dose-finding study showing that insulin and glucagon concentrations probably peaked between 60 and 90 minutes following L-phenylalanine ingestion (Figure S1A,C), as well as providing equivalent preprandial and postprandial periods in which to investigate the effects of L-phenylalanine on hormone release.

The *ad libitum* test meal was a tomato and mozzarella pasta bake ready meal (Sainsbury's, UK) with an energy density of 473 kJ/100 g and containing 3.3 g fat (26% of total energy), 14.9 g carbohydrate (53% of total energy) and 4.5 g protein (16% of total energy) per 100 g. Participants were given 20 minutes to consume the meal in an enclosed area and were instructed to 'eat until comfortably full'. The use of mobile telephones, laptops or other distractions was not permitted during this period.

2.6 | Measurements

2.6.1 | Dose-finding study

Blood samples were analysed for insulin and glucagon concentrations at all time points. Similarly, tolerability was assessed using VAS comprising five questions relating to nausea, warmth, irritability, anxiety and sleepiness at all time points.

2.6.2 | Acute study

Blood samples were analysed for insulin, glucagon, GIP and glucose concentrations for time points *T* = 0 to *T* = 120. Glucose, GLP-1 and PYY measurements were performed for an additional 30 minutes (*T* = 120 to *T* = 150) to investigate possible extended effects of

treatments. Non-targeted metabolomic analysis using nuclear magnetic resonance was performed on samples at $T = 0$ and $T = 45$ to investigate the wider acute effects of L-phenylalanine on metabolism, in particular those pathways not typically associated with phenylalanine metabolism.

Subjective appetite was assessed using VAS comprising four questions relating to hunger, desire to eat, prospective consumption and fullness. This was converted to a composite appetite score (CAS) as previously described.⁸

Absolute energy intake was calculated by measuring the mass of the test meal before and after consumption (Super-SS Bench Scale) and using the difference to calculate total energy consumption using the energy density of the meal. Relative energy intake was also calculated by adding the energy content of the treatments to the energy intake at the test meal.

2.7 | Assays

Insulin, glucagon and total GIP concentrations were measured using a MILLIPLEX MAP human metabolic hormone magnetic bead panel (Merck, USA). Glucose concentrations were measured using a colorimetric enzymatic activity assay (GL364, Randox, UK). Total GLP-1 and total PYY were measured using established in-house radioimmunoassays.^{26,27} Assay protocols (including detection limits and intra-assay coefficients of variation) and details of the non-targeted metabolomic analysis are described in appendices S2 and S3, respectively.

2.8 | Statistical methods

Total AUC for the preprandial (0-70 minutes) and postprandial periods (70-120/150 minutes) were calculated for all outcomes excluding metabolite and energy intake data. Energy intake and AUC data were analysed using a one-way ANOVA with repeated measures.

Time-series data for insulin, glucagon, GIP, GLP-1, PYY, glucose and VAS scales were analysed by fitting a mixed effects model (treatment $\times$ time). If a significant effect was detected, multiple pairwise comparisons were performed using t-tests with the Tukey correction being employed. Separate analyses for time-series data were performed for preprandial (0-70 minutes) and total (0-120/150 minutes) study periods for the acute study to prevent postprandial changes masking smaller preprandial changes. To avoid confusion, a single graphical representation for each outcome is presented, which combines the results from the preprandial and total study periods.

Mixed effects models were also used to assess associations between *ad libitum* energy intake and postprandial hormone and glucose concentrations.

Quantified small metabolites were analysed using Wilcoxon matched-pairs signed rank test and Bonferroni correction was used for multiple test correction.

All statistical analyses were conducted using Stata 16 (StataCorp, USA) and Prism 8.3.0 (Graphpad, USA) with statistical significance based on 95% limits ($P < .05$). All data are mean $\pm$ SEM unless otherwise stated.

3 | RESULTS

3.1 | Participant flow

The numbers of participants who were enrolled, received the intended treatment and were analysed are presented in Figure S3 (dose-finding study) and Figure S4 (acute study).

3.2 | Baseline participant characteristics

3.2.1 | Dose-finding study

Five healthy participants (four males, one female) were recruited into the dose-finding study. The participant characteristics ($N = 5$; mean $\pm$ SD) were age, 23.6 ± 3.0 years, height 1.72 ± 0.14 m, weight 71.9 ± 5.7 kg and BMI 22.6 ± 1.1 kg/m².

3.2.2 | Acute study

Twelve healthy participants were recruited into the acute study. Of the 12 participants recruited, 11 completed all four visits (three males, eight females) with one participant being withdrawn from the study as they no longer satisfied the inclusion criteria. Data from this participant were not included in statistical analyses. The participant characteristics ($N = 11$; mean $\pm$ SD) were age 30.4 ± 13.1 years, height 1.68 ± 0.09 m, weight 64.5 ± 9.1 kg and BMI 22.2 ± 2.1 kg/m².

3.3 | Dose-finding study: tolerability of L-phenylalanine

L-phenylalanine was well tolerated at all doses and by all participants. No side effects were reported during or between visits. No significant main or interaction effects for nausea_{0-150mins} ($P > .05$) and nausea AUC_{0-150mins} ($P > .05$; Figure S5), or for warmth, irritability, anxiety and sleepiness ($P > .05$; data not shown), were detected.

3.4 | Acute study: the effect of L-phenylalanine on gastroenteropancreatic hormones

Insulin, glucagon and GIP data are available for nine of the 11 participants because of a machine error during analysis of samples from two participants.

3.4.1 | Insulin

A significant interaction effect (treatment x time) was detected for insulin_{0-70mins} ($P = .0089$). Multiple pairwise comparisons identified significant differences between L-phenylalanine and other treatments at several time points (Figure 1A). Insulin AUC_{70-120mins} also showed a significant main effect ($P = .0216$) with multiple pairwise comparisons showing a significant difference between placebo and D-phenylalanine (Table 1). However, no significant main or interaction effects were detected when analysing the data for insulin_{0-120mins} ($P > .05$) or insulin AUC₀₋₇₀ ($P = .0646$; Table 1).

3.4.2 | Glucagon

A significant interaction effect (treatment x time) for glucagon_{0-70mins} ($P = .0025$) and glucagon_{0-120mins} ($P = .0008$) was detected. Multiple pairwise comparisons identified a significant difference between L-phenylalanine and other treatments at several time points for both glucagon_{0-70mins} and glucagon_{0-120mins} (Figure 1B). A significant main effect for glucagon AUC_{0-70mins} ($P = .0445$) and glucagon AUC_{70-120mins} was also found ($P = .0063$). Multiple pairwise comparisons revealed a trend towards a significant difference between L-phenylalanine and D-phenylalanine for glucagon AUC_{0-70mins} ($P = .0781$), and a significant difference between L-phenylalanine and D-phenylalanine ($P = .0265$) as well as L-phenylalanine and placebo ($P = .0103$) for glucagon AUC_{70-120mins} (Table 1).

3.4.3 | GIP

A significant interaction effect (treatment x time) for GIP_{0-70mins} was found ($P = .0147$). Multiple pairwise comparisons revealed a significant difference between L-phenylalanine and other treatments at $T = 70$ (Figure 1C). No significant main or interaction effects for GIP_{0-120mins}, GIP AUC_{0-70mins} or GIP AUC_{70-120mins} were detected ($P > .05$; Table 1; Figure 1C).

3.4.4 | GLP-1

No significant main or interaction effects for GLP-1 were detected ($P > .05$; Table 1; Figure 2A).

3.5 | Acute study: the effect of L-phenylalanine on blood glucose

A statistically significant interaction effect (treatment x time) was detected for glucose_{0-150 mins} ($P < .0001$). Multiple pairwise comparisons revealed that both L-phenylalanine and D-phenylalanine significantly lowered several postprandial time points relative to placebo, with L-phenylalanine producing a significantly prolonged reduction (Figure 2C). Glucose AUC_{70-150mins} also showed a significant difference between treatments ($P = .0017$). Multiple pairwise comparisons revealed a significant difference between L-phenylalanine and placebo

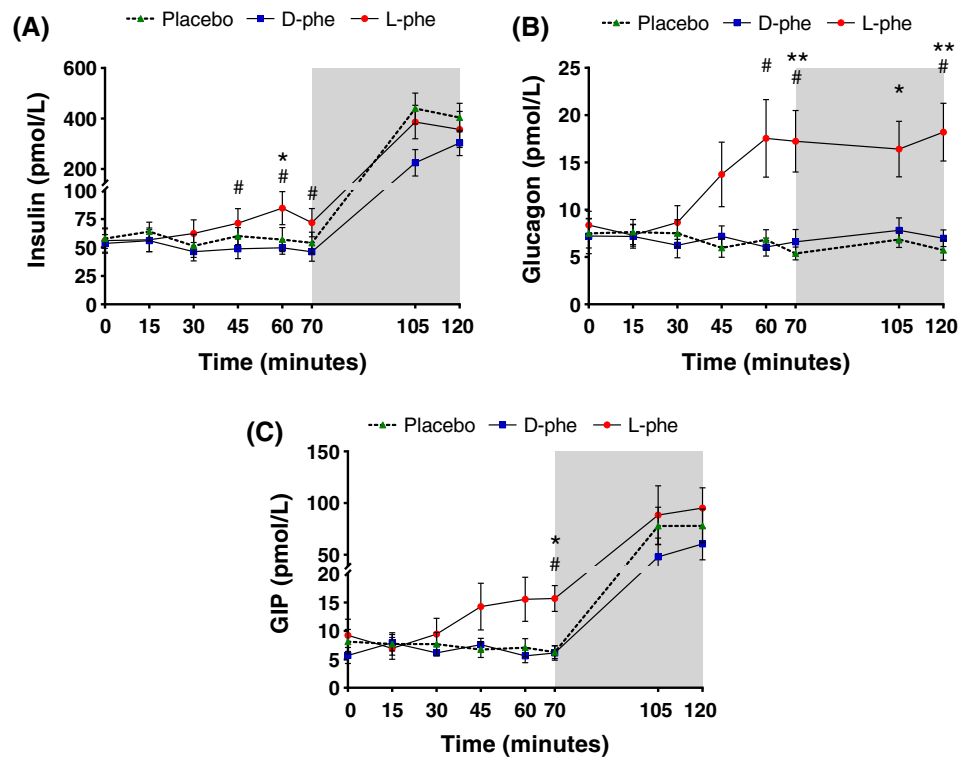


FIGURE 1 The effect of 10 g L-phenylalanine, 10 g D-phenylalanine and placebo on insulin (A; $N = 9$), glucagon (B; $N = 9$) and GIP (C; $N = 9$) concentrations in healthy participants during the acute study. Participants consumed treatment at $T = 0$ and an *ad libitum* meal at $T = 70$. The shaded grey region represents the postprandial period. *L-phenylalanine versus placebo ($P < .05$). **L-phenylalanine versus placebo ($P < .01$). #L-phenylalanine versus D-phenylalanine ($P < .05$). Data are expressed as mean $\pm$ SEM. D-phe, D-phenylalanine; L-phe, L-phenylalanine

TABLE 1 Area under the curve values for preprandial and postprandial periods

Outcome	Preprandial				Postprandial			
	Placebo	D-phe	L-phe	P value	Placebo	D-Phe	L-Phe	P-value
Insulin (pmol/L·min)	4049 ± 595	3522 ± 544	4696 ± 840	.0646	14 971 ± 2036 ^A	8703 ± 1370 ^B	13 582 ± 2159 ^{AB}	.0216 ^a
Glucagon (pmol/L·min)	480 ± 76 ^A	452 ± 79 ^A	813 ± 156 ^A	.0445 ^a	315 ± 38 ^A	363 ± 61 ^A	848 ± 136 ^B	.0063 ^a
GIP (pmol/L·min)	510 ± 109	434 ± 71	797 ± 188	.1269	2640 ± 530	1699 ± 582	3208 ± 890	.2858
GLP-1 (pmol/L·min)	4694 ± 285	4800 ± 353	5021 ± 446	.7347	5473 ± 478	6517 ± 512	6419 ± 435	.1810
PYY (pmol/L·min)	726 ± 163	1029 ± 254	892 ± 244	.5064	1352 ± 693 ^A	2433 ± 405 ^B	1825 ± 347 ^{AB}	.0305 ^a
Glucose (mmol/L·min)	308 ± 4	304 ± 3	321 ± 12	.2359	452 ± 25 ^A	400 ± 15 ^{AB}	397 ± 17 ^B	.0117 ^a

Abbreviations: D-phe, D-phenylalanine; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide-1; L-phe, L-phenylalanine; PYY, peptide tyrosine tyrosine.

Notes: Preprandial: 0-70 minutes; postprandial: 70-120/150 minutes. Means with the same letter are not significantly different from each other ($P > .05$). Data are expressed as mean ± SEM.

^aSignificant effect.

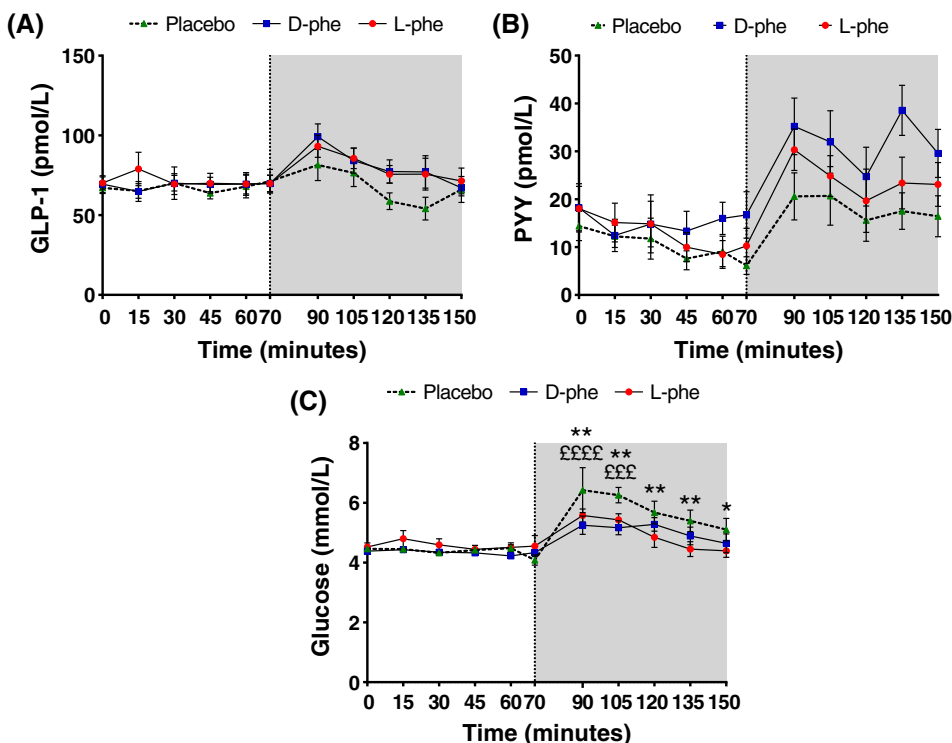


FIGURE 2 The effect of 10 g L-phenylalanine, 10 g D-phenylalanine and placebo on GLP-1 (A; N = 11) PYY (B; N = 11) and glucose (C; N = 11) concentrations in healthy participants during the acute study. Participants consumed treatment at T = 0 and an *ad libitum* meal at T = 70. The shaded grey region represents the postprandial period. *L-phenylalanine versus placebo ($P < .05$). **L-phenylalanine versus placebo ($P < .01$). ***D-phenylalanine versus placebo ($P < .001$). ****D-phenylalanine versus placebo ($P < .0001$). Data are expressed as mean ± SEM. D-phe, D-phenylalanine; L-phe, L-phenylalanine

($P = .0317$) and a trend towards significance for D-phenylalanine and placebo ($P = .0578$; Table 1). No significant main or interaction effects for glucose_{0-70mins} ($P > .05$; Figure 2C) or glucose AUC_{0-70mins} were detected ($P > .05$; Table 1).

3.5.1 | PYY

A significant main effect for PYY AUC_{70-150mins} was detected ($P = .0305$). Multiple pairwise comparisons identified a significant difference between D-phenylalanine and placebo ($P = .0002$; Table 1). Interestingly, a trend for a main treatment effect was found for PYY_{0-150mins} ($P = .0572$;

Figure 2B). Multiple pairwise comparisons identified a significant difference between D-phenylalanine and placebo ($P < .0001$), L-phenylalanine and placebo ($P = .0164$) as well as D-phenylalanine and L-phenylalanine ($P = .0148$). No significant main or interaction effects for PYY_{0-70mins} or PYY AUC_{0-70mins} were detected ($P > .05$; Table 1; Figure 2B).

3.6 | Acute study: the effect of L-phenylalanine on circulating metabolites

Phenylalanine concentrations significantly increased from T = 0 to T = 45 following L-phenylalanine ($P = .0215$) or D-phenylalanine

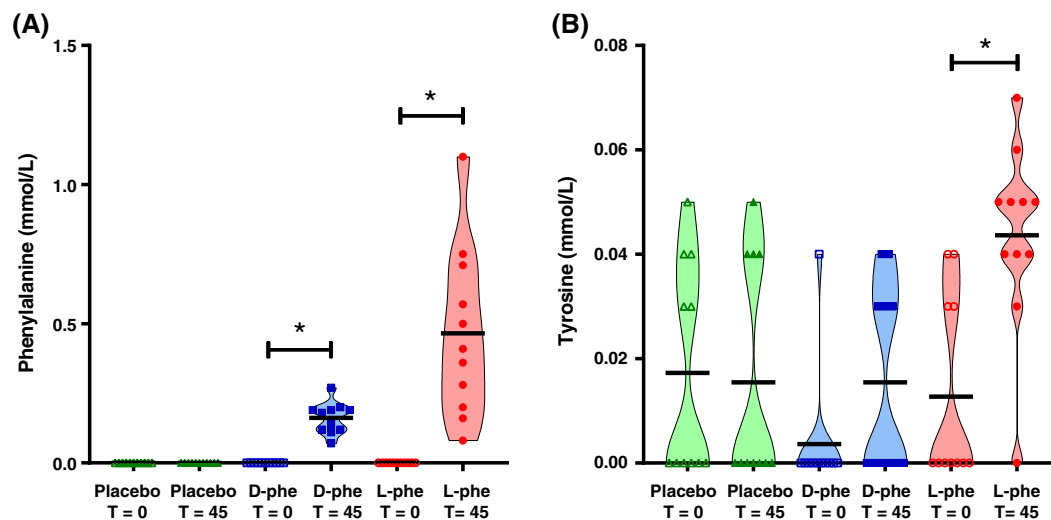


FIGURE 3 The effect of 10 g L-phenylalanine, 10 g D-phenylalanine and placebo on phenylalanine (A; N = 11) and tyrosine (B; N = 11) concentrations in healthy participants at T = 0 and T = 45 during the acute study. *T = 0 versus T = 45 ($P < .05$). Data are depicted as mean (horizontal bar) and violin plots with individual data points. D-phe, D-phenylalanine; L-phe, L-phenylalanine

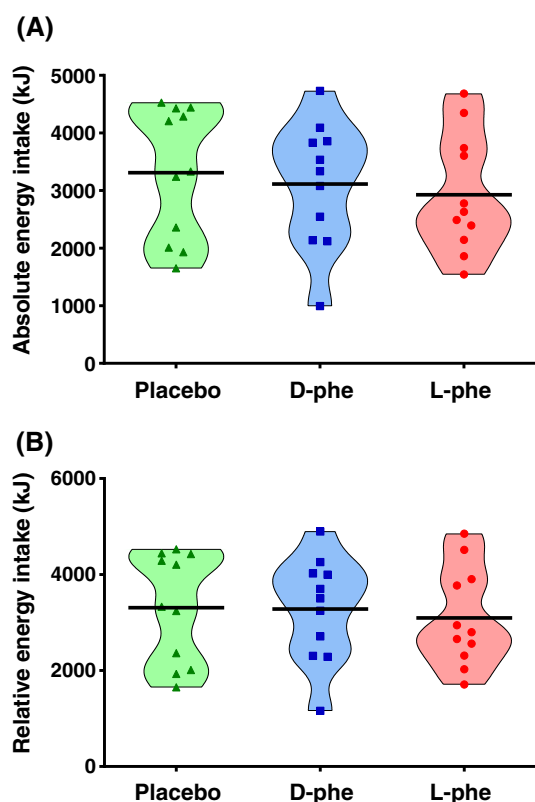


FIGURE 4 The effect of 10 g L-phenylalanine, 10 g D-phenylalanine and placebo on absolute energy intake (A; N = 11) and relative energy intake (B; N = 11) at an *ad libitum* meal in healthy participants. Data are depicted as mean (horizontal bar) and violin plots with individual data points. D-phe, D-phenylalanine; L-phe, L-phenylalanine

treatment ($P = .0215$) (Figure 3A). Tyrosine increased significantly following L-phenylalanine ($P = .0430$; Figure 3B) but not D-phenylalanine treatment. A significant correlation between

phenylalanine and tyrosine was observed before and 45 minutes following L-phenylalanine treatment ($P = 4.65 \times 10^{-5}$, Spearman's Rho = 0.7563), but not following D-phenylalanine treatment ($P > .05$, Spearman's Rho = 0.4030). No significant changes in lactate, alanine, glutamine, histidine, isoleucine, leucine or valine were detected for any treatment ($P > .05$; Figure S6A-G). Additionally, there was no effect of treatment on creatinine, ethanol, creatine, acetic acid, citric acid, formic acid, 3-hydroxybutyric acid, acetoacetic acid, acetone, pyruvic acid or glycerol (data not shown). Concentrations of cholesterol ($P = .0010$), LDL cholesterol ($P = .0199$) and apolipoprotein A1-HDL ($P = .0215$) were significantly lower at T = 45 compared with T = 0 following placebo treatment (Figure S7A-C). The total concentration of apolipoprotein B-carrying particles was also significantly lower at T = 45 compared with T = 0 in all treatments ($P < .0001$; Figure S8A), which were mainly contributed by LDL particles, specifically LDL-5 and LDL-6 particles ($P < .0001$; Figure S8E,F). By contrast, LDL-2, LDL-3 and LDL-4 particles were reduced following placebo treatment ($P = .0006$, $P = .0010$ and $P < .0001$, respectively), but not following L- or D-phenylalanine treatment (Figure S8B-D).

3.7 | Acute study: the effect of L-phenylalanine on subjective appetite

No statistically significant differences between treatments in the CAS were detected ($P > .05$; Figure S9).

3.8 | Acute study: the effect of L-phenylalanine on *ad libitum* energy intake

Absolute energy intake at the *ad libitum* test meal (mean $\pm$ SEM) was not significantly different between the three treatments: (a) placebo

3310 $\pm$ 344 kJ, (b) D-phenylalanine 3114 $\pm$ 325 kJ and (c) L-phenylalanine 2927 $\pm$ 309 kJ ($P > .05$; Figure 4A).

Relative energy intake at the *ad libitum* test meal corrected for the energy content of the treatments (mean $\pm$ SEM) was not significantly different between the three treatments: (a) placebo 3310 $\pm$ 344 kJ, (b) D-phenylalanine 3281 $\pm$ 325 kJ and (c) L-phenylalanine 3094 $\pm$ 309 kJ ($P > .05$; Figure 4B).

4 | DISCUSSION

This study confirms that acute ingestion of 10 g L-phenylalanine is well tolerated and stimulates insulin and glucagon release. We also show for the first time that L-phenylalanine stimulates GIP secretion in humans. As these effects were not observed with the ingestion of D-phenylalanine, they appear to be stereoisomer-specific. Both L-phenylalanine and D-phenylalanine reduced postprandial glycaemia, although this effect appeared prolonged for L-phenylalanine. However, L-phenylalanine at this dose and using this particular study design did not affect subjective appetite or short-term *ad libitum* energy intake.

The initial dose-finding study suggested that 10 g L-phenylalanine was tolerable. We did not administer D-phenylalanine in the dose-finding study, which may have provided greater insight into the effects of D-phenylalanine, although there were no reports of any side effects in the acute study. The metabolomics analyses found L-phenylalanine increased circulating tyrosine levels, as would be expected given the importance of L-phenylalanine in tyrosine synthesis,²⁸ but did not seem to have major effects on other metabolic variables. Minor differences in various lipids appear to reflect the effects of time across groups, which generally show the same pattern, although some changes only achieve significance following placebo treatment. This is reassuring regarding the specificity of the effects of L-phenylalanine, although it would be necessary to analyse samples at later time points and following chronic ingestion to confirm that L-phenylalanine does not have detrimental effects.

4.1 | L-phenylalanine stimulates the release of insulin, glucagon and GIP

L-phenylalanine stimulating the release of insulin and glucagon is consistent with previous human studies.^{16,18,29} To our knowledge, this is the first study to show that acute L-phenylalanine appears to increase GIP concentrations in humans (Figure 1C). There was a clear trend for higher GIP concentrations 30–70 minutes following L-phenylalanine ingestion, although only one time point reached statistical significance ($T = 70$) and further studies are therefore required to confirm this effect. Unfortunately, we were unable to measure GIP in our dose-finding study because of the limited sample volume. L-phenylalanine has been shown to stimulate GIP release in swine and rodent models partly via the CaSR.^{20,30} Congruent with this, no GIP response was observed following D-phenylalanine ingestion

(Figure 1C). Similarly, no significant changes in insulin or glucagon concentrations were observed following D-phenylalanine ingestion in the preprandial period (Figure 1A,B), suggesting these responses may also be CaSR-mediated. These effects are also in accord with previous work showing a stereoselective CCK response with L- but not D-phenylalanine.¹⁷ GIP promotes insulin and glucagon release via the GIP receptor on pancreatic beta- and alpha-cells^{31,32} and may be involved in the L-phenylalanine-induced release of these hormones. Alternatively or additionally, L-phenylalanine may directly interact with the pancreas, as CaSR activation has been shown to stimulate insulin and glucagon secretion from beta- and alpha-cells *in vitro*,³³ or signal via the vagus nerve to modulate nervous control of pancreatic hormone release, given CaSR is expressed in vagal afferent neurons.^{13,14}

In accord with a recently reported human study,¹⁸ L-phenylalanine did not significantly affect GLP-1 or PYY concentrations following ingestion (Figure 2A,B). However, postprandial PYY concentrations following D-phenylalanine were significantly elevated (Table 1). As this is the first study to measure PYY concentrations following D-phenylalanine ingestion, the reason for this increase (and for the absence of a significant response following L-phenylalanine ingestion) is unclear. D-isomers of amino acids are intestinally absorbed more slowly than their L-isomers.³⁴ It is therefore possible that L-phenylalanine is primarily absorbed in the proximal gut, whereas larger amounts of D-phenylalanine reach the distal gut, where PYY-secreting L-cells are most concentrated.³⁵ This is in line with the attenuated rise in phenylalanine concentrations following D-phenylalanine relative to L-phenylalanine at $T = 45$ (Figure 3A). As D-phenylalanine probably does not act via the CaSR, it may stimulate PYY release via other nutrient receptors, for example, the hydroxycarboxylic acid receptor 3 (GPR109B), which is expressed in human colonic epithelium³⁶ and agonized by D-amino acids including D-phenylalanine.³⁷ Alternatively, accumulated D-phenylalanine within the gastrointestinal tract may be converted to L-phenylalanine, which then stimulates PYY release via the CaSR. Enzymes hypothesized to be involved in the conversion of D-phenylalanine to L-phenylalanine have been reported to be expressed in the mammalian gut.^{38–40} The increase in postprandial PYY may in part be driven by the values at $T = 70$ (Figure 2B), suggesting that D-phenylalanine may stimulate PYY release preprandially if measured at later time points without a meal being ingested. These mechanisms are, however, highly speculative at this stage.

4.2 | Prior ingestion of L- and D-phenylalanine reduces postprandial glycaemia

The ingestion of 10 g L- or D-phenylalanine 70 minutes prior to an *ad libitum* meal reduces postprandial glucose at multiple time points relative to placebo (Figure 2C). However, this reduction was particularly prolonged following L-phenylalanine treatment ($T = 120$ – 150 ; Figure 2C) and a significant reduction in glucose AUC_{70–150mins} was only observed with L-phenylalanine ingestion (Table 1).

It is possible that the attenuation in postprandial glycaemia following L-phenylalanine ingestion is related to the insulin and GIP response preceding the *ad libitum* meal, and therefore possibly CaSR-mediated. Prior ingestion of a protein preload improves glycaemic control at a later meal,⁴¹ a phenomenon known as the 'second-meal effect'. This effect is believed to be attributable to the preprandial release of gastroenteropancreatic hormones such as insulin.⁴² Interestingly, the reduction in postprandial glucose concentrations was present despite elevated postprandial glucagon concentrations and the absence of a significant effect on postprandial insulin concentrations.

While preprandial insulin release may explain these effects of L-phenylalanine on postprandial glucose, it is improbable that D-phenylalanine improves glycaemic control in this fashion, as it did not stimulate insulin (Figure 1A) or GIP (Figure 1C) preprandially.

Figure 2A suggests a trend for postprandial concentrations of GLP-1 concentrations to be higher following ingestion of L- or D-phenylalanine relative to placebo, although these effects did not reach statistical significance. Considering that GLP-1 and PYY are co-localized and secreted,³⁵ and the trend towards a main effect of treatment for PYY ($P = .0572$), it is possible that both GLP-1 and PYY are involved in the reduction in postprandial glucose concentrations seen with both amino acid treatments (Figure 2C), and that the absence of a significant effect is attributable to sample size. GLP-1 has a well characterized direct effect on the pancreatic beta cell, potentiating glucose-stimulated insulin release, but insulin concentrations following both L- and D-phenylalanine ingestion were not associated with the glucose levels observed. While L-phenylalanine significantly increases preprandial insulin, D-phenylalanine does not, and results in significantly lower postprandial insulin concentrations compared with placebo. Despite this, both agents reduce postprandial glycaemia compared with vehicle control. Because D-phenylalanine also significantly increased postprandial PYY concentrations, the decrease in insulin may be driven by PYY. PYY has been reported to have several actions that could feasibly mediate this effect,⁴³ including directly inhibiting insulin secretion from the pancreas, increasing insulin sensitivity of skeletal muscle and adipose tissue, and slowing gastric emptying, thereby decreasing the rate of appearance of glucose into systemic circulation and the consequent insulin response.⁴⁴ GLP-1 also inhibits gastric emptying.⁴³ However, while we did not measure gastric emptying rate in the present study, a previous human intervention suggests L-phenylalanine does not affect gastric emptying.¹⁸ It is important to note that postprandial glucose concentrations were measured following an *ad libitum* meal, at which participants ate varying quantities of food. The effects of D- and L-phenylalanine on postprandial glucose control may therefore be secondary to their carbohydrate intake, and not a direct effect of the treatment on glycaemic control. However, this is improbable. There was no significant association between *ad libitum* energy intake and postprandial glucose ($\beta = 0.004$; $P = .7333$). While there was a slight trend for reduced food intake following L- and D-phenylalanine ingestion, these treatments were associated with higher rather than lower GLP-1 and PYY levels, suggesting that

differences in *ad libitum* food intake were not responsible for the differences observed.

4.3 | L-phenylalanine does not affect subjective appetite or short-term *ad libitum* energy intake

L-phenylalanine did not significantly affect subjective appetite or energy intake at an *ad libitum* test meal served 70 minutes post-ingestion. Despite no significant difference being detected, energy intake following L-phenylalanine was ~400 kJ lower than with placebo (Figure 4A). Previous investigations into the anorectic properties of L-phenylalanine have also reported non-significant reductions in energy intake (~500 kJ) following L-phenylalanine relative to placebo,⁴⁵ suggesting possible underpowering. Other human studies investigating nasogastric administration of L-phenylalanine,^{17,18} as well as rodent¹⁹ and non-human primate⁴⁶ L-phenylalanine studies, do report significant reductions in energy intake. The presence of a significant reduction in energy intake may be related to sample size (e.g. if the observed difference reflects a real effect, a sample size of 34 would have been required to detect a significant difference in absolute energy intake between L-phenylalanine and placebo in the present study), choice of placebo (e.g. the placebo selected by Ryan Harshman et al.⁴⁵ also possessed anorectic properties as discussed by Rogers and Blundell⁴⁷) and, in particular, inter-meal interval: previous studies gave the meal 20 minutes after L-phenylalanine ingestion to coincide with peak CCK concentrations¹⁷ compared with our study, in which the meal was given 70 minutes following L-phenylalanine ingestion. Our 70-minute time point was chosen based on the dose-finding study, which suggested that pancreatic hormone release peaked at around this time, and because it gave sufficient time for the phenylalanine to reach more distal parts of the small intestine, where it might be expected to act directly on the PYY- and GLP-1-releasing L cells, which are found at a higher concentration there. However, it may have missed an earlier, larger anorectic effect of L-phenylalanine.

L-phenylalanine-induced satiety has been reported to be modulated by menstrual cycle phase.²³ We did not record the cycle stage when conducting study visits, which may feasibly have affected energy intake, and therefore increased the variation in response to L-phenylalanine. However, when relative energy intake is calculated (i.e. including the 167 kJ provided by L-phenylalanine), the possible clinical significance of the reduction in energy intake with L-phenylalanine seems small (Figure 4B).

In conclusion, our results show that phenylalanine-induced increases in insulin, glucagon and GIP prior to meal ingestion are dependent on its chiral form, in accord with the CaSR mediating these effects. We also show that L-phenylalanine and D-phenylalanine decrease postprandial glucose concentrations following an *ad libitum* test meal, with L-phenylalanine producing a more prolonged suppression. Although it is impractical to regularly consume 10 g of encapsulated amino acids, fortification of foods with L-phenylalanine may represent a possible strategy to prevent or treat glucose dysregulation and diabetes. L-phenylalanine did not significantly affect subjective

appetite or short-term *ad libitum* energy intake, but this may be related to sample size and/or the time point at which the meal was offered. Furthermore, D-phenylalanine—and possibly L-phenylalanine—stimulated the release of PYY. Further work is needed to identify the mechanisms underlying the glucose-lowering effects of L-phenylalanine, for example, by co-administering GLP-1 and GIP receptor antagonists, and appropriately powered studies are required to clarify its effects on energy intake and GLP-1 release. Our work suggests L-phenylalanine plays a role in gastroenteropancreatic hormone secretion and potentially in glycaemic control, and that targeting the systems which sense ingested L-phenylalanine may be therapeutically useful.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

KM and JL designed the study, interpreted data and helped draft the manuscript. Anjali Amin and JF conducted the study and collected and analysed data. Anjali Amin contributed towards drafting the manuscript. JF wrote the manuscript. ZL, GFB, MN and Aoa Alaa carried out sample analysis and helped draft the manuscript. All authors have read and approved the final manuscript.

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DATA AVAILABILITY STATEMENT

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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