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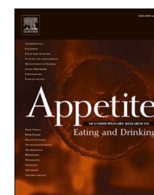

Postprandial glycine as a biomarker of satiety: A dose-rising randomised control trial of whey protein in overweight women

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A R T I C L E I N F O

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A B S T R A C T

This study aimed to identify biomarkers of appetite response, modelled using a dose-rising whey protein preload intervention. Female participants (n = 24) with body mass index (BMI) between 23 and 40 kg/m^2 consumed preload beverages (0 g protein water control, WC; 12.5 g low-dose protein, LP; or 50.0 g high-dose protein, HP) after an overnight fast, in a randomised cross over design. Repeated venous blood samples were collected to measure plasma biomarkers of appetite response, including glucose, glucoregulatory peptides, gut peptides, and amino acids (AAs). Appetite was assessed using Visual Analogue Scales (VAS) and *ad libitum* energy intake (EI). Dose-rising protein beverage significantly changed the postprandial trajectory of almost all biomarkers (treatment*time, $p < 0.05$), but did not suppress postprandial appetite (treatment*time, $p > 0.05$) or EI (ANOVA, $p = 0.799$). Circulating glycine had the strongest association with appetite response. Higher area under the curve ($AUC_{0\text{-}240}$) glycine was associated with lower EI ($p = 0.026$, trend). Furthermore, circulating glycine was associated with decreased Hunger in all treatment groups, whereas the associations of glucose, alanine and amylin with appetite were dependent on treatment groups. Multivariate models, incorporating multiple biomarkers, improved the estimation of appetite response (marginal R^2, range: 0.13–0.43). In conclusion, whilst glycine, both alone and within a multivariate model, can estimate appetite response to both water and whey protein beverage consumption, a large proportion of variance in appetite response remains unexplained. Most biomarkers, when assessed in isolation, are poor predictors of appetite response, and likely of utility only in combination with VAS and EI.

1. Introduction

As evident by the escalating prevalence of obesity over the past four decades (Chooi et al., 2019; Hall et al., 2011), controlling appetite to maintain energy balance has become progressively more challenging. Although appetite can be influenced by psychological events (Best et al., 2018; Blundell, 2017), investigation into physiological mechanisms of appetite is necessary to gain a greater understand of the biological factors that act to modulate total energy intake (EI) by either promoting or inhibit food intake (Horner et al., 2020).

A range of potential biomarkers of appetite response have been proposed to explain the variability of appetite response to food items (Gibbons et al., 2019; Horner et al., 2020). It has long been recognised that foods differ in their satiating capacity (Blundell, 1999), whereby manipulating physicochemical properties of foods changes their satiating properties (Almiron-Roig et al., 2013; Chambers et al., 2015). However, only recently substantial attention was given to the importance of inter-individual appetite response to identical foods (Gibbons et al., 2019), likely in part due to the variable postprandial circulating concentration of metabolites (Berry et al., 2020). Dietary protein, has long been proposed as the most satiating of the macronutrients when EI is matched (Blundell, 1999). Despite this, meta-analyses highlight that multiple studies demonstrate no effect of high versus low protein on

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Abbreviations

%en	percentage of total energy
AA	amino acid
AIC	Akaike Information Criteria
AUC	Area Under the Curve
BCAA	branched-chain amino acid
BMI	body mass index
EAA	essential amino acid
EI	energy intake
GIP	gastric inhibitory polypeptide;
GLP-1	glucagon-like peptide-1
HNU	Human Nutrition Unit
HP	high protein
LP	low protein
NEAA	non-essential amino acid
NPAA	non-proteogenic amino acid
PYY	peptide YY
TOF	Thoughts of Food
VAS	visual analogue scale
WC	water control

either appetite response or EI (Dhillon et al., 2016; Kohanmoo et al., 2020). Circulating biomarkers are commonly cited as possible explanations to underlie differences in appetite response (Lim & Poppitt, 2019).

Postprandial concentrations of glucose, glucoregulatory peptides, gut peptides, and amino acids (AAs) are particularly implicated in mechanisms of appetite regulation. According to the "glucostatic hypothesis", decrease in circulating glucose is associated with hunger (Mayer, 1953). Recently, Wyatt et al. (2021) revisited this hypothesis and demonstrated that postprandial glucose had only a minor effect on hunger, EI, and the initiation of an eating episode. The glucoregulatory peptides, including the gastric inhibitory polypeptide (GIP), glucagon, amylin, and C-peptide were also considered associated with appetite (Lean & Malkova, 2016; Neary & Batterham, 2009; Steinert et al., 2017). Furthermore, gut peptides such as glucagon-like peptide-1 (GLP-1) and peptide YY (PYY), occasionally known as 'satiety' peptides, are routinely measured in dietary and pharmacology interventions as a measure of appetite (Blundell et al., 2010). Mars et al. (2012) initially questioned the utility of measuring dietary-induced increase in circulating gut peptides as physiological markers of appetite. Indeed, we have also shown that whilst pharmacological infusion of gut peptides significantly suppress appetite, these pharmacological-induced increase in circulating concentrations of gut peptides were far more pronounced than what is elicited physiologically in response to consuming a meal (Lim & Poppitt, 2019). Alternatively, the "aminostatic hypothesis" has been implicated in protein-induced satiety, stating that higher circulating concentrations of AAs suppress appetite (Mellinkoff et al., 1956). Recent evidence showed some AAs might be more important than others in explaining postprandial appetite (Korompokis et al., 2016; Veldhorst et al., 2009a), pointing towards the effect of AAs composition on appetite. Despite putative biomarkers of appetite being proposed, with gut peptides routinely measured in appetite studies, the reliability of these biomarkers in estimating appetite is rarely assessed.

Our current study aimed to investigate the association between circulating biomarkers and appetite response to a controlled ingestion of variable doses of whey protein, in a cohort of women part of a larger program examining serum metabolomics of individuals with overweight or obesity and pre-diabetes. Managing appetite to avoid positive energy balance is especially important in this target population who are at high risk towards the development of diabetes from excessive adiposity. Whey protein was selected as a model to investigate protein-induced satiety in our current study. Our choice was based on previous protein beverage appetite studies (Anderson et al., 2004; Astbury et al., 2010; Hutchison et al., 2015), which we hypothesised that 50 g whey protein would significantly suppress appetite and *ad lib* EI in comparison to 0 g protein water control (WC). Furthermore, the highly digestible whey protein induces a substantial change in postprandial plasma glucose, glucoregulatory peptides, gut peptides and AAs (Hall et al., 2003; Veldhorst et al., 2009b), which are putative appetite biomarkers. Since biomarkers assessed in isolation rarely reflect appetite response (Lim & Poppitt, 2019), we proposed to measure multiple biomarkers in a single study, in agreement with Horner et al. (2020). In this study we hypothesised that a suite of blood biomarkers, including glucose, multiple glucoregulatory peptides, multiple gut peptides and AAs can better explain an individual's appetite responses than single blood biomarkers.

2. Methods

2.1. Trial design

The study was an acute, randomised, single-blind, cross-over trial. Participants were assigned to 3 treatments (0 g whey protein water control, WC; 12.5 g low-dose whey protein, LP; 50.0 g high-dose whey protein, HP), with 7-day washout. Randomisation was conducted using a Latin-square design. The study was conducted at the Human Nutrition Unit (HNU), University of Auckland, New Zealand, between September and December 2017. Written informed consent was obtained from each participant, and ethical approval was obtained from Auckland Health and Disabilities Ethics Committee (HDEC, Reference: 17/NTA/172), New Zealand. This study was registered with the Australia New Zealand Clinical Trial Registry (ANZCTR, Reference: ACTRN12618000145202).

2.2. Participants

Advertisements were posted electronically on social media platforms and physically on public notice boards in Auckland, New Zealand. Prior HNU participants were also invited. Interested participants contacted investigators to complete a screening visit. Inclusion criteria were (i) Asian Chinese or Caucasian European, (ii) female, (iii) 18–65 years, (iv) body mass index (BMI) 23–40 kg/m^2, and (v) impaired fasting glucose (5.6–6.9 mmol/L) at screen. Exclusion criteria were (i) gain or loss ≥10% body weight within prior 3 months, (ii) current active diet program, (iii) current medication for weight loss or other conditions known to affect appetite, (iv) depression or anxiety disorders known to affect appetite, (v) dislike, unwilling or unable to consume food items provided in the study as assessed via a Food Preference Questionnaire, (vi) prior bariatric surgery, (vii) other significant diseases, (viii) smokers or ex-smokers ≤ 6 months, (ix) pregnant or breastfeeding, (x) low iron status.

This study was part of a larger research program investigating serum metabolomics associated with pre-diabetes in Asian Chinese and Caucasian European cohorts, hence ethnicity and impaired fasting glucose were inclusion requirements.

2.3. Study design and procedures

24-h prior to study day, participants were requested to refrain from vigorous physical activity, alcohol, and unusually large or small meals. The daily protocol for preload challenge follows a typical "breakfast-lunch" paradigm, summarised in Fig. 1. Participants arrived at the HNU at 0800 h after 10–14 h overnight fast, consumed 250 mL water, followed by venous cannulation. At 0900 h (t = 0 min), baseline blood samples were collected, and subjective feelings of appetite were assessed using visual analogue scales (VAS). Participants then consumed the preload beverage in its entirety within 15 min, seated at individual dining booths. Venous blood samples were collected at t = 15, 30, 60, 90, 120, 180, and 240 min. VAS was also administered at t = 15, 30, 60,

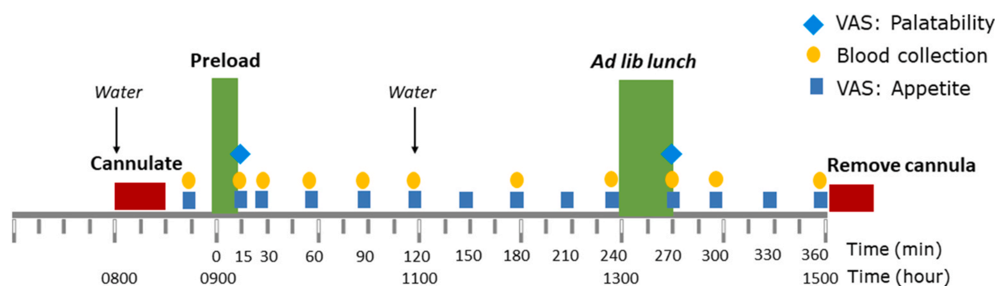

Fig. 1. Daily protocol at HNU.

90, 120, 150, 180, 210, and 240 min, immediately following each blood sample. At 1100 h (t = 120 min) participants consumed 250 mL water to maintain hydration. Since whey protein is absorbed from the small intestine at a rate of 8–10 g/h (Bilsborough & Mann, 2006), at least 5 h is required for complete absorption of 50 g whey protein. Yet, 5-h is longer than a realistic inter-meal interval according to our review (Lim & Poppitt, 2019), hence we decided to assess *ad lib* EI using a 2-item outcome lunch meal at 1300 h (t = 240 min, 4 h after preload). The dietary whey protein is expected to be almost completely absorb from the small intestine into the circulation by lunch time, allowing our study to observe the postprandial dynamics of amino acids. During the *ad lib* EI assessment, participants were requested to eat until comfortably full, seated at individual dining booths for 30 min. Sensory qualities of the preload beverages and outcome lunch meal were also assessed immediately after each meal using VAS. After the outcome lunch meal, participants were then monitored for a further 90 min, blood samples collected (t = 270, 300, and 360 min) and VAS assessed (t = 270, 300, 330, and 360 min) until 1500 h. This extended protocol ensured participants remained in a relaxed state during the outcome lunch meal. Participants were sedentary during study days. The experimental setting adhered to the international standard for appetite research (Blundell et al., 2010). Importantly, participants completed VAS ratings and consumed preload beverages and meals in isolation, with no distraction from investigators, other participants, reading materials or electronic devices.

2.4. Preload beverages

The preload beverages were prepared by blending Whey Protein Isolate powder (WPI 895, Fonterra, Palmerston North, New Zealand) in 380 mL filtered tap water. Our study is interested in modelling the effect of protein-induced change in circulating biomarkers and their associations with appetite, without being confounded by concomitant changes in carbohydrate and fat. Therefore, the energy content increased in parallel with protein content of the preload beverages. WC contained 0 kJ and 0 g protein; LP contained 218 kJ, 12.5 g protein, 0.1 g fat, 0 g carbohydrate; HP contained 871 kJ, 50.0 g protein, 0.5 g fat, 0.2 g carbohydrate. 0.4 mL artificial chocolate flavour (Product number: 143083, Symrise, Auckland, New Zealand) was added to each beverage to mask protein aftertaste and maintain blinding. The thickness/viscosity of the beverage was comparable to a water beverage. Furthermore, to maintain blinding of participants from visual and olfactory stimuli, the beverage was served in an opaque bottle with a small opening for drinking. The AA composition of beverage is presented in Table 1.

2.5. Visual analogue scales

Participants rated subjective feelings of Hunger, Fullness, Satisfaction, and Thoughts of Food (TOF) using VAS. Nausea was also recorded. Additionally, participants rated sensory properties of the preload beverage and the outcome lunch meal as Pleasantness, Visual Appeal, Smell, Taste, Aftertaste, and Palatability. VAS were recorded as a vertical line on 100 mm horizontal paper scale, as detailed previously in Wiessing et al. (2012).

Table 1
AA composition of preload beverages.

Amino acids	Weight per 12.5 g protein, LP (g)	Weight per 50.0 g protein, HP (g)
BCAAs		
Leucine	1.8	7.2
Isoleucine	0.8	3.2
Valine	0.7	2.8
Other EAAs		
Phenylalanine	0.5	1.9
Methionine	0.3	1.2
Lysine	1.4	5.6
Histidine	0.3	1.0
Threonine	0.7	2.7
Tryptophan	0.3	1.2
NEAAs		
Glycine	0.2	0.9
Aspartic acid + asparagine[a]	1.6	6.3
Glutamic acid + glutamine[a]	2.2	8.8
Arginine	0.4	1.5
Alanine	0.7	2.9
Serine	0.6	2.3
Tyrosine	0.5	2.1
Proline	1.6	2.3
Cysteine	0.5	2.0

[a] Asparagine and glutamine are present in whey protein. However, according to the manufacturer specification, asparagine and glutamine were susceptible to hydrolysis using the manufacturer's analysis process, so they were converted to, and captured under, aspartic acid and glutamic acid, respectively.

2.6. Blood samples

Venous blood samples were collected into fluoride oxalate Vacutainer™ tube for plasma glucose, P800 Vacutainer™ tube for plasma GLP-1, PYY, insulin, C-peptide, glucagon, and amylin, and K_2 EDTA Vacutainer™ tube for plasma AAs. Blood samples were centrifuged at 1500×*g* at 4 °*C* for 10 min, and aliquots stored at −80 °*C* for batch analysis. At screening, plasma glucose was measured using Refletron® Plus (Roche Diagnostics, USA). For venous blood samples collected during study days, plasma glucose was measured using Cobas® c311 analyser (Roche, Mannheim, Germany). Plasma GLP-1, PYY, insulin, C-peptide, glucagon, and amylin were measured using MILLIPLEX® MAP Human Metabolic Hormone Magnetic Bead Panel 96-Well Plate Assay (HMHEMAG-34K, Merck Millipore, Germany). Plasma AA was measured using Ultra-High-Performance Liquid Chromatography assay with pre-column derivatisation using AccQ-Tag (Cohen & Michaud, 1993; Prodhan et al., 2020).

2.7. Ad libitum outcome lunch meal

The outcome lunch meal was a previously validated 2-item pasta +

meat sauce meal (Wiessing et al., 2012). Each food item was served in excess to avoid limited portion size from regulating the point at which participants stopped eating. The food items were weighed immediately before and after the lunch meal to calculate EI.

2.8. Statistical analysis

Sample size was based on power calculations using *ad lib* EI data from earlier appetite trials at the HNU (Poppitt et al., 2011). Assuming the within-participant SD of *ad lib* EI was 686 kJ, at least 17 women were required to detect a 500 kJ difference in EI between any two treatments in a cross-over trial with 80% power at 95% significance. The difference in EI between treatments was analysed using one-way analysis of variance (ANOVA), with treatment specified as within-participant factor. Postprandial VAS and biomarkers data (t = 0–240 min) were fitted in a repeated measures linear mixed model (LMM) by specifying baseline (t = 0 min) as covariate. Treatment, time, and treatment*time interaction were included as fixed effects, participant was included as a random effect. Area Under the Curve ($AUC_{0\text{-}240}$) of biomarkers was calculated using the trapezoid method and compared using one-way ANOVA, by specifying baseline concentration as covariate and treatment as within-participant factor. Multicollinearity between biomarkers was checked by Pearson's correlation analysis. Strongly correlated biomarkers ($r > 0.7$) were subsequently analysed as a group. First, *ad lib* EI was modelled from concentrations of biomarkers throughout the morning ($AUC_{0\text{-}240}$) and concentration of biomarkers prior to lunch (t = 240 min) using a univariate LMM. AUC is generally a preferred measure for modelling *ad lib* EI as AUC informs the metabolic status of an individual over the postprandial period. Nevertheless, the concentration of biomarkers prior to lunch was also modelled against *ad lib* EI to understand if a biomarker has an immediate influence on eating behaviour. Similarly, VAS-assessed appetite (t = 0–240 min) was modelled from concentration of biomarkers at their corresponding timepoints (t = 0–240 min) using a univariate LMM. Individual biomarkers which associated with appetite response at $p < 0.05$ in the univariate LMM were collectively included as fixed effects in a multivariate LMM to estimate appetite response. Subsequently, a top-down procedure was employed to eliminate the least significant fixed effect from the full multivariate LMM one at a time, until the best-predictive model was achieved according to the Akaike information criterion (AIC). The proportion of variance explained by biomarkers (fixed effects) was analysed using marginal R^2 (Nakagawa & Schielzeth, 2013). The participant was

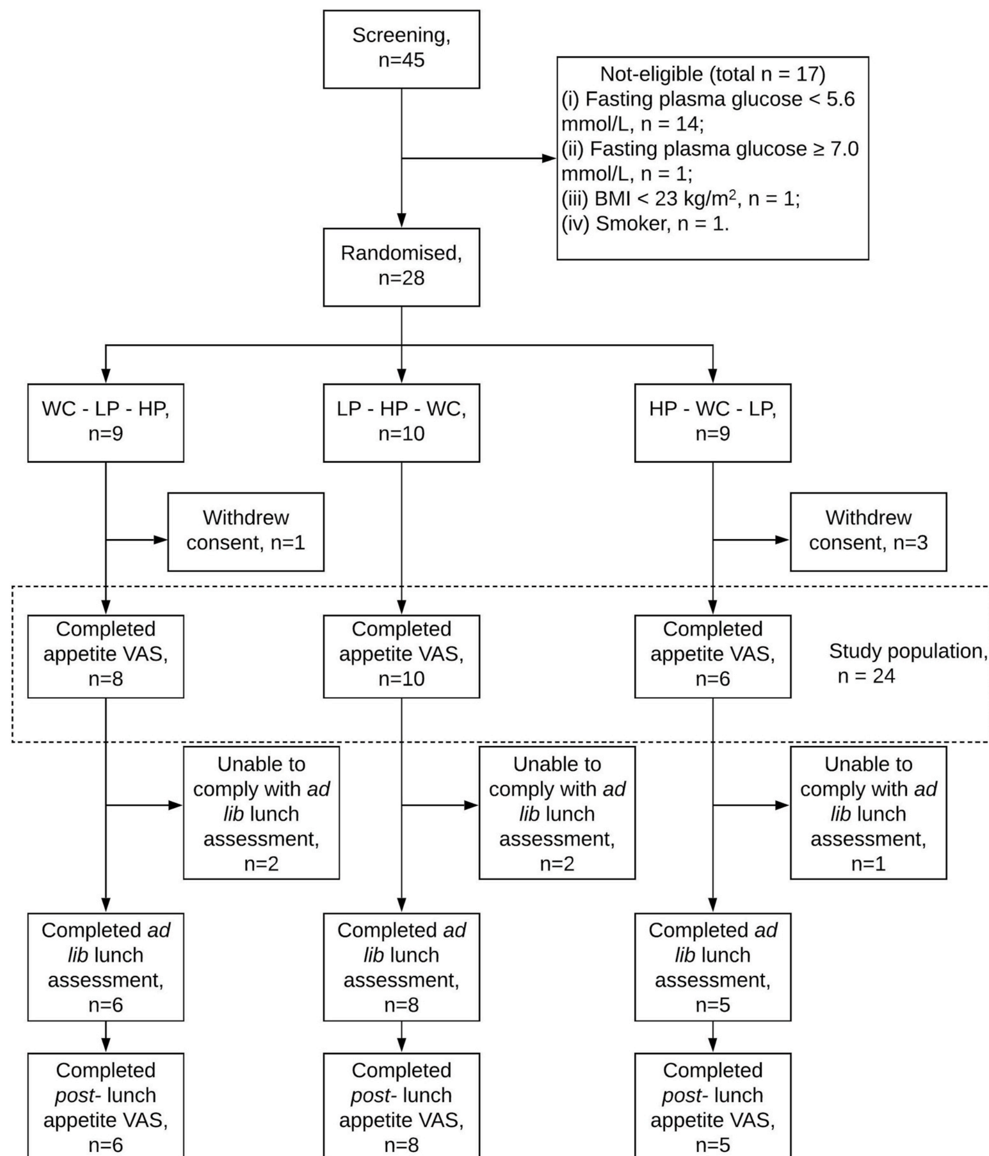

Fig. 2. Flow diagram of participants.

included as random effect in both univariate and multivariate LMMs. Participant characteristics are presented as mean ± SD. Efficacy endpoints are presented as mean ± SEM. Statistical significance was set as $p < 0.05$, except for the univariate LMM, where the significance was set at $p \leq 0.002$ (0.05 divided by 24 tests) following adjustment for multiple testing using the Bonferroni procedure. Data was analysed as observed, missing data was assumed missing at random and was managed by the LMM using the restricted maximum likelihood approach. Most statistical analyses were conducted using Statistical Package for the Social Sciences (SPSS) software (version 25; IBM Corp., Armonk, NY, USA), except marginal R^2 was computed using R (version 4.0.2; R Foundation for Statistical Computing, Vienna, Austria).

3. Results

3.1. Participants

Twenty-eight women were eligible and randomised into the study (Fig. 2), of which four withdrew prior to the intervention. Twenty-four women received the three dietary treatments at three separate visits, whilst 19 participants completed the full 6-h study protocol (t = 0–360 min). Five participants ended the study after 4 h (t = 0–240 min) as they expressed a dislike towards pasta + meat sauce meal and/or an unwillingness to consume. Chinese-to-Caucasian participant ratio was 1:1, and results are presented as a single population. The cohort had a mean (SD) age of 48 ± 15 years, body weight 77.1 ± 13.6 kg, and BMI 29.3 ± 4.8 kg/m^2. Mean (SD) fasting plasma glucose was 6.0 ± 0.4 mmol/L.

3.2. Blood biomarkers

Baseline concentrations (t = 0 min) of plasma glucose, insulin, C-peptide, glucagon, amylin, GLP-1, PYY, and all measured AAs were not significantly different between treatments (ANOVA, $p > 0.05$, all) (Table 2). Postprandial concentrations of glucose, non-essential amino acids (NEAAs), and non-proteogenic amino acids (NPAAs) are presented in Fig. 3. Glucoregulatory peptides, gut peptides, branched-chain amino acids (BCAAs) and other essential amino acids (Other EAAs) are presented in Supplementary Fig. 1. The difference in curve characteristics following *post-hoc* analysis is presented in Supplementary Table 1, and $AUC_{0\text{-}240}$ is presented in Supplementary Table 2.

3.2.1. Glucose

Increasing the dietary bolus of whey protein significantly changed the trajectory of postprandial plasma glucose concentration (treatment*time, $p < 0.001$). Since there was no significant dietary source of carbohydrate in the preload beverages, no postprandial increase in glucose concentration was expected (Fig. 3A). Instead, the postprandial glucose concentration gradually decreased from t = 0 min to t = 240 min in all treatments. A significant drop in glucose concentration at t = 60 min was notable following HP in comparison to WC (post-hoc, p = 0.001), returned to baseline concentration at t = 120 min, but gradually decreased thereafter.

3.2.2. Glucoregulatory peptides

Increasing the dietary bolus of whey protein significantly changed the trajectory of postprandial insulin, C-peptide, glucagon, GIP, and amylin concentrations (treatment*time, $p < 0.001$, all) (Supplementary Fig. 1). WC beverage did not increase peptides concentration, whereas whey protein beverage significantly increased postprandial peptides concentration dose-dependently (Supplementary Table 1).

3.2.3. Gut peptides

Increasing the dietary bolus of whey protein significantly changed the trajectories of postprandial GLP-1 (treatment*time, $p < 0.001$), but not PYY concentrations (treatment*time, p = 0.530) (Supplementary Fig. 1). Similar to the glucoregulatory peptides, WC beverage did not

Table 2
Baseline concentration of biomarkers (t = 0 min).

Biomarkers	WC	LP	HP	*p*-value	Within-participant % CV
Glucose (μM)	5.7 ± 0.6	5.6 ± 0.6	5.6 ± 0.6	0.452	2.8 ± 1.5
Gut peptides					
Insulin (pg/mL)	749.7 ± 423.5	798.9 ± 446.9	760.8 ± 415.8	0.221	14.0 ± 8.1
C-peptide (pg/mL)	1616 ± 447	1657 ± 659	1597 ± 574	0.322	11.5 ± 4.7
Glucagon (pg/mL)	73.2 ± 40.2	75.2 ± 38.0	76.8 ± 47.8	0.766	19.4 ± 14.2
GIP (pg/mL)	78.8 ± 43.1	73.9 ± 37.3	74.2 ± 36.3	0.467	19.8 ± 9.1
Amylin (pg/mL)	32.1 ± 14.4	31.9 ± 15.8	32.4 ± 15.6	0.949	17.7 ± 9.1
GLP-1 (pg/mL)	259.1 ± 64.5	263.1 ± 64.9	263.9 ± 67.8	0.849	7.9 ± 4.5
PYY (pg/mL)	123.7 ± 75.8	139.4 ± 74.1	133.1 ± 78.4	0.179	17.9 ± 15.7
BCAAs					
Leucine (μM)	130.1 ± 43.3	124.9 ± 20.1	121.1 ± 27.4	0.570	12.3 ± 13.6
Isoleucine (μM)	72.3 ± 21.9	70.0 ± 15.0	69.4 ± 18.0	0.818	12.8 ± 14.5
Valine (μM)	260.0 ± 61.6	249.2 ± 35.8	244.4 ± 62.3	0.520	12.0 ± 11.4
Other EAAs					
Phenylalanine (μM)	57.1 ± 11.7	56.2 ± 6.5	54.3 ± 8.4	0.400	9.4 ± 7.5
Methionine (μM)	22.0 ± 7.5	22.9 ± 5.9	21.3 ± 5.5	0.412	14.0 ± 11.0
Lysine (μM)	127.6 ± 43.0	133.0 ± 33.2	124.7 ± 25.4	0.553	16.2 ± 8.5
Histidine (μM)	41.7 ± 11.7	38.9 ± 8.3	37.1 ± 7.0	0.085	14.6 ± 9.6
Threonine (μM)	119.8 ± 33.7	126.5 ± 32.4	119.5 ± 26.2	0.484	14.4 ± 8.6
NEAAs					
Glycine (μM)	235.3 ± 54.8	249.1 ± 76.9	230.9 ± 58.4	0.164	10.2 ± 6.9
Aspartic acid (μM)	3.0 ± 0.9	2.8 ± 0.7	2.7 ± 1.1	0.516	20.4 ± 15.7
Asparagine (μM)	46.0 ± 10.8	47.6 ± 11.8	44.5 ± 10.0	0.191	9.7 ± 7.5
Glutamic acid (μM)	48.5 ± 20.2	42.3 ± 18.1	42.3 ± 20.5	0.153	28.4 ± 20.0
Glutamine (μM)	537.2 ± 48.1	559.0 ± 83.2	539.3 ± 60.5	0.235	7.0 ± 5.2
Arginine (μM)	41.4 ± 14.3	42.2 ± 14.2	40.5 ± 11.8	0.796	16.7 ± 8.7
Alanine (μM)	372.6 ± 99.3	415.1 ± 117.1	385.8 ± 80.0	0.117	14.5 ± 10.1
Serine (μM)	110.9 ± 19.7	113.3 ± 20.7	108.5 ± 17.7	0.263	7.2 ± 5.2
Tyrosine (μM)	64.8 ± 15.9	65.3 ± 11.9	64.0 ± 12.0	0.900	11.4 ± 8.4
Proline (μM)	196.3 ± 76.2	210.6 ± 136.1	187.2 ± 98.2	0.341	15.9 ± 11.3
NPAAs					
Hydroxyproline (μM)	14.4 ± 6.8	17.1 ± 10.9	12.6 ± 4.7	0.056	29.7 ± 16.3
Taurine (μM)	65.2 ± 14.4	64.3 ± 14.8	61.3 ± 11.1	0.231	11.3 ± 6.4
Citrulline (μM)	30.4 ± 5.8	30.3 ± 5.8	29.8 ± 6.4	0.861	10.4 ± 7.3
Ornithine (μM)	35.5 ± 12.0	37.0 ± 11.7	34.0 ± 10.1	0.449	18.1 ± 9.7

Mean (±SD) concentrations and within-participant %CV. Concentrations between treatments were compared using One-Way ANOVA. WC, Water Control; LP, Low Protein; HP, High Protein; GIP, gastric inhibitory polypeptide; GLP-1, glucagon-like peptide-1; PYY, peptide YY; BCAAs, Branch-chained amino acids; Other EAAs, Other essential amino acids; NEAAs, Non-essential amino acids; NPAAs, Non-proteogenic amino acids.

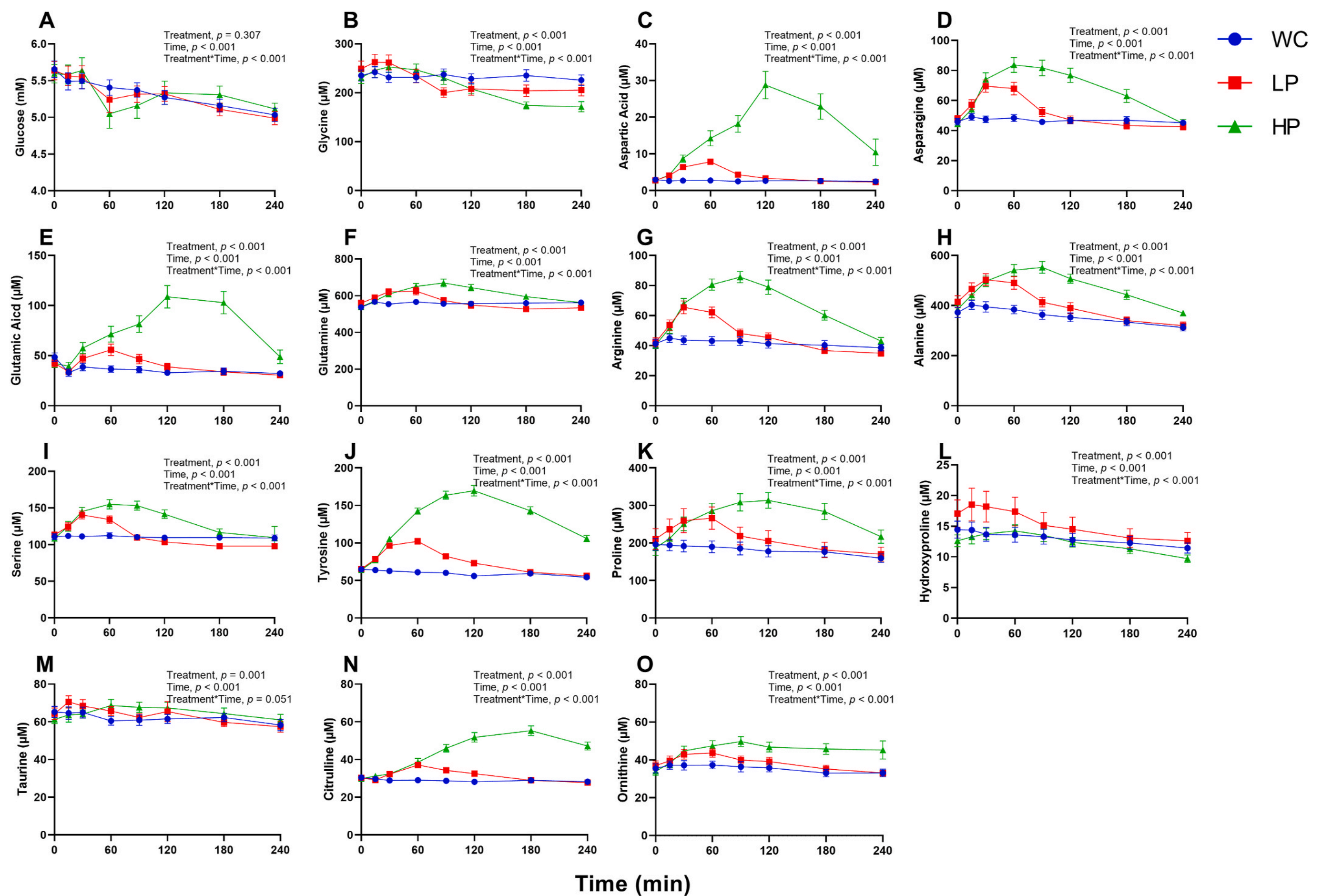

Fig. 3. Postprandial concentration of biomarkers. Mean (±SEM) (A) glucose, (B) glycine, (C) aspartic acid, (D) asparagine, (E) glutamic acid, (F) glutamine, (G) arginine, (H) alanine, (I) serine, (J) tyrosine, (K) proline, (L) hydroxyproline, (M) taurine, (N) citrulline, and (O) ornithine after ingesting Water Control (WC), Low Protein (LP), and High Protein (HP) preload beverages at $t = 0$ min. Postprandial concentrations of biomarkers ($n = 24$, $t = 0$–240 min) were analysed using a linear mixed model with baseline concentration ($t = 0$ min) as covariate.

increase gut peptides concentration, whereas whey protein beverage significantly increased postprandial concentration of GLP-1 dose-dependently (Supplementary Table 1).

3.2.4. *Amino acids*

Overall, increasing the dietary bolus of whey protein significantly changed the trajectories of most postprandial AA concentrations (treatment*time, $p < 0.05$, all), except for taurine, where there was only a trend towards significance (Fig. 3M, treatment*time, $p = 0.051$). As expected, most AA concentrations fluctuated around baseline after consuming the WC beverage, except the major gluconeogenic AA, alanine (Fig. 3H), which decreased (WC, $t = 240$ min vs 0 min, $p < 0.001$). Whey protein beverage significantly increased concentrations of postprandial BCAAs, Other EAAs, most NEAAs except glycine, and two NPAAs including citrulline and ornithine, dose-dependently (Supplementary Table 1). The postprandial response between different BCAAs and Other EAAs (Supplementary Fig. 1) was very similar, but remarkably variable between different NEAAs and NPAAs (Fig. 3). Notably, glycine represents the smallest fraction of whey protein-derived AAs (Table 1). Increasing the dietary load of glycine as part of increasing whey protein in the beverage resulted in an initial increase in postprandial glycine until $t = 60$ min, but, unexpectedly, succeeded by a dose-dependent decrease and reached nadir at $t = 240$ min (Fig. 3B, Supplementary Table 1). Moreover, hydroxyproline reached nadir at $t = 240$ min following both LP and HP beverages, whereas taurine lacked a distinct postprandial peak (Fig. 3M, Supplementary Table 1).

3.3. *Visual analogue scales*

3.3.1. *Sensory ratings*

There was no significant difference in the mean ratings of Pleasantness, Smell, Taste, and Palatability between preload beverages of different protein doses (ANOVA, $p > 0.05$, all). However, HP beverage had significantly stronger Aftertaste than WC (post-hoc, $p = 0.008$) and LP (post-hoc, $p = 0.020$) (Supplementary Fig. 2). Mean ratings of Pleasantness, Visual Appearance, Smell, Taste, Aftertaste and Palatability of the outcome lunch meal were not significantly different between treatments (ANOVA, $p > 0.05$, all) (Supplementary Fig. 3). Mean sensory ratings were not significantly different between Asian Chinese and European Caucasian population (T-test, $p > 0.05$, all).

3.3.2. *Postprandial appetite ratings*

Baseline ratings of VAS-assessed appetite ($t = 0$ min) were not significantly different between treatments (ANOVA, $p > 0.05$, all). Unexpectedly, the treatment did not significantly affect the trajectory of postprandial Hunger, Fullness, TOF, and Satisfaction (treatment*time, $p > 0.05$, all) (Fig. 4). Nevertheless, there was a significant treatment effect in all appetite ratings (treatment, $p < 0.001$, all), whereby HP had lower Hunger and TOF (post-hoc, $p < 0.05$, both), and greater Fullness and Satisfaction (post-hoc, $p < 0.05$, both) compared with LP and WC (Fig. 4). After ingesting the preload beverages, Hunger and TOF dropped transiently at $t = 15$ min, whereas Fullness and Satisfaction peaked at $t = 15$ min. Then, Hunger and TOF progressively increased, whereas

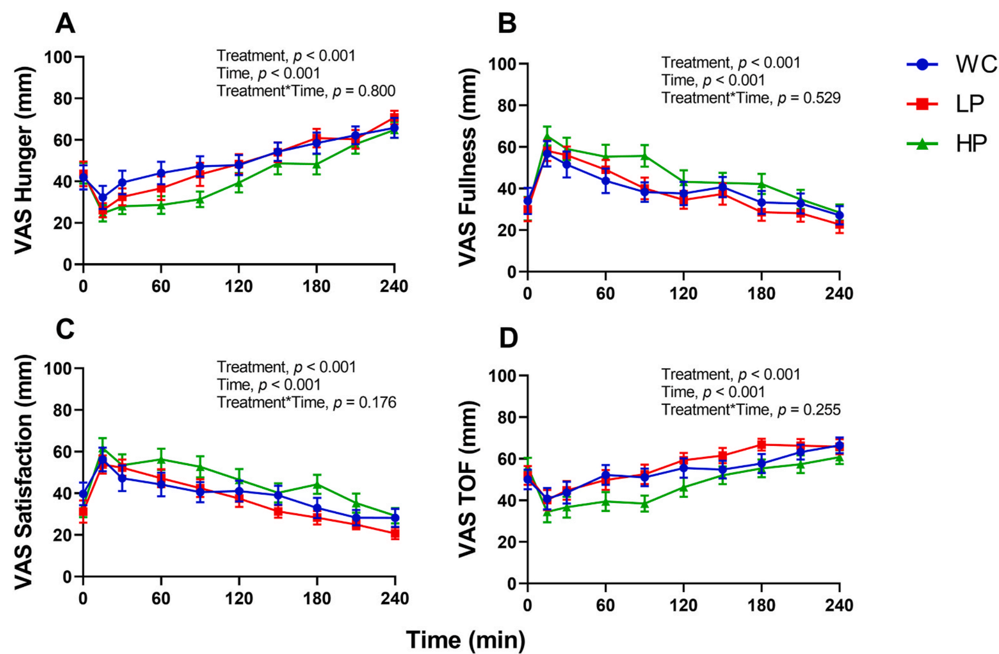

Fig. 4. Postprandial VAS ratings. Mean (±SEM) (A) Hunger, (B) Fullness, (C) TOF, and (D) Satisfaction after ingesting Water Control (WC), Low Protein (LP), and High Protein (HP) preload beverages at t = 0 min. Postprandial appetite ratings (n = 24, t = 0–240 min) were analysed using a linear mixed model with baseline ratings (t = 0 min) as covariate.

Fullness and Satisfaction progressively decreased throughout the morning until lunch at t = 240 min.

Participants tolerated the preload beverages well. Nausea was low at baseline and remained low during all treatments. The treatments did not significantly affect the trajectory of postprandial Nausea (treatment*-time, $p = 0.856$) (Supplementary Fig. 3), hence nausea level was not physiologically meaningful and unlikely to affect appetite responses.

3.4. Ad libitum outcome lunch meal

For the n = 19 participants who completed the outcome lunch meal, mean (±SEM) *ad lib* EI was 3047 ± 314 kJ, 2945 ± 225 kJ and 2961 ± 259 kJ for WC, LP and HP, respectively. In agreement with VAS, increasing the dietary bolus of whey protein in the preload beverage had no significant effect on *ad lib* EI at lunch (ANOVA, $p = 0.799$).

3.5. Within-participant associations between blood biomarkers and appetite

3.5.1. Multicollinearity

Postprandial concentrations of BCAAs (leucine, isoleucine, and valine) had strong positive correlations with each other ($r > 0.9$, all), subsequently analysed as the 'BCAA' group. Similarly, postprandial concentrations of phenylalanine, methionine, lysine, and threonine had strong positive correlations with each other ($r > 0.7$, all), whereas histidine had moderate positive correlations with these EAAs ($r = 0.4$–0.6). Nevertheless, due to their shared physiological characteristic as EAAs, phenylalanine, methionine, lysine, threonine, and histidine were subsequently analysed as the 'Other EAA' group.

3.5.2. Univariate associations between blood biomarkers and energy intake

Ad lib EI was not significantly associated with biomarker concentrations immediately prior to the lunch meal (t = 240 min) ($p > 0.002$, all), or when expressed as $AUC_{0\text{-}240}$ ($p > 0.002$, all). Nevertheless, there was a trend for *ad lib* EI suppression associated with higher $AUC_{0\text{-}240}$ glycine (Estimate = −0.023 ± 0.010, $p = 0.026$, marginal $R^2 = 0.08$, AIC = 912) and higher $AUC_{0\text{-}240}$ taurine (Estimate = −0.106 ± 0.047, $p = 0.029$, marginal $R^2 = 0.07$, AIC = 910). However, fitting both $AUC_{0\text{-}240}$ glycine and $AUC_{0\text{-}240}$ taurine within a multivariate regression analysis did not improve the estimation of *ad lib* EI (AIC = 912).

3.5.3. Univariate associations between blood biomarkers and VAS-assessed appetite

In general, biomarker concentrations were negatively associated with Hunger and TOF, and positively associated with Fullness and Satisfaction. The univariate models revealed that the marginal R^2 varied remarkably between biomarkers as well as between treatment groups. Most biomarkers had very low marginal R^2, notwithstanding its statistically significant association with VAS-assessed appetite (Fig. 5). Notably, of all measured biomarkers, glycine was the most reliable predictor of appetite responses, implicated by a higher marginal R^2 relative to other biomarkers. Following WC beverage, which was equivalent to a prolonged fasted state, circulating glycine was surprisingly identified as the most reliable predictor of appetite responses. It was negatively associated with Hunger and TOF, while positively associated with Fullness and Satisfaction ($p < 0.001$, all) (Table 3). Next to glycine, glucose and alanine were identified to be associated with appetite responses in the same direction as glycine ($p < 0.001$, all), except glucose was not significantly associated with Fullness and Satisfaction ($p > 0.002$, both) (Table 3). Following LP beverage, the univariate models revealed that glucose, all glucoregulatory peptides, GLP-1, BCAAs, Other EAAs, and most NEAAs except glutamic acid, were significantly associated with two or more appetite responses ($p < 0.002$, all). Most biomarkers were more closely associated with appetite responses following the LP beverage in comparison to the WC beverage, as implicated by the higher marginal R^2 (Fig. 5). Interestingly, glycine and alanine were still identified as biomarkers with the highest marginal R^2, negatively associated with Hunger and TOF, while positively associated with Fullness and Satisfaction ($p < 0.001$, all) (Table 3). In contrast, glucose was no longer associated with appetite responses following LP and HP beverages ($p > 0.002$, all). Following the HP beverage, most glucoregulatory peptides except glucagon, some NEAAs including glycine, asparagine, arginine, and alanine, remained significantly associated with two or more appetite responses ($p < 0.002$, all). In agreement with previous analyses, glycine had the highest marginal R^2. It was followed by amylin. Similarly, glycine and amylin were negatively

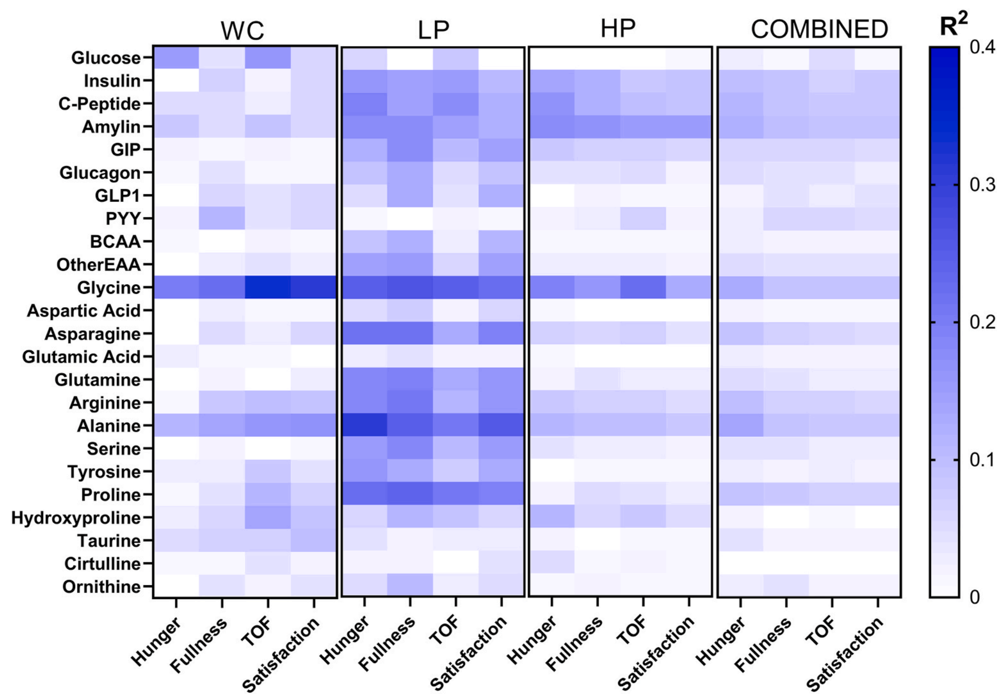

Fig. 5. Heat map. Marginal R^2 of each biomarker when estimating appetite responses in univariate linear mixed model regression analysis, grouped by treatments. WC, Water Control; LP, Low Protein; HP, High Protein; COMBINED, all treatments combined; TOF, Thoughts of Food; GIP, gastric inhibitory polypeptide; GLP-1, glucagon-like peptide-1; PYY, peptide YY; BCAAs, Branch-chained amino acids; Other EAAs, Other essential amino acids.

Table 3
Univariate linear mixed model regression analysis of VAS-assessed appetite.

Biomarkers	Hunger (mm)			Fullness (mm)			TOF (mm)			Satisfaction (mm)		
	Estimates	*p*-value	Marginal R^2	Estimates	*p*-value	Marginal R^2	Estimates	*p*-value	Marginal R^2	Estimates	*p*-value	Marginal R^2
WC												
Glycine (μM)	−0.231 ± 0.051	<0.001	0.20	0.248 ± 0.054	<0.001	0.22	−0.253 ± 0.042	<0.001	0.33	0.281 ± 0.048	<0.001	0.31
Glucose (mM)	−21.441 ± 5.096	<0.001	0.15	11.312 ± 5.240	0.032	0.04	−19.435 ± 4.000	<0.001	0.16	13.374 ± 4.733	0.005	0.06
Alanine (μM)	−0.107 ± 0.029	<0.001	0.11	0.128 ± 0.029	<0.001	0.14	−0.119 ± 0.022	<0.001	0.16	0.136 ± 0.025	<0.001	0.17
LP												
Glycine (μM)	−0.219 ± 0.039	<0.001	0.25	0.204 ± 0.035	<0.001	0.27	−0.190 ± 0.031	<0.001	0.25	0.166 ± 0.030	<0.001	0.23
Alanine (μM)	−0.144 ± 0.018	<0.001	0.31	0.123 ± 0.016	<0.001	0.25	−0.093 ± 0.013	<0.001	0.21	0.108 ± 0.015	<0.001	0.26
HP												
Glycine (μM)	−0.171 ± 0.030	<0.001	0.19	0.172 ± 0.032	<0.001	0.16	−0.171 ± 0.026	<0.001	0.22	0.145 ± 0.030	<0.001	0.13
Amylin (pg/mL)	−0.437 ± 0.082	<0.001	0.18	0.479 ± 0.087	<0.001	0.17	−0.379 ± 0.073	<0.001	0.15	0.410 ± 0.079	<0.001	0.15
COMBINED												
Glycine (μM)	−0.152 ± 0.022	<0.001	0.13	0.125 ± 0.022	<0.001	0.09	−0.108 ± 0.018	<0.001	0.09	0.114 ± 0.020	<0.001	0.09
Alanine (μM)	−0.087 ± 0.010	<0.001	0.14	0.071 ± 0.009	<0.001	0.09	−0.056 ± 0.008	<0.001	0.08	0.063 ± 0.008	<0.001	0.08
Amylin (pg/mL)	−0.454 ± 0.062	<0.001	0.12	0.435 ± 0.059	<0.001	0.10	−0.349 ± 0.049	<0.001	0.09	0.371 ± 0.055	<0.001	0.09

Data presented as mean estimates (±SEM). Proportion of variance in VAS-assessed appetite collectively explained by each biomarker is presented as marginal R^2. WC, Water Control; LP, Low Protein; HP, High Protein; COMBINED, all treatments combined; TOF, Thoughts of Food.

associated with Hunger and TOF, while positively associated with Fullness and Satisfaction ($p < 0.001$, all) (Table 3). However, most biomarkers had decreased ability to estimate appetite responses following HP beverage in comparison to LP beverage. When combining all treatments, most biomarkers except hydroxyproline and citrulline were identified as significant predictors of two or more appetite responses ($p < 0.002$, all). Although univariate models similarly revealed that glycine, alanine, and amylin had the highest marginal R^2 in comparison to other biomarkers, they were much lower than when analysed as individual treatment (Table 3).

3.5.4. *Multivariate associations between blood biomarkers and VAS-assessed appetite*

Table 4 summarises the best-predictive multivariate regression

Table 4
Multivariate linear mixed model regression analysis of VAS-assessed appetite.

Biomarkers	Hunger (mm)		Fullness (mm)		TOF (mm)		Satisfaction (mm)	
	Estimates	*p*-value	Estimates	*p*-value	Estimates	*p*-value	Estimates	*p*-value
WC								
Glucose (mM)	−15.368 ± 4.593	0.001	9.193 ± 4.828	0.060	−12.975 ± 3.977	0.002	4.713 ± 4.210	0.267
Amylin (pg/mL)	−0.430 ± 0.198	0.037	–	–	−0.166 ± 0.183	0.369	–	–
GLP-1 (pg/mL)	–	–	–	–	–	–	0.090 ± 0.040	0.031
PYY (pg/mL)	–	–	0.148 ± 0.042	0.001	–	–	–	–
Glycine (μM)	−0.167 ± 0.056	0.005	0.212 ± 0.063	0.002	−0.180 ± 0.051	0.001	0.209 ± 0.052	<0.001
Aspartic Acid (μM)	–	–	4.488 ± 2.372	0.060	–	–	–	–
Arginine (μM)	–	–	0.446 ± 0.260	0.089	–	–	–	–
Alanine (μM)	–	–	–	–	−0.042 ± 0.027	0.119	0.099 ± 0.033	0.004
Tyrosine (μM)	–	–	−0.347 ± 0.222	0.121	–	–	−0.561 ± 0.196	0.005
Hydroxyproline (μM)	–	–	0.253 ± 0.674	0.710	−0.587 ± 0.449	0.200	0.965 ± 0.501	0.062
Taurine (μM)	−0.261 ± 0.170	0.127	–	–	−0.261 ± 0.139	0.063	0.502 ± 0.159	0.002
Citrulline (μM)	–	–	–	–	0.975 ± 0.496	0.052	–	–
Ornithine (μM)	–	–	–	–	–	–	−0.347 ± 0.251	0.170
Marginal R^2	0.29		0.33		0.34		0.41	
LP								
Glucose (mM)	−9.919 ± 4.017	0.015	–	–	−3.774 ± 3.025	0.214	–	–
C-Peptide (pg/mL)	–	–	–	–	−0.010 ± 0.003	0.001	–	–
Amylin (pg/mL)	−0.151 ± 0.154	0.331	–	–	–	–	–	–
GIP (pg/mL)	–	–	0.044 ± 0.028	0.123	–	–	0.037 ± 0.027	0.169
GLP-1 (pg/mL)	–	–	–	–	–	–	–	–
BCAA (μM)	–	–	0.078 ± 0.025	0.002	–	–	0.046 ± 0.017	0.008
Other EAA (μM)	–	–	–	–	0.112 ± 0.043	0.010	–	–
Glycine (μM)	–	–	0.183 ± 0.052	0.001	–	–	0.172 ± 0.041	<0.001
Aspartic Acid (μM)	0.016 ± 0.782	0.983	−0.162 ± 0.885	0.855	0.025 ± 0.668	0.970	−0.059 ± 0.869	0.946
Asparagine (μM)	−0.591 ± 0.273	0.038	–	–	−0.322 ± 0.222	0.152	–	–
Arginine (μM)	–	–	0.340 ± 0.181	0.062	−0.453 ± 0.169	0.008	–	–
Alanine (μM)	−0.130 ± 0.033	<0.001	–	–	−0.088 ± 0.025	0.001	–	–
Serine (μM)	0.325 ± 0.174	0.069	−0.265 ± 0.151	0.084	–	–	−0.205 ± 0.123	0.102
Tyrosine (μM)	–	–	−0.422 ± 0.235	0.076	–	–	–	–
Hydroxyproline (μM)	−0.810 ± 0.433	0.069	0.313 ± 0.388	0.426	–	–	–	–
Cirtulline (μM)	0.209 ± 0.379	0.581	−0.654 ± 0.363	0.073	–	–	−0.312 ± 0.329	0.346
Ornithine (μM)	0.458 ± 0.300	0.129	–	–	0.358 ± 0.220	0.107	–	–
Marginal R^2	0.35		0.34		0.30		0.31	
HP								
Insulin (pg/mL)	–	–	0.011 ± 0.002	<0.001	–	–	–	–
C-Peptide (pg/mL)	−0.008 ± 0.002	<0.001	–	–	−0.005 ± 0.002	0.002	–	–
Amylin (pg/mL)	–	–	–	–	–	–	0.392 ± 0.092	<0.001
PYY (pg/mL)	–	–	–	–	−0.099 ± 0.030	0.002	–	–
Glycine (μM)	−0.148 ± 0.044	0.001	0.227 ± 0.042	<0.001	−0.270 ± 0.034	<0.001	0.201 ± 0.039	<0.001
Asparagine (μM)	–	–	–	–	–	–	−0.250 ± 0.125	0.048
Glutamine (μM)	–	–	–	–	0.096 ± 0.026	<0.001	–	–
Arginine (μM)	−0.222 ± 0.095	0.022	–	–	−0.401 ± 0.096	<0.001	0.283 ± 0.107	0.009
Serine (μM)	0.125 ± 0.056	0.027	−0.155 ± 0.053	0.004	0.214 ± 0.045	<0.001	−0.136 ± 0.055	0.015
Hydroxyproline (μM)	0.504 ± 0.532	0.349	−1.035 ± 0.596	0.087	1.290 ± 0.470	0.008	−1.051 ± 0.557	0.064
Citrulline (μM)	0.374 ± 0.148	0.013	–	–	–	–	–	–
Marginal R^2	0.29		0.27		0.43		0.32	
COMBINED								
Glucose (mM)	−1.194 ± 2.313	0.606	0.868 ± 2.295	0.706	−5.660 ± 1.855	0.002	−0.215 ± 2.119	0.919
Insulin (pg/mL)	–	–	0.009 ± 0.002	<0.001	–	–	0.006 ± 0.002	<0.001
C-Peptide (pg/mL)	−0.007 ± 0.002	<0.001	–	–	–	–	–	–
GIP (pg/mL)	–	–	–	–	−0.039 ± 0.011	0.001	–	–
Other EAA (μM)	0.078 ± 0.020	<0.001	–	–	–	–	–	–
Glycine (μM)	−0.108 ± 0.034	0.002	–	–	−0.116 ± 0.023	<0.001	0.118 ± 0.025	<0.001
Aspartic Acid (μM)	−0.459 ± 0.140	0.001	–	–	–	–	–	–
Asparagine (μM)	−0.369 ± 0.135	0.007	–	–	–	–	–	–
Glutamine (μM)	0.082 ± 0.025	0.001	–	–	0.076 ± 0.020	<0.001	−0.070 ± 0.023	0.003
Arginine (μM)	−0.523 ± 0.127	<0.001	0.245 ± 0.095	0.010	−0.260 ± 0.076	0.001	0.217 ± 0.086	0.012
Alanine (μM)	−0.085 ± 0.018	<0.001	0.050 ± 0.014	<0.001	−0.035 ± 0.013	0.009	0.047 ± 0.015	0.002
Serine (μM)	0.211 ± 0.059	<0.001	–	–	0.104 ± 0.040	0.009	−0.122 ± 0.045	0.007
Tyrosine (μM)	–	–	−0.165 ± 0.042	<0.001	–	–	–	–
Marginal R^2	0.29		0.13		0.23		0.21	

Data presented as mean estimates (±SEM). Proportion of variance in VAS-assessed appetite collectively explained by biomarkers in the model is presented as marginal R^2. WC, Water Control; LP, Low Protein; HP, High Protein; COMBINED, all treatments combined; TOF, Thoughts of Food; GIP, gastric inhibitory polypeptide; GLP-1, glucagon-like peptide-1; PYY, peptide YY; BCAAs, Branch-chained amino acids; Other EAAs, Other essential amino acids; -, not included in the multivariate model.

models of VAS-assessed appetite. Overall, group of biomarkers that constructed the best-predictive models varied between treatment groups, and when analysed with all treatment groups combined. Importantly, group of biomarkers in multivariate models explained a greater variance of appetite responses than any single biomarker in univariate models. Following the WC beverage, glycine and glucose significantly contributed to decreased Hunger and TOF ($p < 0.05$, all), and increased Fullness and Satisfaction, but the effect was only significant for glycine ($p < 0.05$, both), not glucose ($p > 0.05$, both), in consistent with the univariate models. The marginal R^2 of the multivariate models were 0.29 for Hunger, 0.33 for Fullness, 0.34 for TOF, and 0.41 for Satisfaction, thus higher than any biomarkers alone.

Following the LP beverage, glucose significantly contributed to decreased Hunger ($p = 0.015$), but its association with TOF was not significant after accounting for other biomarkers ($p = 0.214$). Furthermore, BCAAs and glycine significantly contributed to increased Fullness and Satisfaction ($p < 0.05$, all). The marginal R^2 of the multivariate model was 0.35 for Hunger, 0.34 for Fullness, 0.30 for TOF, and 0.31 for Satisfaction, thus higher than any biomarkers alone. Following the HP beverage, glycine, C-peptide, and arginine were the common biomarkers significantly contributed to decreased Hunger and TOF ($p < 0.05$, all). Glycine also significantly contributed to increased Fullness and Satisfaction ($p < 0.001$, both), whereas arginine significantly contributed to increased Satisfaction ($p = 0.009$), but arginine did not contribute to the estimation of Fullness. Unexpectedly, whilst serine significantly contributed to the estimation of appetite responses, it significantly contributed to increased Hunger and TOF, and decreased Fullness and Satisfaction ($p < 0.05$, all). This direction of effect contrasted with other biomarkers and was not previously identified in the univariate model. Nevertheless, the marginal R^2 of the multivariate model was 0.29 for Hunger, 0.27 for Fullness, 0.43 for TOF, and 0.32 for Satisfaction, thus higher than any biomarkers alone. When analysed with all treatments combined, glycine, arginine, and alanine significantly contributed to decreased Hunger and TOF ($p < 0.05$, all); insulin, arginine and alanine significantly contributed to increased Fullness and Satisfaction ($p < 0.05$, all). Additionally, glycine significantly contributed to increased Satisfaction ($p < 0.001$), but did not contribute to the estimation of Fullness. In contrast, serine significantly contributed to increased Hunger and TOF ($p < 0.05$, both), but decreased Satisfaction ($p = 0.007$). Surprisingly, glutamine also significantly contributed to the estimation of appetite responses in the same direction of serine ($p < 0.05$, all), which was again not previously identified in the univariate model. Collectively, the marginal R^2 of the multivariate model was 0.29 for Hunger, 0.13 for Fullness, 0.23 for TOF, and 0.21 for Satisfaction, thus higher than any biomarkers alone.

4. Discussion

In this cohort of women with overweight or obesity, increasing the dietary load of whey protein from zero to 50 g significantly changed the trajectory of postprandial circulating concentrations of glucose, glucoregulatory peptides, GLP-1, and most AAs. These are all putative biomarkers of the postprandial regulation of appetite and satiety. Yet, surprisingly, the increasing load had no effect on trajectories of VAS-assessed appetite or *ad lib* EI. Whilst many individual biomarkers were found to have statistically significant relationships with VAS-assessed appetite in the univariate regression model, these explained only a small proportion of variance in VAS-assessed appetite and were not robust in multivariate regression modelling. Hence, these biomarkers could be regarded as poor predictors of the VAS-assessed appetite. Of the measured biomarkers, it was the AA glycine that was the most reliable biomarker of VAS-assessed appetite and displayed a trend towards suppressing *ad lib* EI. In agreement with our hypothesis, a multi-biomarker multivariate regression model which included concurrent concentrations of glucose, glucoregulatory peptides, gut peptides and AAs significantly improved the estimation of VAS-assessed appetite, potentially highlighting the multiple mechanism that are involved in the complex regulatory pathways that influence human appetite responses.

Our current study measured postprandial circulating concentrations of glucose, glucoregulatory peptides, gut peptides, and AAs as they are relevant to the insulinogenic and glucogenic properties of whey protein (Ang et al., 2019). We confirmed a robust increase in circulating concentrations of glucoregulatory peptides, including insulin, C-peptide, glucagon, GIP, and amylin, following whey protein ingestion. Indeed, GLP-1 can also be classified as a glucoregulatory peptide as it acts as an incretin (Holst, 2007). Interestingly, the whey protein beverage resulted in an early insulin peak (Supplementary Fig. 1a), which was then followed by a later glucagon peak (Supplementary Fig. 1c). The opposing glucoregulatory action between insulin and glucagon demonstrated a notable consequence on circulating glucose concentration. The early insulin peak at t = 30–60 min following HP corresponded to the dip in circulatory glucose at t = 60 min. Circulating glucose then stabilised as glucagon concentration peaked. Whey protein beverage was relatively ineffective in stimulating the postprandial increase in PYY, in agreement with a prior whey protein beverage study (Chungchunlam et al., 2015). Since there is evidence that AAs stimulate secretion of PYY in isolated rat intestine model (Mace et al., 2012), the dietary whey-derived AAs in our current study might have been primarily absorbed from the proximal small intestine before reaching the distal small intestine where PYY-secreting L-cells concentrate (Steinert et al., 2017). Whilst most proteogenic AAs significantly increased after ingesting whey protein beverage, glycine decreased. Circulating glycine is depleted by acting as a carbon donor for the pyruvate-alanine cycle, triggered by an increased deamination of the enlarged BCAA pool following whey protein intake (White et al., 2020). Postprandial glycine is expected to increase when dietary glycine-to-BCAA ratio is higher or during dietary BCAA restriction (White et al., 2016), in agreement with the Veldhorst et al. (2009a) observation that soy and gelatin increased postprandial glycine as these proteins had higher glycine-to-BCAA ratio than dairy proteins, namely whey, casein and α-lactalbumin. Our study also observed a postprandial increase in circulating hydroxyproline, citrulline, and ornithine concentrations, likely due to increased endogenous production, as whey protein contains undetectable levels of these AAs.

Increasing the energy and protein content of preload beverages to 871 kJ and 50 g respectively did not result in a robust dose-dependent effect of protein load on appetite suppression over the 4-h postprandial period. Although the postprandial Hunger ratings were lower following the HP compared to LP and WC, HP did not delay the postprandial progressive increase in Hunger. The *ad lib* EI assessed 4 h after the preload was also not significantly different between the preload beverages. Previous studies from our group (Poppitt et al., 2011; Wiessing et al., 2015) also failed to observe dose-dependent effects of protein-induced satiety, but notably when supplementing up to only 20 g whey protein in a 500 mL water beverage. Even when protein intake was raised to 50 g in our current study, the mean *ad lib* EI of the current study was similar to these previous studies using 20 g whey protein (≈3 MJ) (Poppitt et al., 2011; Wiessing et al., 2015). It must be noted that, protein-induced satiety is weaker in beverage than in solid format (Almiron-Roig et al., 2013). Yet our results are in contrast to others, where a liquid protein load has elicited suppression in appetite responses (Anderson et al., 2004; Astbury et al., 2010; Hutchison et al., 2015). Although differences in our methodology, including the energy and composition of preload beverages, the inter-meal duration and the characteristics of participant may be the contributing factors (Almiron-Roig et al., 2013; Drapeau et al., 2013), variable circulating concentrations of putative appetite biomarkers is hypothesised to provide a unifying explanation to the variability in appetite responses.

Importantly, our current study demonstrated that glycine was the most prominent biomarker of appetite response, investigated in a whey protein model in a cohort of women with overweight/obesity. A lower $AUC_{0\text{-}240}$ glycine increased *ad lib* EI, though the effect was only trending towards significance after adjusting for multiple testing. Interestingly, following both a prolonged fast and whey protein ingestion, the decrease in circulating glycine was significantly associated with increased Hunger and decreased Fullness. Indeed, several studies have suggested that glycine is a potential biomarker of hunger, although the physiological mechanism has not been elucidated. Veldhorst et al. (2009a) previously demonstrated that gelatin, a rich source of dietary glycine, suppressed *ad lib* EI when compared to iso-nitrogenous whey, casein, and soy. Although high circulating glycine concentration may underly the satiating effect of gelatin, it remains speculative whether circulating glycine can retain its biomarker quality following a high-CHO, high-fat or mixed meal models. Interestingly, a recent observational metabolomics study found that of the 124 plasma metabolites analysed, glycine was one of

two metabolites consistently associated with VAS-assessed appetite after consuming a 2 MJ mixed macronutrient meal (55 %en carbohydrate, 30 %en fat, 15 %en protein) (Camacho-Barcia et al., 2021). For the first time, in this study we have shown that glycine significantly contributes to estimates of appetite response when considering concurrent concentrations of other biomarkers using a multivariate model. Our novel finding clearly supports the role of glycine as a potential biomarker of appetite, both in the prolonged fasted state and after whey protein ingestion. Glycine is an inhibitory neurotransmitter in the spinal cord and brain, although little is known of its function in the hypothalamus and other brain regions required for appetite regulation (Gold & Martin, 1983). Glycine receptor agonists hyperpolarize hypocretin/orexin neurons in the hypothalamus, mechanisms linked to arousal and reward seeking behaviour (Karnani et al., 2011). Notably, since glycine metabolism is altered in individuals with obesity, pre-diabetes, and type-2 diabetes (Alves et al., 2019) the potential of glycine to benefit appetite regulation, body weight regulation, and diabetes prevention deserves further investigation.

Our current study also demonstrated that glucose, alanine, and amylin are potential biomarkers of appetite response under specific conditions. The depleting glucose supply in the circulation is associated with increased Hunger during the prolonged fasted state, in agreement with the "glucostatic hypothesis". However, when there was dietary source of protein, circulating glucose was no longer a reliable postprandial biomarker of appetite. Very few studies in recent years have tested the "glucostatic hypothesis" of appetite regulation, partly due to a shift in interest to understand the mechanism of gut peptides. When Wyatt et al. (2021) revisited this hypothesis, they similarly found weak evidence of "glucostatic hypothesis" in the postprandial state. The decrease in circulating alanine was associated with an increase in Hunger. Since alanine is a key glucogenic AA (Chiasson et al., 1975), its association with appetite may be mediated through gluconeogenesis, a process hypothesised to be involved in protein-induced satiety (Veldhorst et al., 2012). The effect of alanine on Hunger was more prominent after ingesting the LP beverage, as its effect remained significant after considering the concurrent concentrations of other biomarkers. Although amylin was associated with appetite following the HP beverage when analysed as an individual biomarker, the multivariate model showed other biomarkers to have a greater influence on appetite response.

Despite GLP-1 and PYY being commonly measured as appetite biomarkers, the substantial change in postprandial GLP-1 in this study was not reflected by a change in either *ad lib* EI or VAS-assessed appetite. In agreement with our previous review (Lim & Poppitt, 2019), our current data questions the physiological role of circulating postprandial GLP-1 as a reliable biomarker of appetite. Conversely, Lemmens et al. (2011) showed GLP-1 and PYY explained 50–60% of the variance of VAS-assessed Fullness following a 5 MJ mixed meal (54 %en CHO, 32 % en fat, 14%en protein). The proportion of variance explained by GLP-1 and PYY individually was much higher than our multivariate models. Furthermore, Gibbons et al. (2013) reported GLP-1 was associated with VAS-assessed appetite following both 2.5 MJ high-fat (38.0 %en CHO, 50.3 %en fat, 11.7 %en protein) and high-CHO (83.6 %en CHO, 3.2 %en fat, 13.2 %en protein) meals. Haddad et al. (2018) also showed VAS-assessed appetite was significantly correlated with circulating GLP-1 and PYY following 4 mixed meals with varied energy content and macronutrient compositions. Given the vast number of appetite studies that assess GLP-1 and PYY as biomarkers of appetite, very few studies have tested the association between gut peptides and appetite response using regression analysis. Rather than GLP-1 and PYY, our current study clearly demonstrated that VAS-assessed appetite was better estimated by circulating glycine. It must be noted of course that this was a dietary protein intervention, and that further studies would be required to test this outcome under different dietary conditions.

The utility of biomarkers to estimate VAS-assessed appetite can be evaluated by the marginal R^2 values. Generally, the ability of an individual biomarker to estimate appetite responses varied between biomarkers and between treatment groups. Indeed, multivariate models improved the estimation of VAS-assessed appetite compared to univariate models, in agreement with the hypothesis. However, the marginal R^2 of the multivariate model improved only modestly, with multivariate models explaining 13–43% of variance in VAS-assessed appetite. Furthermore, the ability of a biomarker to estimate appetite responses can be improved when each individual treatment group is examined separately rather than with all treatment groups combined. However, when translated into real-life settings, the utility of these biomarkers to estimate appetite responses is limited if information on foods consumed prior to blood sampling is not available.

The main strength of our current study was the use of multivariate LMM regression models to test the collective contribution of circulating concentrations of glucose, glucoregulatory peptides, gut peptides, and AAs on appetite response, and the heterogeneity of participants were included as random effect. A multivariate model has the advantage over a univariate model as the former considers the concurrent change in other biomarkers following acute ingestion of whey protein. After considering the concurrent change in biomarker concentrations, serine and glutamine were found to increase Hunger, opposing the effects of other AAs. This positive association was not found in the univariate models. Since our study only investigated the whey-protein-induced satiety, this novel characterisation of "satiety fingerprint" may help leverage the identification of appetite biomarkers in other food models, such as the work carried out by Camacho-Barcia et al. (2021).

There were also some limitations of this study. First, all participants were female who exhibited evidence of impaired glucose tolerance, including elevated fasting plasma glucose and altered fasting AA profile (Mook-Kanamori et al., 2016). Therefore, the generalisability of the identified "satiety fingerprint" to a wider population is unknown. Nevertheless, women with overweight or obese and prediabetes is a relevant target population as women generally shows a greater interest in weight loss interventions (Crane et al., 2017), and their metabolic health may benefit greatly from interventions that improve appetite and body weight regulation. It is important to test our "satiety fingerprint" in other populations, including males. Second, since our aim was to model the associations between appetite and blood biomarkers, we did not include age and body weight as covariates. Nevertheless, it is noteworthy that older adults had a slower gastric motility, higher concentration of gut peptides, but less suppression of *ad lib* EI than younger adults following a 70 g high protein beverage, although the *ad lib* EI was significantly lower than younger adults (Giezenaar et al., 2015, 2018, 2020). Body weight may also regulate appetite via differential resting metabolic rate which affect energy requirement (Hopkins & Blundell, 2016; Weise et al., 2014). Yet, cross-sectional observation did not consistently support obesity is associated with altered appetite biomarkers, and it is debatable whether alteration in appetite biomarkers is a cause or consequence of weight gain (Lean & Malkova, 2016). Third, the laboratory was not equipped to measure tryptophan at the time of measurement, an AA hypothesised to be involved in appetite regulation (Steinert et al., 2014; Teff et al., 1989). Indeed, the appetite model also did not include other classical appetite biomarkers, such as ghrelin, leptin, and β-hydroxybutyrate. The potential effect of ovarian hormones on appetite was also not characterised (Roney & Simmons, 2017). Therefore, there is a potential to expand the model and to further identify novel biomarkers of appetite using advanced plasma metabolomics technologies (Camacho-Barcia et al., 2021; Horner et al., 2020).

In conclusion, glycine was the single most prominent biomarker of appetite identified both in the prolonged fasted state and following whey protein ingestion. Whilst multivariate modelling improved the estimation of appetite response, a large part of the variance in VAS-assessed appetite remained unexplained, notwithstanding the tightly controlled laboratory environment. Continued discovery methodologies, including metabolomics and proteomics, are required to more precisely identify the mechanistic pathways that are integral in the

complex regulation of human appetite. Our finding reflects the complexity of appetite regulation, stemming from both psychological events and physiological mechanisms. Measuring only a selection of putative biomarkers, especially when analysed in isolation, does not accurately capture the appetite response of an individual and should only be used in conjunction with subjective appetite ratings or objective assessment of EI.

Contribution of authors

JJL – Protocol design, human ethics approval, data collection, laboratory analysis, statistical analysis, data interpretation, and drafting manuscript; IRS – Protocol design, oversight of trial, data interpretation, and critical review manuscript draft; WCYY – Protocol design, human ethics approval, and data collection; LWL – Protocol design, oversight of trial; DB – Statistical analysis; DCS – Data interpretation, and critical review manuscript draft; SDP – funding, PI on project, protocol design, data interpretation, and critical review manuscript draft.

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Data availability

The lead author has full access to the data reported in the manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.appet.2021.105871.

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