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Original article

Postprandial concentration of circulating branched chain amino acids are able to predict the carbohydrate content of the ingested mixed meal☆

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SUMMARY

Background: The amount of the macronutrients protein and carbohydrate (CHO) in a mixed meal is known to affect each other's digestion, absorption, and subsequent metabolism. While the effect of the amount of dietary protein and fat on the glycemic response is well studied, the ability of postprandial plasma amino acid patterns to predict the meal composition is unknown.
Objective: To study the postprandial plasma amino acid patterns in relation to the protein, CHO, and fat content of different mixed meals and to investigate if these patterns can predict the macronutrient meal composition.
Design: Ten older adults were given 9 meals with 3 different levels (low, medium, and high) of protein, CHO, and fat in different combinations, taking the medium content as that of a standardized western meal. We monitored the postprandial plasma response for amino acids, glucose, insulin, and triglycerides for 8 h and the areas under the curve (AUC) were subsequently calculated. Multiple regression analysis was performed to determine if amino acid patterns could predict the meal composition.
Results: Increasing meal CHO content reduced the postprandial plasma response of several amino acids including all BCAA (leucine; $q < 0.0001$, isoleucine; $q = 0.0035$, valine; $q = 0.0022$). The plasma BCAA patterns after the meal significantly predicted the meal's CHO content (leucine; $p < 0.0001$, isoleucine; $p = 0.0003$, valine; $p = 0.0008$) along with aspartate ($p < 0.0001$), tyrosine ($p < 0.0001$), methionine ($p = 0.0159$) and phenylalanine ($p = 0.0332$). Plasma citrulline predicted best the fat content of the meal ($p = 0.0024$).
Conclusions: The postprandial plasma BCAA amino acid patterns are lower with increasing meal CHO content and are strong predictors of a mixed meal protein and CHO composition, as are plasma citrulline for the fat content. We hypothesize that postprandial plasma amino acid concentrations can be used to predict the meal's macronutrient composition.

1. Introduction

Metabolic monitoring and meal prediction are gaining increasing attention [1,2]. It is shown to be a tool for improvement health, nutrition and medical treatment, especially for patients with metabolic dispositions or under nutritional risks [2]. It is also suggested to improve nutrition and treatment in the intensive care unit [3,4] Overall, predicting meals through metabolic monitoring is shown to be particularly useful research tool in a variety of metabolic studies and interventions [2].

☆ Data described in the manuscript, code book, and analytic code will be made available upon request pending approval of the principal investigator (nep.deutz@ctral.org).

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Meal prediction through monitoring of just a single postprandial metabolite is possible, because during meal ingestion, the macronutrients protein, carbohydrate (CHO) and fat have a complex interplay which affects each other's digestion, absorption and subsequent metabolism [5–8]. Therefore, the postprandial plasma concentration patterns of the macronutrient products (amino acids (protein), glucose (CHO) and triglycerides (fat)) can differ depending on the content of the other two macronutrients in the meal. Thus, the macronutrient composition of the mixed meal can be predicted from the different postprandial plasma concentration patterns. This can then be applied to multiple regression and machine learning, ultimately predicting both the meal content and the postprandial metabolic response.

Glucose, mainly through the use of continuous glucose monitors (CGM), are almost exclusively the metabolite used for metabolic monitoring and meal prediction [1,9]. It is well established that the postprandial increase in blood glucose concentration and the glycemic index of a mixed meal are modified by the protein and fat content of smaller mixed meals [7,8,10,11]. Thus, the glycemic response of a mixed meal with equal CHO composition but different protein and fat contents can yield different glycemic indexes [7,8,10,11].

In line, the amount of ingested CHO in a mixed meal could affect the postprandial response of plasma amino acid concentrations. Previous studies showed that ingesting CHO alone or together with casein protein reduces the plasma response of amino acids like leucine, isoleucine, valine and tyrosine [6,12]. This supports the notion that increasing CHO content in a mixed meal reduces the postprandial response of circulating amino acids, suggesting plasma amino acid patterns as potential predictors of the macronutrient composition of a mixed meal. The effect of a meal's fat content on amino acid patterns are less explored, but previous studies have shown that CHO and fat combined inhibit the postprandial amino acid response [13,14].

We hypothesize that the macronutrient composition of a mixed meal can be predicted from postprandial amino acids plasma concentration patterns. In this study, we investigated how different levels of CHO, protein and fat in a mixed meal affect postprandial amino acid patterns in older adults, designing different meal compositions with a low, medium and high level of each macronutrient. We aimed to determine if the CHO, protein and fat content of a meal affects the postprandial patterns of plasma amino acids. We further aimed to determine which of the plasma amino acids can predict the meal content of protein, CHO, and fat.

2. Materials and METHODS

2.1. Subjects

We recruited 10 healthy older adults, 6 females and 4 males (Table 1). Recruitment was done by advertising efforts in the local community. We selected subjects through a screening visit where medical history and medications were assessed. Inclusion criteria were older adults from 60 to 85 years with no obesity and who were otherwise considered healthy and not suffering from any acute or chronic disease or condition that may influence protein and amino acid metabolism. Written informed consent was obtained, and the study was approved by the Local Institutional Review Board (IRB).

2.2. Study design

The study was conducted as a paired prospective clinical study consisting of 9 different study days for each subject, all performed in the Clinical Research Unit of the Center for Translational Research on Aging and Longevity at Texas A&M University between June 2018 and July 2019. Subjects were allowed at least 3 days between each study day and all study days for a subject were within a 5 weeks period. The primary endpoint was set as the calculated predictive values for each amino acid for the meals CHO content based on the measurements from all study days. The secondary endpoints were considered as the predictive values for protein and fat content along with the comparison of the amino acid postprandial plasma patterns of different meal content for each macronutrient.

Table 1
Subject characteristics.

Gender (f/m)	(6/4)
Age (yr)	68 (0.5)
Body weight (kg)	74.1 (3.8)
Body mass index (kg/m^2)	27.0 (1.3)
Lean Body Mass (LBM, kg)	43.8 (3.0)
Free fat mass (FFM, kg)	26.9 (2.2)
Glucose (mg/dl)	89.8 (2.6)
Triglycerides (mg/dl)	113.3 (12.9)
Insulin (μU/mL)	5.9 (2.2)
HOMA-IR	1.35 (0.54)

General characteristics of included subjects (n = 10) displayed as mean (SEM). Glucose, triglycerides, insulin, and HOMA-IR are average baseline characteristics. HOMA-IR; Homeostatic Model Assessment for Insulin Resistance.

2.3. Anthropometrics and body composition

Body weight and height were measured by a digital beam scale and stadiometer, respectively, and body mass index (BMI) was calculated. Body-composition was assessed by dual-energy X-ray absorptiometry [Hologic QDR 4500/Version 12.7.3.1 (Bedford, MA)] to obtain whole body fat-free mass (FFM) and lean body mass (LBM). These measures were standardized for height to obtain FFM index and LBM index.

2.4. Meal composition

We composed 9 meals with predefined amounts of 3 levels (low, medium, and high) of protein, carbohydrates (CHO), and fat (the compositions of the 9 meals are summarized in Table 2). The medium level of each macronutrient was taken as the content of a standardized western diet, meal 2 being a standardized control meal as all macronutrients were at medium level. The meal was prepared as a liquid drink mixed to ensure a fresh meal consisting of the precise macronutrient composition that could be consumed within a fixed time frame. It was made with a pudding base (Jell-O

Table 2
Experimental meal compositions.

	Protein (g)	CHO (g)	Fat (g)	Total Energy (kcal)
Meal 1 (P1C1F1)	15	52.25	13	386
Meal 2 (P2C2F2)	**30**	**94.75**	**26**	**733**
Meal 3 (P3C3F3)	60	179.75	52	1427
Meal 4 (P1C2F2)	15	52.25	26	673
Meal 5 (P3C2F2)	60	52.25	26	853
Meal 6 (P2C1F2)	30	52.25	26	673
Meal 7 (P2C3F2)	30	179.75	26	1073
Meal 8 (P2C2F1)	30	94.75	13	616
Meal 9 (P2C2F3)	30	94.75	52	967

Macronutrient content in meals administered of protein (P), carbohydrate (C) and fat (F) at three different levels (1; low, 2; medium, and 3; high), displayed in grams (g). Macronutrient medium levels, and meal 2 (P2C2F2) represents content in a standardized western diet.

Vanilla, Kraft Food, IL, USA) containing 0.75 g CHO and with different levels of protein (low P1: 15 g, medium P2: 30 g, high P3: 60 g), CHO (low C1: 42.5 g, medium C2: 85 g, high C3: 170 g) and fat (low F1: 13 g, medium F2: 26 g, high F3: 52 g). Whey protein (BiPro 9500 Whey Protein Isolate, Agropur, MN, USA) was used as the protein nutrient, containing a mixture of essential and non-essential amino acids displayed in Table 3. Maltodextrin (Polycose, Abbott Nutrition, IL, USA) was used as the carbohydrate (CHO) nutrient, and sunflower oil (Great Value, Wal-mart, AZ, USA) as the fat nutrient. Meals were prepared early on each study day. The protein hydrolysates and maltodextrin were dissolved in 250 ml water, mixed with the pudding base, and then thoroughly mixed with sunflower oil within 30 min of ingestion.

2.5. Study day

Subjects were fasted overnight for at least 8 h prior to the study day. Before administering the meal, subjects received a venous catheter in the hand which was kept in a hot box throughout the experiment to allow collection of arterialized venous blood samples. The subjects were kept in upright sitting position throughout the day. Baseline fasted blood samples were drawn (time 0). Subjects then received one of the 9 meals, in a randomized order on each study day. We then collected blood samples for a total of 8 h at time = 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, 420, and 480 min.

2.6. Sample processing and biochemical analysis

Blood samples were collected in pre-chilled, EDTA or li-heparinized tubes (Becton Dickinson Vacutainer system, Franklin Lakes, NJ, USA) and kept on ice. Plasma was obtained by centrifugation of whole blood at 4 °C for 10 min at 3120 *g*, and was aliquoted with vortexing to tubes containing either 0.1 vol of 33% (w/w) trichloroacetic acid or the residue after evaporation of 0.17 vol of 33% (w/w) 5-sulfosalicylic dihydrate to denature proteins. All samples were frozen in liquid nitrogen and stored at −80 °C until analysis.

All analyses were performed in plasma. Insulin was measured with an electrochemiluminescent immunoassay, and glucose and triglycerides with standardized enzymatic assays, all assessed by LabCORP. Plasma amino acids were analysed batchwise with LC/MS/MS, as described in depth earlier [15]. Branched chain amino acids (BCAA) are defined as the sum of leucine (LEU), isoleucine (ILE) and valine (VAL). Essential amino acids (EAA) are defined as the sum of histidine (HIS), ILE, LEU, lysine (LYS), methionine (MET), phenylalanine (PHE), threonine (THR), tryptophan (TRP) and VAL. The non-essential amino acid (NEAA) are defined as aspartate (ASP), glutamate (GLU), hydroxyproline (hPRO), asparagine (ASN), glutamine (GLN), citrulline (CIT), serine (SER), glycine (GLY), arginine (ARG), tau-methylhistidine (t-MHIS), alanine (ALA), taurine (TAU), proline (PRO), ornithine (ORN) and tyrosine (TYR).

Table 3
Whey protein amino acid content.

Amino Acid	(mg/g Whey protein)
Alanine	49
Arginine	24
Aspartic Acid + Aspargine	114
Cysteine	28
Glutamic Acid + Glutamine	161
Glycine	17
Histidine	20
Isoleucine	56
Leucine	127
Lysine	102
Methionine	23
Phenylalanine	35
Proline	47
Serine	29
Threonine	47
Tyrosine	36
Valine	54

Amino acid content given as mg/g Whey protein (BiPro® Whey protein isolate) added to each meal.

2.7. Calculations and statistical analysis

The statistical package within Graphpad Prism Version 8.3.1 for Windows (GraphPad Software La Jolla, CA, USA, www.graphpad.com) was used for all data analysis. Area under the curve (AUC) over 8 h (480 min) for plasma glucose, insulin, triglycerides, and amino acids were calculated by using the trapezoid method. Both total AUC and incremental AUC (including both positive and negative peaks) were evaluated. We observed that total AUC gave the best prediction, and therefore was chosen for further analysis. AUC are displayed per minute for glucose ($mg \cdot dL^{-1} \cdot$ min), insulin ($\mu U \cdot dL^{--1}$*min), TG ($mg \cdot dL^{-1} \cdot$ min) and all amino acids ($\mu mol \cdot L^{-1}$*min). Results are expressed as mean ± standard error (SE).

To evaluate the effect of altering the level of the 3 macronutrients (protein, CHO and fat), we compared meals with low, medium and high levels of each macronutrient while keeping the other two macronutrients at a fixed standardized medium level. Statistics were run with repeated measures one-way ANOVA. For multiple comparisons, we corrected p-values for false discovery rate (FDR) [16] using the two-stage step-up method of Benjamini, Krieger and Yekutieli [17], calculating FDR-adjusted p-values as q-values. The level of significance was set at $p < 0.05$ and $q < 0.05$ was considered a true discovery after correcting for FDR.

Multiple regression analysis was performed by calculating ordinary least squares for plasma glucose, TG and each amino acid as the dependent variable with the meal content of the 3 macronutrients (protein, CHO and fat) as the independent variables. Values for all 9 meals were included in the analysis. Coefficients were estimated along with SE and coefficient intervals. [t]-values (coefficients/SE) were calculated to estimate how good each amino acid could predict the macronutrient meal content of protein, CHO and fat, respectively.

3. Results

3.1. Plasma glucose, insulin, and triglycerides

When comparing the effect of different levels of protein content in the meals (Fig. 1A), no effects were observed on plasma glucose and TG concentrations. Plasma glucose increased when comparing meals with increasing CHO content, as was the case for plasma triglycerides (TG) with increasing meal fat content (Fig. 1C). Insulin (Fig. 1B) increased between the low and median amount of CHO but did not further increase with the high CHO amount. Insulin was not affected by the amount of whey protein. The insulin response tended to decrease in meals with high fat or protein content ($p = 0.016$, $p = 0.078$ respectively), likely because of the known effect of reduced gastric emptying prolonging insulin release [18].

3.2. Plasma amino acids

When comparing different protein levels, plasma concentrations of all individual amino acids in Whey protein increased (Table 4) and returned to baseline within 7–8 h for high and 5–6 h for low and medium protein content meals (Fig. 2). The total AUCs were calculated for all individual amino acids (Fig. 3), as well as for

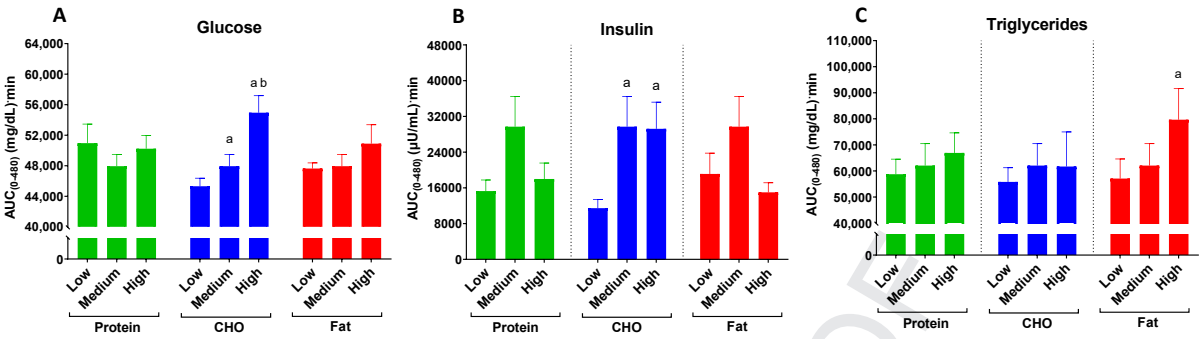

Fig. 1. Area under the curve (AUC) over 480 min for plasma levels of glucose (A), insulin (B), triglycerides (TG) (C) comparing meals where each macronutrient content is altered with a low, medium and high level while keeping the other two macronutrients at a standard medium level. Data are displayed as mean (SEM). Statistics were performed with repeated measure one-way ANOVA correcting for false discovery rate FDR. Q < 0.05 was considered a true discovery with a) vs. low level and b) vs. medium level.

the sum of the individual BCAA, (non)essential amino acids and all individual amino acids (Fig. 4).

When comparing low vs high protein content, the AUC increased as expected for all amino-acids including BCAA (leucine; isoleucine; valine) (Fig. 3A–C and Table 4) along with the other essential amino acids aspartate (185%), glutamate (29%); hydroxyproline (78%); glutamine (7%); serine (22%); arginine (28%); threonine (67%), alanine (37%), valine (73%), methionine (86%), isoleucine (113%), leucine (63%), tryptophan (63%) (Table 4). We also observed an increase in the non-essential amino acid citrulline (37%) and ornithine (24%) (Table 4). The sum of all BCAA, EAA, NEAA and all amino acids (SUMAA) also increased with increasing meal protein content (Fig. 4).

Differences in amino acid concentration caused by different levels of CHO ingestion were observed for approximately 4 h (Fig. 2). We found that all BCAA were significantly reduced when comparing meals with increasing CHO level (leucine 30% isoleucine 26%; valine 55%; total BCAA 27%, Fig. 2). We also observed a significant reduction for some other essential amino acids (tryptophan 22%, methionine 22%) as well as when calculating the sum of all essential amino acids (17%) and the sum of all amino acids (9%) (Fig. 4 and Table 4). This reduction was also evident for some non-essential amino acids (aspartate 43%; glutamate 23%; citrulline 18%; serine 13%; proline 9%; tyrosine 20%) (Table 4). When comparing meals with altering levels of fat content, there were no differences for neither essential nor nonessential amino acids, with the exception of the clear difference in AUC for citrulline (20%) (Fig. 3D and Table 4). Several of the non-essential amino acids had an overall reduced AUC from baseline (hydroxyproline, glutamine, citrulline, serine, glutamate, arginine, tau-methylhistidine, and

Table 4
Statistically differences in amino acid AUC_{0-480} comparing macronutrient levels.

	Protein (levels)			CHO (levels)			Fat (levels)		
	Low vs Medium	Medium vs High	*Low vs* High	*Low vs* Medium	Medium vs High	*Low vs* High	*Low vs* Medium	Medium vs High	*Low vs* High
Aspartate	0.1101	↑**<0.0001***	↑**<0.0001***	↓ **0.002***	↓**0.0058***	↓**0.0002***	>0.9999	>0.9999	>0.9999
Glutamate	0.3357	↑**0.0050***	↑**0.0178***	↓**0.0072***	0.2798	↓**0.0053***	0.3812	0.3812	0.3812
Hydroxyproline	0.1873	0.0726	0.3741	0.1473	0.3864	0.1473	0.4077	0.4077	0.4077
Asparagine	↑**0.0304***	↑**0.0003***	↑**0.0003***	0.1855	0.8361	0.2832	0.6588	0.8489	0.9951
Glutamine	0.7231	0.2941	0.2941	0.3277	0.3277	0.1325	0.2632	0.8300	0.5767
Citrulline	0.0636	↑**0.0001***	↑**<0.0001***	↓**0.0410***	↓**0.0158***	↓**0.0112***	0.0721	↓**0.0036***	↓**0.0036***
Serine	0.2457	↑**0.0012***	↑**0.0012***	0.0609	0.2772	↓**0.0018***	0.6544	0.9911	0.6890
Glycine	0.5497	0.9524	0.5497	0.5433	0.0802	↑**0.0173***	0.4285	0.4285	0.4194
Arginine	0.1010	↑**0.0023***	↑**0.0002***	0.4911	0.3951	0.2379	>0.9999	0.5615	0.5615
Threonine	↑**0.0394***	↑**0.0002***	↑**<0.0001***	0.1824	0.7457	0.1824	0.8432	0.8550	0.8432
Tau-methylhistidine	0.8011	0.8011	0.8011	0.2745	0.5837	0.3430	0.6627	0.6627	0.1648
Alanine	↑**0.0460***	↑**0.0078***	↑**0.0003***	0.2204	0.2204	0.0870	0.3028	0.3828	0.3028
Taurine	0.4436	0.1810	0.5605	0.8227	0.9722	0.8267	0.8929	0.8626	0.8626
Proline	↑**0.0306***	↑**0.0086***	↑**0.0007***	0.4825	↓**0.0409***	↓**0.0038***	0.2584	0.2584	0.4007
Valine	↑**0.0018***	↑**<0.0001***	↑**<0.0001***	↓**0.0441***	↓**0.0031***	↓**0.0022***	0.6071	0.2172	0.1216
Methionine	↑**0.0056***	↑**0.0056***	↑**0.0023***	↓**0.0235***	↓**0.0288***	↓**0.0023***	0.3083	0.3568	0.9586
Isoleucine	↑**0.0010***	↑**<0.0001***	↑**<0.0001***	↓**0.0054***	↓**0.0220**	↓**0.0035***	0.4491	0.8568	0.2451
Leucine	↑**0.0032***	↑**<0.0001***	↑**<0.0001***	↓**0.0084***	↓**0.0084***	↓**0.0001***	0.2590	0.4398	0.0976
Tryptophan	↑**0.0065***	↑**0.0003***	↑**<0.0001***	0.1765	0.6863	0.1965	0.5152	>0.9999	0.5152
Phenylalanine	↑**0.3483***	↑**<0.0001***	↑**<0.0001***	0.2362	0.2158	0.0629	0.5873	0.2395	0.1352
Ornithine	0.3483	↑**0.0003***	↑**0.0003***	0.2901	0.2901	0.2318	0.7551	0.8724	0.7551
Histidine	0.1167	↑**0.0160***	↑**0.0014***	0.9370	>0.9999	0.9370	0.6605	0.6605	0.6605
Lysine	↑**0.0037***	↑**<0.0001***	↑**<0.0001***	0.7735	0.4243	0.1306	0.3762	0.3762	0.3762
Tyrosine	↑**0.0045***	↑**<0.0001***	↑**<0.0001***	↓**0.0203***	↓**0.0275***	↓**0.0011***	0.1941	0.4268	0.2515
BCAA	↑**0.0001***	**0.0011**	↑**<0.0001***	↓**0.0060***	↓**0.0060***	↓**0.0005***	0.3921	0.3731	0.1391
EAA	↑**0.0015***	↑**<0.0001***	↑**<0.0001***	↓**0.0069***	↓**0.0430***	↓**0.0004***	0.5424	0.9397	0.5424
NEAA	0.1151	↑**0.0003***	↑**0.0001***	0.6712	0.6712	0.5338	0.9926	>0.9999	>0.9999
SUMAA	↑**0.0396***	↑**<0.0001***	↑**<0.0001***	0.0765	0.1388	↓**0.0053***	>0.9999	>0.9999	>0.9999

Statistically significant differences displayed as the calculated q-values when comparing altered levels of each macronutrient in meals where the other two were kept at a fixed medium level. A significant increase is indicated with ↑ and a decrease indicated with ↓. Statistics are performed using Repeated measures one-way ANOVA correcting for false discovery rate (FDR) were applied calculating q-values for true discoveries. *$q < 0.05$ was considered a true discovery.

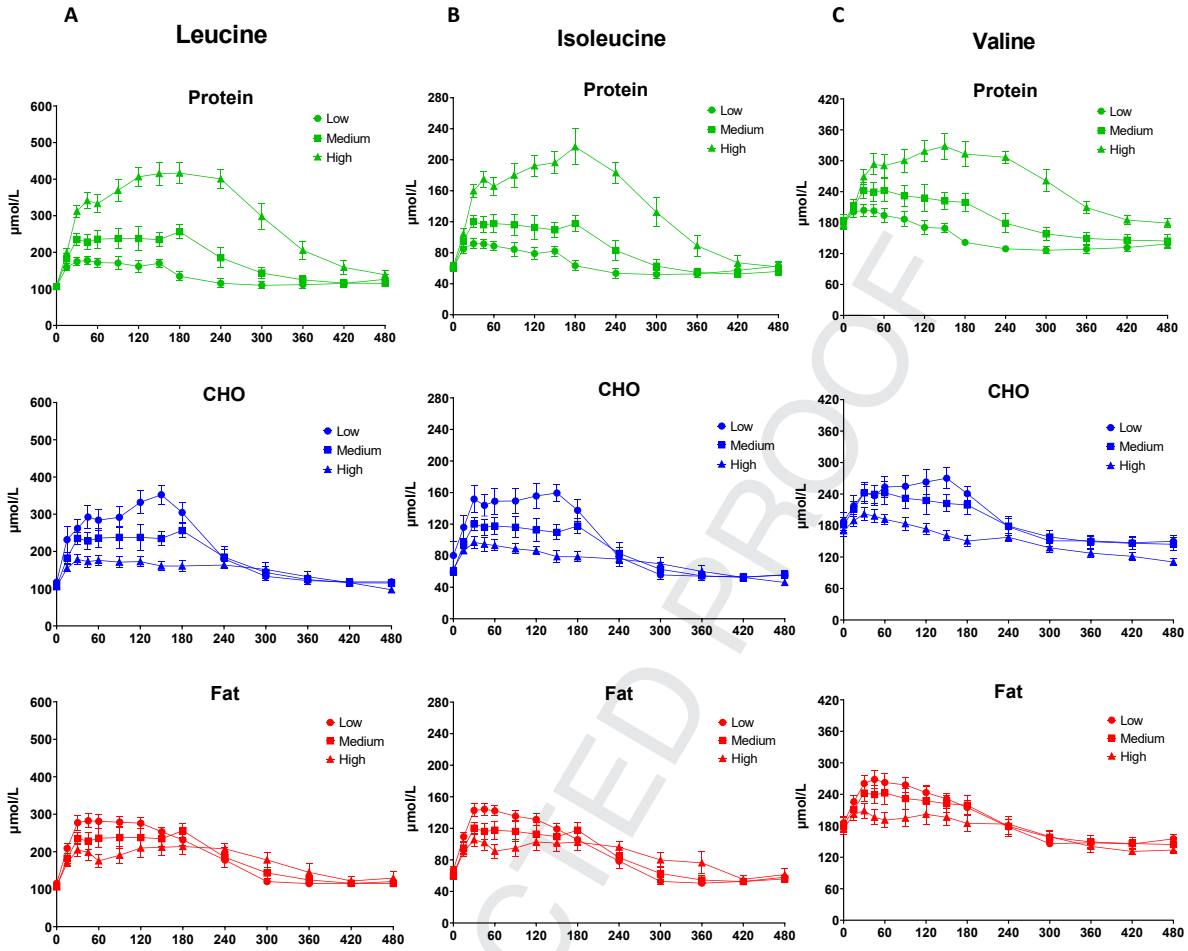

Fig. 2. Postprandial plasma levels of branched chain amino acid for leucine (A), isoleucine (B) and (C) valine for each timepoint over 8 h comparing the 3 levels (low, medium and high) for each macronutrient while the other two are kept at a fixed medium level. Data are displayed as mean (SE). Statistics were performed with repeated measure one-way ANOVA correcting for false discovery rate FDR. *q < 0.05 was considered a true discovery with a) vs. low level and b) vs. medium level.

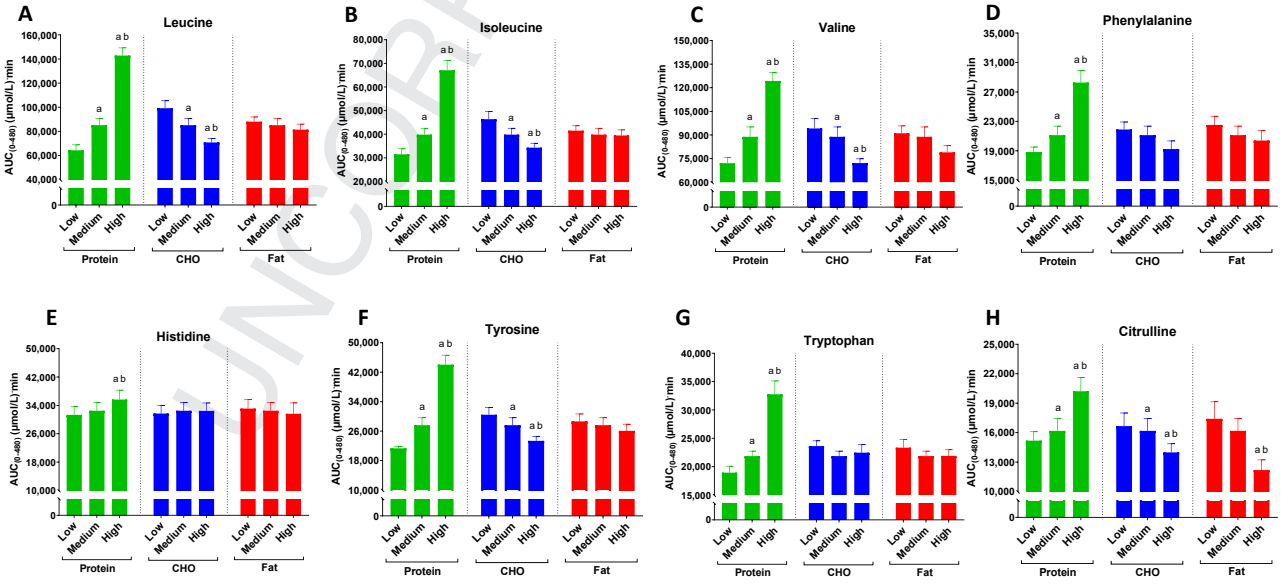

Fig. 3. Area under the curve (AUC) over 480 min for plasma levels of branched chain amino acids (BCAA) (A–C), aromatic amino acids (D–G) and citrulline comparing meals where each macronutrient content is altered with a low, medium and high level while keeping the other two macronutrients at a standard medium level. Data are displayed as mean (SE). Statistics were performed with repeated measure one-way ANOVA correcting for false discovery rate (FDR). *q < 0.05 was considered a true discovery with a) vs. low level and b) vs. medium level.

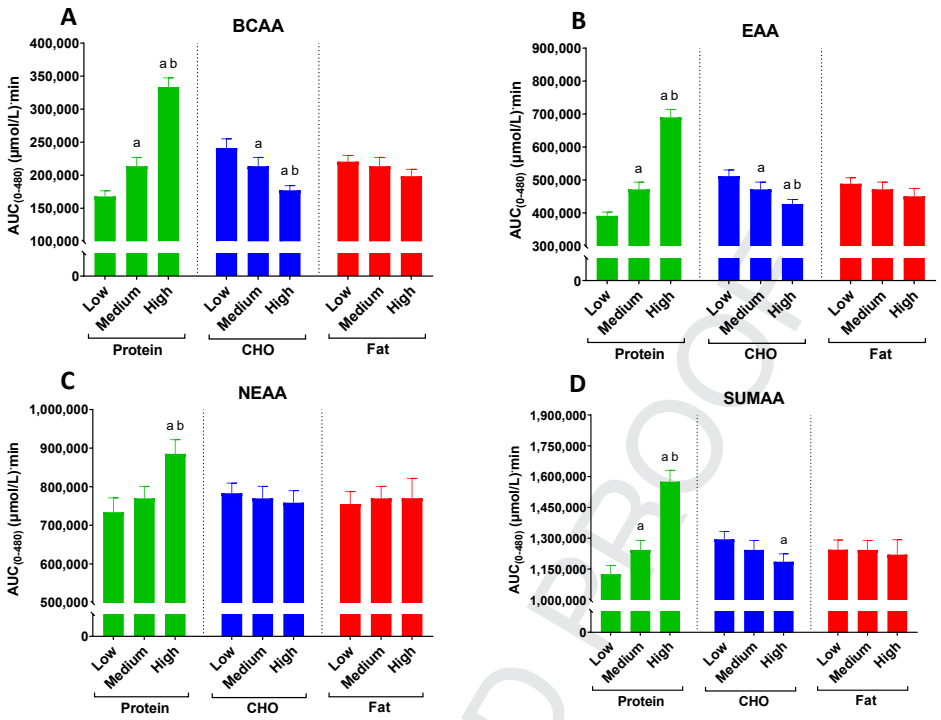

Fig. 4. Area under the curve (AUC) over 480 min comparing meals where each macronutrient content is altered with a low, medium, and high level while keeping the other two macronutrients at a fixed medium level. Graphs for the sum branched chain amino acids (BCAA), sum of essential amino acids (EAA) sum of non-essential amino acids (NEAA) and sum of all amino acids are shown. Statistics were performed with repeated measure one-way ANOVA correcting p-values for false discovery rate (FDR) as q-values. *q < 0.05 was considered a true discovery with a) vs. low level and b) vs. medium level.

taurine) for all meals (Suppl.). Graphs and curves for every time point for all amino acids as well as graphs for all meals can be found in supplementary material (Suppl.).

3.3. Multiple regression analysis

The regression coefficients and confidence interval for BCAA are shown in Table 5a–c and in Table 5d for citrulline. Regression data for all other metabolites can be found in supplementary material (Suppl.). Calculated [t]-values are shown in Fig. 5, sorted from highest to lowest to show the most predictive amino acids that significantly could predict each macronutrient in the meal. We found that all amino acids, except for glutamate, histidine, glycine, taurine, glutamine, tau-methylhistidine and hydroxyproline were significantly predictive for protein content, whereas leucine had the strongest association. For meal CHO content, all BCAA along with some other essential amino acids (phenylalanine and methionine) as well as total BCAA, total essential amino acids and the sum of all amino acids, were all significantly associated with the CHO content. Some non-essential amino acids (aspartate and tyrosine) were also highly predictive for the CHO intake and were in fact, along with leucine, the most predictive amino acids for the CHO content of the meal. Only citrulline was predictive for fat content of the meal with a [t]-value higher than triglycerides.

4. Discussion

In the present study, we showed that the amount of carbohydrates (CHO) in a liquid mixed meal reduces postprandial plasma concentration of several amino acids, notably the branched chain amino acids (BCAA). We also show that circulating BCAA as well as other essential (tryptophan and methionine) and non-essential amino acids (aspartate and tyrosine) are able to predict the CHO content as well as protein content of a mixed meal. The fat content of the meal, however, did not affect postprandial amino acid patterns in plasma, except for that increasing fat content reduced the citrulline response and citrulline was additionally highly predictive for the fat content of the meal. These distinct postprandial amino acid patterns can be used to predict the macronutrient composition of a mixed meal even better than the metabolite itself (plasma glucose and TG).

4.1. High CHO content of a mixed meal reduces postprandial amino acid responses

Earlier studies have shown that combined infusion of CHO and lipids reduces the postprandial response of several amino acids compared with fasting state-levels [13], and that dietary intake of CHO reduces circulating levels of phenylalanine [12]. Another study showed that co-ingesting CHO mixed with essential amino acids reduced postprandial plasma BCAA and tyrosine levels compared with dietary intake of essential amino acids alone [6], in agreement with results. A more recent study did show that leucine and total essential amino acids concentrations were suppressed when leucine was ingested with a meal containing both fat and CHO compared to dietary intake of only leucine, without examining the effect of the individual macronutrient components [19]. Our results also emphasize that the meal CHO content is highly influential on the postprandial amino acid response which needs to be accounted for when optimizing dietary intake of proteins.

4.2. Postprandial amino acids predict the CHO content of a mixed meal

The most important novelty of our findings is that postprandial plasma amino acid patterns can be used to predict a meal's

Table 5
Multiple regression.

	Coefficients	SEM	CI	[t]	P-value
a. Leucine					
Intercept	56,101	4873	46,413 to 65,789	11.51	**<0.0001**
Protein	1814	128.4	1559 to 2070	14.13	**<0.0001**
CHO	−201.9	45.33	−292.0 to −111.8	4.453	**<0.0001**
Fat	−161.3	148.2	−455.9 to 133.3	1.088	0.2796
b. Isoleucine					
Intercept	73,022	4965	63,153 to 82,892	14.71	**<0.0001**
Protein	1229	130.9	968.9 to 1489	9.393	**<0.0001**
CHO	−160.4	46.18	−252.3 to −68.64	3.474	**0.0008**
Fat	−267.5	151	−567.6 to 32.64	1.772	0.0800
c. Valine					
Intercept	27,451	2633	22,217 to 32,684	10.43	**<0.0001**
Protein	812.9	69.39	674.9 to 950.8	11.71	**<0.0001**
CHO	−91.49	24.49	−140.2 to −42.81	3.736	**0.0003**
Fat	−65.14	80.06	−224.3 to 94.02	0.8136	0.4181
d. Citrulline					
Intercept	16,038	1238	13,576 to 18,499	12.95	**<0.0001**
Protein	141.5	32.64	76.64 to 206.4	4.336	**<0.0001**
CHO	−15.62	11.52	−38.51 to 7.283	1.356	0.1788
Fat	−117.9	37.66	−192.8 to −43.04	3.131	**0.0024**

Regression coefficients, SEM, confidence interval of 95% (CI), calculated for tAUC μmol/L*min0-480 for each unit (g) of macronutrient (Protein, CHO or Fat). Multiple regression was performed for each amino acid calculating ordinary least squares using the macronutrients as the independent variables. Results are displayed as mean (SEM) with a confidence interval of (CI 95%). [t]-values calculated as coefficients/SEM. $P < 0.05$ was considered significant.

macronutrient content. The plasma response of the BCAAs, along with aspartate, tyrosine, valine, methionine, phenylalanine, and serine were able to predict the meal's CHO content, as good or even better than the plasma glucose concentration itself. This underpins the metabolic interplay between ingested CHO and circulating amino acids, but also demonstrates a possible use for plasma amino acids measurements that could either alone, or in combination with plasma glucose predict the macronutrient content of an entire ingested mixed meal. This could be applied during metabolic monitoring for patients, dietary meal evaluation or for research purposes. In contrast, altering the meal's fat content did not have the same effect, nor were amino acids predictive for the fat content, except for a surprising reduction in postprandial citrulline levels when the meal's fat content is increased. In fact, postprandial citrulline came out as a better predictor for the fat content than triglycerides (TG), which suggest a practical use of citrulline measurements.

4.3. Duration of the postprandial amino acid response

The postprandial amino acid response after ingesting Whey protein has been thoroughly studied [6,8,20–23]. However, our study differs in that the postprandial period was monitored for a total of 8 h. Studies often measure the postprandial response for only 2–4 h, showing that concentrations do not return to baseline during this observation window. We show that for meals with a high protein content (60 g), almost none of the amino acids returned to baseline within 7–8 h after meal intake, whereas for meals with low and medium protein content (15 and 30 g) this was 5–6 h. Further, while the postprandial response of amino acids in meals with low and medium protein content reached their peak plasma level after approximately 2 h, meals with high protein content did not peak within 4 h, most likely because of delayed gastric emptying [23]. It should be noted that the glycemic response was also prolonged in our study (with values returning to

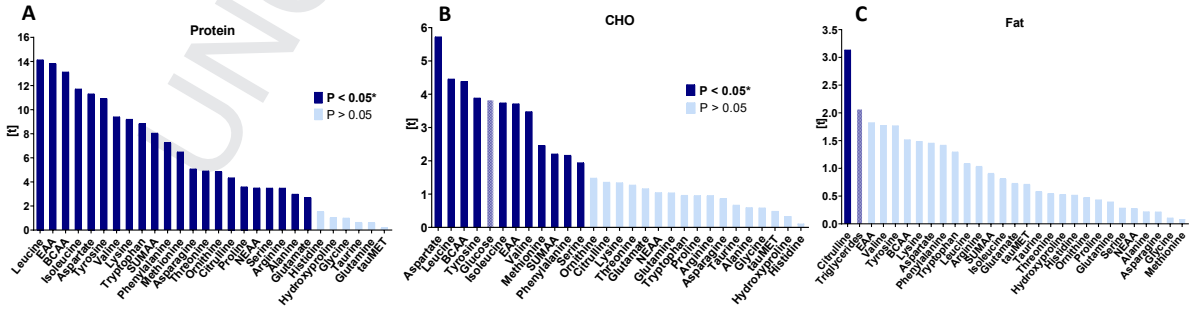

Fig. 5. [t]-values calculated from multiple regression for each amino acid sorted from highest to lowest for each of the mixed meal's 3 macronutrients to show the amino acid giving the best predictability for each macronutrient. The amino acids that could significantly predict the macronutrient content are displayed in dark blue, while the non-significant values are displayed in light blue. For CHO and fat, the [t]-values for the corresponding metabolite (glucose and triglycerides, respectively) are graphed with patterns. Multiple regression was performed for each amino acid calculating ordinary least squares using the macronutrients as the independent variables. [t]-values are calculated as coefficients/SEM. *$p < 0.05$ was considered significant. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

baseline after approximately 4–5 h), but for meals with high protein content, plasma glucose used the entire 8 h before completely returning to baseline. Oral CHO load given as a monocomponent generally returns to baseline within 2–3 h, so the prolonged glucose curve we observed can probably also be ascribed to delayed gastric emptying by fat and proteins [23,24].

Hence, we show that postprandial amino acid responses are of considerable duration that is often not accounted for in studies and interestingly also suggests that amino acid levels are not necessarily returning to baseline between larger meals in a regular western diet. We want to underpin the importance of monitoring blood level metabolites for a sufficient time frame if the entire postprandial responses of mixed meals is to be observed.

4.4. Glycemic responses in high protein content mixed meals

Glycemic responses and the glycemic index for meals with combined CHO and protein or fat are shown to be lower than when ingesting CHO alone [7,8,10,11,25]. The exact mechanism behind this has not been elucidated, but earlier studies suggest an effect *via* insulin [26] and insulin clearance [7]. Delayed gastric emptying and reduced release of incretin hormones from the gut are suggested as two additional possible mechanisms [7,8]. We did not find any clear reduction in AUC of glucose when comparing meals with increasing amounts of protein content. However, our design included higher amounts of protein (15–60 g) to resemble the content of larger western meals [27], while the above studies compared smaller meal protein content of 0–18 g [7,8]. In their studies the effect of meal proteins was investigated on meals with substantially lower protein content than in our study and their effect was evident when comparing 15–18 g of proteins to 0–5 g of proteins. Thus, our results do not contradict these studies, but suggest that the inhibiting effect of protein content on the glycemic response is not as clear in larger mixed meals with higher protein content as it is in smaller meals. Thus, in a larger western meal, like a dinner that compares to the meals in our study design, the described effect of ingested protein on glycemic responses is most likely diminished.

4.5. Mechanisms underlying the effect of ingested CHO on postprandial amino acids

What are the possible underlying metabolic mechanisms behind the interplay between ingested CHO and circulating amino acids? Adding CHO to a meal is well known to reduce gastric emptying [28,29], as is the effect of adding fat [24]. CHO also reduces the appearance of amino acids in the portal drained viscera when ingested with protein [5,30]. However, the mechanism can not only be due to reduced absorption as its been demonstrated that when comparing CHO and amino acid infusion to an amino acid infusion alone, various plasma amino acids concentrations are lowered, including all BCAA [13,14]. It is well established that insulin is an important stimulator of peripheral (and splanchnic) uptake for protein synthesis and oxidation of amino acids [31,32]. Amino acids themselves, especially BCAA, can also augment pancreatic insulin secretion [21,33] and insulin reduces plasma amino acids [34]. This implies that the CHO meal content could increase amino acid oxidation. When investigated individually, studies find a clear stimulating effect on net protein synthesis by ingested CHO [35,36] in fact by all macronutrients [35]. A similar response of CHO on net protein synthesis in mixed meals has been confirmed in later studies, but they interpreted it to be negligible compared to that of ingesting amino acid alone [12,37]. More recent studies suggest that any additional insulinotropic or other metabolic effect of co-ingesting CHO with proteins do not affect the net protein balance in muscle [6,38], questioning whether the effect of CHO as a monocomponent is further augmenting net protein synthesis in a mixed meal. Interestingly, an effect of ingested CHO on amino acids could be trough glycolysis within the muscle cell. Decreased glycolysis directly increase the conversion from keto acids to BCAA [39]. In turn, ingested CHO could then increase glycolysis and reduce the conversion and release of BCAA from muscle. In summary, these studies suggest that co-ingesting CHO with protein increases gut-retention, reducing conversion of BCAA due to altered glycolysis, and reduces the appearance of amino acid in the portal circulation with a possible insulinotropic effect on protein synthesis and/or breakdown in muscle of undetermined importance.

4.6. Limitations of the study

Our study has some important considerations and limitations that should be considered when interpreting the results. Various ways of evaluating areas under the curve (AUCs) to evaluate postprandial responses have been applied. For glycemic responses and indexes, positive incremental AUC is more accurate than total or net AUC over a shorter time-interval [40]. As pointed out by the authors, such calculation assumes that the metabolic response is purely positive and further, that the baseline values do not, from a physiological view, affect the magnitude of the postprandial response. AUC calculations also then rely heavily on the accuracy of the baseline value. We calculated the AUC with both total and incremental area, and total area came out as most precise (Suppl.). Concerning limitations, the study groups are small in this study so the prediction could be different if applied to a population-based study or when applied to groups with underlying metabolic conditions as well as differences between age groups that could affect amino acid availability [41]. Further, Whey protein was chosen to best mimic a standard mixed meal protein content, but we cannot exclude that the predictive accuracy could be affected by the amount of the amino acids, as Whey protein is particularly high on some EAA. Our study also consisted of a non-heated liquid meal that could affect the digestion and absorption, compared to that of a warm solid meal, as could the study design with subjects without any dietary control except for fasting before study days. During measurements, the subjects was kept in upright sitting position due to the necessity of arterialized blood flow and to avoid any difference of gastric emptying between subjects by physical activity, however, this could have affected amino acid availability [42]. It was also unavoidable designing meals with different caloric content to keep the macronutrient contents fixed, that also could affect gastric emptying. Finally, from this study we cannot determine whether it is the digestion, absorption, oxidation, or elimination of circulating amino acids that are affected by the ingested CHO (and fat). Future studies with both ingested and intravenous amino acid isotopes are necessary to determine this.

In conclusion, we show that the postprandial plasma response of several amino acids depends on the meal composition, especially the CHO content. This study has implications for optimized nutritional care for subjects with metabolic risk factors as older adults, and suggests that plasma amino acids are strong predictors, not only of the protein content, but also the CHO and fat content of a mixed meal that could be applied to metabolic monitoring to improve nutritional care.

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Authors contributions

NED, MJE, RGO RBM AND GLC contributed to the conception and design of research; LER, SYS, NED organized and conducted the studies; MH, NED analysed the data and performed statistical analysis; MH, NED, MJE interpreted results. MH prepared figures and drafted manuscript; MH, NED, MJE revised manuscript; MH, NED, MJE, LER, SYS, RGO, RBM, GLC edited and approved final version of manuscript. MH had primary responsibility for final content.

Conflict of 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.clnu.2021.07.016.

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