

Amino Acid Plasma Profiles from a Prolonged-Release Protein Substitute for Phenylketonuria: A Randomized, Single-Dose, Four-Way Crossover Trial in Healthy Volunteers

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Article

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Abstract: Several disorders of amino acid (AA) metabolism are treated with a protein-restricted diet supplemented with specific AA mixtures. Delivery kinetics impacts AA absorption and plasma concentration profiles. We assessed plasma profiles after ingestion of an AA mixture engineered to prolong AA absorption with Physiomimic Technology™ (Test) in a randomized, single-dose, four-way crossover trial in healthy volunteers (Trial Registration: ISRCTN11016729). In a two-step hypothesis, the primary endpoints were (i) significant reduction in peak plasma concentrations (C_{max}) of essential amino acids (EAAs) while (ii) maintaining EAA bioavailability ($AUC_{0-300\text{ min}}$) compared to a free AA mixture (Reference). Secondary endpoints included effects on plasma profiles of other AA groups and effects on several metabolic markers. Thirty subjects completed the study. Both co-primary endpoints were met: C_{max} for EAAs was 27% lower with the Test product compared to the Reference product (ratio, 0.726, $p < 0.0001$); overall plasma EAA levels from the two AA mixtures was within the pre-specified bioequivalence range ($AUC_{0-300\text{ min}}$ ratio, 0.890 (95% CI: 0.865, 0.915)). These findings were supported by the results of secondary endpoints. Prolongation of AA absorption was associated with modulation of several metabolic markers. It will be important to understand whether this can improve the long-term management of disorders of AA metabolism.

Keywords: disorders of amino acid metabolism; phenylketonuria; dietary management; amino acid absorption; bioequivalence; prolonged release

1. Introduction

The estimated global incidence of all inherited disorders of amino acid (AA) metabolism is 15/100,000 live births. Of these, phenylketonuria (PKU) represents the most common and significant disease entity, with an estimated global incidence of 7/100,000 [1], although birth incidence varies by ethnicity and consanguinity, and estimates are influenced by the level of ascertainment [2]. Many inherited disorders of AA metabolism require prompt initiation of treatment at birth and lifelong

adherence to a protein-restricted diet supplemented with defined AA mixtures that lack specific AAs [3–8]. Important products downstream of a metabolic block may need to be supplemented, such as tyrosine in PKU, phenylalanine in tyrosinemia and cysteine in some forms of homocystinuria. The diet must be engineered to provide adequate vitamins, minerals and other nutrients normally obtained from natural protein-containing foods. These are usually provided through appropriate supplementation of the AA mixture. In all, more than 70 disorders of AA metabolism are managed with AA mixtures [9].

The plasma AA profile after ingesting free AAs is different from that of AAs administered as intact proteins: plasma levels peak faster and higher but decrease more quickly with free AAs [10]. The rate of absorption influences the fate of the ingested AAs. Fast absorption is associated with more AA oxidation, higher levels of blood urea nitrogen (BUN), and less protein accretion compared to slowly digested proteins [11–13]. This results in lower biological value for free AA mixtures compared to intact proteins [14]. In an effort to compensate for these differences, treatment guidelines for PKU recommend daily AA intake of up to 150% of the amounts recommended for healthy subjects, distributed through the day [15,16], with the objective of providing sufficient nitrogen assimilation and minimising protein catabolism.

The absorption kinetics of free AA mixtures used for the dietary treatment of disorders of AA metabolism have not been extensively investigated. Modifying the “immediate-release” absorption kinetics typically observed with free AAs to a more gradual kinetic profile with a less prominent peak may improve the efficiency of protein metabolism, reduce AA oxidation and the increase in BUN that occurs when large amounts of free AAs are ingested. Prolonging the uptake of AAs may also reduce the incidence and severity of catabolic episodes, thereby reducing fluctuations in blood phenylalanine levels [17].

Key kinetic parameters for investigating the effect of replacing “immediate-release” free AAs with prolonged-release AAs in the diet of such patients include the peak blood concentration (C_{max}), which should be lower, and the overall extent of absorption expressed as the area under the concentration/time curve (AUC), which should be equivalent (Supplementary Figure S1). Prolonged release is also typically reflected by higher blood concentrations at later time points after intake (C_{last}).

Physiomimic Technology™ (PT™) is a pharmaceutical process that results in small granules coated with functional additives—ethylcellulose and sodium alginate—that allow gradual release of their contents in the small intestine. PT™ modifies the release and absorption of AAs, while masking their taste and odour, with positive effects on the typically unpleasant aftertaste of traditional AA formulations. This study was based on preliminary evidence obtained from a porcine model, where application of PT™ to free AA mixtures reduced C_{max} by 18% while maintaining a similar overall increase in plasma AAs [18].

We conducted the present study in healthy adult volunteers to determine the effects on plasma AA profiles of a prolonged-release AA mixture formulated with PT™, comparing it with an immediate-release formulation of the same AA mixture, a commercially available free AA mixture and a natural intact protein, casein. We also measured blood urea nitrogen (BUN), insulin and glucose levels to explore whether the AA release kinetics modulate these metabolic pathways.

2. Materials and Methods

2.1. Subjects

Enrolled subjects were healthy non-smoking men or women between the ages of 18 and 45 years with body weight between 55 and 85 kg and body mass index (BMI) ≤ 30 kg/m²; pregnant and breast-feeding women were excluded; subjects were excluded if they had health conditions that investigators considered might interfere with the study aims including, but not limited to gastrointestinal, renal or hepatic diseases, diabetes or other metabolic conditions. Full enrolment criteria are available in Supplementary Table S1.

At the screening visit, subjects underwent a physical examination, 12-lead ECG, comprehensive laboratory blood tests and received nutritional counselling for diet standardization. All study participants provided written informed consent. The study was conducted at CRST Oy in Turku, Finland, and was approved by the Ethics Committee of the Hospital District of Southwest Finland, Turku, Finland; Ref: 78/1801/2017. Trial Registration: ISRCTN11016729.

2.2. Study Design

This randomized, single-blind, four-way crossover trial tested the effects of a novel prolonged-release coating technology on the plasma profiles and bioavailability of AAs from a protein substitute for PKU. The primary comparison was between (i) the Test product, a prolonged-release AA formulation engineered with PTTM containing vitamins, minerals and other nutrients, and (ii) the Reference product, a mixture containing identical proportions and quantities of free AAs, vitamins, minerals, other nutrients and functional additives (Supplementary Table S2). Sample size was calculated on both primary endpoints to detect a 20% change in C_{max} and bioequivalent overall increase in plasma EAAs with 90% power.

Secondary comparisons were made with (i) a commercially available AA mixture (MP) with a composition comparable to the Test and Reference products, and (ii) an intact slowly digested protein derived from cow's milk (food-grade acid casein; 80 MESH; A.C.E.F., Italy). These comparisons are considered secondary because MP and casein have AA compositions that are quantitatively different from the Test and Reference products; casein differs also qualitatively from the other products, in that it lacks added micronutrients and minerals.

Subjects were assigned to one of 3 body weight categories: 60 kg (55–65.4), 70 kg (65.5–75.4), 80 kg (75.5–85). For 2 days before each test day, subjects were provided a standardized diet with daily energy and protein content corresponding to their body weight (60 kg: 1988 kcal/56.6 g protein, 70 kg: 2292 kcal/65.8 g protein, 80 kg: 2784 kcal/77.6 g protein). On the evening before each test day, subjects consumed a light meal with a standardised, low protein content (0.24 g/kg).

On each test day, after overnight fasting (10–12 h), subjects were assigned to receive one of the study products based on the randomisation sequence (Supplementary Figure S2).

A nominal dose of 0.4 g AA/kg, estimated to provide one third of the daily protein equivalent (PE) requirement generally prescribed to adults with PKU, was administered as a suspension in 300 mL of water. Dosing was adjusted by body weight: the 55–65.4 kg group receiving 24 g AAs (20 g PE), the 65.5–75.4 kg group receiving 28 g AAs (23.3 g PE) and the 75.5–85 kg group receiving 32 g AAs (26.6 g PE). A 300-kcal light snack containing mainly carbohydrates was provided 5 h after intake of the study products to prevent protein catabolism and consequent release of AAs into the bloodstream. The schedule of each test day is illustrated in Figure 1.

Fifteen timed venous blood samples were collected via an indwelling catheter up to 7 h after product intake. Concentrations of AAs, BUN, insulin and glucose were determined. Baseline values were derived from samples collected 30 min and just before dosing (0 min). Urine was collected on the morning of the test day (12 h over-night urine), and subsequent fractions were collected between 0 and 150 min and between 150 and 300 min to measure urea and other laboratory parameters; a fourth sample was collected between 300 and 420 min for safety laboratory assessments.

Test days were planned to be separated by washout periods of 9 to 14 d. The total study duration for each subject was planned to be approximately 38–70 d (screening period, 4 test days separated by 3 washout periods and a follow-up visit, if required).

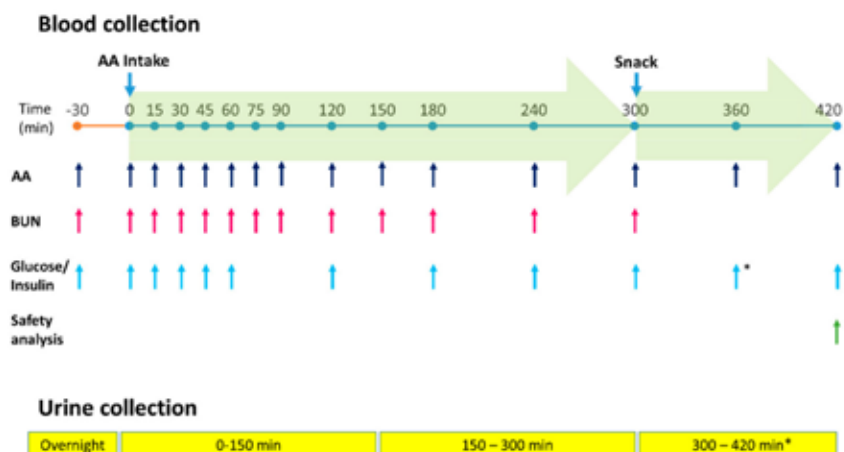


Figure 1. Sample collection and analysis schedule. AA, amino acids; BUN, blood urea nitrogen; *for safety assessment only.

2.3. Primary Endpoints

Two important aspects of prolonged release are the reduction of peak blood AA levels (C_{\max}) together with maintenance of the overall extent of absorption of an ingested dose, also expressed as bioavailability. Therefore, two co-primary endpoints were evaluated to compare the Test product vs. the Reference product:

- 1) Superiority in terms of the C_{\max} in plasma essential amino acids (EAAs) and
- 2) Bioequivalence in terms of plasma AUC of the EAAs over the first 5 h after product intake ($AUC_{0-300\text{min}}$); the AUC cut-off was set at 5 h to avoid potential changes in plasma AAs after the snack was administered.

The criteria for evaluating the primary endpoints were established a priori, with analysis of the second being contingent on meeting the first: (i) significantly lower C_{\max} for EAAs with the Test product vs. Reference product; (ii) an equivalent EAA bioavailability over the first 5 h defined as a log-transformed $AUC_{0-300\text{min}}$ ratio with a 90% CI in the range 0.80–1.25, based on guidelines for bioequivalence studies with drug products [19,20].

EAAs included tyrosine and arginine; tyrosine was included because it is conditionally essential in PKU and arginine because it is necessary for adequate growth in patients on diet regimens that are low in natural proteins.

2.4. Secondary Endpoints

Secondary endpoints addressed the plasma profiles for up to 7 h of EAAs and additional groups of AAs, including single AAs, large neutral AAs (LNAA), branched-chain AAs (BCAAs) and total AAs. Secondary endpoints evaluated to compare the Test product vs. the Reference product were:

- 1) C_{\max} of these AA groups in plasma;
- 2) Their bioequivalence in terms of plasma AUC over the first 7 hours after product intake ($AUC_{0-420\text{min}}$);
- 3) Their plasma concentration at the last measured time point (C_{last}).

C_{\max} and the $AUC_{0-420\text{min}}$ ratio were compared as above.

2.5. Secondary Comparisons

Formal comparisons between the Test product and the MP comparator or casein were not performed due to differences in their AA compositions; however, we did make secondary comparisons between their plasma profiles, as described for the secondary endpoints.

We compared trends in blood levels of phenylalanine and tyrosine among the 3 phenylalanine-free AA preparations, because these AAs are critical in the treatment of patients with PKU. We also explored possible differences in blood insulin, glucose, BUN and urea excretion in urine.

Overall acceptability/palatability and subjective satiety were assessed 2 h after study products were ingested using questionnaires adapted from Flint et al. [21] and translated into Finnish.

2.6. Safety Assessments

On the first 3 test days, blood parameters for liver and kidney function were measured (aspartate aminotransferase, alanine aminotransferase, γ -glutamyl transferase, bilirubin, alkaline phosphatase, creatinine). Additionally, concentrations of albumin and prealbumin in serum were evaluated at the end of each of the first three test days. After the fourth test day, the examinations and tests used at screening were repeated. On all test days, urine parameters (creatinine, total protein, urate, calcium, phosphate, pH, urea) were evaluated. Subjects maintained a diary that was given to them on each test day and collected at the next visit. Adverse events were recorded. Tolerability of all study products was evaluated.

2.7. Statistical Analysis

Sample size calculation was based on both co-primary endpoints (C_{\max} and $AUC_{0-300\min}$ of EAAs). With 90% power, 24 evaluable subjects were needed to demonstrate a $\geq 20\%$ decrease in C_{\max} and bioequivalence of $AUC_{0-300\min}$ for EAAs comparing the Test product with the Reference product. Assuming a drop-out rate of 25%, this corresponded to randomising 32 subjects.

AUC was calculated using the trapezoidal rule. Equivalent or better bioavailability of the Test vs. Reference product corresponded to a log-transformed AUC ratio with a 90% CI of ≥ 0.80 . Primary endpoints were analysed on log-transformed data. A linear mixed effect model appropriate for a four-period crossover design was fitted for the parameters. The statistical model included sequence, period and treatment as fixed effects, and “subject within sequence” and residual error term as random effects. The p -value was used for superiority testing for C_{\max} ; in addition, a 90% CI for $AUC_{0-300\min}$ was calculated from the model for equivalence testing. CI estimates were converted by anti-log transformation to obtain ratios of geometric least square means. Comparable statistical methodology was used for the secondary parameters. All results are described as mean, standard deviation, geometric mean, median, and coefficient of variation. Comparisons of kinetic parameters between the Test product and both commercially available comparator products, MP and casein, and between the Reference product and MP and casein were calculated from the statistical models described above. Effects on the metabolic markers BUN, urine urea, insulin and glucose were compared with repeated measures of covariance (ANCOVA). Responses to the questionnaire on overall acceptability of the products were compared with Wilcoxon exact test.

3. Results

Of the 35 healthy volunteers enrolled, 30 completed the study according to the protocol: 15 women and 15 men, mean \pm SD age 27.4 ± 8.2 years (range 18–43 years), mean \pm SD body weight 70.0 ± 8.5 kg. Four subjects discontinued without receiving any dose of any product, and one subject was excluded from the per-protocol analysis because of a major protocol violation. This subject's results were included in the safety evaluation.

3.1. Kinetic Outcomes

3.1.1. Primary Endpoints

Both co-primary endpoints were met (Figure 2; Table 1). The C_{\max} for EAAs was 27% lower with the Test product compared to the Reference product (ratio 0.726, $p < 0.0001$) whereas the overall increase

in plasma EAAs (AUC_{0-300}) from the two AA mixtures was within the pre-specified bioequivalence range (ratio, 0.890 (90% CI: 0.865, 0.915)).

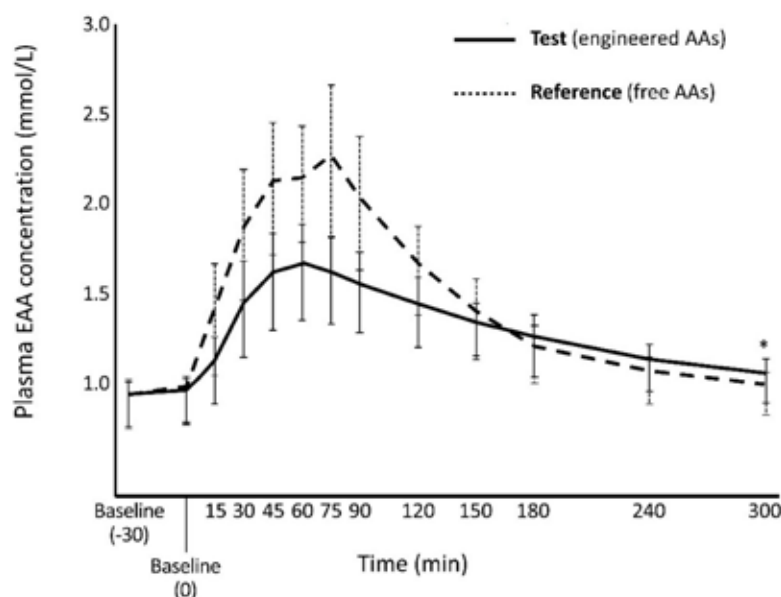


Figure 2. Plasma EAA concentrations after ingesting Test and Reference products. * Mean (SD) concentration after 5 h (C_{last}) was significantly higher with the Test product, 1.06 (0.124) vs. 0.996 (0.117) mmol/L; $p = 0.0012$. ANCOVA test.

Table 1. Peak blood EAA concentration (C_{max}) and overall increase in plasma EAAs up to 300 min ($AUC_{0-300min}$) after intake of Test and Reference products (co-primary endpoints).

EAA C_{max} mmol/L, Mean (SD)		C_{max} Ratio	p -Value [‡]
Test	Reference		
1.77 (0.253)	2.44 (0.368)	0.726	<0.0001
EAA $AUC_{0-300min}$ (mmol/L) min, Mean (SD)		$AUC_{0-300min}$ Ratio	90% CI
Test	Reference		
396 (45.0)	444 (46.2)	0.890	0.865, 0.915 *

* Value within the predefined bioequivalence range. † Linear mixed effect model EAA, essential amino acids; SD, standard deviation; AUC, area under the time-concentration curve; CI, confidence interval.

3.1.2. Secondary Endpoints

These findings were supported by the results of secondary endpoints on the plasma profiles of different classes of AAs. The C_{max} values for EAAs, LNAAs, BCAAs and total AAs were consistently significantly lower after intake of the Test product compared to the Reference product (Table 2), while the overall increase in plasma levels (AUC_{0-420}) was in the bioequivalence range over the entire 7 h of observation (Table 3).

Table 2. The peak plasma amino acid concentrations (C_{\max}) after ingesting the Test or Reference products (secondary endpoints).

AA Group	C_{\max} mmol/L, Mean (SD)		C_{\max} Ratio	p -Value ‡
	Test	Reference		
LNAAs	1.27 (0.175)	1.87 (0.301)	0.677	<0.0001
BCAAs	0.693 (0.106)	1.20 (0.221)	0.579	<0.0001
Total AAs	3.57 (0.468)	4.59 (0.576)	0.775	<0.0001

‡ Linear mixed effect model AA, amino acid; LNAA, large neutral amino acid; BCAA, branched-chain amino acid; SD, standard deviation.

Table 3. Overall increase in plasma levels of the indicated amino acids up to 7 h after ingesting the Test or Reference products (secondary endpoints).

AA Group	$AUC_{0-420\text{min}}$ (mmol/L) *min, Mean (SD)		$AUC_{0-420\text{min}}$ Ratio	90% CI
	Test	Reference		
EAAAs	509 (57.1)	549 (55.1)	0.924	0.900, 0.950 *
LNAAs	385 (43.2)	424 (43.9)	0.908	0.885, 0.932 *
BCAAs	206 (28.3)	240 (29.6)	0.860	0.836, 0.885 *
Total AAs	1106 (129)	1176 (97.2)	0.937	0.909, 0.966 *

* value within the predefined bioequivalence range. AA, amino acid; EAA, essential amino acid; LNAA, large neutral amino acid; BCAA, branched-chain amino acid; SD, standard deviation; AUC, area under the time-concentration curve; CI, confidence interval.

C_{last} values 7 h after intake of the Test product were significantly higher compared to C_{last} after the Reference product for all classes of AAs but not for total AAs (Table 4).

Table 4. Plasma amino acid concentrations 7 h after administration (C_{last}).

AA Group	C_{last} mmol/L, Mean (SD)		C_{last} Ratio	p -Value ‡
	Test	Reference		
EAAAs	0.822 (0.119)	0.780 (0.0882)	1.054	0.0158
LNAAs	0.628 (0.0920)	0.595 (0.0677)	1.055	0.0123
BCAAs	0.316 (0.0596)	0.281 (0.0525)	1.126	0.0001
Total AAs	2.09 (0.275)	2.06 (0.207)	1.012	0.5658

‡ Statistical test RM-ANCOVA. AA, amino acid; EAA, essential amino acid; LNAA, large neutral amino acid; BCAA, branched-chain amino acid; SD, standard deviation.

3.1.3. Secondary Comparisons

Comparisons between the Test product and the comparator products MP and casein were considered secondary due to differences in their compositions. The Test product consistently showed significantly lower C_{\max} values compared to both MP and casein (Table 5).

Table 5. Peak plasma AA concentrations after ingesting the Test product or the indicated secondary comparators.

AA Group	C _{max} mmol/L, Mean (SD)			Comparisons			
	Test	MP	Casein	Test vs. MP		Test vs. Casein	
				C _{max} Ratio	p-Value ‡	C _{max} Ratio	p-Value ‡
EAAAs	1.77 (0.253)	2.12 (0.314)	2.18 (0.379)	0.835	<0.0001	0.818	<0.0001
LNAAs	1.27 (0.175)	1.64 (0.226)	1.68 (0.282)	0.775	<0.0001	0.759	<0.0001
BCAAs	0.693 (0.106)	1.043 (0.149)	1.03 (0.189)	0.664	<0.0001	0.676	<0.0001
Total AAs	3.57 (0.468)	4.11 (0.585)	4.16 (0.613)	0.870	<0.0001	0.860	<0.0001

‡ Linear mixed effect model AA, amino acid; EAA, essential amino acid; LNAA, large neutral amino acid; BCAA, branched-chain amino acid; SD, standard deviation; C_{max}, maximum plasma concentration; MP, marketed product.

When compared to MP, the Test product demonstrated bioequivalent increases in plasma levels of EAAs, LNAAs and total AAs at 420 min (Table 6).

Table 6. Overall increase in plasma AA levels over 7 h after ingesting the Test product or the indicated secondary comparators.

AA Group	AUC _{0-420min} (mmol/L) *min, Mean (SD)			Comparisons			
	Test	MP	Casein	Test vs. MP		Test vs. Casein	
				AUC _{0-420min} Ratio	90% CI	AUC _{0-420min} Ratio	90% CI
EAAAs	509 (57.2)	556 (54.6)	543 (55.4)	0.917	0.892, 0.942 *	0.936	0.910, 0.961 *
LNAAs	385 (43.2)	435 (39.0)	422 (40.3)	0.887	0.864, 0.910 *	0.911	0.888, 0.935 *
BCAAs	207 (28.3)	263 (26.1)	248 (27.3)	0.758	0.763, 0.807	0.829	0.806, 0.852 *
Total AAs	1106 (129)	1170 (123)	1160 (122)	0.945	0.917, 0.974 *	0.952	0.923, 0.981 *

* Value within the predefined bioequivalence range. AA, amino acid; EAA, essential amino acid; LNAA, large neutral amino acid; BCAA, branched-chain amino acid; SD, standard deviation; AUC, area under the concentration-time curve; MP, marketed product; CI, confidence interval.

We evaluated effects on plasma tyrosine levels because, although all marketed AA mixtures for PKU contain high levels of tyrosine, it is not highly soluble and its bioavailability represents a critical point. Analyses showed that the Test and Reference products were equivalent (AUC₀₋₄₂₀ ratio 1.052 (90% CI: 0.984, 1.125)), meaning that PTTM did not alter the bioavailability of this AA, which is conditionally essential in PKU patients. Comparisons of plasma tyrosine profiles with MP and casein are not informative because of their different AA compositions compared to the Test and Reference products.

Although phenylalanine was not present in the Test and Reference products, their effects on the kinetic profile of plasma phenylalanine were compared to determine if administering a prolonged release AA mixture influences this important parameter. Plasma phenylalanine fluctuations were less prominent after intake of the Test product vs. the Reference product ($p = 0.0046$, ANCOVA; Figure 3).

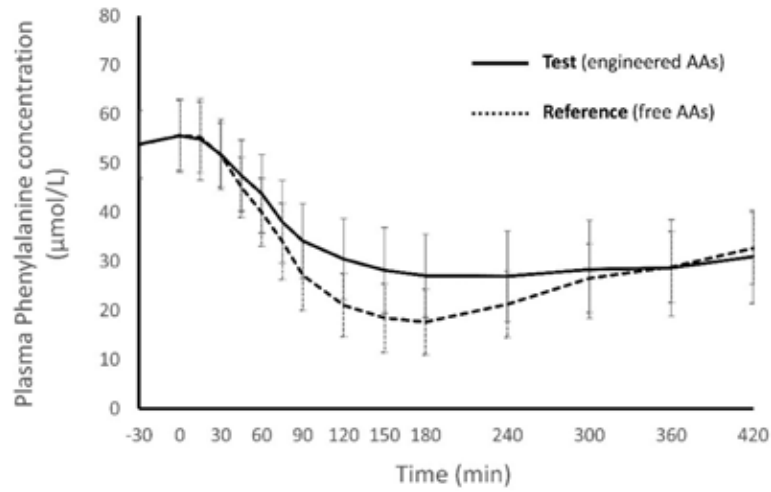


Figure 3. Plasma phenylalanine concentrations after intake of Test and Reference products. There was less variation in phenylalanine concentration after Test ($p = 0.0046$, ANCOVA).

3.2. Markers of Metabolism

We measured changes in BUN and urine urea, two indicators of AA catabolism. A statistically significant difference emerged between the Test and the Reference products for both BUN (ANCOVA test: $p < 0.0001$; Figure 4) and urine urea excretion (ANCOVA test: $p = 0.0074$; Table 7).

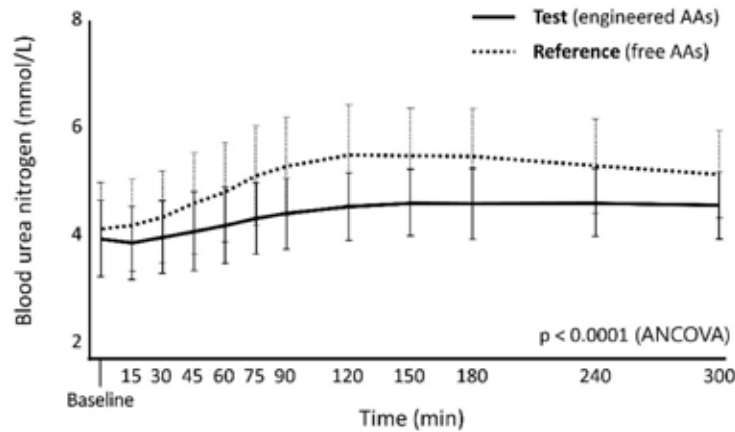


Figure 4. Blood urea nitrogen (BUN) measured at the indicated times after ingestion of Test and Reference products as an indicator of nitrogen utilization.

Table 7. Total urea excreted in urine in the first 5 h after ingesting study products.

Statistics	Test	Reference	Casein	MP
LSM, mmol	40.1	45.6	43.7	39.9
95% CI	36.0, 44.2	41.6, 49.6	39.7, 47.7	35.8, 43.9
Comparisons with Test	LSM Difference	95% CI	p-Value	
vs Reference	-5.52	-9.52, -1.52	0.0074 *	
vs Casein	-3.62	-7.61, 0.37	0.075	
vs MP	0.203	-3.85, 4.25	0.92	

* statistically significant (RM-ANOVA) CI, confidence interval; LSM, least square mean; MP, marketed product.

This was supported by a finding of significantly lower mean (SD) quantity of urea excreted with the Test product vs. Reference (Table 7).

We measured plasma levels of insulin and glucose to explore whether administering a prolonged-release AA mixture might influence these biomarkers of metabolism (Table 8). Analysing insulin curves over time for the products and constructing an RM-ANCOVA model revealed a statistically significant difference between the Test and the Reference products (Figure 5A; ANCOVA test: $p < 0.0001$). The curves for glucose mirror the curves for insulin: after insulin release from pancreatic beta cells, blood levels of glucose start to decrease as glucose is taken into muscle, fat and liver cells. The lower the insulin peak, the smaller the decrease of glucose levels.

Table 8. Plasma levels of metabolic markers over the first 5 h following ingestion of the indicated products.

Parameter	AUC _{0-300min} Mean (SD)				Ratio of Geometric LSM (90% CI)		
	Test	Reference	MP	Casein	Test vs. Reference	Test vs. MP	Test vs. Casein
BUN, (mmol/L) *min	1357 (201)	1573 (266)	1423 (241)	1476 (292)	0.868 (0.837, 0.900)	0.957 (0.923, 0.993)	0.927 (0.894, 0.962)
Insulin, (U/L) *min	2.14 (1.01)	2.70 (1.38)	2.59 (1.08)	2.60 (1.34)	0.785 (0.716, 0.861)	0.803 (0.732, 0.880)	0.819 (0.748, 0.898)
Glucose, (mmol/L) *min	1621 (91)	1609 (93)	1610 (86)	1600 (111)	1.005 (0.992, 1.019)	1.006 (0.992, 1.019)	1.012 (0.999, 1.026)

AUC, area under the concentration-time curve; LSM, least squares mean; BUN, blood urea nitrogen; SD, standard deviation; MP, marketed product.

After intake of the Test product, glucose levels did not decrease as much and were more constant over time compared to levels after intake of the Reference product. By analysing glucose curves over time for the products and constructing an RM-ANCOVA model, a statistically significant difference emerged between the Test and the Reference products (Figure 5B; ANCOVA test: $p = 0.0068$).

Regarding the subjects’ assessment of the products, there were no significant differences between products for subjectively evaluated palatability or sense of satiety, except for a higher rating on the item “overall liking” for casein compared to the Test product ($p < 0.0006$; Wilcoxon exact test).

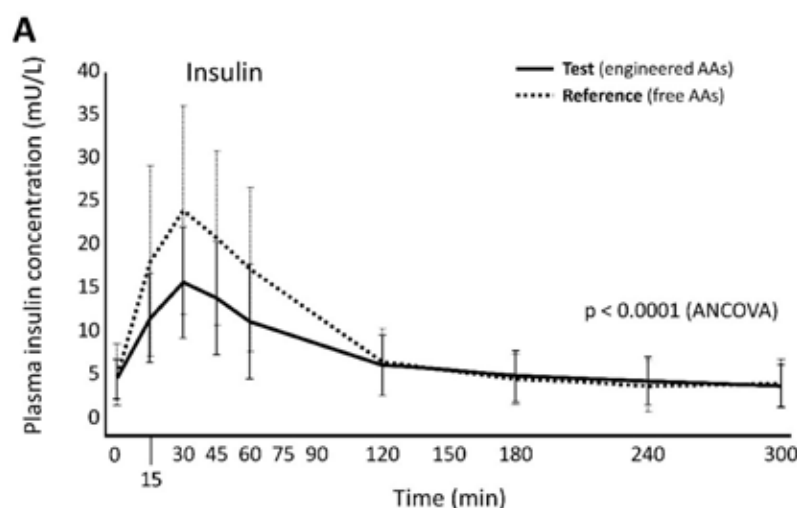


Figure 5. Cont.

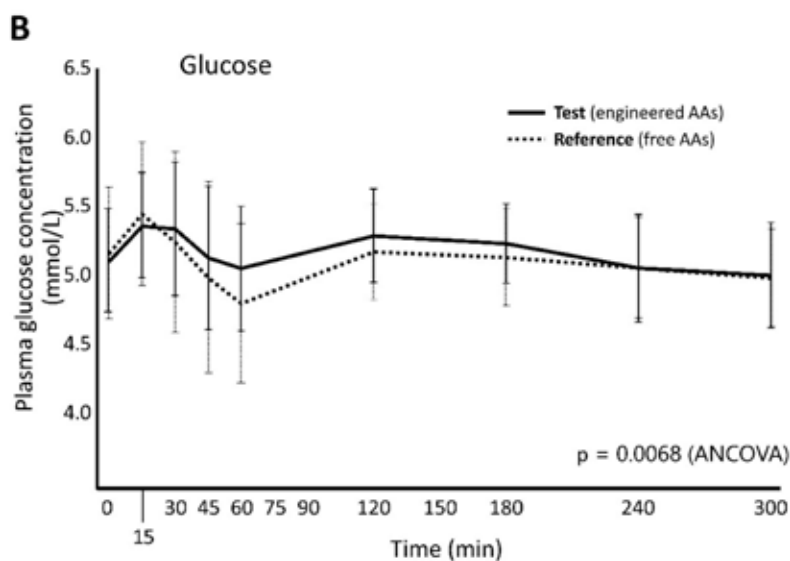


Figure 5. Plasma levels of (A) insulin and (B) glucose measured at the indicated times after ingestion of Test and Reference products.

3.3. Safety Profile

In the safety population ($n = 31$), 27 subjects (87%) reported at least one adverse event (AE). Most AEs (97.5%) were not considered related to intake of the study products (most common AEs were headache and nasopharyngitis). One AE was classified as definitely related to the Test product (headache) and one AE was classified as probably related to the Reference product (nausea).

4. Discussion

In this single-dose study conducted in healthy adults, an AA mixture formulated with Physiomic Technology™ significantly prolonged the release of AAs, lowering peak EAA levels in plasma (first co-primary endpoint), while maintaining an equivalent overall increase in plasma EAAs (second co-primary endpoint), compared to a reference amino acid mixture of similar composition but formulated without the novel coating technology. The aim of prolonging AA release and absorption is to avoid an AA C_{max} that exceeds anabolic capacity and promotes metabolic breakdown of AAs, while maintaining overall absorption for sustained protein synthesis. Avoiding excessively high AA C_{max} and prolonging absorption improves AA utilization for protein synthesis [10,22,23]. This improvement in nitrogen balance results from both increased protein synthesis and decreased metabolic breakdown of AAs [24]. A positive nitrogen balance after AA ingestion could be particularly important for PKU patients, and generally for all such patients with disorders of amino acid metabolism, where AA supplementation represents the major nitrogen source. To overcome the known limitations of immediate-release free AAs, several measures have been introduced in the dietary management of PKU patients, such as increasing the daily intake of phenylalanine-free protein substitute (i.e., at least 150% of normal dietary requirements) and administering multiple doses over the day. Nitrogen retention is improved when free AA mixtures are given in multiple doses over the day, rather than as a single bolus [25], and there is less fluctuation of blood phenylalanine concentrations. AA mixtures that mimic physiological absorption kinetics are expected to improve the rate of assimilation into protein and minimise oscillations of amino acid levels [17]. PT™ could be one means to achieve this.

We chose EAAs for the primary endpoints because they are not synthesised endogenously and because they are often chronically deficient in the diet of patients with metabolic disorders such as PKU, making the patients strongly dependent on phenylalanine-free proteins or protein substitutes. The results obtained for the primary endpoints—lower peak levels of EAAs in plasma and sustained overall bioavailability of EAAs—were corroborated by similar results from the secondary

This was supported by a finding of significantly lower mean (SD) quantity of urea excreted with the Test product vs. Reference (Table 7).

We measured plasma levels of insulin and glucose to explore whether administering a prolonged-release AA mixture might influence these biomarkers of metabolism (Table 8). Analysing insulin curves over time for the products and constructing an RM-ANCOVA model revealed a statistically significant difference between the Test and the Reference products (Figure 5A; ANCOVA test: $p < 0.0001$). The curves for glucose mirror the curves for insulin: after insulin release from pancreatic beta cells, blood levels of glucose start to decrease as glucose is taken into muscle, fat and liver cells. The lower the insulin peak, the smaller the decrease of glucose levels.

Table 8. Plasma levels of metabolic markers over the first 5 h following ingestion of the indicated products.

Parameter	AUC _{0-300min} Mean (SD)				Ratio of Geometric LSM (90% CI)		
	Test	Reference	MP	Casein	Test vs. Reference	Test vs. MP	Test vs. Casein
BUN, (mmol/L) *min	1357 (201)	1573 (266)	1423 (241)	1476 (292)	0.868 (0.837, 0.900)	0.957 (0.923, 0.993)	0.927 (0.894, 0.962)
Insulin, (U/L) *min	2.14 (1.01)	2.70 (1.38)	2.59 (1.08)	2.60 (1.34)	0.785 (0.716, 0.861)	0.803 (0.732, 0.880)	0.819 (0.748, 0.898)
Glucose, (mmol/L) *min	1621 (91)	1609 (93)	1610 (86)	1600 (111)	1.005 (0.992, 1.019)	1.006 (0.992, 1.019)	1.012 (0.999, 1.026)

AUC, area under the concentration-time curve; LSM, least squares mean; BUN, blood urea nitrogen; SD, standard deviation; MP, marketed product.

After intake of the Test product, glucose levels did not decrease as much and were more constant over time compared to levels after intake of the Reference product. By analysing glucose curves over time for the products and constructing an RM-ANCOVA model, a statistically significant difference emerged between the Test and the Reference products (Figure 5B; ANCOVA test: $p = 0.0068$).

Regarding the subjects’ assessment of the products, there were no significant differences between products for subjectively evaluated palatability or sense of satiety, except for a higher rating on the item “overall liking” for casein compared to the Test product ($p < 0.0006$; Wilcoxon exact test).

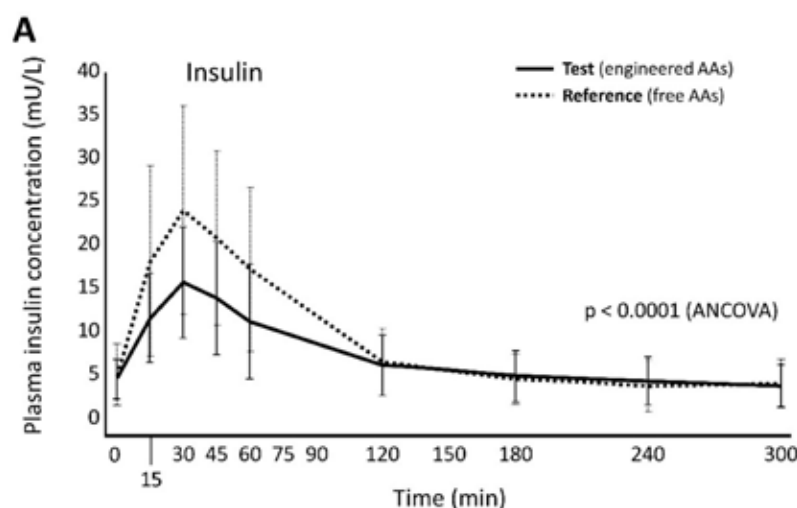


Figure 5. Cont.

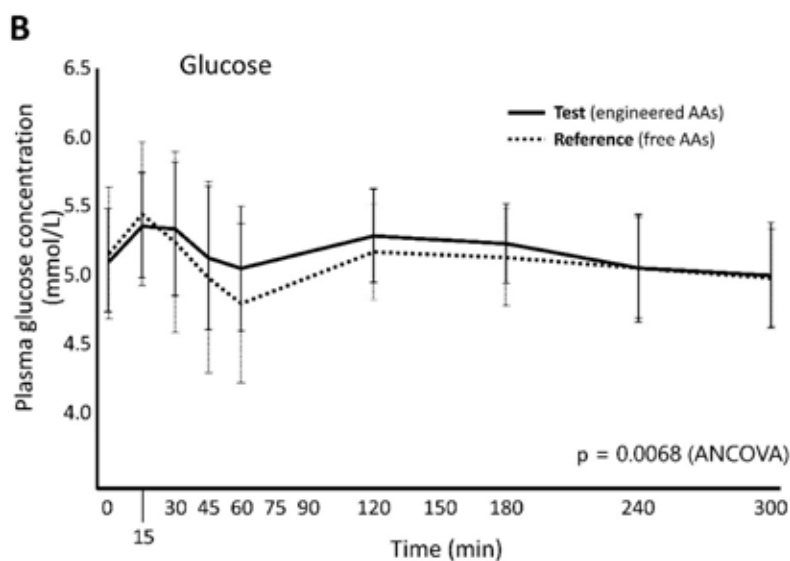


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References

1. Waters, D.; Adeloje, D.; Woolham, D.; Wastnedge, E.; Patel, S.; Rudan, I. Global birth prevalence and mortality from inborn errors of metabolism: A systematic analysis of the evidence. *J. Glob. Health* **2018**, *8*, 021102. [[CrossRef](#)] [[PubMed](#)]
2. Sanderson, S.; Green, A.; A Preece, M.; Burton, H. The incidence of inherited metabolic disorders in the West Midlands, UK. *Arch. Dis. Child.* **2006**, *91*, 896–899. [[CrossRef](#)] [[PubMed](#)]
3. Van Wegberg, A.M.J.; Macdonald, A.; Ahring, K.; Bélanger-Quintana, A.; Blau, N.; Bosch, A.M.; Burlina, A.; Campistol, J.; Feillet, F.; Giżewska, M.; et al. The complete European guidelines on phenylketonuria: Diagnosis and treatment. *Orphanet J. Rare Dis.* **2017**, *12*, 162. [[CrossRef](#)] [[PubMed](#)]
4. Morris, A.A.M.; Kozich, V.; Santra, S.; Andria, G.; Ben-Omran, T.I.M.; Chakrapani, A.B.; Crushell, E.; Henderson, M.J.; Hochuli, M.; Huemer, M.; et al. Guidelines for the diagnosis and management of cystathionine beta-synthase deficiency. *J. Inherit. Metab. Dis.* **2016**, *40*, 49–74. [[CrossRef](#)]
5. Frazier, D.M.; Allgeier, C.; Homer, C.S.; Marriage, B.J.; Ogata, B.; Rohr, F.; Splett, P.L.; Stembridge, A.; Singh, R. Nutrition management guideline for maple syrup urine disease: An evidence and consensus-based approach. *Mol. Genet. Metab.* **2014**, *112*, 210–217. [[CrossRef](#)]
6. Van Spronsen, F.J.; Van Rijn, M.; Meyer, U.; Das, A. Dietary Considerations in Tyrosinemia Type I. In *Plant Promoters and Transcription Factors*; Springer Science and Business Media: Berlin/Heidelberg, Germany, 2017; Volume 959, pp. 197–204.
7. Gokmen-Ozel, H.; Macdonald, A.; Daly, A.; Ashmore, C.; Preece, M.A.; Hendriks, C.; Vijay, S.; Chakrapani, A. Dietary practices in glutaric aciduria type 1 over 16 years. *J. Hum. Nutr. Diet.* **2012**, *25*, 514–519. [[CrossRef](#)]
8. Molema, F.; Gleich, F.; Burgard, P.; Van Der Ploeg, A.T.; Summar, M.L.; Chapman, K.A.; Barić, I.; Lund, A.M.; Kölker, S.; Williams, M.; et al. Evaluation of dietary treatment and amino acid supplementation in organic acidurias and urea-cycle disorders: On the basis of information from a European multicenter registry. *J. Inherit. Metab. Dis.* **2019**, *42*, 1162–1175. [[CrossRef](#)]
9. Ney, D.M.; Etzel, M.R. Designing medical foods for inherited metabolic disorders: Why intact protein is superior to amino acids. *Curr. Opin. Biotechnol.* **2017**, *44*, 39–45. [[CrossRef](#)]
10. Gropper, S.S.; Gropper, D.M.; Acosta, P.B. Plasma Amino Acid Response to Ingestion of L-Amino Acids and Whole Protein. *J. Pediatr. Gastroenterol. Nutr.* **1993**, *16*, 143–150. [[CrossRef](#)]
11. Jones, B.J.; Lees, R.; Andrews, J.; Frost, P.; Silk, D.B. Comparison of an elemental and polymeric enteral diet in patients with normal gastrointestinal function. *Gut* **1983**, *24*, 78–84. [[CrossRef](#)]
12. Boirie, Y.; Dangin, M.; Gachon, P.; Vasson, M.-P.; Maubois, J.-L.; Beaufrère, B. Slow and fast dietary proteins differently modulate postprandial protein accretion. *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 14930–14935. [[CrossRef](#)] [[PubMed](#)]
13. Dangin, M.; Boirie, Y.; Garcia-Rodenas, C.; Gachon, P.; Fauquant, J.; Callier, P.; Ballèvre, O.; Beaufrère, B. The digestion rate of protein is an independent regulating factor of postprandial protein retention. *Am. J. Physiol. Metab.* **2001**, *280*, E340–E348. [[CrossRef](#)] [[PubMed](#)]
14. Hennermann, J.B.; Roloff, S.; Gellermann, J.; Vollmer, I.; Windt, E.; Vetter, B.; Plöckinger, U.; Mönch, E.; Querfeld, U. Chronic kidney disease in adolescent and adult patients with phenylketonuria. *J. Inherit. Metab. Dis.* **2012**, *36*, 747–756. [[CrossRef](#)] [[PubMed](#)]
15. Singh, R.; Cunningham, A.C.; Mofidi, S.; Douglas, T.D.; Frazier, D.M.; Hook, D.G.; Jeffers, L.; McCune, H.; Moseley, K.D.; Ogata, B.; et al. Updated, web-based nutrition management guideline for PKU: An evidence and consensus based approach. *Mol. Genet. Metab.* **2016**, *118*, 72–83. [[CrossRef](#)] [[PubMed](#)]
16. Van Spronsen, F.J.; Van Wegberg, A.M.; Ahring, K.; Bélanger-Quintana, A.; Blau, N.; Bosch, A.M.; Burlina, A.; Campistol, J.; Feillet, F.; Giżewska, M.; et al. Key European guidelines for the diagnosis and management of patients with phenylketonuria. *Lancet Diabetes Endocrinol.* **2017**, *5*, 743–756. [[CrossRef](#)]
17. Macdonald, A.; Singh, R.H.; Rocha, J.C.; Van Spronsen, F.J. Optimising amino acid absorption: Essential to improve nitrogen balance and metabolic control in phenylketonuria. *Nutr. Res. Rev.* **2018**, *32*, 70–78. [[CrossRef](#)]
18. Giarratana, N.; Gallina, G.; Panzeri, V.; Frangi, A.; Canobbio, A.; Reiner, G. A New Phe-Free Protein Substitute Engineered to Allow a Physiological Absorption of Free Amino Acids for Phenylketonuria. *J. Inborn Errors Metab. Screen.* **2018**, *6*, 232640981878378. [[CrossRef](#)]

19. European Medicines Agency. Guideline on the Investigation of Bioequivalence. Available online: <https://www.ema.europa.eu/en/investigation-bioequivalence> (accessed on 15 January 2020).
20. US FDA. Bioavailability and Bioequivalence Studies Submitted in NDAs or INDs—General Considerations FDA. Available online: <https://www.fda.gov/regulatory-information/search-fda-guidance-documents/bioavailability-and-bioequivalence-studies-submitted-ndas-or-inds-general-considerations> (accessed on 15 January 2020).
21. Flint, A.; Raben, A.; Blundell, J.; Astrup, A. Reproducibility, power and validity of visual analogue scales in assessment of appetite sensations in single test meal studies. *Int. J. Obes.* **2000**, *24*, 38–48. [[CrossRef](#)]
22. Gropper, S.S.; Acosta, P.B. Effect of Simultaneous Ingestion of L-Amino Acids and Whole Protein on Plasma Amino Acid and Urea Nitrogen Concentrations in Humans. *J. Parenter. Enter. Nutr.* **1991**, *15*, 48–53. [[CrossRef](#)]
23. Lacroix, M.; Bos, C.; Léonil, J.; Airinei, G.; Luengo, C.; Daré, S.; Benamouzig, R.; Fouillet, H.; Fauquant, J.; Tomé, D.; et al. Compared with casein or total milk protein, digestion of milk soluble proteins is too rapid to sustain the anabolic postprandial amino acid requirement. *Am. J. Clin. Nutr.* **2006**, *84*, 1070–1079. [[CrossRef](#)]
24. Metges, C.C.; E El-Khoury, A.; Selvaraj, A.B.; Tsay, R.H.; Atkinson, A.; Regan, M.M.; Bequette, B.J.; Young, V.R. Kinetics of L-[1-(13)C]leucine when ingested with free amino acids, unlabeled or intrinsically labeled casein. *Am. J. Physiol. Metab.* **2000**, *278*, E1000–E1009. [[CrossRef](#)]
25. Mönch, E.; Herrmann, M.E.; Brösicke, H.; Schöffner, A.; Keller, M. Utilisation of amino acid mixtures in adolescents with phenylketonuria. *Eur. J. Nucl. Med. Mol. Imaging* **1996**, *155*, S115–S120. [[CrossRef](#)] [[PubMed](#)]
26. Pennings, B.; Boirie, Y.; Senden, J.M.; Gijsen, A.P.; Kuipers, H.; Van Loon, L.J.C. Whey protein stimulates postprandial muscle protein accretion more effectively than do casein and casein hydrolysate in older men. *Am. J. Clin. Nutr.* **2011**, *93*, 997–1005. [[CrossRef](#)]
27. Reitelseder, S.; Agergaard, J.; Doessing, S.; Helmark, I.C.; Lund, P.; Kristensen, N.B.; Frydberg, A.; Schjerling, P.; Van Hall, G.; et al. Whey and casein labeled with l-[1-13C]leucine and muscle protein synthesis: Effect of resistance exercise and protein ingestion. *Am. J. Physiol. Metab.* **2011**, *300*, E231–E242. [[CrossRef](#)]
28. Van Spronsen, F.J.; Van Dijk, T.; Smit, G.P.; Van Rijn, M.; Reijngoud, D.-J.; Berger, R.; Heymans, H.S. Large daily fluctuations in plasma tyrosine in treated patients with phenylketonuria. *Am. J. Clin. Nutr.* **1996**, *64*, 916–921. [[CrossRef](#)]
29. Van Spronsen, F.J.; Van Rijn, M.; Bekhof, J.; Koch, R.; Smit, P.G. Phenylketonuria: Tyrosine supplementation in phenylalanine-restricted diets. *Am. J. Clin. Nutr.* **2001**, *73*, 153–157. [[CrossRef](#)] [[PubMed](#)]
30. Grünert, S.C.; Brichta, C.M.; Krebs, A.; Clement, H.-W.; Rauh, R.; Fleischhaker, C.; Hennighausen, K.; Sass, J.O.; Schwab, K.O. Diurnal variation of phenylalanine and tyrosine concentrations in adult patients with phenylketonuria: Subcutaneous microdialysis is no adequate tool for the determination of amino acid concentrations. *Nutr. J.* **2013**, *12*, 60. [[CrossRef](#)]
31. MacDonald, A.; Rylance, G.W.; Asplin, D.; Hall, S.K.; Booth, I.W. Does a single plasma phenylalanine predict quality of control in phenylketonuria? *Arch. Dis. Child.* **1998**, *78*, 122–126. [[CrossRef](#)]
32. Giordano, M.; Castellino, P.; DeFronzo, R.A. Differential responsiveness of protein synthesis and degradation to amino acid availability in humans. *Diabetes* **1996**, *45*, 393–399. [[CrossRef](#)]
33. Atherton, P.; Etheridge, T.; Watt, P.W.; Wilkinson, D.; Selby, A.; Rankin, D.; Smith, K.; Rennie, M.J. Muscle full effect after oral protein: Time-dependent concordance and discordance between human muscle protein synthesis and mTORC1 signaling. *Am. J. Clin. Nutr.* **2010**, *92*, 1080–1088. [[CrossRef](#)]
34. Floyd, J.C.; Fajans, S.S.; Conn, J.W.; Knopf, R.F.; Rüll, J. Stimulation of insulin secretion by amino acids. *J. Clin. Invest.* **1966**, *45*, 1487–1502. [[CrossRef](#)] [[PubMed](#)]
35. Weigel, C.; Rauh, M.; Kiener, C.; Rascher, W.; Knerr, I. Effects of Various Dietary Amino Acid Preparations for Phenylketonuric Patients on the Metabolic Profiles along with Postprandial Insulin and Ghrelin Responses. *Ann. Nutr. Metab.* **2007**, *51*, 352–358. [[CrossRef](#)] [[PubMed](#)]
36. Flint, A.; Gregersen, N.T.; Gluud, L.L.; Møller, B.K.; Raben, A.; Tetens, I.; Verdich, C.; Astrup, A. Associations between postprandial insulin and blood glucose responses, appetite sensations and energy intake in normal weight and overweight individuals: A meta-analysis of test meal studies. *Br. J. Nutr.* **2007**, *98*, 17–25. [[CrossRef](#)]

37. Rosén, L.A.; Ostman, E.; Björck, I. Effects of cereal breakfasts on postprandial glucose, appetite regulation and voluntary energy intake at a subsequent standardized lunch; focusing on rye products. *Nutr. J.* **2011**, *10*, 7. [[CrossRef](#)] [[PubMed](#)]
38. Krog-Mikkelsen, I.; Sloth, B.; Dimitrov, D.; Tetens, I.; Björck, I.; Flint, A.; Holst, J.J.; Astrup, A.; Elmståhl, H.; Raben, A. A Low Glycemic Index Diet Does Not Affect Postprandial Energy Metabolism but Decreases Postprandial Insulinemia and Increases Fullness Ratings in Healthy Women. *J. Nutr.* **2011**, *141*, 1679–1684. [[CrossRef](#)] [[PubMed](#)]
39. US FDA. Select Committee on GRAS Substances (SCOGS) Opinion: Cellulose Acetate (Packaging) and Ethyl Cellulose (Packaging). Available online: <http://wayback.archive-it.org/7993/20171031061136/https://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/SCOGS/ucm261248.htm> (accessed on 15 January 2020).
40. US FDA. Select Committee on GRAS Substances (SCOGS) Opinion: Alginates. Available online: <http://wayback.archive-it.org/7993/20171031063235/https://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/SCOGS/ucm260857.htm> (accessed on 15 January 2020).
41. Van Rijn, M.; Hoeksma, M.; Sauer, P.; Szczerbak, B.; Gross, M.; Reijngoud, D.-J.; Van Spronsen, F. Protein metabolism in adult patients with phenylketonuria. *Nutrition* **2007**, *23*, 445–453. [[CrossRef](#)]
42. Thompson, G.N.; Pacy, P.J.; E Watts, R.W.; Halliday, D. Protein Metabolism in Phenylketonuria and Lesch-Nyhan Syndrome. *Pediatr. Res.* **1990**, *28*, 240–246. [[CrossRef](#)] [[PubMed](#)]



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