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Randomized control trials

Protein type and caloric density of protein supplements modulate postprandial amino acid profile through changes in gastrointestinal behaviour: A randomized trial☆

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SUMMARY

Background & aims: The requirement of leucine and essential amino acids (EAA) to stimulate muscle protein synthesis increases with age. To target muscle anabolism it is suggested that higher postprandial blood levels of leucine and EAA are needed in older people. The aim was to evaluate the impact of oral nutritional supplements with distinct protein source and energy density, resembling mixed meals, on serum amino acid profiles and on gastrointestinal behaviour.

Methods: Four iso-nitrogenous protein (21 g) supplements were studied containing leucine-enriched whey protein with 150/320 kcal (W150/W320) or casein protein with 150/320 kcal (C150/C320); all products contained carbohydrates (10 or 32 g) and fat (3 or 12 g). Postprandial serum AA profiles were evaluated in twelve healthy, older subjects who participated in a randomized, controlled, single blind, cross-over study. Gastrointestinal behaviour was studied *in vitro* by looking at gastric coagulation and cumulative intestinal protein digestion over time.

Results: The peak serum leucine concentration was twofold higher for W150 vs. C150 (521 ± 15 vs. 260 ± 15 μmol/L, $p < 0.001$), higher for W320 vs. C320 (406 ± 15 vs. 228 ± 15 μmol/L, $p < 0.001$), and higher for low-caloric vs. high-caloric products ($p < 0.001$ for pooled analyses; $p < 0.001$ for interaction protein source*caloric density). Similar effects were observed for the peak concentrations of EAA and total AA (TAA). *In vitro* gastric coagulation was observed only for the casein protein supplements. Intestinal digestion for 90 min resulted in higher levels of free TAA, EAA, and leucine for W150 vs. C150, for W150 vs. W320, and for C150 vs. C320 ($p < 0.0125$).

Conclusions: A low caloric leucine-enriched whey protein nutritional supplement provides a higher rise in serum levels of TAA, EAA and leucine compared to casein protein or high caloric products in healthy, elderly subjects. These differences appear to be mediated in part by the gastrointestinal behaviour of these products.

Clinical trial registration: ClinicalTrials.gov: NCT02013466.

1. Introduction

Aging often coincides with loss of muscle mass, strength and function, known as sarcopenia [1]. Sarcopenia, a geriatric syndrome closely linked to physical frailty, has a substantial impact on the quality of life of the individual and increases the risk of disability and hospitalization [1]. Sarcopenia has been linked to a decreased

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muscle anabolic response to insulin and dietary essential amino acids (EAA) [2–5].

Muscle protein synthesis has been shown to respond to extracellular EAA concentration [6], subsequent transport, and intracellular amino acid rate of deposition in muscle [7,8]. A high amount of EAA and leucine in one single bolus has also been shown to enable stimulation of muscle protein synthesis in older adults [9–12]. Accordingly, higher postprandial blood levels of EAA and leucine correlate to a higher muscle protein synthesis rate [13,14]. This suggests that the postprandial profile by which amino acids and especially EAA and leucine appear in blood and become available for muscle, is relevant for subsequent stimulation of muscle protein synthesis. This appears to be even more important with advancing age, conditions of inflammation and insulin resistance. The threshold needed to trigger intracellular translation initiation pathways to activate protein synthesis is likely higher under these conditions [15].

The quantity and source of dietary proteins have been shown to impact postprandial blood levels of EAA and leucine [16]. As a result, the concept of "fast/slow" protein was implemented to indicate the postprandial profile of amino acids appearing in the systemic circulation [17,18]. As an example, the dairy proteins whey and casein contain a similar amount of EAA, but blood EAA levels increased faster and to a higher level after the intake of whey protein [14,19]. Therefore, whey is considered a "fast" protein, while casein is a "slow" protein. A difference in gastric emptying and digestion and absorption kinetics between casein and whey [14,20,21] are suggested as an underlying factor. Distinct factors have been shown to modulate gastric emptying and the gastrointestinal digestion of nutritional compositions. Gastric coagulation of casein with formation of solid particles [17], is an example of a physiological phenomenon that is known to delay gastric emptying of casein [21,22]. Another example is the difference in sequential release of casein and whey-derived peptides in the jejunum [23], that is indicative of distinct hydrolysis kinetics of casein and whey. Moreover, it is known that the higher the caloric density of nutrition the more it delays gastric emptying [24,25]. Supplementing sucrose to a casein or milk protein indeed delayed protein digestion and absorption kinetics [26] and increased the oro-ileal transit time [27]. However, the relevance of gastric coagulation and of intestinal digestion rate for postprandial amino acid profiles and systemic amino acid bioavailability of casein and whey protein-containing supplements, i.e. resembling mixed meals of different caloric densities, is less well known.

Our primary study aim was to evaluate postprandial blood amino acids profiles in healthy older people after the intake of a leucine-enriched whey protein nutritional supplement compared to an iso-caloric and iso-nitrogenous control product containing casein protein. While distinct effects of intact whey and casein protein on blood amino acid profiles in older people have been reported, the use of carbohydrates and fat besides protein is less well known. This is relevant as it represents conventional products or a 'mixed meal'. Secondly, we aimed to study the impact of caloric density of the whey and casein products on postprandial amino acid profiles and systemic amino acid bioavailability, which was not studied like this before. Third, we aimed to understand the relevance of distinct gastrointestinal behaviour of the nutritional supplements for postprandial amino acid profiles. While *in vivo* digestion studies with jejunal sampling and/or intrinsically labelled proteins are preferred, these measures are also invasive and require that nutritionally supplements are produced with intrinsically labelled proteins. Therefore, we applied *in vitro* models to compare the coagulation behaviour and protein digestion rate (initial and overall cumulative digestion over time) of the nutritional supplements, under conditions closest to those found in a healthy, elderly population.

2. Materials and methods

2.1. Subjects in clinical study

Fifteen healthy adults that were 65 years or older were screened. A total of 12 subjects (5 male) were enrolled in the study. Subjects who signed the informed consent had a Body Mass Index (BMI) between 21 and 30 kg/m^2, and were willing and able to comply with the protocol. The protocol included adhering to a fasting state from 22:00 h the day prior to the study visits, refraining from alcohol consumption (24 h) and intense physical activities (24 h) before the study visits and not changing dietary habits for the duration of the study. Subjects with a (history of) gastrointestinal disease, or those that had been diagnosed with, or were suspected of having, diabetes mellitus (fasting glucose $\geq$ 7.0 mmol/L) were excluded from participation. Other exclusion criteria were: infection or fever in the past 7 days, medication use (antibiotics within 3 weeks of study entry, current use of corticosteroids or hormones, and the use of antacids or any medication influencing gastric acid production), known allergy to milk or milk products, lactose intolerance and known galactosaemia. Moreover, subjects were excluded when they currently participated in a weight loss or muscle strengthening program or used nutritional supplements that contained proteins or amino acids within one week of study entry, as well as those who had smoked for the past 3 months or abused alcohol or drugs. The Modified Baecke Questionnaire for Older People [28] was completed to measure the normal physical activity level. Body weight and height were measured. Supplementary Fig. 1 shows the Consort flow diagram.

All subjects were informed of the study procedures and possible risks before signing informed consent. The subject enrolment and study conduction was done by Ampha B.V. (clinical research unit, Nijmegen, the Netherlands) according to ICH-GCP principles, and in complied with the principles of the 'Declaration of Helsinki' (59th WMA General Assembly, Seoul, October 2008) and the local laws and regulations. The Independent Review Board Nijmegen (IRBN), the Netherlands, approved the study. This trial is registered at the ClinicalTrials.gov Trial Register under number NCT02013466.

2.2. Study products

Four study products, differing in protein source and caloric density (Table 1), were tested *in vivo* and *in vitro*. Two products composed a leucine-enriched whey protein nutritional supplement

Table 1
Composition of the study products.

Nutrients	W150	C150	W320	C320
Energy (kcal)	150	150	320	320
Protein (En%)	56	56	26	26
Carbohydrates (En%)	26	26	40	40
Fat (En%)	18	18	34	34
Total protein (g)	21	21	21	21
Whey protein (g)	20	–	20	–
Casein protein (g)	–	21	–	21
Free BCAA (g)	1	0	1	0
Total leucine (g)[a]	3	2	3	2
Total EAA (g)[a]	10	9	10	9
Carbohydrates (g)	11[b]	10	33[b]	32
Fat (g)	3	3	12	12

BCAA, branched chain amino acids; EAA, essential amino acids.
[a] Provided by protein and free BCAA.
[b] Includes citric acid to obtain an acidic product.

(20 g whey protein (from whey protein isolate), 3 g total leucine) with 150 kcal (W150) or 320 kcal (W320), respectively. The other products contained an iso-nitrogenous amount of casein (21 g casein protein (from calcium caseinate)) with 150 kcal (C150) or 320 kcal (C320), respectively. Additional calories were added as fat and carbohydrates, similar to conventional medical nutrition supplements. The osmolality of the 320 kcal products was higher (239–248 mOsm/kg) compared with the 150 kcal products (131–139 mOsm/kg). The pH of the whey products was acidic (4.0), while pH was neutral for the casein products (6.6). Subjects consumed the complete products within 5 min. Site staff, not involved in the study, mixed the study products (as powders) with water to a volume of 300 ml before consumption.

2.3. Clinical study design (in vivo study)

This clinical study was a randomised, controlled, single-blinded, cross-over, single centre study. Subjects eligible for participation were randomly allocated, by using three 4 × 4 Latin squares, to obtain a unique order of the study products. Study products were packaged indistinguishably and coded. Aside from a noticeable difference in weight between the low and high-caloric sachets, study staff was blinded to the origin of the study products until completion all data were included in the study database.

Subjects visited the research location in a fasting state on four separate mornings. A flexible 18-22 gauge catheter was placed into a forearm vein for blood sampling. Fasting blood samples were collected at baseline for serum C-reactive protein (CRP), albumin, glucose, insulin, and amino acid concentrations (t = −15 min), and a second basal sample for amino acid, glucose and insulin concentrations was taken just before product intake. After consumption of the study product (t0), repeated blood samples were drawn over the next 4 h, at 15, 30, 45, 60, 75, 90, 105, 120, 150, 180, 210, and 240 min for analyses of amino acids, glucose and insulin concentrations. Before product consumption and at 240 min after product intake, blood pressure and heart rate were measured, and a tolerance questionnaire was completed to measure the intensity of gastrointestinal symptoms (heartburn, belching, nausea, vomiting, abdominal distension, flatulence, diarrhoea, and constipation), light-headedness, headache, thirst and dry mouth.

Visits were planned with intervals of 7–10 days between the visits. A follow-up phone call was scheduled approximately one week after the last study visit to identify adverse events and evaluate the tolerance of the intervention. Throughout the study, the use of relevant concomitant medication and nutritional supplement intake was recorded. Supplementary Fig. 2 illustrated the clinical study design.

Stopping guidelines with premature discontinuation of participation in the study included cases when further participation was considered a health risk for a subject (at supervising clinicians discretion), and cases in which a subject had decided to resign from further participation in the study.

2.4. In vitro digestion modelling (in vitro study)

A semi-dynamic two-step gastrointestinal model was used, as illustrated in Fig. 1. The model has continuous pH control, digestive juice addition and separate pre-defined gastric and intestinal steps. The model consists of eight parallel bioreactors that are computer controlled. Each is equipped with a pH electrode and four dosing-lines with separate substrate pumps (Dasgip AG, Jülich, Germany) (Fig. 1a and b). Gastrointestinal conditions were simulated by pH and substrate pump control at 37 °C as described previously [22]. The flow, volumes and compositions of secretions were based on what is used in a dynamic model of the gastro-intestinal tract

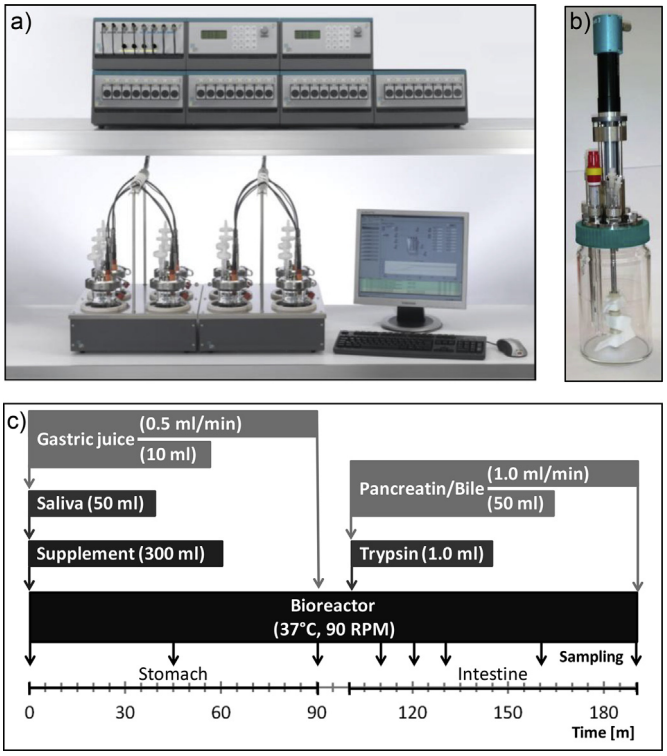

Fig. 1. Semi-dynamic model of the gastrointestinal tract. The model consisted of a) Computer controlled parallel bioreactor system, and b) Single bioreactor with overhead agitation. c) Time schedule.

[29,30], as described earlier in detail [22]. For the current study, the approach was adapted: a) using reactors with a larger volume (to fit 300 ml), therefore no scaling was necessary, b) adding a small intestinal phase, c) adapting gastric secretion to elderly conditions by lowering pepsin and lipase concentration based on the decreased peak pepsin output observed in elderly, i.e. 52%, as compared to healthy young adults [31], and d) using overhead agitation at 90 rpm with three perpendicularly placed 45° two-blade impellers, instead of magnetic stirring, for adequate mixing of the study product and digestive juices with lower shear. The four different substrates were a) 1 M HCl and b) 1 M $NaHCO_3$ and 3 M NaOH to adjust the pH, and c) artificial gastric juice and d) pancreatin/bile mixture containing 2% w/v porcine bile extract (Sigma B8631), 1.75% w/v pancreatin (Pancrex V, Pfizer) supernatant, 0.25% w/v NaCl, 0.03% KCl, and 0.015% w/v $CaCl_2 \cdot H_2O$. Artificial saliva was added manually.

Experiments were done in triplicate (n = 3) with the investigator blinded for the composition of the nutritional supplements. Gastric digestion was simulated for 90 min. During this period the pH was lowered according to a predefined curve that is based on the acid secretion capacity of older people and the pH of the supplements [32]. The pH was lowered from 4.0 to 2.2 within 90 min in whey compositions and from 6.6 to 3.0 in casein supplements within 90 min. In one set of the experiments, the simulation was discontinued and the gastric contents were analysed for solid particles, i.e. coagulate fractions. In another set of experiments, the first 90-min phase of gastric digestion was followed by a 90-min simulation phase of small intestinal digestion, allowing the system ten minutes between the 2 phases to bring the pH to the set start pH (6.5) of the intestinal phase. The set intestinal pH curve was the same for all supplements, i.e. an increase to pH 7.2 in 90 min. At the start of the experiment, 50 ml of saliva was added manually (Fig. 1c). The flow rate for gastric juice was 0.5 ml/min. In

the first two minutes of gastric digestion, an additional 10 ml was added as a shot to simulate the gastric juice already present in the stomach. The secretion of the pancreatin/bile mixture was at a rate of 1.0 ml/min. At the start of the intestinal phase, 1 ml of trypsin solution (0.2% w/v (sigma T9201)) was added manually to simulate the action of enterokinase on trypsinogen from the pancreatin. In the first 5 min, 50 ml of pancreatin/bile mixture was added as a shot to simulate the intestinal contents at the time of ingestion.

2.5. Sample processing, analyses, and calculations

2.5.1. Blood samples (in vivo)

Blood was sampled in serum tubes. Serum was obtained by centrifuging whole blood at room temperature for 15 min at 1000 *g*. All serum samples were stored at −80 °C until further analyses.

2.5.2. Coagulate fractions (in vitro)

After 90 min of simulated gastric digestion, the content of the reactors was poured over three analytical sieves. The mesh widths of the sequential sieves was 2 mm, 1 mm, and 0.25 mm (Retsch, VWR, Amsterdam, Netherlands) in order to separate insoluble particles by particle diameter (D) in three fractions: larger than 2 mm ($D_{>2}$), between 1 and 2 mm ($D_{<1-2>}$), between 0.25 and 1 mm ($D_{<0.25-1>}$). The dry matter content of each fraction was determined as described previously [20].

2.5.3. Protein digestion rate (in vitro)

Sampling over time in the intestinal phase enables us to gain insights into the initial digestion rate (first one-two samples (t10 and t20), early intestinal phase) and the overall digestion rate (later samples, representing cumulative digestion over a maximum 90-min intestinal period). Measurement of the accumulation of protein digestion products was done with two distinct, complementary methods. The analysis was focused on the end products of pancreatic enzymes. It has been found *in vivo* that the absorbable fraction of protein digestion products consists of free amino acids and peptides built of two to six amino acids, in a ratio of 25:75 mol% of free and peptide-bound amino acids respectively [33]. Therefore, 1kD was chosen as the upper limit as done by others [34], and peptides smaller than 1kD and free amino acids were quantified.

2.5.4. Peptide analysis (from in vitro study)

Peptides were quantified using size exclusion chromatography (SEC). The HPLC system (Shimadzu) was equipped with a Superdex Peptide 10/300 column (GE Healthcare 17-5176-01). Detection of peptide bonds in the eluent (25%wt acetonitrile, 0.16%wt trifluoracetic acid) was by done by UV absorption, $\lambda = 200$ nm. The relationship between elution time and molecular weight was calibrated using ten distinct small proteins and peptides ranging from 12kD to 188D, of which five were smaller than 1 kD. The correlation coefficient of linear fit between elution time and molecular weight was 0.980 between 9.2 and 19.2 min. The AUC was calculated from 15.3 min (1kD) to 23 min to exclude contribution of free aromatic amino acids that absorb at this wavelength.

2.5.5. Analysis of free amino acids (from in vivo and in vitro studies)

Proteins and large peptides were precipitated with perchloric acid. After filtration, the amount of the free amino acids in the supernatant is determined by UFLC using a pre-column derivatization with o-phtaldialdehyde and fluorimetry as detection [35].

2.5.6. Analysis of other blood parameters (from in vivo study)

Glucose and albumin concentrations were measured using colorimetry. Turbidimetry was used for analysis of CRP. Insulin concentrations were measured by immunoluminometric assay.

2.5.7. Calculations and primary/secondary outcome parameters (in vivo)

For serum free leucine, EAA (sum of histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine), and the sum of all amino acids (TAA: alanine, arginine, asparagine, aspartic acid, citrulline, cysteine, glutamic acid, glutamine, glycine, serine, taurine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine; tyrosine was excluded because of technical problems in the analysis) the following parameters were calculated: the maximum (or peak) concentration, the maximum increment from baseline (delta), the iAUC (from baseline, during 4 h after product intake) and the time to reach half the serum leucine iAUC ($t_{1/2}$). For serum glucose and insulin, the maximum (or peak) and iAUC values were calculated.

The primary outcome parameter of the clinical study was the maximum leucine level; secondary parameters were maximum levels of EAA, TAA, glucose and insulin, the iAUC of leucine, glucose and insulin, and $t_{1/2}$ of leucine. As a deviation to the protocol but before data analyses, iAUC and $t_{1/2}$ of EAA and TAA were added as secondary parameters.

2.6. Statistical analysis

Power analysis for the *in vivo* study was based on an estimated 100 μmol/L difference in peak leucine level between W150 and C150 with a standard deviation of difference of 60 μmol/L for whey and 54 μmol/L for casein as estimated from Dangin et al. [19], an α of 0.05, a power of 80% and dropout rate of 33%. All *in vivo* serum variables (amino acids, glucose and insulin concentrations) were analysed using a mixed model with a random effect for subjects, a fixed effect for the factors protein (2 levels: whey, casein) and caloric density (2 levels: low, high), and a fixed interaction of protein*caloric density. Statistical significance of these pooled analyses was defined as a two-tailed $p < 0.05$. In separate mixed model analyses, the difference between W150 and C150, between W150 and W320, between C150 and C320 and between W320 and C320 were analysed. Statistical significance was defined as a two-tailed $p < 0.0125$ in order to correct for multiple comparisons. Baseline value (for leucine, EAA, AA, insulin, or glucose), serum albumin, and serum CRP were included as covariates in the respective mixed model. The concentration at t0 was used for baseline value when concentrations at t-15 and t0 were statistically significantly different using mixed model analysis. In case of no statistical difference, the mean concentration of the 2 time points at baseline was taken. For peak leucine, additional analyses were performed using age, physical activity level, sex or BMI (categories $\leq$25 and >25 kg/m^2) as additional covariates in the mixed model. SAS 9.1.3 (SAS Institute; PROC MIXED procedure) software was used. *In vivo* data are presented as estimated marginal mean (EMM) with SE, as derived from the mixed model, unless otherwise stated.

In vitro gastric coagulation data were analysed with Student's t-test, 2-tailed, equal variances assumed, and differences were considered significant when $p < 0.05$. *In vitro* protein digestion kinetics data was analysed using ANOVA univariate analysis with LSD post hoc test. One on the triplicate measures for W150 was excluded from the dataset because of technical malfunction of the bioreactor. Because there were no more supplements available for the study, the statistical interaction model was used to determine estimated means. Differences were considered significant when $p < 0.0125$, in order to correct for multiple comparisons. Statistics were performed using SPSS 15.0 for Windows. Data are shown as raw mean with SEM, unless stated otherwise.

3. Results

3.1. Subjects

The first subject was included in the study on October 27, 2008. The last subject completed the study on February 19, 2009. Ten out of 12 subjects completed all visits. Two subjects terminated the study early, but had at least 1 study visit completed. None of the randomized subjects violated the eligibility criteria during the study or had other protocol violations. Therefore, all subjects were included in the population analysis, and descriptive and statistical analyses were performed on the 12 subjects (Supplementary Fig. 1). Subjects' characteristics are listed in Table 2. No related serious adverse events were observed during the study. A few mild to moderate adverse events were reported with no clinically relevant difference in occurrence between the products. There were no clinically relevant differences in incidence of GI symptoms between treatments.

3.2. Serum concentrations of amino acids, glucose, and insulin

Profiles for serum leucine, EAA, and TAA are illustrated in Fig. 2, and for serum glucose and insulin in Fig. 3. Values and statistical comparison between groups at baseline, peak, delta, iAUC and $t_{1/2}$ for serum leucine, EAA, and TAA are listed in Table 3. Baseline values for serum amino acids, glucose and insulin were well balanced between the study visits.

Pooled analysis of the low-caloric vs. the high-caloric products (W150 and C150 vs. W320 and C320) showed a significantly higher peak and iAUC of serum leucine, EAA, and TAA for the low-caloric vs the high-caloric products ($p < 0.001$). The caloric density effect was stronger for the leucine-enriched whey protein supplements (W150 vs. W320) than for the casein protein supplements (C150 vs. C320) on peak levels of serum leucine (effect size (EMM ± SE) of 114 ± 14.3 μmol/L ($p < 0.001$) vs 32 ± 14.8 μmol/L ($p = 0.042$)), serum EAA (effect size of 395 ± 49.2 μmol/L ($p < 0.001$) vs 120 ± 49.6 μmol/L ($p = 0.023$), and serum TAA (effect size of 546 ± 79.0 μmol/L ($p < 0.001$) vs 247 ± 76.3 μmol/L ($p = 0.003$)). This is also reflected by the significant interaction effect between protein source and caloric density ($p < 0.001$ for peak levels of leucine and EAA, $p = 0.015$ for TAA). Including age, physical activity level, gender or BMI in the mixed model for peak leucine did not change the significance of the effect.

Peak serum glucose concentration was 5.54 ± 0.18 and 6.05 ± 0.19 mmol/L after intake of W150 and C150, respectively ($p = 0.013$), and 6.42 ± 0.18 and 6.66 ± 0.18 mmol/L after W320 and C320, respectively ($p = 0.195$). Serum glucose iAUC did neither differ between W150 and C150 ($p = 0.152$), nor between W320 and C320 ($p = 0.577$). Pooled analysis of the low-caloric vs. the high-caloric products showed significantly lower peak and iAUC serum glucose concentration for the low-caloric vs the high-caloric products ($p < 0.001$). There was no significant interaction effect between protein source and caloric density for the peak serum glucose concentration ($p = 0.314$), nor for serum glucose iAUC ($p = 0.494$).

Peak and iAUC serum insulin concentration did not differ between W150 and C150 ($p = 0.915$ for peak; $p = 0.782$ for iAUC), nor between W320 and C320 ($p = 0.989$ for peak; $p = 0.261$ for iAUC). Pooled analysis of the low-caloric vs. the high-caloric products showed a significantly lower peak and iAUC serum insulin concentration for the low-caloric vs the high-caloric products ($p < 0.001$). The interaction effect between protein source and caloric density for peak serum insulin concentration ($p = 0.933$), or for serum insulin iAUC ($p = 0.335$) was not significant.

Table 2
Subject characteristics.

Characteristic	Baseline	W150	C150	W320	C320
Gender:					
Male (number (%))	5 (42)				
Female (number (%))	7 (58)				
Age (y)	67.4 ± 1.8				
Body weight (kg)	73.1 ± 7.5				
BMI (kg/m^2)	26.0 ± 2.6				
Serum albumin (g/L)		41.2 ± 1.89	39.8 ± 1.32	40.3 ± 2.23	40.9 ± 1.87
Serum CRP (mg/L)		3.4 ± 2.58	5.3 ± 9.27	1.7 ± 0.89	3.1 ± 2.12
Physical activity level[a]	13.57 ± 6.65				

All data are means ± SD, except for sex; n = 12 (ITT).
[a] Total score, derived from the Modified Baecke Questionnaire for Older People [28].

3.3. In vitro gastric coagulation

The leucine-enriched whey protein supplements did not yield any measurable coagulates after gastric digestion, while casein supplements did. Gastric digestion of C320 yielded a significantly higher total amount of coagulates than C150 ($p = 0.005$). This was due to a higher amount of coagulates of the middle diameter fraction ($D_{<1-2>}$) ($p = 0.021$), and a tendency for a higher amount of coagulates of the smallest diameter fraction ($D_{<0.25-1>}$) ($p = 0.051$). The largest diameter fraction, however, was not significantly different ($p = 0.691$), (Fig. 4). The percentage of total coagulate matter in $D_{>2}$ was significantly lower in C320 vs C150 (62.3 ± 9.0 vs 91.0 ± 1.4%, respectively; $p = 0.035$), while the percentage in $D_{<1-2>}$ was significantly higher (15.5 ± 3.6 vs 4.6 ± 0.8%, respectively; $p = 0.043$) and in $D_{<0.25-1>}$ tended to be higher (22.2 ± 6.4 vs 4.4 ± 0.6% respectively; $p = 0.051$).

3.4. In vitro protein digestion rate

The vast majority of small peptides were released during the intestinal phase of digestion for all supplements tested. The amount of small peptides present early in the intestinal phase (t10 min) was significantly higher in the casein supplements than the leucine-enriched whey protein supplements of the same caloric density (150 kcal: $p = 0.003$ and 320 kcal $p < 0.001$). For the later samples in the intestinal phase, these amounts were no longer significantly different ($p > 0.0125$). At t90, larger amounts of peptides tended to be present in samples of low caloric supplements; W150 vs W320 ($p = 0.072$) and C150 vs C320 ($p = 0.049$) (Fig. 5A).

There were free AA present in the gastric phase of the leucine enriched, whey protein supplements. These are most likely the free branched chain amino acids (leucine, isoleucine, valine; all EAA) that are present in the initial supplement. There was negligible release of amino acids in the gastric phase.

In the early phase of intestinal digestion (t10 and t20), the total amount of free AA was significantly higher for C320 than both C150 ($p < 0.0125$) and W320 ($p < 0.0125$). From t30 onwards, W150 was significantly higher than W320 ($p < 0.0125$) and from t60 onwards also higher than C150 ($p < 0.0125$). At the end of the intestinal phase (t90), both C150 and W320 were significantly higher than C320 ($p < 0.001$) (Fig. 5B).The amount of free EAA at t10 was significantly higher for C320 than for C150 and W320 ($p < 0.001$). From t20 onwards, W150 was significantly higher than C150 ($p < 0.0125$) and from t30 onwards also significantly higher than W320 ($p < 0.0125$). W320 was significantly higher than C320 at t60

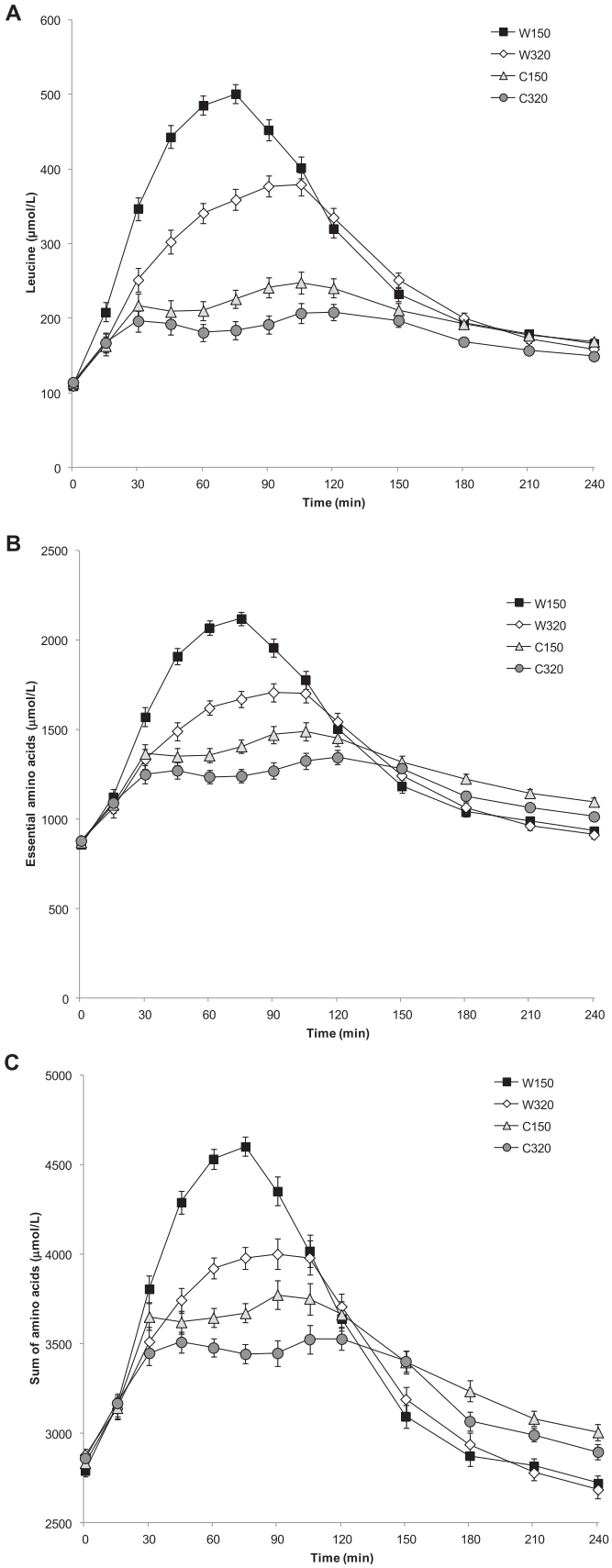

Fig. 2. Serum amino acids. Serum levels (EMM ± SE) of A) leucine, B) essentials amino acids (EAA), and C) sum of amino acids (TAA) before and after ingestion (at T = 0) of the study products: leucine-enriched whey protein, 150 kcal (W150); casein protein, 150 kcal (C150); leucine-enriched whey protein, 320 kcal (W320); and casein protein, 320 kcal (C320), in healthy older subjects (n = 12).

($p = 0.0013$) and t90 ($p < 0.001$) (Fig. 5C). After correction for the initial difference in free EAA level between the leucine-enriched whey protein supplements and casein supplement caloric equivalents, the amount of free EAA for W150 was still significantly higher than W320 from t30 onwards ($p < 0.0125$), and also higher than C150 at t60 ($p = 0.006$) and t90 ($p < 0.001$) (Fig. 5E).

The amount of free leucine was significantly higher at all time points for both leucine-enriched whey protein supplements than for their casein supplement caloric equivalents ($p < 0.0125$). From t30 onwards, W150 was also significantly higher than W320 ($p < 0.0125$). For the casein supplements at t10, C320 was higher than C150 ($p = 0.008$), at t20 and t30 there was no significant difference ($p = 0.046$ and $p = 0.865$, respectively). At t60 and t90, C150 was significantly higher than C320 ($p = 0.008$ and $p < 0.001$, respectively) (Fig. 5D). After correction for the initial higher leucine level, the amount of leucine in W150 was also significantly higher than W320 from t30 onwards ($p < 0.0125$), and also significantly higher than C150 at t90 ($p < 0.001$), but not at t60 ($p = 0.057$) (Fig. 5F).

SEC chromatograms of t90 samples indicate that peptides smaller than 1kD constitute about 70% (68–72) of all soluble protein and peptides (data not shown). At that time point, there is on average 4.5 g of free amino acids (Fig. 5B). Assuming all proteins were dissolved at this point and free AA do indeed not contribute to the signal, the estimated degree of digestion at t90 of the total 21 gr of food protein and 1.3 gr of protein from digestive juices is 76% (calculated as ((22.3–4.5)*0.7 + 4.5)/22.3*100%), of which about 70% are peptides and 30% are amino acids.

4. Discussion

In the current study, blood AA profiles after consumption of a leucine-enriched whey protein nutritional supplement or an iso-caloric and iso-nitrogenous casein protein supplement were compared in healthy older men and women. In addition, the impact of the caloric density of the products on the blood AA profiles was analysed. Higher levels of TAA, EAA, and leucine were observed after the leucine-enriched whey protein supplements compared to the casein protein supplements. This effect was further augmented by the low-caloric density of the formulation, both for the casein and whey protein-containing supplements. As far as we are aware, this is a novel observation as it resembles realistic protein amounts in mixed meals. In accordance with *in vivo* data, gastric physico-chemical behaviour *in vitro* was profoundly protein type dependent; only the casein supplements clotted. The initial intestinal protein digestion rate of casein supplements was higher than that of whey protein supplements. The overall protein digestion rate and cumulative release of AAs over 90-min, however, was higher for whey supplements than their casein counterparts. In addition to the protein type dependency, this was also caloric density dependent, i.e. higher for 150 kcal than 320 kcal. These overall differences in the *in vitro* data thus nicely fit with the observed differences in *in vivo* blood AA profiles. The results suggest that gastric coagulation, likely impacting gastric emptying, and/or overall protein digestion rate are determinants of amino acid profiles, while initial enzymatic hydrolysis rate in the intestine is less important. The observed differences in postprandial amino acid profiles *in vivo*, therefore, appear to be at least partly mediated by the gastrointestinal behaviour of the products.

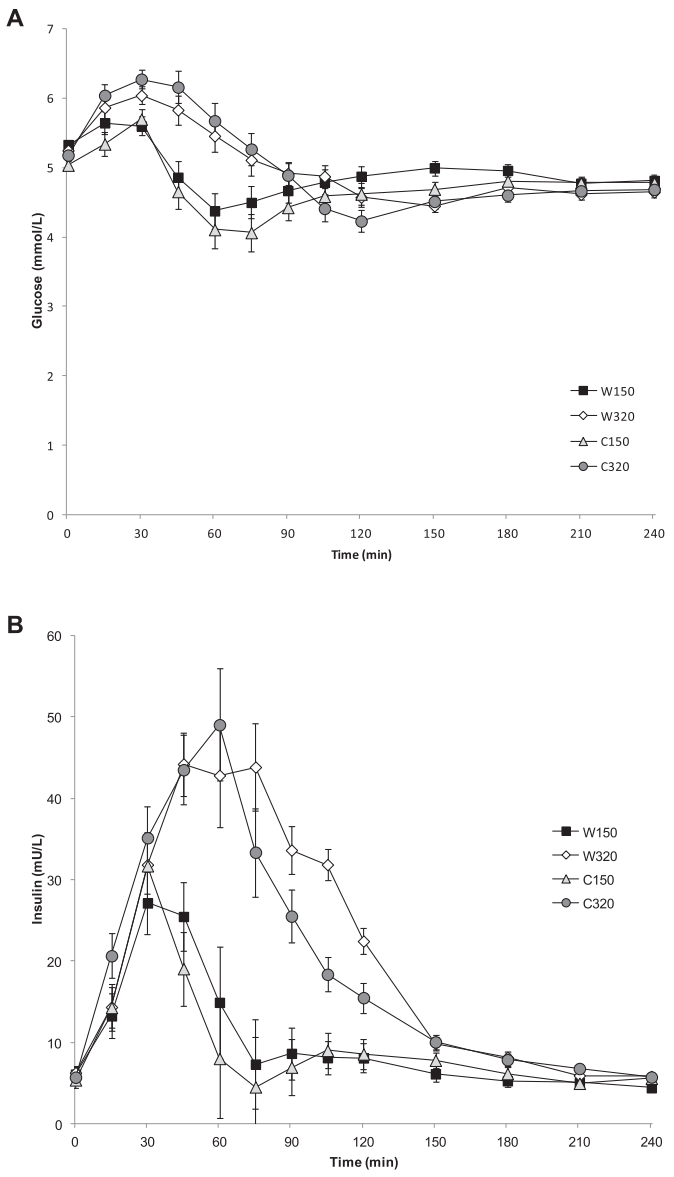

Fig. 3. Serum glucose and insulin. Serum levels (EMM ± SE) of A) glucose and B) insulin before and after ingestion (at T = 0) of the study products: leucine-enriched whey protein, 150 kcal (W150); casein protein, 150 kcal (C150); leucine-enriched whey protein, 320 kcal (W320); and casein protein, 320 kcal (C320), in healthy older subjects (n = 12).

4.1. Effect of protein source

The leucine content in the leucine-enriched whey protein supplements was 1.5-fold higher compared to the casein protein supplements. This contributed to the difference in serum leucine levels. However, despite the equal contents of EAA and TAA, these profiles differed between the products and indicate the contribution of other aspects. Delayed gastric emptying of casein due to gastric coagulation of the protein has been demonstrated [22], and subsequent differences in intestinal behaviour between casein and whey have been shown with intra-jejunal sampling [20,21,23] and with the use of intrinsically labelled proteins [14]. It has been demonstrated that the particle size has an impact on gastric emptying. Solid matter needs to be broken down to <1–2 mm particles prior to their stomach emptying [36]. Our *in vitro* data confirm gastric coagulation with casein specifically, but also indicate different digestion kinetics for whey and casein protein supplements.

To our knowledge, the intestinal digestive properties of casein and whey protein have not been extensively studied, especially in mixed meals. We observed that the initial intestinal digestion rate of casein-containing supplements was higher. This is in line with other *in vitro* digestion studies, using human gastric and duodenal aspirates. In these studies, caseins in milk from several species were digested very rapidly after being exposed to either human gastric or duodenal juices, as compared to whey proteins [37,38].

Moreover, we observed in our study that the overall protein digestion rate, as reflected by the cumulative levels of TAA, EAA and leucine over the total 90-min intestinal digestion period, was higher with the whey-containing supplements. Thus, digestive properties of the whey and casein-containing supplements differ, independent from metabolism and feedback mechanisms in the body.

4.2. Effect of caloric density

Interestingly, a significant interaction effect between protein source and caloric density for the peak level of leucine and (essential) amino acids was observed. This means that the effect of calories is even more pronounced for the whey protein supplement than for the casein protein supplement. Most previous studies compared whey and casein products with no added calories from fat and carbohydrates [14,17,18], and only one study used a mixed meal [19]. So far, it has only been suggested that, by comparing between studies, the difference in digestion rate for whey versus casein in a mixed meal is less pronounced than when the proteins are given without added calories [16,19]. In line with our observations, the co-ingestion of carbohydrates with casein protein lowered plasma AA levels in older adults [39], and also delayed digestion and absorption kinetics using intrinsically labelled casein [26]. Only one study in young adults showed lower peak plasma levels of leucine, branched chain amino acids (BCAA) and EAA when whey protein was co-ingested with carbohydrates [40]. Two studies, in young adults with intrinsically labelled milk protein, showed that the addition of carbohydrates and fat delays gastric emptying of milk protein and slows the release of dietary nitrogen to the periphery [27,41]. A larger caloric load is indeed known to slow gastric emptying [24,25], but the impact on digestion and absorption is less well known. *In vitro*, we observed that a higher caloric density increased the amount of casein coagulates, and especially the number of smaller sized particles. In addition to this distinctive gastric behaviour, we observed that a higher caloric density decreased the cumulative release of AA from the whey protein supplements significantly over the total 90-min intestinal digestion period. It is possible that the smaller coagulates facilitated the early phase of intestinal digestion with the high-caloric casein product, as reflected in the initial higher level of small peptides and amino acids. This effect was diminished later in the intestinal phase when coagulates had disappeared. Thus, as well as potentially having an effect on gastric emptying rate *in vivo*, caloric density 'slows' overall protein digestion rate *in vitro*.

4.3. Limitations and alternative explanations

While our data demonstrate that the observed differences in serum amino acid profiles are mediated, at least in part, by the gastrointestinal behaviour of the products, we cannot exclude other metabolic effects that impact on serum amino acid levels. This is relevant as the rate of dietary protein digestion and amino acid absorption from the gut also modulate the postprandial metabolic response [17,42]. This includes a larger inhibition of endogenous

Table 3
Serum amino acid concentrations at baseline and after intake of the study products in healthy older subjects (n = 12).

Parameter	W150	C150	W320	C320	p-Value W150 vs C150	p-Value W150 vs W320	p-Value C150 vs C320	p-Value W320 vs C320
Leucine								
Baseline (μmol/L)	109 ± 6.3	113 ± 6.5	110 ± 6.3	115 ± 5.7				
Maximum (μmol/L)[1]	521 ± 15.1	260 ± 15.4	406 ± 14.5	228 ± 15.2	<0.001	<0.001	0.042	<0.001
iAUC (μmol/L*min)[2]	44,588 ± 1439	22,207 ± 1473	35,952 ± 1386	15,793 ± 1451	<0.001	<0.001	<0.001	<0.001
$t_{1/2}$ (min)[3]	87 ± 5.4	119 ± 5.6	101 ± 5.2	118 ± 5.5	<0.001	0.015	0.867	0.003
EAA								
Baseline (μmol/L)	859 ± 31.2	868 ± 35.0	887 ± 29.1	883 ± 30.6				
Maximum (μmol/L)	2187 ± 57.0	1540 ± 57.5	1420 ± 56.8	1792 ± 55.0	<0.001	<0.001	0.023	<0.001
iAUC (μmol/L*min)	129,793 ± 5690	100,516 ± 5765	75,181 ± 5685	101,181 ± 5471	<0.001	<0.001	<0.001	<0.001
$t_{1/2}$ (min)	83 ± 5.0	115 ± 5.1	117 ± 5.0	94 ± 4.8	<0.001	0.042	0.762	<0.001
TAA								
Baseline (μmol/L)	2786 ± 46.5	2824 ± 37.3	2880 ± 55.1	2848 ± 60.1				
Maximum (μmol/L)	4687 ± 111	3946 ± 111	4141 ± 108	3699 ± 110	<0.001	<0.001	0.003	<0.001
iAUC (μmol/L*min)	162,702 ± 12,979	143,018 ± 12,994	128,047 ± 12,684	105,525 ± 12,872	0.032	<0.001	<0.001	0.008
$t_{1/2}$ (min)	78 ± 5.2	101 ± 5.3	87 ± 4.9	103 ± 5.1	<0.001	0.143	0.769	0.007

EAA, essential amino acids; AA, sum of amino acids.
Study products: leucine-enriched whey protein, 150 kcal (W150); casein protein, 150 kcal (C150); leucine-enriched whey protein, 320 kcal (W320); and casein protein, 320 kcal (C320).
[1]Maximum (or peak) level. [2]Maximum minus baseline difference. [2]Incremental area under the curve. [3]Time to reach half the serum iAUC.
Statistical analysis was done by mixed model including serum albumin, serum CRP, and baseline serum outcome parameter concentration as covariates and subject as random factor.
Baseline values were not different between the study products.

protein breakdown [14,17], and greater splanchnic sequestration [14,17] of casein compared with whey protein. Both may reduce the systemic appearance of amino acids with casein. Similarly, the addition of carbohydrates and fat lowers whole body protein breakdown, which is even more pronounced in combination with whey than with casein protein [17,19]. The observed higher insulin levels after ingestion of the high caloric products could have inhibited protein breakdown to a larger extent [43]. Moreover, added carbohydrates have been shown to reduce protein oxidation [27] and to increase splanchnic sequestration of dietary nitrogen [41,44]. Thus, we cannot exclude that, besides and maybe partly due to distinct gastrointestinal behaviour, inhibition of protein breakdown and increased splanchnic sequestration with casein and higher caloric content have also contributed to the lower serum amino acid levels. Based on the amino levels only, we cannot quantify how many amino acids came directly from the products over time. Another limitation of the *in vivo* study could be that results have been obtained in a healthy group of people that are 65 years and older. Given that the prevalence and incidence of gastrointestinal dysfunction, i.e. in the form of dysmotility, may increase with age and in disease [45], it could be expected that the differences would be augmented in a population that is not healthy.

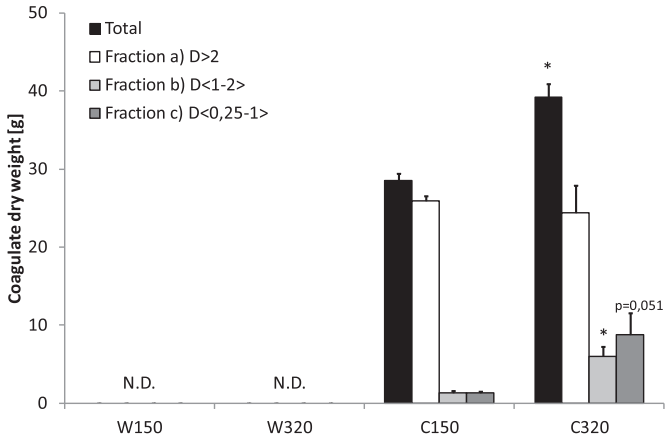

Fig. 4. *In vitro* gastric coagulation. Coagulates formed during the gastric phase and differentiated by their particles size (in mm). Means ± pooled standard error (n = 3). * significantly different from C150 (Student's t-test; $p < 0.05$). N.D – not detected.

In vitro models are an approximation of the dynamic situation *in vivo*. When the conditions of gastric and intestinal phases of *in vitro* digestion are fixed, they make it possible to compare between products for gastric coagulation properties and hydrolysis kinetics. Measurement of digestion and absorption *in vivo* is much more invasive or requires the use of intrinsically labelled protein. In our study, the pH was set to be equal for all products in the intestinal phase. However, the 10 min of neutralization between the gastric and intestinal phases were not sufficient to bring the pH to 6.5; the casein products needed 20 min and the whey products 30 min to reach this value. This difference will likely have negatively impacted the initial protein digestion kinetics, especially of the whey products, since the pH was suboptimal for a longer period. Moreover, it is known that the accumulation of products of digestion in a closed system can limit enzymatic hydrolysis to considerably less than 100%. At the end of the intestinal phase (t90), we estimated that 76% was digested to end products, i.e. the absorbable fraction of <1kD [33]. When comparing *in vitro* and *in vivo* observations, the pattern of cumulative *in vitro* digestion of free AA in our study resembled that of the *in vivo* serum AA profile. Besides the free AA, a large abundance of peptides in the absorbable fraction will also and even faster appear as free AA in blood *in vivo* [46] for which we cannot discriminate its origin. However, we did not observe a difference in the cumulative peptide release *in vitro* between the products in our study. A final limitation of the study is that we cannot discriminate between the effect of calories originating from carbohydrates or from fat.

4.4. Relevance of the data

Postprandial blood levels of EAA and leucine are considered relevant to stimulate muscle protein synthesis [13,14]. With advancing age and disease, the threshold for anabolic stimuli to increase muscle protein synthesis is likely to become higher [8,15]. The high postprandial levels of EAA and leucine that we observed after ingestion of a leucine-enriched whey protein supplement in healthy older subjects, therefore, seems preferable to provoke

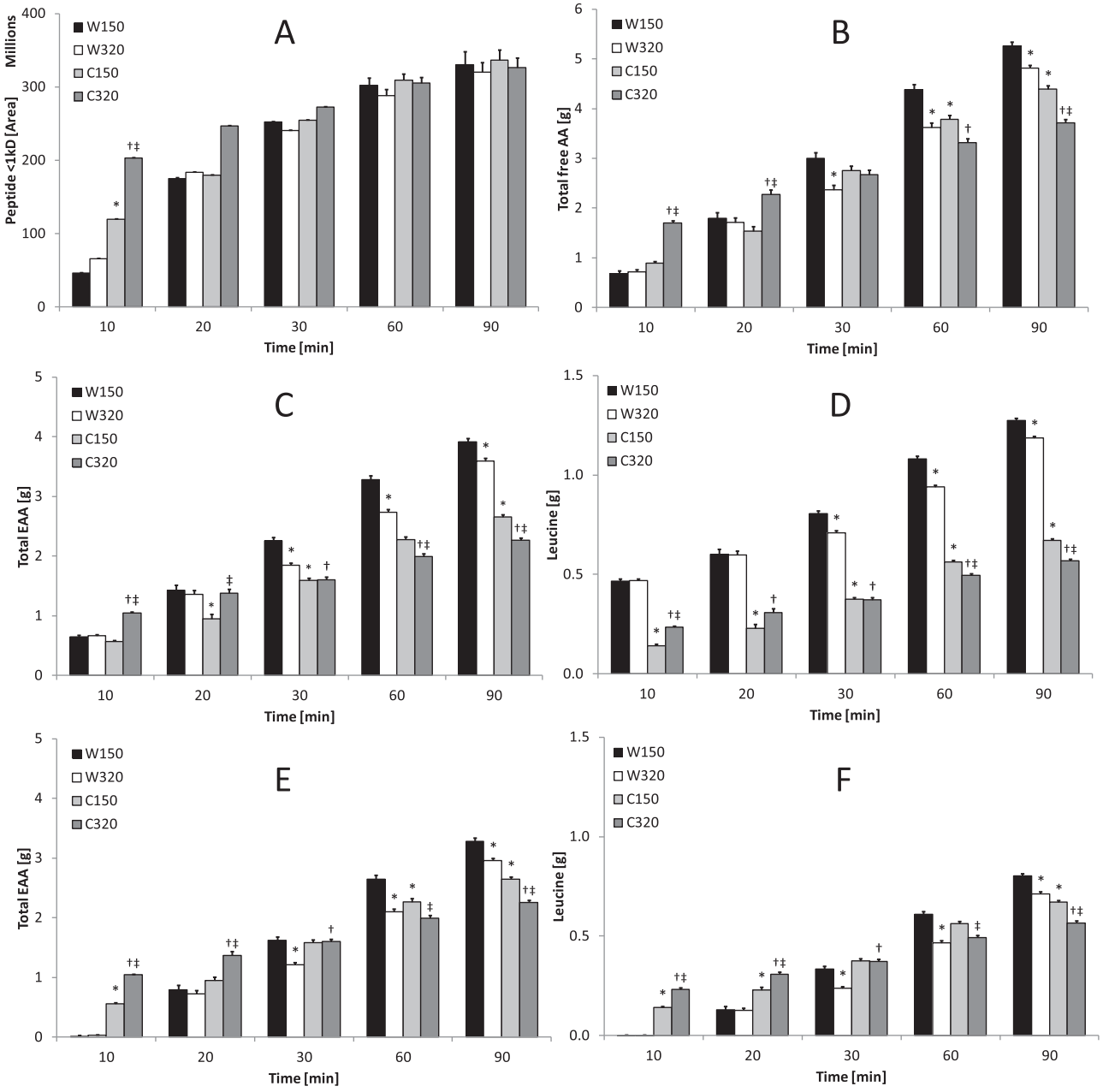

Fig. 5. ***In vitro*** **protein digestion**. Accumulation of protein digestion products formed in the intestinal phase over time: A) peptides smaller than 1kD, B) total free AA, C) total free EAA, D) free leucine, E) total free EAA corrected for the initial difference in free EAA in the supplements, and F) total leucine corrected for the initial difference in free leucine in the supplements. Means ± pooled standard error.* significantly different from W150, † significantly different from W320, ‡ significantly different from C150 (ANOVA univariate analysis with LSD post hoc test and correction for multiple comparisons; $p < 0.0125$). The stomach phase is not shown, since negligible amounts of products were formed in this phase.

subsequent muscle protein synthesis also in older sarcopenic adults. Moreover, a low caloric product favours higher postprandial blood leucine and EAA levels and faster digestion. The impact of energy density on serum amino acid profiles and gastrointestinal digestion is likely relevant for muscle anabolism and subsequent improvement of muscle mass, strength and function in older sarcopenic adults, in order to counteract the negative impact on quality of life, disability and hospitalization.

In conclusion, it was demonstrated in a healthy population of people of 65 years and older, that consumption of a low caloric leucine-enriched whey protein supplement provides a higher rise in serum levels of TAA, EAA and leucine compared to casein or high caloric products. These differences appear to be mediated at least in part by the gastrointestinal behaviour of these nutritional compositions. This effect on postprandial amino acid profiles is promising for stimulation of muscle protein synthesis in sarcopenic older people with a low caloric leucine-enriched whey protein supplement and for potential long-term effects on muscle mass, strength and function. This requires further study.

Statement of authorship

Y.C. Luiking was involved in writing of the protocol, the statistical analysis plan, data interpretation and writing of the manuscript. S. Verlaan initiated the clinical study and was involved in data interpretation. E. Abrahamse and T. Ludwig designed and

supervised the *in vitro* experiments, their analysis and interpretation, and wrote parts of the manuscript. Y Boirie was involved in data interpretation. All authors critically reviewed the manuscript.

Source of funding

This study was financially supported by Nutricia Research, Utrecht, the Netherlands.

Conflict of interest

Y.C. Luiking, E. Abrahamse, T. Ludwig and S. Verlaan are employees of Nutricia Research. Y. Boirie is an advisor for Nutricia Research.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.clnu.2015.02.013.

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