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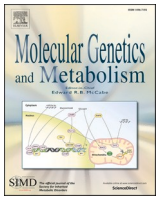

The effects of casein glycomacropeptide on general health status in children with PKU: A randomized crossover trial

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ABSTRACT

In PKU, it is suggested that casein glycomacropeptide based protein substitute (GMP) may have physiological advantage when satiety, oxidative stress, renal function and inflammation are considered. Its prebiotic properties may also help gastrointestinal (GI) tolerance.

In children with PKU, a randomized/crossover trial comparing phenylalanine-free amino acids (AA) vs GMP as the single source of protein substitute for 12-weeks in each arm was conducted. There was a 4-week wash out period with AA in-between. At baseline and end of each intervention, blood and fecal samples were taken to monitor gut health, oxidative stress, renal function, inflammatory markers and plasma amino acids. Satiety and Pediatric Quality of Life (PedsQL) GI symptoms questionnaires were completed. Usual weekly blood spots for phenylalanine and tyrosine were done.

Twelve patients (8 males; aged 4-9y) with PKU participated. GMP improved the following GI symptoms: stomach pain ($p = 0.003$), heartburn and reflux ($p = 0.041$) wind and bloating ($p = 0.018$). With GMP, there was also a trend for less constipation ($p = 0.068$), discomfort with eating ($p = 0.065$) and nausea and vomiting ($p = 0.087$). There were no changes on stool gut health markers (IgA, short chain fatty acids and fecal calprotectin). There were no statistically significant differences for renal, oxidative stress, inflammatory and gut health markers or measures of satiety except for adiponectin ($p = 0.028$) and total antioxidant capacity ($p = 0.049$), although the latter was possibly without clinical significance. Mean dried blood spot phenylalanine (Phe) was 114 μmol/L higher with GMP vs AA ($p < 0.001$). There was no difference in tyrosine levels. In conclusion, GI symptoms statistically significantly improved with GMP versus AA. The Phe content of GMP may present challenges when it is used as the only protein substitute in children with classical PKU with low Phe tolerance.

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1. Background

Phenylketonuria (PKU) is a rare autosomal recessive disorder caused by variants in the phenylalanine hydroxylase (PAH) gene. The PAH enzyme hydroxylates the amino acid phenylalanine (Phe) into tyrosine (Tyr) and requires tetrahydrobiopterin (BH4) as a co-factor [1,2]. Defects in PAH may result in high levels of Phe in blood and body fluids [3]. If PKU remains untreated, it causes global intellectual disability, significant delays in neurodevelopment, hyperactive behavior with autistic features, seizures, and movement problems. The neuropathology of PKU is complex and is associated with impaired cerebral protein synthesis [4,5], white matter disruption [2,3], neurotransmitter and amino acid deficiency in the nervous system [2,3] and reduced cerebral glucose [6]. Early treatment following newborn screening should prevent major cognitive and neurological deficits, resulting in a broad normal range of general ability [7], attainment of expected educational standards and [2,4] an independent lifestyle in adulthood. PKU is heterogenous, depending on enzyme activity and severity may be defined by variant analysis using the BIOPKU database [8].

A Phe-restricted diet is the main treatment strategy. Phe is found in all food protein sources including milk, meat, fish, eggs, cheese, nuts, seeds, soya, wheat and oats. Children with classical PKU (associated with no enzyme activity) usually tolerate less than 10 g/day of natural protein [9]. Consequently, supplementation with a Phe-free/low-Phe protein substitute is essential. Protein substitutes contain a balance of both essential and non-essential amino acids providing up to 70 % of the total nitrogen intake, and almost all Tyr, an indispensable amino acid in PKU due to the inability to convert Phe into Tyr by PAH [9]. Protein substitutes contain large neutral amino acids that compete at the blood brain barrier with Phe, modulating its transport into the brain, preventing the preferential absorption of Phe [10]. The low Phe/Phe-free nitrogen sources in protein substitutes also help promote anabolism, protein synthesis and ultimately helps support optimal growth if given in amounts as recommended by the European PKU guidelines [11]. The amount of protein equivalent from protein substitute may also affect body composition. Sailer et al. reported an inverse correlation between protein equivalent from protein substitute and fat mass [12]. Protein substitutes are traditionally sourced from Phe-free L-amino acids (AA), but in recent years modified low Phe casein glycomacropeptide (GMP), also called caseinomacropeptide has been incorporated into routine practice [10,13,14].

GMP is a small milk-derived bioactive peptide weighing approximately 7 kDa. It is comprised of 64 amino acids in the hydrophilic C-terminal portion of kappa-casein (κ-CN). This peptide is formed by the chymosin (or pepsin) cleavage of κ-casein between Phe105-Met106 during the manufacture of cheese. It undergoes glycosylation with 1:1 different O-linked glycan structures attached primarily to the threonine and serine residues in the peptide [15]. It contains galactose, *N*-acetylgalactosamine, and N-neuraminic acid (the predominant sialic acid found in human cells). Commercially available GMP with a protein content of 78–83.7 % contains 7–9 % of sialic acid [14]. Extraction of GMP from sweet whey involves processes such as ultrafiltration and ion exchange chromatography [16].

It was as early as 1965 [17] that Delfour first established that milk contains a sialic acid bound protein called κ-CN and GMP was made during the manufacture of cheese. It took many more years before GMP was considered as an alternative protein source in PKU. In 2009, the first case report was described of a 29-year-old male with classical PKU given GMP. He had stopped dietary treatment during adolescence which led to spastic quadriparesis and a seizure disorder that was treated with standard anticonvulsant therapy. Dietary treatment was recommenced with the addition of GMP as the primary source of protein. He was given a variety of GMP based beverages, puddings and snack bars and consumed 3 doses/day over 10 weeks. This led to a 13–14 % reduction in blood Phe levels and it gave an early suggestion of GMP's safety and acceptability in PKU [18].

In its pure form, GMP has an unusual amino acid sequence, high in isoleucine, valine, threonine but containing only small/residual amounts of aromatic amino acids (tryptophan, Tyr, Phe), histidine, arginine, leucine and cysteine. All GMP-based protein substitutes designed for PKU do require adaptation of the original unmodified GMP, supplemented with rate limiting amino acids (histidine, leucine, methionine, Tyr, arginine and tryptophan) to ensure their nutritional suitability. There is no consistent amino acid profile (amount of each amino acid per 1 g protein equivalent), and different formulations contain variable amounts of arginine, Tyr, lysine, and valine [13]. GMP-based protein substitutes mainly contain residual Phe between 1.5 and 1.8 mg/g protein equivalent [13]. A patient with PKU taking 60 g/day of protein from GMP supplements (the average adolescent /adult dose) will take an extra 90 to 108 mg Phe/day.

Literature suggests that GMP may have distinct nutritional and health beneficial biological properties including antimicrobial [19–21], anticariogenic [22], prebiotic [23], anti-oxidative, anti-inflammatory [24], and immunoregulatory [25–27]. GMP has shown potential in modulating digestion by its suppression of gastrin-triggered secretion and its impact on gastric motility [21,24]. It is thought to attenuate skeletal fragility [28] and exhibits anti-colorectal cancer properties [24]. Many of the biological and functional properties of GMP have been attributed to sialic acid [29], but others have been linked to the peptide backbone or both the peptide and glycan.

Although many studies have been conducted with GMP in animal and cell models, there is still limited research in patients with PKU (mainly in adults with PKU), particularly examining the potential nutritional, biological, and quality of life (breath malodor, palatability) benefits in PKU [13]. Daly et al. [30] studied the effect of GMP on bone mineral density in children with PKU in a longitudinal study over 36 months. Although the median z-scores were below the population mean, there were no differences in bone mineral apparent density, bone turnover markers and body-less head body mass density between GMP and AA. In the same study, there was a trend for patients to be taller, having an improved lean body mass and decreased fat mass when using GMP as their single source of protein equivalent, even though this did not reach statistical significance. GMP is associated with better palatability, less breath malodor and improved satiety in patients with PKU [31,32]. However, Tiele et al. [33] found similar amounts of volatile organic compounds in breath samples of 20 children with PKU and matched controls comparing GMP and AA, although 95 % of patients with PKU noticed that their breath was worse with AA compared to GMP based protein substitute. A positive effect on gut microbiota has been found with GMP in patients with PKU with a possible prebiotic role on specific taxa and promoting bacterial diversity and systemic health [34].

In this randomized, controlled, crossover study in children with PKU we aimed to investigate if a GMP-based protein substitute compared to L-AA supports any long-term health benefits particularly concerning gut health, inflammation, renal function, oxidative stress and impact on blood Phe levels in patients with PKU.

2. Methods

2.1. Study product

The GMP based study product was powdered PKU sphere™ (Vitaflo International Ltd.®, UK) produced with Lacprodan® CGMP-20 (Arla Foods Ingredients Group P/S, DK). PKU sphere™ is supplemented with essential and non-essential amino acids, carbohydrates, vitamins and minerals, trace elements and docosahexaenoic acid (DHA). It was available in 2 pack sizes providing 15 g or 20 g of protein equivalent with a red berry, vanilla or chocolate flavor. Table 1 describes the amino acid, micro- and macronutrient composition of the GMP product and the usual AA protein substitutes used in this study.

Table 1
Nutritional and amino acid composition of the study product (GMP; PKU sphere powder; Vitaflo International Ltd.®) and AA-based protein substitutes prescribed to patients.

Energy, macronutrients and micronutrients	Protein substitutes used in the randomized controlled trial				
	GMP PKU sphere 20 35 g powder	AA PKU Gel 10 24 g powder	AA PKU Cooler 20 174 mL	AA PKU Air 20 174 mL	AA PKU Lophlex LQ 20 125 mL
Energy (kcal)	120	76	130	100	120
Protein (g)	20	10	20	20	20
Carbohydrate (g)	6.3	8.9	8.9	2.6	8.8
Sugar (g)	2.5	4.8	5.9	0.3	8.8
Fat (g)	1.6	0.02	1.6	1.0	0.44
Vitamin A (μg)	259	144	261	261	285
Vitamin D (μg)	5.0	3.5	10	10	8
Vitamin E (mg)	5.3	2.2	5.2	5.2	3.2
Vitamin K (μg)	23	9.8	24	24	25
Vitamin C (mg)	29	15	37	37	17.8
Thiamin (mg)	0.6	0.24	0.70	0.70	0.42
Riboflavin (mg)	0.6	0.29	0.77	0.77	0.5
Niacin (mg)	3.2	3.4	3.5	3.5	7.1
Vitamin B6 (mg)	0.6	0.26	0.87	0.87	0.58
Folic acid (μg)	102	50	101	101	120
Vitamin B12 (μg)	1.6	0.48	1.6	1.6	1.8
Biotin (μg)	13	6.0	13	13	53.4
Pantothenic acid (mg)	2.0	1.2	1.9	1.9	1.8
Sodium (mg)	283	91	104	104	<25
Potassium (mg)	234	225	244	244	100
Chloride (mg)	7.0	140	139	139	<25
Calcium (mg)	399	260	400	400	356
Phosphorus (mg)	413	198	357	365	276
Magnesium (mg)	115	40	110	110	107
Iron (mg)	7.4	3.4	7.3	7.3	5.3
Zinc (mg)	7.4	2.6	5.6	5.6	3.9
Copper (mg)	0.60	0.19	0.73	0.73	0.53
Manganese (mg)	0.4	0.41	0.50	0.50	0.53
Selenium (μg)	30	8.4	26	26	26.7
Chromium (μg)	12	17	14	14	10.6
Molybdenum (μg)	20	12	23	23	25
Iodine (μg)	84	33.1	85	85	58.4
Choline (mg)	200	67	200	200	152
		Amino acids			
Phenylalanine (mg)	36	0	0	0	0
Alanine (g)	0.83	0.43	0.92	0.92	1.16
Arginine (g)	0.96	0.70	1.50	1.50	2
Aspartic acid (g)	1.31	1.10	2.37	2.37	1.75
Cystine (g)	0.24	0.29	0.61	0.61	0.51
Glutamine (g)	2.70	0.86	0	0	0
Glycine (g)	0.71	1.10	2.35	2.35	1.88
Histidine (g)	0.70	0.43	0.92	0.92	0.79
Isoleucine (g)	1.42	0.74	1.62	1.62	1.4
Leucine (g)	3.02	1.13	2.54	2.54	2.13
Lysine (g)	0.95	0.77	1.67	1.67	1.63
Methionine (g)	0.28	0.22	0.45	0.45	0.34
Proline (g)	1.60	0.79	1.69	1.69	2
Serine (g)	1.01	0.48	1.04	1.04	1.09
Threonine (g)	2.29	0.77	1.62	1.62	1.04
Tryptophan (g)	0.40	0.24	0.50	0.50	0.41
Tyrosine (g)	2.25	1.10	2.38	2.38	1.88
Valine (g)	1.14	0.86	1.86	1.86	1.38

Abbreviations: GMP, glycomacropeptide-based protein substitute; AA, L-amino acid-based protein substitute; PKU, phenylketonuria.

2.2. Patient selection

Continuously treated children with PKU, diagnosed by newborn screening, aged between 4 and 12 years were eligible to participate in the study. The inclusion criteria were: 1) taking more than half of the total daily protein intake provided by a protein equivalent from a protein substitute and 2) a dietary Phe tolerance including fruit and vegetables and special low protein sources of ≤1000 mg/day (≤20 g/day from natural protein). Prior to study entry, 3 of 4 consecutive blood Phe levels were within the European PKU Guidelines (2017) target therapeutic range (120–360 μmol/L) [11]. At the study entry, patients were naïve to GMP and historically had only been prescribed AA based protein substitutes.

Exclusion criteria included: previous use of GMP and concomitant diseases/disorders such as renal, gastrointestinal (GI) and diabetes. Patients being treated with adjunct therapies (e.g. sapropterin) and diagnosed with food allergies or intolerances (e.g. soya, milk, fish, nut, wheat, and lactose) were also excluded.

2.3. Study design

Fig. 1 shows the detailed study design. This was a randomized, controlled, crossover trial 32 weeks in duration. During the 28-day screening period, one daily dose of GMP protein substitute was trialled for 3 days to test tolerance and acceptability. Patients were then randomly allocated into two arms:

- **Arm A:** 12-week intervention period with usual AA with a 4-week wash out period with the same AA based protein substitute, followed by a 12-week intervention period with GMP based protein substitute as their single protein substitute source.
- **Arm B:** 12-week intervention period with GMP based protein substitute with a 4-week wash out period with pre-study AA based protein substitute, followed by a 12-week intervention period with the same AA formula as their single protein substitute source.

The participants and caregivers were only informed of the treatment arm assignment before baseline of the first intervention. People assessing the outcomes were blinded to the participants` group assignments.

The dose of protein equivalent from protein substitute was standardized for each subject (3 or 4 doses/day taken at standard times, the same as before entering the study). The prescribed intake of dietary Phe was consistent during the study. However, Phe intake from food was decreased (50 to 100 mg/day) by the patient's dietitian if there was a consistent increase in blood Phe levels above the upper therapeutic target range for more than 2 consecutive levels, unrelated to illness.

A hospital study visit was performed for screening (day −31), baseline/commencement of first intervention (day 0), end of first intervention (day 84), second intervention baseline (day 112) and end of second intervention (day 196). During the screening visit, eligibility of study participants was confirmed, and medical assessments were performed. During each visit, the following assessments were done:

1). fasting venous samples to analyze nutritional status, endocrine, inflammation, renal, oxidative stress and gut health markers;
2). questionnaires to assess satiety and GI symptoms (Pediatric Quality of Life Inventory [PedsQL] GI Symptom Scale) [35];
3). product acceptability (at the end of each intervention);
4). medical history and medications;
5). anthropometric measurements (height and weight) assessed by trained and experienced dietitians using a calibrated scale (Seca, Medical Measuring Systems and Scales, UK—Model 875) and a stadiometer (Seca 360 Wireless Wall-Mounted Stadiometer Height Measure). UK-WHO growth charts were used to calculate z-scores [36].

During each study visit, after collection of venous blood samples, a standard breakfast chosen by each patient was provided and repeated at each visit. A satiety questionnaire (Teddy Bear Visual Analogue Scale) [37] was completed by the children before breakfast and then every 15 min for 2 h post breakfast.

During each intervention, blood Phe and Tyr were monitored weekly

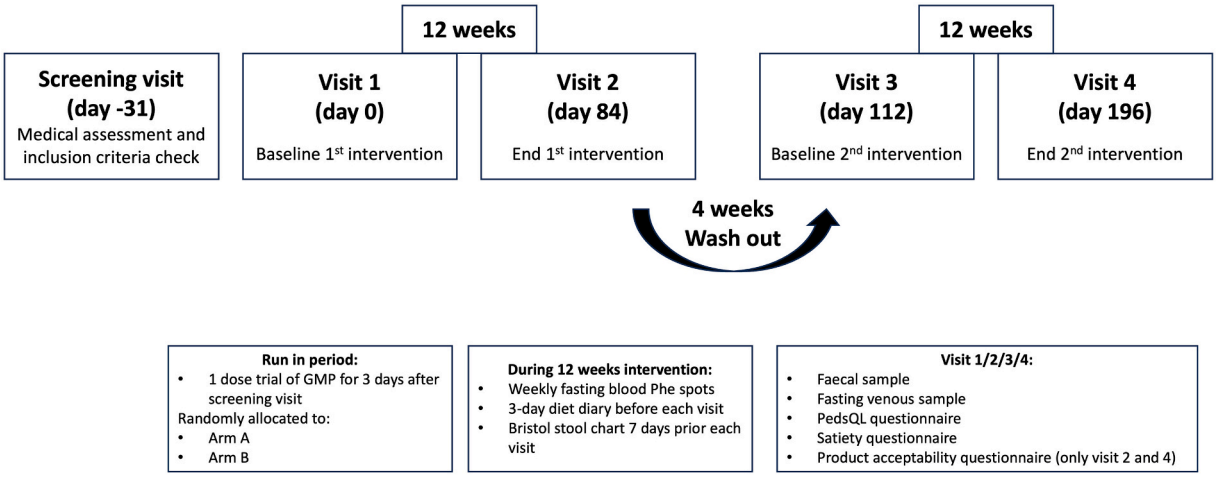

Fig. 1. Study design. Abbreviations: AA, amino acid-based protein substitute; GMP, glycomacropeptide based protein substitute; Phe, phenylalanine; PedsQL, pediatric quality of life gastrointestinal symptoms scale.

by home blood spots. A 3-day food diary was performed before each study visit, together with a Bristol Stool Form Scale [38] recorded by the parents/caregivers for 7 days prior to the study visit. Daily protein substitute adherence forms were completed by caregivers during the study.

Fecal samples were also collected by the participants a maximum of 72 h prior to each study visit (Visit 1/2/3/4). They were given a fecal sample collection kit composed of gloves, fecal sample collector paper that could be placed in the toilet, tubes with an incorporated scoop for fecal collection, ice packs and zip-lock cooling bags. Two tubes were filled with approximately 300 mg of fecal samples, placed in the provided zip-lock bag, and stored immediately after collection in the freezer at −18 to −20 °C together with ice packs. They were then transported in the zip-locked cold bag with ice packs that were provided to keep the samples cold in transit.

2.4. Data collection

2.4.1. Blood spot samples, venous and fecal samples

Fasting blood spots for Phe and Tyr were performed by caregivers weekly for each 12-week intervention. Caregivers were trained by metabolic pediatric nurses on blood spot collection. One central lab performed blood spot card analysis by tandem mass spectrometry. At day 0, 84, 112 and 196, fasting venous blood samples were performed and fecal samples collected (72 h prior to each study visit) to analyze data on gut markers (blood sample: immunoglobulin A [IgA]; fecal sample: acetate, propionate, isovalerate, butyrate, methylbutyrate, valerate, fecal calprotectin and total short chain fatty acids [SCFA]). From venous samples the following markers for nutritional and biochemical status were performed: plasma amino acids, albumin, prealbumin, transferrin, retinol binding protein (RBP), glucose, hemoglobin A1C (HbA1c), insulin, insulin-like growth factor 1 (IGF-1) and C-peptide, inflammation markers (adiponectin, myeloid-related protein [MRP], sCD25 and C-reactive protein [CRP]), renal function (urea, creatinine, cystatin C) and oxidative stress (total antioxidant reactivity [mM trolox equivalent], glutathione [GSH] and malondialdehyde [MDA]). All the venous and fecal samples were analyzed by Great Ormond Street Hospital for Children NHS Foundation Trust Laboratory, Great Ormond Street, London, UK.

2.4.2. Food diaries and questionnaires on GI symptoms, satiety and product acceptability

Age-appropriate versions of the PedsQL (5–7 years and 8–12 years) were completed at each visit (day 0, 84, 112 and 196) [35]. PedsQL assessed the following domains regarding GI symptoms: stomach pain, discomfort when eating, trouble swallowing, heartburn and reflux, nausea and vomiting, wind and bloating, constipation, blood in bowel movements and diarrhea, at the beginning and end of each study intervention. Several items of each domain were analyzed with a total of 74 items. The domain '*food and drinks restriction*' was not applicable to PKU due to the nature of dietary management. A 5-point Likert response scale was used in each domain answering each sentence with 0 = "*never*", 1 = "*almost never*", 2 = "*sometimes*", 3 = "*often*" or 5 = "*almost always*". A higher score in each domain indicated an improvement with less symptoms.

At each study visit, a validated satiety questionnaire based on a validated "teddy bear" visual analogue scale [37] was completed with children every 15 min for 2 h pre- and post- standardized breakfast. No further food or drink (except water) was permitted in this 2-h window. The children rated their satiety on a 5-point Likert scale as follows: 1= "*I am really hungry, my belly feels very empty and is rumbling*", 2=" *I am quite hungry and my belly feels a little empty*", 3= "*I feel just right, not too hungry and not too full*", 4 = "*I am quite full, but there is still a little room in my belly*", and 5= " *I am not hungry at all! My belly feels very full and I cannot eat any more food*!".

Prior to each clinic visit, 3-day food diaries including 2 weekdays and 1 weekend day were collected to obtain detailed data on dietary intakes. All records were checked by a research dietitian (A.P.), and daily energy, macro- and micronutrient intakes were analyzed by the same dietitian using the software *Nutritics*® (v5.09*, Dublin, Ireland). Nutritional information on amino acid content of foods and protein substitutes were added into the software manually.

A product acceptability questionnaire including five domains (smell, taste, aftertaste, texture and packaging) evaluating protein substitutes was developed by the authors and performed at the end of each intervention. Participants were asked to rate each domain using a 5-point Likert scale (0= "*I really didn't like it*", 1= "*I didn't like it*", 2= "*I neither liked nor disliked it*", 3= "*I liked it*" or 4= "*I loved it*". Difficulties in preparation and consumption of the protein substitutes were also assessed ranging from "0= *very difficult*", to "4= *very easy*".

2.5. Statistical analysis

A clinically relevant difference in blood Phe was characterized as an effect size of 0.6 (equivalent to observing a difference in blood Phe levels of 60 μmol/L between the two dietary therapies based on a standard deviation of 150 μmol/L). Based on a one-sided alpha level of 0.05 and a power of 80 %, there was a recruitment target of 16 participants.

Table 2
Mean daily dietary intakes of participants during each study period (AA vs. GMP).

Daily dietary Intakes	Type of PS		*P*
	AA[a]	GMP[b]	
	Median (range)	Median (range)	
Energy (kcal)	1762 (1298–1677)	1646 (1544–2233)	0.76
Protein (g)	65 (59–69)	65 (63–69)	0.17
Protein (% of energy)	16 (15–19)	16 (13–18)	1.00
Natural protein (g)	9 (7–11)	8 (8–10)	0.90
Protein equivalent from PS (g)	56 (45–60)	56 (45–60)	0.14
Phe from foods (mg)	453 (346–567)	389 (363–490)	0.66
Extra Phe from PS (mg)	–	102 (90–106)	0.91
Tyr from PS (mg)	6358 (5310–7140)	6368 (5873–7125)	0.47
Carbohydrates (g)	232 (182–240)	272 (240–315)	0.34
Carbohydrates (% of energy)	59 (54–61)	57 (55–60)	0.68
Fat (g)	50 (36–57)	51 (47–76)	0.83
Fat (% of energy)	26 (22–27)	27 (25–32)	0.17

Abbreviations: AA, L-amino acid-based protein substitute; GMP, glycomacropeptide based protein substitute; PS, protein substitute; Phe, phenylalanine; Tyr, tyrosine.

[a] The AA protein substitutes used were: PKU Gel (Vitaflo®), PKU Cooler (Vitaflo®), PKU Air (Vitaflo®), PKU Lophlex LQ (Nutricia®).

[b] PKU Sphere (Vitaflo®).

Randomization lists were produced by a statistician external to the study prior to the recruitment of the first participant. Participants were randomized using a 1:1 ratio. The list was produced based on the principle of randomly permuted blocks with random block sizes of 2 and 4.

All statistical analyses were performed with R (version 3, R Foundation for Statistical Computing, Vienna, Austria). A p-value <0.05 was used to define statistical significance and results are reported with 95 % confidence intervals. Median (interquartile range [IQR]) or mean (±standard deviation [SD]) were used to summarize data depending on normality tests. Regression analysis techniques were used including period and randomization of the group as well as therapy effect. Longitudinal mixed modelling was used to compare blood Phe measurements during interventions.

2.6. Ethical aspects

Informed consent was obtained from parents/caregivers prior to starting the study and age-appropriate assents for participating patients. The principles of the "Declaration of Helsinki" (52nd WMA General Assembly, Edinburgh, Scotland, 3–7 October 2000) and Good Clinical Practice guidelines were used to conduct this study. The project was registered, and the protocol is available at clinicaltrials.gov with the ID: NCT04076176.

Approval to conduct the study was given on the 20th February 2019 by the North West - Liverpool East Research Ethics Committee with the Integrated Research Application System (IRAS) number 239520 and reference 19/NW/0032.

3. Results

3.1. Participants

Twelve patients ($n = 8$ males; 67 %) were recruited from 2 study sites between July 2019 and April 2022. The median age was 6.5 years (range: 4–9 years). Eleven patients were recruited from Birmingham Children's Hospital and one patient from Bristol Children's Hospital. Five subjects commenced the AA intervention followed by the GMP (Arm A); 7 subjects commenced GMP followed by the AA (Arm B). All subjects completed the study.

Ethnic origins of patients were Eastern European ($n = 6$), Asian ($n = 4$), mixed race ($n = 1$), and white, British ($n = 1$). All were identified by newborn screening and median diagnostic blood Phe was 1460 μmol/L

Table 3
Results of all symptoms analyzed on the stomach pain scale comparing AA vs. GMP.

Symptoms		Arm A: AA followed by GMP Median (IQR)	Arm B: GMP followed by AA Median (IQR)		Overall	*P*
Feels pain in stomach	Baseline of 1st intervention	50 [50–50]	50 [50–50]	AA	50 [50–75]	**0.009***
	End of 1st intervention	50 [25–75]	100 [75–100]			
	Baseline of 2nd intervention	25 [0–75]	50 [50–63]	GMP	100 [75–100]	
	End of 2nd intervention	100 [75–100]	50 [50–75]			
Gets stomach aches	Baseline of 1st intervention	50 [50–50]	50 [50–75]	AA	50 [50–75]	**0.010***
	End of 1st intervention	50 [25–75]	100 [75–100]			
	Baseline of 2nd intervention	25 [0–75]	50 [50–63]	GMP	100 [69–100]	
	End of 2nd intervention	100 [75–100]	50 [50–63]			
Stomach hurts	Baseline of 1st intervention	50 [50–75]	50 [50–75]	AA	50 [50–75]	**0.003***
	End of 1st intervention	50 [25–75]	100 [75–100]			
	Baseline of 2nd intervention	25 [0–75]	50 [50–88]	GMP	100 [94–100]	
	End of 2nd intervention	100 [100–100]	50 [50–63]			
Wakes up at night with stomach aches	Baseline of 1st intervention	100 [75–100]	100 [100–100]	AA	100 [75–100]	0.119
	End of 1st intervention	100 [75–100]	100 [100–100]			
	Baseline of 2nd intervention	100 [75–100]	100 [100–100]	GMP	100 [100–100]	
	End of 2nd intervention	100 [100–100]	100 [88–100]			
Has an uncomfortable feeling in stomach	Baseline of 1st intervention	50 [25–100]	50 [50–100]	AA	50 [50–81]	**0.013***
	End of 1st intervention	50 [50–100]	100 [75–100]			
	Baseline of 2nd intervention	50 [25–50]	50 [50–88]	GMP	100 [75–100]	
	End of 2nd intervention	100 [75–100]	50 [50–63]			
Gets an upset stomach	Baseline of 1st intervention	50 [25–50]	50 [50–88]	AA	50 [50–100]	0.104
	End of 1st intervention	50 [25–50]	100 [75–100]			
	Baseline of 2nd intervention	25 [0–75]	50 [50–88]	GMP	100 [69–100]	
	End of 2nd intervention	100 [75–100]	75 [50–100]			
Total score for stomach pain	Baseline of 1st intervention	50 [50–58]	58 [58–81]	AA	69 [56–76]	**0.003***
	End of 1st intervention	58 [33–79]	100 [79–100]			
	Baseline of 2nd intervention	38 [17–75]	67 [58–77]	GMP	100 [80–100]	
	End of 2nd intervention	92 [83–100]	71 [63–75]			

Arm A: AA followed by GMP; Arm B: GMP followed by AA.
A Likert scale from 0 (never a problem) to 5 (almost always a problem) was translated to a value of 0 to 100. Higher scores show improvement in symptoms and less problems. Fisher's and Wilcox test compared differences between AA and GMP for individual questions. *$p < 0.05$.
Abbreviations: AA, amino acid-based protein substitute; GMP, glycomacropeptide base protein substitute; IQR, interquartile range.

(range:1039–1950 μmol/L). The dietary treatment started at a median of 9 days (range: 8–12 days). Most patients (83 %; $n = 10$) were classified by variant analysis as classical PKU. The remaining two patients had mild PKU.

3.2. Dietary intakes and protein substitute adherence

All patients took their daily dose of protein substitute as prescribed, except for one patient who took 75 % of the prescribed dose on day 3 of the intervention period due to falling asleep before the final dose was completed. Dietary intakes remained unchanged during the study period (Table 2).

3.3. PedsQL gastrointestinal symptoms scale

A Likert scale from "0=never a problem" to "5=almost always a problem" was used to assess stomach pain, discomfort when eating, trouble swallowing, heartburn and reflux, nausea and vomiting, wind and bloating, constipation, blood in bowel movements and diarrhea. This was translated to frequency of counts with values from 0 to 100 for each symptom, whereby 0 shows "*almost always a problem*" (no improvement in GI symptoms) and 100 shows "*never a problem*" (improvement in GI symptoms and less issues).

3.3.1. Stomach pain score

The overall total stomach pain score was significantly higher with GMP (i.e., less pain) than AA (GMP: 100; IQR: 80.2–100 vs. AA: 68.8; IQR: 56.2–76.0; $p = 0.003$) (Table 3). Eight of 12 participants showed individual scores which were at least 10 points higher on GMP, indicating lower stomach pain symptoms. Supplementary Fig. 1 presents stomach pain scores comparing AA vs. GMP at each of the intervention study visits.

3.3.2. Discomfort when eating

There was a trend for less "*discomfort when eating*" with GMP compared with AA (total score for GMP: 100; IQR: 90–100 vs. AA: 88; IQR: 70–93; $p = 0.065$). Forty-two per cent of patients ($n = 5/12$) scored 10 points higher with GMP. However, the only individual symptom of this domain "*discomfort when eating*" that showed a clear improvement was "*feels full as soon as he/she starts to eat*" ($p = 0.035$) (Supplementary Table 1).

3.3.3. Trouble swallowing

There were no differences found in scores of "*trouble swallowing*" (GMP: 100; [IQR: 100–100] vs. AA: 100 [IQR: 79–100]; $p = 0.153$) (Supplementary Table 2) and subdomain scores.

3.3.4. Heartburn and reflux

Overall, patients on GMP had a significantly higher total score (associated with less symptoms) compared with AA (median: 94 [IQR: 81–100] vs. 81 [IQR: 73–88, respectively; $p = 0.041$) (Supplementary Table 3). Fifty per cent of patients ($n = 6/12$) reported a score of at least

Table 4
Scores for the specific symptoms of the "*wind and bloating*" domain are presented during the study comparing AA vs. GMP.

Symptoms		Arm A: AA followed by GMP Median (IQR)	Arm B: GMP followed by AA Median (IQR)	Overall		*P*
Stomach feels full of wind	Baseline of 1st intervention	0 [0–75]	50 [13–50]	AA	50 [25–50]	0.265
	End of 1st intervention	25 [0–50]	100 [50–100]			
	Baseline of 2nd intervention	25 [0–50]	75 [50–100]	GMP	63 [44–100]	
	End of 2nd intervention	25 [0–75]	50 [50–75]			
Stomach feels very full	Baseline of 1st intervention	25 [0−100]	25 [13–63]	AA	25 [25–63]	**0.013***
	End of 1st intervention	25 [25–100]	100 [50–100]			
	Baseline of 2nd intervention	25 [25–50]	50 [38–63]	GMP	88 [50–100]	
	End of 2nd intervention	75 [75–100]	25 [25–50]			
Stomach gets big and hard	Baseline of 1st intervention	50 [25–50]	100 [38–100]	AA	50 [44–100]	**0.020***
	End of 1st intervention	50 [25–50]	100 [75–100]			
	Baseline of 2nd intervention	100 [25–100]	50 [38–75]	GMP	100 [94–100]	
	End of 2nd intervention	100 [100–100]	75 [50–100]			
Has a lot of wind	Baseline of 1st intervention	0 [0–25]	25 [0–50]	AA	0 [0–50]	0.074
	End of 1st intervention	0 [0–0]	75 [25–100]			
	Baseline of 2nd intervention	0 [0–50]	0 [0–38]	GMP	63 [0–100]	
	End of 2nd intervention	50 [0–100]	25 [0–50]			
Passes a lot of wind	Baseline of 1st intervention	25 [0–25]	25 [0–38]	AA	0 [0–50]	0.119
	End of 1st intervention	0 [0–0]	75 [25–88]			
	Baseline of 2nd intervention	0 [0–50]	0 [0–38]	GMP	50 [19–81]	
	End of 2nd intervention	25 [25–50]	25 [0–50]			
Stomach feels windy	Baseline of 1st intervention	25 [0–100]	25 [13–50]	AA	50 [0–75]	0.074
	End of 1st intervention	0 [0–50]	100 [50–100]			
	Baseline of 2nd intervention	0 [0–50]	0 [0–38]	GMP	75 [50–100]	
	End of 2nd intervention	50 [25–100]	50 [38–88]			
Stomach makes noises	Baseline of 1st intervention	25 [0–50]	50 [25–50]	AA	50 [19–57]	**0.015***
	End of 1st intervention	0 [0–50]	50 [50–100]			
	Baseline of 2nd intervention	50 [0–50]	50 [13–50]	GMP	63 [50–100]	
	End of 2nd intervention	75 [50–100]	50 [38–63]			

		Arm A: AA followed by GMP Median (IQR)	Arm B: GMP followed by AA Median (IQR)	Overall		*P*
Total score for wind and bloating	Baseline of 1st intervention	28 [14–46]	39 [21–57]	AA	38 [27–51]	**0.018***
	End of 1st intervention	21 [21–39]	86 [46–98]			
	Baseline of 2nd intervention	21 [21–46]	32 [25–50]	GMP	70 [41–97]	
	End of 2nd intervention	54 [36–89]	39 [36–55]			

Arm A: AA followed by GMP; Arm B: GMP followed by AA.
Fisher's and Wilcox test compared differences between AA and GMP for individual questions. *$p < 0.05$.
Abbreviations: AA, amino acid-based protein substitute; GMP, glycomacropeptide base protein substitute; IQR, interquartile range.

10 points higher on GMP. Only one item under this domain, "*burps a lot*", significantly improved with GMP (median: 75 [IQR: 44–100]) compared to AA (median: 38 [IQR: 25–50]; $p = 0.025$). Supplementary fig. 2 presents overall scores for the domain "*heartburn and reflux*".

3.3.5. Nausea and vomiting

The median total score was higher with GMP (GMP: 100 [IQR: 84–100] vs. AA: 84 [IQR: 59–95]) for nausea and vomiting, however, the difference was not statistically significant ($p = 0.087$) (Supplementary Table 4). Fifty-eight per cent ($n = 7/12$) had more than 10 points improvement with GMP. There was a significant improvement in "*feels like throwing up when he/she eats*" on GMP (median: 100 [IQR: 100–100]) compared with AA (median: 75 [IQR: 50–100] ($p = 0.019$).

3.3.6. Wind and bloating

Table 4 shows specific symptoms from the domain of "*wind and bloating*" in all the study visits, and Supplementary Fig. 3 compares the scores obtained with AA and GMP during the study. Seventy-five per cent ($n = 9/12$) reported a score at least 10 points higher on GMP than on AA associated with less wind and bloating.

3.3.7. Constipation

There was a trend for improved constipation symptoms with GMP (median: 86 [IQR: 80–93]) compared with AA (median: 71 [IQR: 56–88]) ($p = 0.068$) (Supplementary Table 5). The item "*spends lot of time on the toilet having a bowel movement*" improved with GMP (GMP: 100 [IQR: 100–100] vs. AA: 75 [IQR: 50–100]); $p = 0.041$). There was also a trend for improvement with "*has to push hard to have a bowel movement*" (GMP: 75 [IQR: 50–100] vs. AA: 50 [IQR: 25–81]; $p = 0.064$). Overall, 75 % ($n = 9/12$) scored at least 10 points higher with GMP ($p < 0.001$).

One patient who had been using laxatives for constipation for two years prior to entering this study was able to stop after commencing GMP.

Table 5
Differences in gut health markers comparing each hospital visit and overall differences between AA and GMP.

Gut health markers	Hospital visit	Arm A: AA followed by GMP Median (IQR)	Arm B: GMP followed by AA Median (IQR)		Overall	Estimate (SD)	*P*
IgA^{+} (g/L)	Baseline of 1st intervention	1.16 [1.12–1.21]	1.20 [0.93–1.34]	AA	1.36 [1.03–1.44]	−0.02 (0.05)	0.698
	End of 1st intervention	1.40 [1.36–1.44]	1.12 [0.89–1.32]				
	Baseline of 2nd intervention	1.44 [1.39–1.50]	1.32 [0.88–1.39]	GMP	1.29 [1.05–1.45]		
	End of 2nd intervention	1.40 [1.36–1.52]	1.11 [0.95–1.46]				
Acetate* (mmol/g)	Baseline of 1st intervention	95.70 [34.50–98.20]	107.90 [51.26–115.54]	AA	120 [63.93–156.75]	−14.13 (21.2)	0.508
	End of 1st intervention	120.00 [95.60–135.90]	112.69 [86.89–122.31]				
	Baseline of 2nd intervention	73.90 [50.10–93.50]	75.31 [47.90–98.16]	GMP	107.58 [72.85–126.91]		
	End of 2nd intervention	95.50 [61.90–130.00]	132.00 [48.45–199.40]				
Propionate* (mmol/g)	Baseline of 1st intervention	12.50 [11.053–18.3]	21.61 [14.84–32.69]	AA	22.80 [13.33–33.46]	2.22 (4.54)	0.626
	End of 1st intervention	22.80 [17.901–30.2]	27.17 [20.26–34.22]				
	Baseline of 2nd intervention	26.80 [15.25–32.8]	20.69 [13.47–35.87]	GMP	22.47 [20.16–28.64]		
	End of 2nd intervention	21.22 [20.8–24.3]	24.71 [11.10–43.16]				
Isovalerate* (mmol/g)	Baseline of 1st intervention	2.00 [0.73–3.20]	4.35 [2.76–4.95]	AA	2.75 [1.45–3.27]	−0.1 (0.62)	0.878
	End of 1st intervention	1.98 [1.10–2.75]	2.935 [2.35–4.27]				
	Baseline of 2nd intervention	2.60 [1.85–3.30]	1.75 [1.47–2.36]	GMP	2.30 [1.10–3.47]		
	End of 2nd intervention	1.20 [1.00–1.90]	2.86 [2.04–3.81]				
Butyrate* (mmol/g)	Baseline of 1st intervention	11.30 [9.32–13.50]	14.61 [7.83–22.30]	AA	14.70 [9.69–24.00]	−0.83 (4.02)	0.837
	End of 1st intervention	14.50 [12.83–23.90]	17.65 [15.15–21.90]				
	Baseline of 2nd intervention	14.30 [8.74–14.30]	10.17 [9.06–17.72]	GMP	16.61 [12.63–21.54]		
	End of 2nd intervention	16.55 [10.60–20.10]	19.40 [8.59–32.95]				
2-methylbutyrate* (mmol/g)	Baseline of 1st intervention	2.30 [1.10–2.70]	4.23 [2.94–4.99]	AA	2.60 [2.06–3.01]	−0.07 (0.58)	0.899
	End of 1st intervention	2.12 [2.10–2.60]	3.12 [1.88–4.46]				
	Baseline of 2nd intervention	2.01 [2.00–3.00]	1.654 [1.53–2.41]	GMP	2.00 [1.00–3.65]		
	End of 2nd intervention	1.00 [1.00–2.00]	2.99 [2.23–3.33]				
Isovalerate* (mmol/g)	Baseline of 1st intervention	2.00 [0.73–3.20]	4.35 [2.76–4.95]	AA	2.75 [1.45–3.28]	−0.1 (0.62)	0.878
	End of 1st intervention	1.98 [1.10–2.75]	2.94 [2.35–4.27]				
	Baseline of 2nd intervention	2.60 [1.85–3.30]	1.75 [1.47–2.36]	GMP	2.30 [1.10–3.47]		
	End of 2nd intervention	1.20 [1.00–1.90]	2.86 [2.04–3.81]				

Gut health markers	Hospital visit	Arm A: AA followed by GMP Median (IQR)	Arm B: GMP followed by AA Median (IQR)		Overall	Estimate (SD)	*P*
Valerate* (mmol/g)	Baseline of 1st intervention	0.37 [0.30–2.45]	2.97 [2.30–4.09]	AA	2.01 [1.49–2.77]	0.58 (0.46)	0.206
	End of 1st intervention	1.38 [1.10–1.60]	3.37 [2.63–3.93]				
	Baseline of 2nd intervention	0.88 [0.50–1.39]	2.04 [0.98–3.23]	GMP	3.10 [2.29–3.64]		
	End of 2nd intervention	3.10 [2.10–3.20]	2.77 [2.07–3.70]				
Fecal Calprotectin* (ug/g)	Baseline of 1st intervention	63 [46, 85]	82 [42, 121]	AA	15 [15, 47]	−32.9 (38.86)	0.401
	End of 1st intervention	47 [38, 57]	15 [15, 15]				
	Baseline of 2nd intervention	33 [15, 119]	15 [15, 34.5]	GMP	15 [15, 15]		
	End of 2nd intervention	15 [15, 15]	15 [15, 15]				
Total SCFA* (mmol/g)	Baseline of 1st intervention	131.60 [69.86–155.10]	163.14 [94.10–175.91]	AA	185.27 [102.67–222.45]	−12.27 (27.91)	0.662
	End of 1st intervention	185.27 [151.30–186.00]	177.14 [143.13–194.55]				
	Baseline of 2nd intervention	132.20 [80.57–163.00]	119.28 [79.89–153.57]	GMP	171.56 [117.23–190.61]		
	End of 2nd intervention	151.20 [100.80–182.70]	201.40 [80.25–295.65]				

Comparisons were performed using linear mixed models including random effects for participants and fixed effects for randomization and intervention.
Arm A: AA followed by GMP; Arm B: GMP followed by AA.
Abbreviations: AA, L-amino acid-based protein substitute; GMP, glycomacropeptide based protein substitute; SCFA, short chain fatty acids; IQR, interquartile range; IgA, immunoglobulin A, SD, standard deviation.
+ Venous sample; * Fecal sample.

3.3.8. Blood in stools

No differences were found for blood in the stools (median GMP: 100 [IQR: 100–100] vs. median AA: 100 [IQR: 94–100]; $p = 0.243$) (Supplementary Table 6). Four patients identified blood in the stools but only one when taking GMP.

3.3.9. Diarrhea

There was no difference in median diarrhea scores with GMP (median: 96 [IQR: 85–100]) vs. AA (median: 93 [IQR: 86–95]; $p = 0.884$) (Supplementary Table 7).

3.4. Gut health markers

Table 5 shows data on gut health markers (serum IgA, and fecal acetate, propionate, isovalerate, butyrate, 2-methylbutyrate, isovalerate, valerate, total SCFA, and fecal calprotectin) at each intervention study visit with AA and GMP. No statistically significant differences were found.

3.5. Nutritional status blood markers

All blood markers of nutritional status except for albumin levels were similar (Table 6) between GMP and AA. Albumin levels were only slightly but significantly higher in favor of GMP ($p = 0.009$). There was also a trend for a higher RBP with GMP ($p = 0.061$).

3.6. Renal, inflammation, and oxidative stress blood markers

Results on renal, inflammation and oxidative stress markers comparing GMP and AA groups are presented in Table 7. Renal markers including serum urea, creatinine and cystatin C were similar between GMP and AA ($p > 0.05$). Although there was no statistically significant difference, markers of inflammation (MRP 8/14, sCD25, and CRP) had a higher trend with AA compared to GMP. Adiponectin was significantly higher with GMP (GMP: 14.3, IQR: [9.9–17.8]; AA: 13.7, IQR: [8.4–17.2]; p = 0.049). The oxidative stress marker, GSH, a major contributor to body's antioxidant defence system, was lower with GMP, but the difference did not reach statistical importance (GMP: 2.200, IQR: [1.433–7.647]; AA: 8.998, IQR: [4.834–24.071]; $p = 0.080$). Total antioxidant capacity was statistically significantly higher with GMP although probably without clinical significance: GMP: 0.308, IQR: [0.291–0.343]; AA: 0.299, IQR: [0.131–0.330]; p = 0.028). MDA was similar (Table 7).

Table 6
Nutritional and endocrine markers at each hospital visit and overall comparison between AA and GMP.

Nutritional and endocrine markers+	Hospital visit	Arm A: AA followed by GMP Median [IQR]	Arm B: GMP followed by AA Median (IQR)	Overall Median (IQR)		Estimate (SD)	*P*
Albumin (g/L)	Baseline of 1st intervention	45 [44–47]	46 [44–47]	AA	45 [44–45]	1.38 (0.51)	**0.009***
	End of 1st intervention	44 [43–45]	46 [45–46]				
	Baseline of 2nd intervention	46 [45–46]	44 [44–45]	GMP	46 [45–47]		
	End of 2nd intervention	47 [46–47]	45 [45–46]				
Prealbumin (mg/L)	Baseline of 1st intervention	237 [222–251]	189 [183–209]	AA	197 [185–216]	12.15 (9.33)	0.199
	End of 1st intervention	194 [179–205]	204 [190–235]				
	Baseline of 2nd intervention	201 [195–221]	186 [178–210]	GMP	211 [198–228]		
	End of 2nd intervention	215 [204–226]	197 [186–241]				
Transferrin (g/L)	Baseline of 1st intervention	2.66 [2.52–2.92]	2.76 [2.65–2.86]	AA	2.72 [2.44–2.89]	0.08 (0.05)	0.101
	End of 1st intervention	2.72 [2.50–2.93]	2.78 [2.55–2.97]				
	Baseline of 2nd intervention	2.60 [2.52–2.81]	2.71 [2.70–2.74]	GMP	2.81 [2.56–3.22]		
	End of 2nd intervention	2.81 [2.64–3.22]	2.72 [2.35–2.85]				
Retinol binding protein (mg/L)	Baseline of 1st intervention	41 [30–43]	37 [34–41]	AA	29 [26–35]	5.23 (2.73)	0.061
	End of 1st intervention	26 [23–29]	36 [34–38]				
	Baseline of 2nd intervention	36 [30–37]	32 [27–40]	GMP	36 [35–40]		
	End of 2nd intervention	36 [35–41]	35 [30.5–42]				
Glucose (mmol/L)	Baseline of 1st intervention	4.9 [4.9–5.0]	4.6 [4.5–5.1]	AA	5.0 [4.8–5.1]	0.01 (0.12)	0.953
	End of 1st intervention	5.0 [4.9–5.0]	4.6 [4.6–5.2]				
	Baseline of 2nd intervention	5.1 [4.8–5.3]	4.7 [4.7–4.8]	GMP	5.0 [4.6–5.3]		
	End of 2nd intervention	5.0 [4.9–5.3]	4.9 [4.8–5.1]				
Hemoglobin A1c (mmol/mmol)	Baseline of 1st intervention	30 [28–31]	32 [31−32]	AA	30 [29–32]	1.45 (1.34)	0.285
	End of 1st intervention	29 [29–30]	30 [29–32]				
	Baseline of 2nd intervention	33 [32−33]	29 [28–29]	GMP	31 [30–34]		
	End of 2nd intervention	33 [32–34]	31 [30−31]				
Insulin (mU/L)	Baseline of 1st intervention	6.1 [4.8–9.4]	6.0 [4.2–6.1]	AA	5.7 [4.8–7.7]	−0.01 (1.06)	0.994
	End of 1st intervention	4.8 [4.7–5.3]	7.5 [3.6–10.4]				
	Baseline of 2nd intervention	3.1 [3.0–6.7]	5.7 [4.1–9.2]	GMP	7.2 [2.8–10.2]		
	End of 2nd intervention	6.7 [2.8–9.2]	7.2 [5.6–8.6]				

Nutritional status marker	Hospital visit	Arm A: AA followed by GMP Median (IQR)	Arm B: GMP followed by AA Median (IQR)	Overall Median (IQR)		Estimate (SD)	*P*
Insulin-like growth factor 1 (ng/mL]	Baseline of 1st intervention	153 [143–161]	125 [111−132]	AA	141 [119–179]	−1.71 (9.13)	0.852
	End of 1st intervention	134 [123–148]	158 [110−231]				
	Baseline of 2nd intervention	162 [122–178]	194 [112–239]	GMP	160 [122–194]		
	End of 2nd intervention	160 [129–165]	153 [115–217]				
C-peptide (pmol/L]	Baseline of 1st intervention	362 [302–478]	328 [258–365]	AA	339 [204–442]	−23.91 (41.29)	0.565
	End of 1st intervention	190 [143–336]	402 [282–596]				
	Baseline of 2nd intervention	189 [152–401]	401 [385–633]	GMP	325 [265–440]		
	End of 2nd intervention	278 [205–325]	397 [339–622]				

Arm A: AA followed by GMP; Arm B: GMP followed by AA.
Comparisons were performed using linear mixed models including random effects for participants and fixed effects for randomization and intervention. *p < 0.05.
Abbreviations: AA, amino acid-based protein substitute; GMP, glycomacropeptide base protein substitute; SD, standard deviation; IQR, interquartile range.
+ Venous sample.

Table 7
Renal, inflammatory and oxidative stress markers in Arm A and B for comparing AA vs. GMP.

Markers +		Hospital visit	Arm A: AA followed by GMP Median (IQR)	Arm B: GMP followed by AA Median (IQR)	Overall Median (IQR)		Estimate (SD)	*P*
Renal markers	Urea (mmol/L)	Baseline of 1st intervention	4.8 [4.5–4.9]	5.6 [5.0–5.8]	AA	5.0 [4.0–5.2]	−0.35 (0.26)	0.185
		End of 1st intervention	5.2 [5.0–5.7]	4.0 [3.6–4.4]				
		Baseline of 2nd intervention	5.8 [4.8–5.9]	4.0 [3.9–4.7]	GMP	4.3 [3.8–4.8]		
		End of 2nd intervention	4.6 [4.6–5.0]	4.6 [4.0–5.1]				
	Creatinine (μmol/L)	Baseline of 1st intervention	33 [30–36]	37 [36–39]	AA	34 [30–40]	−0.01 (0.77)	0.992
		End of 1st intervention	35 [31–41]	36 [35–38]				
		Baseline of 2nd intervention	34 [29–36]	35 [35–38]	GMP	35 [33–37]		
		End of 2nd intervention	33 [32–37]	34 [30–40]				
	Cystatin C (mg/L)	Baseline of 1st intervention	0.79 [0.68–0.79]	0.81 [0.75–0.81]	AA	0.86 [0.76–0.93]	−0.02 (0.03)	0.516
		End of 1st intervention	0.78 [0.76–0.91]	0.81 [0.77–0.90]				
		Baseline of 2nd intervention	0.86 [0.8–0.90]	0.87 [0.86–0.90]	GMP	0.82 [0.77–0.89]		
		End of 2nd intervention	0.82 [0.78–0.86]	0.87 [0.75–0.93]				
Inflammation markers	Adiponectin (mg/L)	Baseline of 1st intervention	17.4 [14.4–19.5]	9.5 [6.6–12.9]	AA	13.7 [8.4–17.2]	−1.09 (0.54)	**0.049***
		End of 1st intervention	15.8 [13.7–17.3]	11.7 [9.7–14.4]				
		Baseline of 2nd intervention	14.1 [13.8–19.5]	8.2 [7.5–10.8]	GMP	14.3 [9.9–17.8]		
		End of 2nd intervention	17.8 [14.6–18.2]	10.0 [7.6–16.8]				
	MRP 8/14 (ng/mL)	Baseline of 1st intervention	500 [500–500]	2409 [1160–2574]	AA	1126 [500–1728]	−446.12 (394.44)	0.263
		End of 1st intervention	1126 [1120–4354]	500 [500–1768]				
		Baseline of 2nd intervention	1137 [500–1190]	809 [500–1413]	GMP	500 [500–1493]		
		End of 2nd intervention	500 [500–1042]	905 [500–1448]				
	sCD25 (pg/mL)	Baseline of 1st intervention	1404 [1286–1416]	1441 [1361–15,278]	AA	1489 [1324–2121]	−273.33 (340.59)	0.426
		End of 1st intervention	1718 [1489–2121]	1476 [783–1809]				
		Baseline of 2nd intervention	1526 [1387–1880]	1584 [1362–1833]	GMP	1486 [1031–1781]		
		End of 2nd intervention	1495 [1322–1566]	1442 [156–1724]				
	CRP (mg/L)	Baseline of 1st intervention	0.17 [0.17–0.59]	0.68 [0.43–0.84]				
		End of 1st intervention	1.58 [0.58–1.74]	0.17 [0.17–1.30]	AA	0.58 [0.17–1.74]		
		Baseline of 2nd intervention	0.38 [0.17–0.65]	0.17 [0.17–2.52]				
		End of 2nd intervention	0.17 [0.17–0.34]	0.51 [0.17–1.25]	GMP	0.17 [0.17–0.88]	−0.91 (0.56)	0.111

Markers +		Hospital visit	Arm A: AA followed by GMP Median (IQR)	Arm B: GMP followed by AA Median (IQR)	Overall median (IQR)		Estimate (SD)	*P*
Oxidative stress markers	Total antioxidant capacity (mmol/L)	Baseline of 1st intervention	0.313 [0.295–0.320]	0.309 [0.294–0.369]	AA	0.299 [0.131–0.330]	0.09 (0.04)	**0.028***
		End of 1st intervention	0.281 [0.112–0.315]	0.365 [0.294–0.424]				
		Baseline of 2nd intervention	0.311 [0.263–0.372]	0.276 [0.265–0.300]	GMP	0.308 [0.291–0.343]		
		End of 2nd intervention	0.303 [0.292–0.314]	0.323 [0.210–0.345]				
	GSH (mmol/L)	Baseline of 1st intervention	2.321 [1.807–4.334]	1.488 [1.199–4.400]	AA	8.998 [4.834–24.071]	−7.04 (3.95)	0.080
		End of 1st intervention	14.061 [5.889–23.679]	1.660 [1.398–2.989]				
		Baseline of 2nd intervention	3.453 [0.911–6.368]	3.602 [2.556–4.611]	GMP	2.200 [1.433–7.647]		
		End of 2nd intervention	6.924 [2.36–9.815]	5.858 [4.81–18.677]				
	uM MDA (mmol/L)	Baseline of 1st intervention	0.267 [0.219–0.296]	0.325 [0.251–0.345]	AA	0.292 [0.260–0.385]	−0.02 (0.04)	0.588
		End of 1st intervention	0.263 [0.251–0.267]	0.411 [0.370–0.424]				
		Baseline of 2nd intervention	0.324 [0.272–0.349]	0.277 [0.258–0.381]	GMP	0.370 [0.260–0.420]		
		End of 2nd intervention	0.263 [0.253–0.303]	0.376 [0.311–0.436]				

Arm A: AA followed by GMP; Arm B: GMP followed by AA.
Comparisons were performed using linear mixed models including random effects for participants and fixed effects for randomization and intervention.
Abbreviations: AA, amino acid based protein substitute; GMP, glycomacropeptide based protein substitute; GSH, glutathione; MDA, malondialdehyde; MRP, myeloid related protein; CRP, c-reactive protein; sCD 25 Serum soluble interleukin-2 receptor.
+ Venous sample.

3.7. Plasma Phe and Tyr from venous samples

Plasma Phe and Tyr levels from fasting venous samples were collected at each study visit (Table 8). Based on regression modelling, mean plasma Phe with GMP was 104 ± 64 μmol/L higher than with AA. This is clinically relevant in children even without reaching statistical significance ($p = 0.12$). There was no difference in median Tyr levels with GMP and AA (43 vs. 37 μmol/L, $p = 0.601$).

Median fasting venous plasma Phe and Tyr levels for each study visit are presented in Supplementary Table 8.

3.8. Blood Phe and Tyr levels from blood spots

Fig. 2 presents results for weekly blood Phe levels in each arm of the study. Overall median blood Phe levels for the study were 298 μmol/L and 188 μmol/L for GMP and AA, respectively. The linear regression

Table 8
Median (IQR) fasting venous plasma concentrations of Phe and Tyr on GMP vs. AA.

Protein substitute	Plasma Phe (μmol/L)			Plasma Tyr (μmol/L)		
	Median [IQR]	Mean Difference (SD)	*P*	Median [IQR]	Mean Difference (SD)	*P*
GMP-based	427 [399–478]	102 (64)	0.120	43 [31–50]	2 (5)	0.601
AA-based	387 [161–465]			37 [34–46]		

Abbreviations: IQR, interquartile range; SD, standard deviation; GMP, casein glycomacropeptide based protein substitute; AA, L-amino acid-based protein substitute; Phe, phenylalanine; Tyr, tyrosine.

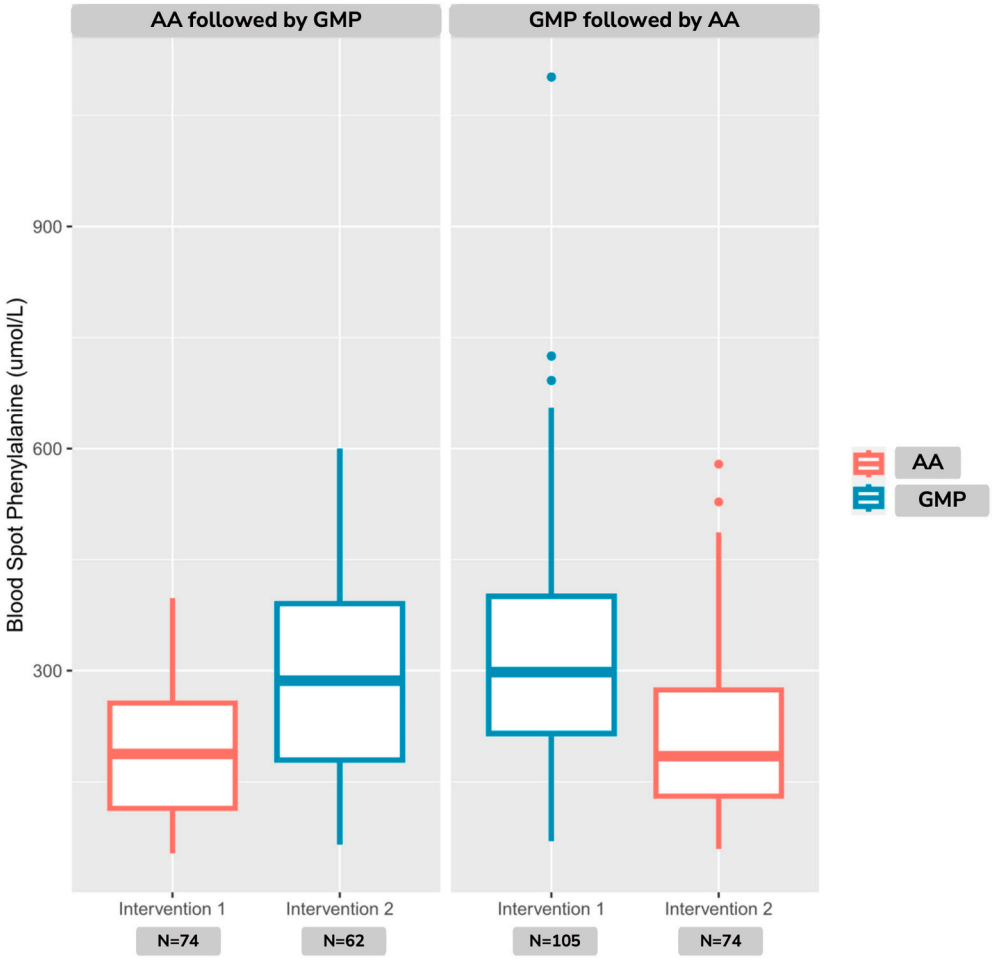

Fig. 2. Blood phenylalanine levels (μmol/L) measured on blood spots during the study comparing AA and GMP. Abbreviations: GMP, glycomacropeptide based protein substitute;AA, L-amino acid based protein substitute; Interverntion 1 and 2 are the first and second protein substitute intervention used in each of the study

model showed a significant mean of 114 ± 14 μmol/L ($p < 0.001$) higher blood Phe with GMP. There was also a higher fluctuation of blood phenylalanine levels for GMP when compared with AA (IQR: 193–395 vs. 121–262 μmol/L).

Median blood Phe and Tyr levels for each arm of the study are presented in Supplementary Table 9. Median blood Tyr was 46 μmol/L for AA and 52 μmol/L for GMP. The regression model showed no statistical differences ($p = 0.96$).

3.9. Satiety

Fig. 3 presents the results of satiety ratings comparing GMP vs AA. No statistical differences were found when comparing the distribution of scores using Fisher's test.

3.10. Anthropometry

No statistical differences in height-for-age ($p = 0.12$) and weight-for-age ($p = 0.20$) were found and z-scores remained stable over the course of the study (Supplementary Table 10).

3.11. Product acceptability

No differences between GMP and AA were observed for any domains for acceptability: smell ($p = 1$), taste ($p = 1$), aftertaste ($p = 0.4$), texture ($p = 1$), packaging ($n = 1$), difficulties with preparation ($p = 0.59$) and consumption/intake ($p = 0.64$) (Supplementary Fig. 4).

4. Discussion

Although there are several studies that have investigated the potential biological health and nutritional properties of GMP based protein substitutes in PKU, controlled clinical studies are still lacking. We performed a comprehensive randomized controlled trial in children comparing GMP with AA protein substitutes, assessing a wide range of biological parameters.

In our study, there was a significant improvement in overall scores in 4 of 9 domains using a validated questionnaire assessing GI symptoms (stomach pain, discomfort when eating, heartburn, and reflux, wind and bloating). Several other symptoms associated with discomfort with eating and constipation showed trends for improvement. No differences for renal markers, and gut health markers were found between the two protein substitutes. Oxidative stress markers were contradictory with a trend for lower GSH with GMP but higher total antioxidant activity. Most inflammation markers were not different except for adiponectin that was higher with GMP compared with AA. Albumin significantly improved on GMP but this is unlikely to be clinically relevant. In contrast with other studies, we did not observe any differences regarding satiety and acceptability between AA and GMP protein substitutes.

Interestingly, similar results for GI symptoms were reported in a randomized, controlled, crossover study [39] when older subjects with PKU reported less diarrhea, constipation, heartburn, nausea and abdominal pain with GMP compared with AA, even though symptoms were self-reported and over a short period of time (3 weeks). Daly et al. also found similar improvements in a group of patients with tyrosinemia

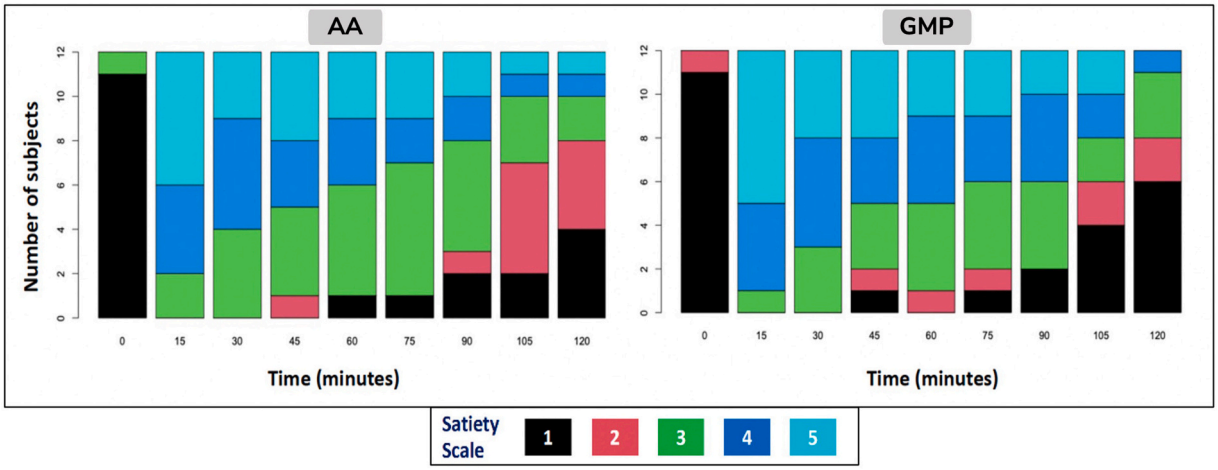

Fig. 3. Satiety scale results are shown at each time during 2 h post-breakfast comparing AA and GMP. A validated satiety questionnaire based on a "teddy bear" visual analogue scale was applied every 15 min for 2 h post-breakfast (no further food or drinks except water were permitted) [37]. **Scale: 1 (Black):** I am really hungry, my belly feels very empty and is rumbling; **2 (Red):** I am quite hungry and my belly feels a little empty; *3 (Green):* I feel just right, not too hungry and not too full; **4 (Dark blue):** I am quite full, but there is still a little room in my belly; **5 (Light blue):** I am not hungry at all! My belly feels very full and I cannot eat any more food!". Abbreviations: AA, L-amino acid-based protein substitute; GMP: glycomacropeptide-based protein substitute. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

using a GMP based protein substitute [40].

Patients with PKU on dietary treatment are expected to take their protein substitute at least 3 times each day and any associated recurrent GI symptoms causing discomfort may impact their overall wellbeing, quality of life, joy of eating and even limit their social interactions. Although not systematically evaluated in routine practice, GI symptoms have long been described in children and adults with PKU [41–43]. In a recent survey of 33 PKU centers in Europe, health professionals reported GI discomfort in patients with PKU. The most reported complaints were GI reflux followed by flatulence, constipation, and diarrhea. It was noted that GI symptoms were not usually discussed as part of the routine PKU clinic review [44]. Burton et al. [45] also found that GI issues such as gastritis, gastroesophageal reflux disease, diverticulosis and irritable bowel syndrome were higher in patients with PKU compared with the general population.

Data from experimental studies suggested structural alterations within the small intestine and possible alterations of the human Caco-2 cells with GMP [34]. These cells have been used to study the absorption mechanism of foods through the intestinal epithelium, representing the morphologic characteristics of small intestine cells [46]. Lamni et al. [34] showed that AA worsened intestinal hydrogen peroxide and lipopolysaccharides induced oxidative and inflammatory cytokines in these cells. These were restored to physiological conditions with GMP. This may explain the improvement in GI symptoms with GMP in our study, even though we did not find any differences in gut markers.

Calprotectin was below the limit of quantification in the fecal samples on either AA or GMP. Fecal calprotectin, a calcium- and magnesium-binding protein primarily produced in neutrophils is a biomarker of intestinal inflammation. When found in the faeces, it indicates the presence of neutrophil migration to the inflamed intestinal mucosa [47].

There is evidence that gut microbiota in PKU is characterized by a lower microbial diversity with decreased amounts of some beneficial microbiota genera such as *Bifidobacteria* and *Romboutsia* [48]. GMP has the potential to ameliorate dysbiosis but data supporting the beneficial impact of GMP on microbiota is limited. Montanari et al. [19] evaluated the effect of 6 months of GMP supplementation on gut microbiota in 9 patients with PKU but showed no overall significant microbiota changes except for a positive effect on butyrate production. Ney et al. [49] showed an altered bioavailability of microbiome derived components that may impact on the bioavailability of specific amino acids, e.g., Tyr and tryptophan. It was suggested that these amino acids were less bioavailable in AA based protein substitutes compared with GMP, leading to a higher preferability of degradation by intestinal microbes. Ney et al. [49] also studied microbiota derived metabolites and found that serotonin and dopamine, which are both synthetized by Tyr and tryptophan, improved with GMP, despite a 50 % higher intake of Tyr from AA. In our study we did not find any significant improvement in gut health measures related to the use of GMP in contrast to what was expected. It may be that the small sample size and 12-week intervention may not be enough to clearly show a difference in SCFA of children with PKU. The positive effects found in pre-clinical studies with increased SCFA may not be found in clinical studies as many other environmental and dietary factors may play a role. Possible changes in fiber intake or environmental factors such as exercise were not considered but may have an impact.

Most of the nutritional and endocrine markers (prealbumin, transferrin, RBP. glucose, HbA1c, insulin, IGF, C-peptide) analyzed in our study were similar between AA and GMP. We did not find a significant difference for fasting insulin, although median results were higher with GMP (7.2 vs 5.7, $p = 0.994$). GMP based protein substitutes have a higher amount of leucine which can potentially explain the result. Postprandial insulin was not measured. Van Calcar and MacLeod et al. [32,50] found significantly higher insulin levels post-prandially with GMP compared with AA in adolescents and adults with PKU. Similar to our results, 2 studies from Portugal [51,52], studying some of the same patient cohort, found a trend for higher insulin levels with GMP although the differences were not statistically significant. In a systematic review of 104 studies [53], it was shown that insulin had a role in regulating muscle protein synthesis in the presence of elevated amino acids.

Albumin, a plasma protein, significantly increased with GMP (45 vs 46, $p = 0.009$), but possibly without clinical relevance. Albumin has been associated with improved relative muscle mass in young healthy participants [54]. Higher albumin levels together with higher insulin may suggest a possible beneficial effect on an improved amino acid absorption with GMP. Daly et al. [55] did show a trend for improved lean body mass with long-term use of GMP (36 months) but further studies with a larger patient cohort with GMP are necessary.

Previously, GMP has been associated with a reduction in proinflammatory cytokines and had a disease modifying effect in patients with ulcerative colitis [56]. Findings describing inflammation status in

patients with PKU are contradictory. Proinflammatory cytokines IL-6 and IL-1 β have been found in higher levels in treated patients with PKU compared to controls [57]. However, Rocha et al. showed in a group of adult PKU patients that IL-8, IL-10 and CRP were similar to healthy controls [58]. Recently, 20 French adults with PKU "on diet" and "off diet" were compared with a control group, and no differences were found in plasma CRP and cytokine profile (IFN- γ, IL-1α, IL-1β, IL-2, IL-6, IL-10, and TNF-α) [59]. In an animal study, Sawin et al. [60] found reduced plasma concentrations of IFN-γ, TNF-α, IL-1, and IL-2 as well as the percentage of stimulated spleen cells producing IFN-γ in mice fed with GMP compared with casein. Preclinical studies using GMP reported increased systemic inflammation in PKU based on findings of splenomegaly and elevated plasma concentrations of inflammatory cytokines with AA, that normalized when using GMP [28]. Our study did not find significant differences in the inflammatory markers except for a higher adiponectin with GMP. Adiponectin has been associated with anti-inflammatory properties, being thought to decrease intracellular ceramide, a sphingolipid associated with insulin resistance, cell death, inflammation, and atherosclerosis. Adiponectin also stimulates fatty acid oxidation in skeletal muscle and insulin induced glucose utilization [61]. It is unclear if this increase is clinically significant and may have some positive long-term effects both on inflammation, body composition and glucose metabolism which should be addressed in future studies.

Chronic exposure to oxidative damage may lead to the development of various chronic (e.g., cancer, respiratory diseases) and neurodegenerative diseases (e.g., Alzheimer's disease), and may contribute to the aging process [62,63]. It has been well established that oxidative stress may be involved in the pathophysiology of PKU. Patients with PKU are also prone to increased oxidative damage characterized by an increase in free radical production and a depletion in antioxidant capacity [64]. Both nutritional (e.g., deficiencies of nutrients with antioxidant properties such as selenium, zinc, co-enzyme Q10 and perhaps L-carnitine) [64] and disease-related factors (e.g., chronic exposure to high Phe levels and metabolites) may contribute to the increased oxidative stress. Kumru et al. [65] found that patients with PKU with poor adherence had higher levels of oxidative stress markers compared with a healthy control group and the patient group with good metabolic control. Glutathione peroxidase, co-enzyme Q10, co-enzyme Q10/cholesterol and L-carnitine levels were significantly lower in the poor adherence group. However, no differences were found in paraoxonase 1, total antioxidant status, total oxidant status and oxidative stress index [65].

Few studies have examined antioxidant status in patients with PKU using GMP [65,66]. In this study, we found that antioxidant/oxidative status biomarkers were contradictory with total antioxidant capacity higher with GMP although this may not be statistically significant (median 0.308 vs 0.299). In contrast, MDA was similar and there was a trend for lower GSH with GMP compared with AA. We studied a group of early and continuously treated well-controlled young children with PKU and abnormalities in oxidative stress biomarkers would not be expected. In preclinical studies, GMP has been associated with antioxidant properties [67]. One recent preclinical study in human Caco-2 cells suggested that AA significantly worsened intestinal hydrogen peroxide (H_2O_2) and lipopolysaccharides (LPS)-induced oxidative status but GMP reduced or reversed the negative influences of increased oxidative stress [34]. Impact on oxidative stress may be more affected by blood Phe control than source of the protein equivalent in the protein substitute.

In our study, we examined a small cohort of young patients for a short period but there were no early signs of renal dysfunction. Renal insufficiency, with hypertension has been reported to be higher in patients with PKU [45,68], and this has been attributed to the relatively higher protein intake and high renal load of AAs [69,70]. Hence, it is speculated that GMP based protein substitutes may be beneficial for renal function compared with synthetic AA. Previously, Stroup et al. [69] showed that mice fed with GMP had an improved renal status compared with AA. Moreover, in a study with 8 participants, potential renal acid load increased with AA, and subsequently a higher calcium and magnesium excretion was observed which could impact bone mineral density [70]. In contrast, Daly et al. [30] has measured renal urinary calcium showing this was no different and had no impact on bone mineral density.

Renal insufficiency is multi-factorial, and it is important all potential risk factors are carefully considered in studies. In a long-term study of 41 adults (early treated patients with PKU) over 10 years, there were no changes in creatine levels and estimated glomerular filtration in patients with good metabolic control, compared with increased creatinine and estimated glomerular filtration in patients with poor metabolic control, [71]. In contrast, Henneman et al. [72] found in a cross-sectional study in 67 patients with early and late diagnosed PKU aged 15–43 years that glomerular filtration rate was low in 19 % of the patients. Proteinuria was detected in 31 % and arterial hypertension in 23 % of the patients. However, these results need careful interpretation as 25 patients (37 %) were overweight, and 10 patients did not adhere to the prescribed diet.

Satiety following protein substitute consumption has an important role in the management of obesity as it should lessen hunger and help control energy intake and is therefore important in weight management [73]. Although there is no conclusive evidence showing any clear differences in satiating properties between different protein sources, animal protein-based meals [74] have been associated with increased satiety. Whey and casein, the major proteins of milk were also linked with increased satiety in the short- and long-term, respectively [75]. As GMP is one of the main bioactive peptides found in whey protein (20–25 %), it may promote satiety. Solverson et al. [28] showed in a PKU mouse study that GMP was associated with a lower percentage body fat and reduced food intake when compared with AA and casein. However, evidence from human studies was inconclusive. Macleod et al. in a cross over study [50] reported in 11 patients with PKU (8 adults), that postprandial levels of ghrelin were lower and feelings of fullness were greater with a breakfast including GMP compared with AA. However, all subjects completed a 'motivation to eat' visual analogue scale questionnaire and there were no differences between the two study diets. In contrast, Ahring et al. [76] did not demonstrate any differences in ghrelin profile after taking GMP, and there were also no significant differences when applying a visual scale for satiety. Similarly, in a 3-year controlled study, Daly et al. found no difference in satiety with GMP compared with AA in a group of children with PKU. The results from the latter studies are in line with findings in our study after a standard breakfast, with a controlled amount of energy (individualized for each child) and GMP. Satiety is a complex, multifactorial behavioral and physiological process impacted by nutritional intake, hormonal regulation, environment, and level of activity; so, to find a direct correlation with protein substitute intake without controlling for other confounding variables is not expected.

In short term studies, GMP based protein substitutes have been described as having a better smell, taste, mouth feel and after taste compared with traditional AAs [28]. However, in our study we did not find a preference for GMP. Our cohort were school aged children (4–9 years), and they were well established on AA and were not considering changing their type of protein substitute prior to the clinical trial. Children may have an imprinted taste preference for AA associated with their early introduction to Phe-free AA based infant formula and repeated exposure, modulating their taste preference [77]. In contrast, other GMP studies have mainly focused on teenagers or adults with PKU. This is at a time when dietary adherence is commonly lower, particularly with protein substitute and an alternative source of protein substitute is sought [78].

Consistent with the findings of other studies conducted in children with PKU, in this study the sole use of GMP based protein substitute as the main protein source led to an increase in blood Phe levels when weekly blood spots were taken [79]. In contrast, fasting plasma Phe levels of patients taken during the intervention study visits showed no statistical difference between AA and GMP. This may be explained by a small number of samples with venous blood (only performed in hospital

visits) compared with blood Phe spots, and therefore, not reaching a statistical significance. The overall difference between blood Phe levels on GMP vs. AA was similar (114 μmol/L blood spots vs 104 μmol/L venous sample). This is clinically relevant and in clinical practice, particularly in children with classical PKU, the dietary Phe intake from food should be reduced to compensate for the Phe content of the GMP. In our study, if blood Phe levels exceeded upper target blood level on GMP, the Phe intake was reduced by 50 to 100 mg/day but some patients found it challenging due to very low natural protein tolerance. In the future, lower Phe GMP based protein substitutes should be developed.

5. Limitations

Patients were not blinded to the interventions as it was not feasible to disguise the type of protein substitute used. The recruitment/data collection period coincided with COVID-19, so the study had to be slightly adapted so patients spent a minimum time at the hospital, although none of the study procedures were compromised. It has been shown in some studies that blood Phe control was negatively impacted during COVID-19 [80,81], and it is possible the uncertainty and conditions surrounding COVID-19 may have affected the quality of the blood Phe control in our study. Due to the COVID-19 pandemic, only 12 participants were recruited which lowered the study power to approximately 70 % but this did not impact the level of significance used in the study. This was a very intensive and demanding study for children with PKU, which explains the difficulties with recruitment. The sample size was small and some of our results may not have reached statistical significance due to this limitation. The washout period of 4 weeks may have been too short, as we observed some carry over effect on GI symptoms. When GMP was given as the first intervention in Arm B, any improvement identified, was still observed at the baseline of the second intervention and only appeared to return to the usual pattern by the end of the second intervention with AA. Most biomarkers of oxidative stress, inflammation and gut health did not reach statistical significance and this may have been related to the intervention time that could have been too short to detect real changes in the biomarkers assessed. Either an extended intervention period (> 3 months) or alternative biomarkers could be considered in future studies.

6. Strengths

This was a well-designed randomized, controlled, crossover trial conducted in well controlled children with PKU. We analyzed an extensive number of biochemical markers that are rarely assessed in PKU, comparing two different protein substitutes. The results describing GI symptoms were significant even in such a small number of patients which strengthens the power and clinical significance of our results. Patients were naïve to GMP at study commencement.

7. Conclusions

In this randomized, crossover study in a group of children with PKU we found a significant improvement in stomach pain, discomfort when eating, heartburn and reflux, wind and bloating, and constipation with the use of a GMP protein substitute when compared with AA based protein substitutes. These are clinically relevant showing benefit in several GI issues which may have a positive impact on social interactions, quality of life and even adherence to dietary treatment. We found no difference for renal function or oxidative stress between GMP or AA based protein substitutes.

The Phe content of GMP may present challenges when used as the only protein substitute in children with PKU with minimal natural protein tolerance. This should be considered, and dietary Phe intake may need adjustment.

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CRediT authorship contribution statement

Alex Pinto: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Data curation, Conceptualization. **Anne Daly:** Writing – review & editing, Investigation. **Camille Newby:** Writing – review & editing, Investigation. **Abigail Robotham:** Writing – review & editing, Investigation. **Simon Heales:** Writing – review & editing, Investigation. **Simon Eaton:** Writing – review & editing, Investigation. **Helen Aitkenhead:** Writing – review & editing, Investigation. **Kimberly Gilmour:** Writing – review & editing, Investigation. **Richard Jackson:** Writing – review & editing, Formal analysis. **Catherine Ashmore:** Writing – review & editing, Investigation. **Sharon Evans:** Writing – review & editing, Investigation. **Júlio Cesar Rocha:** Writing – review & editing, Supervision, Investigation. **Fatma Ilgaz:** Writing – review & editing, Investigation. **Mary Hickson:** Writing – review & editing, Supervision, Investigation. **Anita MacDonald:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Data curation, Conceptualization.

Declaration of competing interest

A.P. received an educational grant from Cambrooke Therapeutics and Biomarin and grants from Vitaflo International, Nutricia, Biomarin, Mevalia, Galen, PIAM, and Applied Pharma Research to attend scientific meetings. This project is also part of A.P.'s PhD, which is funded by Vitaflo International®. A.D. received research funding from Vitaflo International and financial support from Nutricia, Mevalia, and Vitaflo International to attend study days and conferences. C.N. received honoraria from Nutricia and Vitaflo International to attend study days and conferences.

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Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymgme.2024.108607.

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