THE NUTRITIONAL PROFILING OF SPECIAL FEEDS FOR PHENYLKETONURIA

By

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ABSTRACT

In children with classical phenylketonuria (PKU), a lifelong low phenylalanine diet is the only treatment option with natural protein (phenylalanine) intake restricted to < 20% of safe recommended intake [1] to minimise neurological damage. The diet must be supplemented with artificial protein or protein substitutes. These are traditionally based on L-amino acids (AA), providing phenylalanine free nitrogen, essential amino acids, tyrosine, vitamins and minerals supporting growth, physiological pathways and protein synthesis. Casein glycomacropeptide (CGMP) is a bioactive sialylated glycophosphopeptide, a derivative from κ casein, and it is used as an alternative protein replacement in PKU. As a glycopeptide it offers some unique biological properties and in PKU, there is suggestion it may improve bone mass and density and stabilise blood phenylalanine concentrations. Unlike phenylalanine free AA, CGMP contains a small amount of residual phenylalanine. The effect of CGMP has not been investigated in children with PKU. In this thesis the primary objectives were to examine the efficacy and impact of CGMP compared to AA via a series of controlled studies in children with PKU on: 1) stability of blood phenylalanine concentrations; 2) 24-hour blood phenylalanine variability with a preliminary investigation on postprandial blood amino acid absorption and 3) body composition, bone density, mass and geometry over 3 years. The secondary objectives were to compare the effects of CGMP versus AA on: 1) satiety and 2) breath volatile organic compounds.

Findings from these studies consistently showed that residual phenylalanine in CGMP destabilised blood phenylalanine control, particularly in children who have

minimal phenylalanine tolerance. When blood phenylalanine was monitored over 24 hours, there was less variability in blood phenylalanine concentrations with CGMP. This only became evident after phenylalanine intake was lowered in the diet so the overall daily phenylalanine intake was not increased. In children taking CGMP only, there was a trend for improved body composition and height growth. At 36 months independent of age the bone formation marker procollagen type 1 N terminal propeptide (P1NP) was increased in those taking CGMP only compared to the AA group. Satiety and breath volatile organic compounds were not influenced by CGMP compared to AA.

Evidence suggesting a potential positive clinical benefit associated with this alternative glycosylated phosphopeptide was only shown in those exclusively taking CGMP. Its residual phenylalanine content caused metabolic instability principally in those with low phenylalanine tolerance. Therefore, only 48% of children could use CGMP exclusively. Future research to develop a phenylalanine-free CGMP, available to all PKU children is a priority. A greater understanding of the structural and functional properties of CGMP in particular the sialic acid, phosphopeptide and glycan chain composition, will help consolidate the potential positive clinical benefits found by these studies.

1. van Wegberg, A.M.J., et al., *The complete European guidelines on phenylketonuria: diagnosis and treatment.* Orphanet J Rare Dis, 2017. **12**(1): p. 162.

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LIST OF PAPERS ABSTRACTS AND CONFERENCE PRESENTATIONS

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2. Daly A, Evans S, Chahal S, Santra S, MacDonald A: Glycomacropeptide in children with phenylketonuria: does its phenylalanine content affect blood phenylalanine control? J Hum Nutr Diet 2017, 30(4):515-23.

3. Daly A, Evans S, Chahal S, Santra S, Pinto A, Jackson R, Gingell C, Rocha JC, Van Spronsen FJ, MacDonald A: Glycomacropeptide: long-term use and impact on blood phenylalanine, growth and nutritional status in children with PKU. *Orphanet J Rare Dis.* 2019; 14(1):44.

4. Daly A, Evans S, Chahal S, Santra S, Pinto A, Gingell C, Rocha JC, van Spronsen FJ, Jackson R, MacDonald A: The Effect of Glycomacropeptide versus Amino Acids on Phenylalanine and Tyrosine Variability over 24 Hours in Children with PKU: A Randomized Controlled Trial. Nutrients. 2019; 11(3).

5. Daly A, Evans S, Pinto A, Jackson R, Ashmore C, Rocha JC, MacDonald A: Preliminary Investigation to Review If a Glycomacropeptide Compared to L-Amino Acid Protein Substitute Alters the Pre- and Postprandial Amino Acid Profile in Children with Phenylketonuria. Nutrients. 2020; 12(8).

6. Daly A, Högler W, Crabtree N, Shaw N, Evans S, Pinto A, Jackson R, Ashmore C, Rocha JC, Strauss BJ, Wilcox G, MacDonald A. Growth and body composition in PKU children. A 3 year prospective study comparing the effects of L-amino acids to glycomacropeptide protein substitutes. Nutrients. 2021; 13(4).

7. Daly A, Evans S, Pinto A, Jackson R, Ashmore C, Rocha JC, MacDonald A: The Impact of the Use of Glycomacropeptide on Satiety and Dietary Intake in Phenylketonuria. Nutrients. 2020; 12(9).

8 Tiele FA, Daly A, Hattersley J, Pinto A, Evans S, Ashmore C, MacDonald A, Covington JA: Investigation of paediatric PKU breath malodour, comparing glycomacropeptide with phenylalanine free L-amino acid supplements. *J Breath Res.* 2019; 14(1):016001.

During the period of postgraduate study additional papers were published but have not been included in the main thesis:

Pena MJ, Pinto A, Daly A, MacDonald A, Azevedo L, Rocha JC, Borges N: The Use of Glycomacropeptide in Patients with Phenylketonuria: A Systematic Review and Meta-Analysis. Nutrients. 2018; 10(11).

Daly A, Evans S, Pinto A, Ashmore C, Rocha JC, MacDonald A: A 3 Year Longitudinal Prospective Review Examining the Dietary Profile and Contribution Made by Special Low Protein Foods to Energy and Macronutrient Intake in Children with Phenylketonuria. Nutrients, 2020; 12(10).

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Daly A, Högler W, Crabtree N, Shaw N, Evans S, Pinto A, Jackson R, Ashmore C, Rocha JC, Strauss BJ, Wilcox G, Fraser WD, Tang JCY, MacDonald A. A three year longitudinal study comparing bone mass, density and geometry measured by DXA, pQCT and bone turnover markers in children with PKU taking either L-amino acid or glycomacropeptide protein substitutes

ABSTRACTS GENERATED FROM THE RESEARCH

2019 BIMDG (British Inherited Metabolic Disease Group) Cambridge 26th to 27th June

Daly A, Tiele FA, Pinto A, Evans S, Ashmore C, Hattersley J, Covington J, MacDonald A. A randomised cross over study examining volatile organic compounds sampling glycomacropeptide and phenylalanine free L-amino acid protein substitute.

2018 BIMDG Nottingham UK 21st to 22nd June

Daly A, Evans S, Chahal S, Santa S, Pinto A, Gingell C, Rocha JC, van Spronsen FJ, MacDonald A. Glycomacropeptide compared to phenylalanine free L-amino acids evaluation of a peptide based protein substitute on nutritional status in children with PKU.

2017 BIMDG London UK 29th to 30th June

Daly A, Chahal S, Evans S, Pinto A, Gingell C, Rocha JC, MacDonald A. Does the additional phenylalanine in CGMP-AA protein substitute lead to destabilisation of blood phenylalanine concentrations compared to conventional amino acid protein substitutes?

2020 ESPKU (European Society for Phenylketonuria) Virtual Online 30th Oct to 1st Nov

Daly A, Evans S, Pinto A, Ashmore C, Rocha JC, MacDonald A. Is there any differences in body shape in children taking glycomacropeptide or traditional amino acid protein substitutes?

Daly A, Evans S, Pinto A, Ashmore C, Rocha JC, MacDonald A. Does the type of protein substitute change how quickly amino acids are absorbed in the body?

Daly A, Evans S, Pinto A, Ashmore C, Rocha JC, MacDonald A. The importance of special low protein foods in a PKU diet.

Daly A, Evans S, Pinto A, Ashmore C, Rocha JC, MacDonald A. Does a glycomacropeptide protein substitute make you less hungry?

2019 ESPKU Izmir Turkey 31st Oct - 3rd Nov

Daly A, Tiele FA, Pinto A, Evans S, Ashmore C, Hattersley J, Covington J, Rocha JC, MacDonald A. A pilot randomised cross over study in PKU investigating the exhaled breath signature of organic compounds comparing glycomacropeptide with phenylalanine free L-amino acid supplements.

2018 ESPKU Venice Italy 1st to 4th Nov

Daly A, Evans S, Chahal S, Santra S, Pinto A, Gingell C, Rocha JC, van Spronsen FJ, MacDonald A. Prospective longitudinal controlled parallel study over 12 months comparing glycomacropeptide and conventional amino acid protein substitutes in children: effect on blood phenylalanine, tyrosine, phenylalanine:tyrosine ratio and growth.

Daly A, Evans S, Chahal S, Santra S, Pinto A, Gingell C, Rocha JC, van Spronsen FJ, Jackson R, MacDonald A. Variability of phenylalanine concentrations over 24 hours using two different protein substitutes and changing the dietary phenylalanine intake.

Daly A, Evans S, Chahal S, Santra S, Pinto A, Gingell C, Rocha JC, van Spronsen FJ, MacDonald A. Glycomacropeptide compared to phenylalanine free L-amino acids evaluation of a peptide based protein substitute on nutritional status in children with PKU.

2016 GMDI (Genetics Metabolic Dietitians International) Arizona USA 27th to 30th April Metabolic Nutrition in the 21st century

Daly A, Evans S, Chahal S, MacDonald A. Can we ignore the residual amount of phenylalanine in CGMP when given as a dominant form in medical foods in children with PKU? Poster #12 p19

2019 SSIEM (Society for the Study of Inborn Errors of Metabolism) Rotterdam Netherlands 3rd to 6th Sept. Building bridges

Daly A, Pinto A, Evans S, Ashmore C, Rocha JC, Jackson R, MacDonald A. Body composition and bone mineral density in PKU children:- interim results from a 3y longitudinal study. J Inherit Metab Dis 2019; 42 S1

Daly A, Evans S, Pinto A, Ashmore C, Rocha JC, MacDonald A. Food patterns in children with PKU a 12-month prospective review profiling dietary habits. J Inherit Metab Dis 2019; 42 S1

Daly A, Tiele AF, Evans A, Ashmore C, Hattersley J, Covington J, Rocha JC, MacDonald A. A crossover study investigating volatile breath compounds, comparing glycomacropeptide with phenylalanine free L amino acid supplements. J Inherit Metab Dis 2019; 42 S1

Pena M.J, Pinto A, Daly A, MacDonald A, Azevedo L, Rocha J.C, Borges N. The use of glycomacropeptide in patients with phenylketonuria. Evidence from systematic review and meta-analysis. J Inherit Metab Dis 2019; 42 S1

2018 SSIEM Athens Greece 4th to 7th Sept. Old roads, new connections

Daly A, Evans S, Chahal S, Pinto A, Gingell C, Rocha JC, van Spronsen FJ, Jackson R, MacDonald A. Variability of phenylalanine concentrations over 24 hours using two different protein substitutes and changing dietary phenylalanine intake. J. Inherit Metab Dis 2018; 41, S1

Daly A, Evans S, Chahal S, Santra S, Pinto A, Rocha JC, van Spronsen FJ, MacDonald A. Nutritional status in children with PKU: Glycomacropeptide compared to phenylalanine free amino acids. J. Inherit Metab Dis 2018; 41, S1

Daly A, Evans S, Chahal S, Santra S, Pinto A, Gingell C, Rocha JC, van Spronsen FJ, MacDonald A. Prospective study: Glycomacropeptide and conventional amino acid protein substitutes in children: effect on blood phenylalanine and growth. J. Inherit Metab Dis 2018; 41, S1

2017 ICIEM (International Conference of Inherited Errors of Metabolism) Rio de Janeiro Brazil 5th to 8th Sept. 13th International Congress together with ASIEM, JSIM, SIMD and SLEIMPN

Daly A, Chahal S, Evans S, Pinto A, Gingell C, Rocha JC, MacDonald A. Post prandial amino acid profiles in children with phenylketonuria using CGMP-AA or Phe-free protein substitutes. Journal of Inborn Errors of Metabolism and Screening. JIEMS special edition 2017

Daly A, Chahal S, Evans S, Pinto A, Gingell C, Rocha JC, MacDonald A. Does the additional phenylalanine in CGMP-AA protein substitute lead to destabilisation of blood phenylalanine concentrations compared to conventional amino acid protein substitutes? JIEMS special edition 2017

2016 SSIEM Rome Italy 6th to 9th Sept. Metabolic pathways, cellular networks and beyond

Daly A, Chahal S, Evans S, MacDonald A. Normal growth and bone mineral density in 38 PKU children taking conventional amino acid protein substitutes. J. Inherit Metab Dis 2016; 39, S1

Daly A, Chahal S, Evans S, MacDonald A. Impact of the amino acid profile of casein glycomacropeptide on metabolic control in children with PKU. J. Inherit Metab Dis 2016; 38, S1

2015 SSIEM Lyon France 1st to 4th Sept. Les confluences

Daly A, Evans S, Chahal S, MacDonald A. Glycomacropeptide: Can we safely advocate its use in children with PKU? J.Inherit Metab Dis 2015; 38, S1

CONFERENCE PRESENTATIONS

ESPKU European Society for Phenylketonuria

2020 Virtual online conference 30th Oct - 1st Nov

Presentation 1: CGMP: Results of a 3-year longitudinal study examining body composition, satiety and amino acid absorption

Presentation 2: Special low protein foods: An essential component in a low phenylalanine diet

2018 Venice Italy 1st - 4th Nov

Presentation 1: Prospective longitudinal controlled parallel study over 12 months comparing glycomacropeptide and conventional amino acid protein substitutes in children; effect on blood phenylalanine, tyrosine and phenylalanine to tyrosine ratio and growth

Presentation 2: PKU: effects of CGMP

GMDI Genetics Metabolic Dietitians International

2021 Virtual conference 28th April to 1st May

Bone density, mass and geometry overview of a 3 year study comparing glycomacropeptide and conventional amino acid protein substitutes

2018 Florida USA 26 - 28th April

Advances in the nutritional management of PKU: The story unfolds

NSPKU National Society for Phenylketonuria

2020 Kent UK 28th Feb to 1st March

Special low protein foods the importance in a low phenylalanine diet

2018 Lincolnshire UK 2nd to 4th March

Does a small amount of phenylalanine in CGMP affect blood phenylalanine control

2017 Oxford UK 7th to 9th April

PKU research update: Bone mineral density in children with PKU

2016 Cheltenham UK 11th to 13th March

Preliminary research findings using a novel glycomacropeptide protein substitute

SSIEM Society for the Study of Inborn Errors of Metabolism

2020 Virtual online conference Dec 3rd

Preliminary results on bone mineral density in children taking CGMP compared to phenylalanine free amino acids

2018 Athens Greece 4th to 7th Sept

A prospective study: Glycomacropeptide and conventional amino acid protein substitutes in children effect on blood phenylalanine and growth.

2015 Lyon France 1st to 4th Sept

Glycomacropeptide can we safely advocate its use in children with PKU?

Other conferences

2020 Virtual online conference 27th Nov

Vitaflo dietitians meeting Presentation of research finding CGMP v AA

2019 Webinar on line presentation 16th March

The latest CGMP research in PKU

2019 Palma de Mallorca Spain 27th to 28th Sept. SEA IEM (South European Academy Nutrition Education of IMD)

Phenylalanine variability using three different protein substitutes and adjusting for the phenylalanine dietary intake

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LIST OF ABBREVIATIONS

AA	amino acids
BBB	blood brain barrier
BCA	branched chain amino acids
BH4	tetrahydrobiopterin
BIA	bioelectrical impedance analysis
BMC	bone mineral content
BMD	bone mineral density
BMI	body mass index
CCK	cholecystokinin
CGMP	casein glycomacropeptide
DNA	deoxyribonucleic acid
GTPCH	guanosine triphosphate cyclohydrolyase 1
CMP	casein macropeptide
CRP	C reactive protein
DHPR	dihydropteridine reductase
EAR	estimated average requirements
FM	fat mass
Gal	galactose
Gal1NAc	N acetylgalactosamine
HDTc	high density triglycerides
IGF 1	insulin-like growth factor
IQ	intelligent quotient
IQR	interquartile range
LAT1	L type amino acid 1
LBM	lean body mass
LCPUFA	long chain polyunsaturated fatty acid
LNAA	large neutral amino acids
MPH	mild hyperphenylalaninemia
MRI	magnetic resonance imaging
mTORC	mammalian target of rapamycin
Neu5Ac	N acetylneuraminic acid
PAH	L phenylalanine hydroxylase
PCD	pterin 4a carbinolamine dehydratase
Phe	phenylalanine
Phe-free AA	phenylalanine free amino
PKU	phenylketonuria
PS	protein substitute
PTS	6 pyruvoyl tetrahydropterin synthase
PYY	peptide YY
REE	resting energy expenditure
RNA	ribonucleic acid
SCFA	short chain fatty acids
TC	total cholesterol
Tyr	tyrosine
VAS	visual analog scale
VOC	volatile organic compounds

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CHAPTER 1

GENERAL INTRODUCTION

1.1 BACKGROUND

Phenylketonuria (PKU; McKusick #261600) is the most prevalent inborn error of amino acid metabolism. It results from mutations in the phenylalanine hydroxylase gene, which encodes for the enzyme L-phenylalanine hydroxylase (PAH) [1, 2]. Hillert [3] estimates a predicted global prevalence of 0.45 million people with PKU. Phenylalanine hydroxylase is a pterin dependent amino acid hydroxylase and is part of the monooxygnase family, which includes tryptophan and tyrosine hydroxylase. All three utilise tetrahydrobiopterin 4 (BH4) as a cofactor [4]. Phenylalanine hydroxylase converts phenylalanine into tyrosine and is dependent on the BH4 cofactor, molecular oxygen and non- haem iron [5]. A reduction or absence in phenylalanine hydroxylase (or one of the enzymes involved in the synthesis or regeneration of tetrahydrobiopterin) increases blood phenylalanine levels causing neurological damage [6-8].

1.2 PATHOLOGY OF NEUROLOGICAL DAMAGE

In untreated PKU, brain development is severely disturbed. The most common outcome is irreversible intellectual disability, [9, 10] together with seizures, neuropsychiatric and cognitive impairment [11, 12]. This altered brain pathology is caused by hyperphenylalaninemia and hypotyrosinemia which changes brain neuro-functioning [13]. The synthesis of the neurotransmitters dopamine and serotonin are dependent on tyrosine, tryptophan and other large neutral amino acids (LNAA) [14]. These LNAA cross the blood brain barrier via one transporter: L type amino acid 1 (LAT1). High levels of phenylalanine disturb this balanced entry of amino acids into the brain. This results in decreased concentrations of neurotransmitters, which alters brain chemistry causing neuropsychological problems such as memory difficulties, mood disturbances, intellectual

impairment and behavioural difficulties [15, 16]. Other disturbances associated with high brain phenylalanine concentrations include cholesterol biosynthesis, important for myelination of neurones [17], morphological synaptic and dendritic changes. These change communication between cells, as a result of altered cytoskeletal proteins [18-20]. Demyelination and altered white brain matter are evident on magnetic resonance imaging (MRI) and are significantly related to high blood and brain phenylalanine concentrations [21-24]. Human and animal studies have demonstrated abnormalities in myelination in subjects with PKU [25-27]. Smith et al [28] showed that for every 4 weeks delay in starting dietary treatment, intelligent quotient (IQ) decreased by approximately 4 points. The precise pathogenesis of brain dysfunction is still unclear. High blood and brain phenylalanine concentrations are detrimental to neurodevelopmental sequelae, and they result in intellectual disability, poor growth and seizures [10, 14]. These can be avoided by early and continuous dietary intervention.

1.2.1 BEHAVIOURAL AND COGNITIVE CHANGES

Biochemical defects in PKU cause behavioural and cognitive changes. Their prevalence and severity correlate strongly with the degree and timing of exposure to elevations in blood phenylalanine [29, 30]. Abnormalities include psychiatric disorders [31-33], depression [34, 35], anxiety [34, 36, 37], psychosis [38, 39] and attention deficit hyperactivity disorders [40, 41]. Neurocognitive deficits have been described in a range of domains including:- lower intelligence [42, 43], attention [44, 45], processing speed [12, 46], working memory [44, 47], immediate verbal and visual memory [48], motor skills, coordination [49] and executive functions [50, 51]. This leads to a decreased ability to plan, organise and poor conceptual reasoning.

1.3 DEFINING PHENOTYPES AND GENOTYPES

There are different clinical PKU phenotypes, which are determined by the enzyme defect, the genotype and the severity of the disorder [3, 52]. There is no consensus regarding classification [53]. Guttler suggested pre-treatment blood phenylalanine concentrations and dietary phenylalanine tolerance to classify PKU [54]. However, this is flawed as many infants start treatment before a maximum blood phenylalanine concentration is reached, and phenylalanine tolerance is difficult to determine [15]. The European PKU guidelines [53] propose a classification system recommended by Blau [55] (Table1.1). Hillert et al [3] in a detailed study reviewed the global genetic landscape and epidemiology of PKU. He compared both genotypes and metabolic phenotypes from 16,092 subjects with PKU, demonstrating different disease severities from 51 countries in 17 world regions. The global phenotypical distribution was: 62% classical PKU, 22% mild PKU and 16% mild hyperphenylalaninemia. A genotype/ phenotype pattern was identified across Europe with classical PKU in the east, mild PKU in the southwest and mild hyperphenylalaninemia in the south. The phenylalanine hydroxylase gene located on chromosome 12q22-24.1 [56, 57] has more than 1,180 bi-allelic variants leading to a high degree of heterogeneity [14]. About 2% of all elevated phenylalanine levels found by newborn screening are due to non-PKU BH4 disorders (tetrahydrobiopterin defects) [58].

1.3.1 EPIDEMIOLOGY

PKU is one of the most frequent inherited metabolic disorders although the prevalence varies significantly among ethnicities and countries [57]. Global prevalence is estimated to be 1:23,930 live births with a range of 1:4,500 to 1:125,000. PKU prevalence is highest in Europe (Italy; 1:4,000 and Ireland; 1:4,545), but occurring less frequently in the UK;

1:10,000. The lowest PKU prevalence is reported in south Asia in Japan 1:125,000 although China has a similar prevalence to Europe [3]

Table 1.1 Classification of phenylketonuria

Classification Type	**Phenylalanine concentrations pre treatment commencement**	**Phenylalanine tolerance with dietary treatment only (mg/ kg/ day or mg/ day)**
Mild hyperphenylalaninemia (MHP)[53]	< 360 μmol/L	No dietary treatment required
MHP/mild PKU[53]	360-600 μmol/L	Phenylalanine restricted diet, sapropterin or both. Monitoring necessary
Mild PKU[55]	600-900 μmol/L	25-50 mg/ kg/ day (400-600 mg/ day)
Moderate PKU (mPKU)[55]	900-1200 μmol/L	20-25 mg/ kg/ day (350-400 mg/ day)
Classical/ severe PKU (cPKU) [55]	> 1200 μmol/L	< 20 mg/ kg/ day (250-300 mg/ day)
Tetrahydrobiopterin defects [58-61]	1-2% of hyperphenylalaninemia are due to mutations in genes coding for enzymes in the biopterin biosynthesis or regeneration Guanosine triphosphate cyclohydrolase I (GTPCH) 6-pyruvoyl tetrahydropterin synthase (PTPS) Dihydropteridine reductase (DHPR) Pterin 4a carbinolamine dehydratase deficiency (PCD)	Dietary treatment depends on defect. DHPR can be detected by NBS requiring dietary restriction GTPCH, PTPS, PCD are detected by specific pterin patterns in the urine and require pharmacological treatment (L-dopa, 5, hydroxytryptophan)

Legend: MPH, mild hyperphenylalaninemia; mPKU, moderate phenylketonuria; GTPCH, guanosine triphosphate cyclohydrolase 1; PTPS, 6-pyruvoyl tetrahydropterin; DHPR, dihydropteridine reductase; PCD, pterin 4a carbinolamine dehydratase deficiency

1.4 DISCOVERY OF PKU

In 1934, Asjbørn Følling identified an excess of phenylpyruvic acid as the cause of an odd musty odour from the urine of two Norwegian children who were severely developmentally delayed [62, 63]. The condition was initially named *imbecilitas phenylpyruvica* and later phenylketonuria (PKU). Penrose [64, 65] made the link between dietary phenylalanine and urine phenylpyruvic acid. The first successful low phenylalanine diet was designed by Louis Woolf a biochemist from Great Ormond Street Children's Hospital (UK). In 1953 it was used by Bickel and colleagues to treat 2 year old Sheila Jones at Birmingham Children's Hospital, UK. Before treatment Sheila was unable to walk or talk. After a period of following the artificial diet, Sheila could walk and engage in her surroundings, changing the treatment paradigm for PKU [66].

Chapter 2 *Protein substitutes in PKU; their historical evolution*

1.4.1 PRINCIPLES OF DIETARY TREATMEMNT

A strict lifelong low phenylalanine diet remains the foundation of treatment in PKU [67]. It may be prescribed as the sole treatment or combined with drug therapy. The goals of dietary management are to prevent intellectual disability and achieve normal nutritional status. This includes bone mass and density, growth (weight, height, body mass index), and body composition.

In classical PKU, natural protein or phenylalanine is restricted to ≤ 25% of a safe recommended intake [68]. This is necessary to minimise neurological damage, prevent phenylalanine deficiency and maintain phenylalanine concentrations within the European PKU Guideline target ranges [53]. In order to help clarification and consistency of dietary

prescriptions, in the UK protein cut off values are used to classify the suitability of foods permitted in the diet [69, 70]. The dietary restriction (Table 1.2) can be considered in three principal parts:

Table 1.2. Classification of foods allowed in a PKU diet

1. Foods high in protein best avoided • Animal protein: e.g., milk, meat, fish, cheese, eggs, chocolate, yoghurt; plant protein: nuts, soya, pulses, wheat based products (bread, flour, pasta, cakes, biscuits), corn, oats, Quorn ™; gelatin, plant algae and spirulina • Aspartame E951 (an artificial sweetener) is a source of phenylalanine
2. Foods permitted without restriction • Fruits and vegetables containing ≤ 75 mg/100g (except potatoes) • Most foods containing protein ≤ 0.5 g/100g • Plant milks containing protein ≤ 0.1 g/100g • Low protein special foods containing phenylalanine ≤ 25 mg/100g (low protein bread, pasta, milk replacements) • Sugar, jams, fats and oils
3. Foods given in calculated and measured amounts (1g protein/ 50 mg exchanges of phenylalanine) • A prescribed daily amount of phenylalanine/natural protein that is titrated according to blood phenylalanine concentrations is allocated on an individual basis. In the UK an exchange system is used to allocate protein/phenylalanine • One exchange is the amount of natural protein calculated to provide 1g protein or 50 mg of phenylalanine. Different upper cut off points are used to categorise foods as exchange foods. Any of the following foods with a protein/ phenylalanine above the cut off point is an exchange food: o milk/ plant milks: protein content > 0.1 g/100g o breakfast cereals, savoury spreads: protein content > 0.5 g/100g o sauces/ dressings: protein content > 1 g/100g o soya sauces: protein content > 1.5 g/100g o fruits and vegetables: phenylalanine content > 75 mg/100g

A replacement source of protein, obligatory to maintain growth and metabolic function is provided by a low/ free phenylalanine protein substitute or medical formula. Nitrogen is provided as amino acids or peptides; they are usually supplemented with vitamins, minerals and trace elements, and may contain essential and/ or long chain fatty acids and prebiotics. Protein substitutes have several pharmacological functions:

- provide large neutral amino acids, which act in competition at the blood brain barrier for the large neutral amino acid transporter (LAT1). This prevents excess phenylalanine entering the brain leading to neurocognitive damage [71, 72]
- promote normal growth and cellular function [73]
- provide a source of nitrogen for manufacturing smaller molecular substances e.g., nitric oxide [74]
- provide a source of tyrosine, a surrogate essential amino acid in PKU [75]
- optimise blood phenylalanine control [76, 77]
- prevent protein deficiency.

In Chapter 2, the history of protein substitute evolution is described. In the late 1960s, low phenylalanine protein hydrolysates were slowly replaced with phenylalanine amino acid preparations, which are still used today. Protein substitute composition, bioavailability and long term metabolic efficacy has received little scrutiny. In 2008, a new class of protein substitutes were designed based on a bioactive glycosylated caseinglycomacropeptide (CGMP) [78, 79].

1.5 BIOACTIVE PROPERTIES OF GLYCOMACROPEPTIDE

The two major proteins in milk, casein and whey have unique physiochemical and biological properties [80-82]. During the production of cheese, bovine casein treated with chymosin is cleaved into two peptides: para casein (residue 1-105) and casein macropeptide (CMP) (residue 106-169). CMP is found in the soluble whey elute [83]. CMP is a 64 amino acid phosphoglycoprotein, which undergoes glycosylation forming a glycoprotein, named casein glycomacropeptide (CGMP) [84]. Five oligosaccharides (glycans) have been identified as part of the glycomacropeptide structure [83]. The structural properties and beneficial effects of CGMP are linked to the glycan chains on the macropeptide [85]. CGMP is an incomplete peptide, lacking in phenylalanine and other aromatic and sulphur containing amino acids. Nielsen and Tromhold in 1994 recognised the potential of CGMP as a source of protein in a low phenylalanine diet [86]. However, they also recognised that supplementation of CGMP with some essential amino acids was necessary to make it viable in PKU. So far it has been commercially prohibitive to produce a phenylalanine-free source of CGMP as there is a residual amount of a phenylalanine left after the manufacturing process (35mg/ 20g of protein equivalent) [87].

1.5.1 GLYCOME OF BOVINE MILK PROTEIN

There are two types of glycosylation that occur in casein macropeptide: *N* linked when the glycan chain is attached to the amide side chain of the asparagine residue, and *O* linked when the glycan is attached to the oxygen of a serine or threonine residue [88, 89]. These linkages are associated with the unique bioactive properties of CGMP. Approximately 60% of CGMP is glycosylated [90] with exclusively *O* linking glycans. The glycosylation is variable and depends on the lactation and genetic phenotype of the casein preparation [87,

91, 92]. The main glycans in mature bovine milk are: *N*-acetylgalactosamine (Gal1NAc), galactose (Gal) and *N*-acetylneuraminic acid which is also called sialic acid (Neu5Ac). There is evidence to suggest glycosylation is a controlled hierarchical process which influences the biological associated activities of CGMP [87, 93]. The sialic content of CGMP is vital for most of the biological roles associated with this bioactive glycoprotein. Table 1.3 gives examples of the beneficial uses of CGMP.

Table1.3 Beneficial properties associated with glycomacropeptide

Glycoprotein	Properties	Evidence
Modulation of immune response	• Mice splenocytes were used to test for immune responses. CGMP was found to bind to the mitogen producing cytokines blocking inflammatory responses • A lymphoctye model of colitis in mice compared CGMP with a common medication used to treat colitis (dextran sodium sulphate). CGMP produced an anti-inflammatory effect in the lymphocyte reducing colitis, a similar and sometimes better response than conventional medical treatment	[94] [83] [88] [95] [96, 97]
Probiotic properties	• Sialic acid promotes bifidobacterial growth. These are important in inhibiting pathogenic bacteria. Human studies suggest some benefits include producing a higher diversity of microbiota and short chain fatty acid concentrations, which have positive effects on gut health • Studies in PKU mice demonstrate a positive effect of CGMP compared to casein and AA diets on gut health. Desulfovibrio bacteria decreased, and SCFA increased with a lower level of inflammation markers	[98] [99] [100]
Satiety and stimulation of cholecystokinin release	• CGMP triggers incretin receptors on the intestinal mucosa • Cholecystokinin is stimulated by CGMP • Human studies show CGMP influences hormone responses to satiety, but energy intake and weight loss have not been demonstrated	[101-107]

Anti cariogenic effect	• Prevents dental erosion. There is a positive action of CGMP showing a protective effect and suppressing demineralization of teeth. It inhibits the adhesion and modifies the composition of cariogenic plaque bacteria. Xylitol has a synergistic action with CGMP and is used as an ingredient in toothpaste	[108-110]
Inhibition of cholera toxins	• CGMP binds cholera toxin in mice. 1 mg of CGMP prevented diarrhoea in mice infected with the cholera toxin	[111]
Improved learning behaviour	• Piglets were given different amounts of CGMP and had brain gangliosides and sialoprotein concentrations associated with learning and memory measured. Learning improved in the group given the highest concentration of CGMP • Sialic acid was associated with increased learning and an increased concentration in the genes associated with learning	[112, 113]
Nutraceutical use	• Used in food products, CGMP has a good stability and is soluble in acid • Infant formula prepared with galactooligosaccharides (GOS) together with CGMP as a sialyl donor has been produced, but further research is needed to investigate its safety and the impact of using sialyated GOS infant formula	[114-116]

Legend: CGMP, casein glycomacropeptide; GMP, glycomacropeptide; PKU, phenylketonuria; AA, amino acid; SCFA, short chain fatty acids; GOS, galactooligosaccharides

1.5.2 ADAPTATION OF CGMP FOR USE AS A LOW PHENYLALANINE PROTEIN SUBSTITUTE IN PKU

In addition to containing a residual amount of phenylalanine [83], commercially prepared CGMP has inadequate amounts of five indispensable amino acids. They are histidine, tryptophan, methionine, leucine and tyrosine. When CGMP is supplemented with these five amino acids, it is a safe alternative to phenylalanine-free L-amino acids [79]. CGMP has also been incorporated into foods without altering their palatability [117].

1.5.3 IS CGMP BETTER THAN L-AMINO ACIDS WHEN USED AS A PROTEIN SUBSTITUTE SOURCE IN PKU?

Various authors suggest CGMP offers potential advantages compared to traditional protein substitutes based on L-amino acids. It improves phenylalanine control [78, 118], growth [79], body composition, bone density, and neurocognition. [119]. It also has a better palatability, which helps dietary adherence [117, 120]. However, no long-term studies have been conducted in children, pregnancy or adults to confirm these findings. The short-term studies and those using animal models clearly do not measure the long-term efficacy of CGMP and they fail to meet rigorous scientific scrutiny. The European PKU guidelines [53] did not issue guidance about the use of CGMP due to inadequate evidence. This thesis aims to address these shortcomings by prospectively examining over a 3 year period, the efficacy of CGMP compared to traditional L-amino acid protein substitutes in children with PKU. The following questions will be addressed.

1.6 QUESTION 1. DOES THE PHENYLALANINE CONTENT OF CGMP AFFECT BLOOD PHENYLALANINE CONTROL IN PKU?

Studies using CGMP have suggested no significant alteration in blood phenylalanine concentrations [118, 121-124]. In all the studies with one exception [124], phenylalanine concentrations were higher (although not statistically significant) in the CGMP groups. However, all these studies have their limitations, subjects were studied for ≤ 6 months duration, blood phenylalanine control was suboptimal at study entry and some of the subjects had been on adjunctive pharmacology treatment. One study [118] suggested that the residual phenylalanine in the CGMP they investigated was too high at 0.4 g/100g of product. This was only used in three subjects all with a higher phenylalanine tolerance. In the remaining 9 subjects, the CGMP was further refined providing phenylalanine at 0.2 g/100g of product. This adaptation suggested that caution and further investigations were necessary before the wider introduction of CGMP to subjects with classical PKU. Table 1.4 lists the studies using CGMP in PKU and their outcomes.

Table 1.4 Studies using CGMP compared to L-amino acid protein substitutes in PKU

Author/ Year	Country	Study design Age (range)	Nos of subjects/ gender	PKU Phenotype	Study intervention	Mean/ median phenylalanine concentrations in AA compared to CGMP-AA (µmol/L)
Van Calcar 2009[118]	United States	Cross sectional 23 y ± 7 (11-31)	11 4F, 7M	10 Classical 1 Variant	100% AA v 100% CGMP 4 days on each product	AA = 619 CGMP = 676 *p=ns*
MacLeod 2010[125]	United States	Cross sectional 23 y ± 7 (11-31)	11 4F, 7M	11 Classical	100%AA v 100% CGMP 4 days on each product	AA = 619 CGMP = 676 *p=ns*
Ney 2016[122]	United States	Randomised cross over clinical study (15-49)	30 18F, 12M	20 Classical 10 Variant	21 days: 100% GMP or 100% AA	AA = 655 CGMP =777 *p=ns*
Zaki 2016[124]	Egypt	Clinical study 6.7 y (5.0-11.8)	10 4F, 6M	10 Classical	9 weeks: 50% CGMP + 50% AA 9 weeks: 100% AA	100%AA =490 CGMP50%+50%AA= 376 *p=ns*
Pinto 2017[126]	Portugal	Retrospective longitudinal study 27 y ± 10 (13-42)	11 8F, 3M	6 Classical, 4 Mild, 1 HPA	20 months: n=11 CGMP, n=11 AA	AA =516 CGMP =540 *p=ns*
Ahring 2018[121]	Denmark	Randomised cross over clinical study 33.3 y ± 11.2 (15-48) 4 PS given over 4 visits	8 7F, 1M	8 Classical	PS1= CGMP, PS2 = AA PS1 and PS2 same AA profile PS3 = CGMP+AA, PS4=AA PS3 and PS4 same AA profile but no Phe	AA=688 CGMP=819, *p=ns*

Legend: AA, amino acid; CGMP, casein glycomacropeptide; PS, protein substitute; ns, not significant; F, female; M, male

Chapter 3A. *Glycomacropeptide in children with phenylketonuria: does its phenylalanine content affect blood phenylalanine control?*

Chapter 3B. *is a follow-on study as a result of the findings from the pilot study (3A). Long term use of modified casein glycomacropeptide (CGMP2) compared to amino acid protein substitutes on phenylalanine, growth and nutritional status*

1.7. QUESTION 2. DOES A PROTEIN SUBSTITUTE BASED ON A BIOACTIVE AMINO ACID SEQUENCE GLYCOMACROPEPTIDE IMPROVE PROTEIN BIOAVAILABILITY BY CHANGING THE PHENYLALANINE AND TYROSINE VARIABILITY?

Amino acids have several nutritional, biochemical and physiological roles linked to growth, health and prevention of disease [74, 127, 128]. Functional amino acids (essential or non-essential) regulate key metabolic pathways. They provide nitrogen, hydrocarbon skeletons and sulphur, which cannot be substituted by other nutrients in the diet [129]. Both nitrogen and sulphur are unable to be synthesised de novo. Table 1.5 references some of the functional roles of amino acids, which regulate body composition and bone health. Other functions include modulating bacterial flora, glucose homeostasis and inflammatory responses. Amino acids are also involved in cell signalling (including mammalian target of rapamycin complex 1 (mTORC1), and the interaction and generation of small peptides, glucagon like peptide 1(GLP-1), peptide–YY (PYY), serotonin and insulin. Insulin plays a key regulatory role in amino acid metabolism, and amino acids alter insulin action by regulating glucose and protein metabolism [130]. Therefore, protein substitutes have a central physiological purpose in addition to providing a source of high quality protein. Achieving this balance is essential to maintaining health. It is vital in children where there is an 80% dependence

on a protein which supplies daily nitrogen requirements. Designing a protein substitute to meet these criteria is challenging.

Table 1.5. Functional role of amino acids

Amino acid	Function	Publication
Branched chain amino acids (BCAA) Leucine, valine, isoleucine	• BCAAs are involved in protein synthesis and breakdown • Leucine/isoleucine stimulate protein synthesis via (mammalian target of rapamycin) mTOR pathway • BCAAs effect neurotransmission of chemicals in the brain by modifying large neutral amino acid (LNAA) transport at the blood brain barrier	[131-136]
Leucine	• Stimulates m RNA translation via insulin dependent and independent pathways stimulating muscle protein synthesis • Leucine activates glutamate dehydrogenase activity in pancreatic β cells, this stimulates the tricarboxylic acid activity of the pancreatic cells leading to increased insulin production. This response can be increased when carbohydrate is present	[137, 138]
Alanine	• Main gluconeogenic amino acid	[139]
Glutamine	• Most abundant amino acid in the body • Glutamine plays an active role in the immune system providing 50% of ATP in lymphocytes and 35% in macrophages sustaining immune responses • Regulates ammonia formation • Production of glutathione maintains acid base balance	[127]
Glutamine, glutamate, aspartate	• Energy for enterocyte proliferation, protects cells from apoptosis • Major energy substrate for the small intestine	[140-143]

Glycine	• Makes serine the coenzyme of glutathione, purines and haem. Acts as an inhibitory neurotransmitter	[144]
Glycine, tryptophan, tyrosine	• Regulation of neurological development and function	[145, 146]
Methionine	• Major donor of methyl group affecting DNA and protein methylation in cells	[147]
Arginine, glutamine, glutamate, leucine, proline	• Modulates gene expression and enhances growth of small intestine and skeletal muscle	[74, 148, 149]
Arginine	• Produces nitric oxide which has a vasodilatory effect • Synthesis of creatine, used in muscle metabolism and polyamines regulating gene expression • Pathway for ammonia detoxification • Stimulates growth hormone and insulin	[150-152]
Cysteine	• Necessary for skin and hair formation • Sulphur containing amino acid. It is key in forming a disulfide bridge between two peptide chains e.g., insulin	[153, 154]
Histidine	• Involved in the metabolism of carbohydrate, protein and nucleic acids	[155]
Tryptophan	• Source of nicotinic acid • Precursor of serotonin	[16, 17, 75, 156-158]
Tyrosine	• Precursor of catecholamine neurotransmitters epinephrine, norepinephrine, and dopamine. Precursor of thyroxine and melatonin • Tyrosine residues have active sites which can be altered and change the enzyme specificity • Usually, a semi-essential amino acid but essential in PKU due to the biochemical absence/ reduced activity of phenylalanine hydroxylase	[16, 17, 75, 157, 158]

Legend: BCAA, branched chain amino acid; mTOR, mammalian target of rapamycin; LNAA, large neutral amino acids; m RNA, messenger ribonucleic acid; DNA, deoxyribonucleic acid

L-amino acids are incapable of replicating the physiological actions of whole protein. Amino acids are directly absorbed from the small intestine [74]. A meal based on L amino acids results in higher plasma amino acid concentrations compared to a meal based on whole protein. Amino acids are rapidly absorbed but have a shorter duration in the plasma compared to amino acids slowly released from whole protein. This difference influences their utilization [159-161]. Herrmann et al [162] demonstrated that ingestion of large doses of protein substitute derived from amino acid, increased amino acid oxidation and nitrogen excretion decreasing their cellular availability. For effective protein synthesis all essential amino acids must be available to the tissues in appropriate amounts simultaneously [163]. In children with PKU, protein substitutes provide 80% or more of their total protein needs. Therefore, any protein substitute that will imitate the physiological absorption of whole protein, will theoretically improve growth, body composition, bone density and may possibly influence inflammatory responses and appetite.

Chapter 4A. *Variability of phenylalanine concentrations over 24 hours using two different protein substitutes and changing the dietary phenylalanine intake*

Chapter 4B. *A pilot study to review if a glycomacropeptide compared to L amino acid protein substitutes alters the pre and postprandial amino acid profile in children with phenylketonuria*

1.8 QUESTION 3. DOES THE USE OF GLYCOMACROPEPTIDE IMPACT GROWTH AND BODY COMPOSITION IN CHIDREN WITH PKU?

The impact of using an artificial diet on physical growth was first reported in the late 1970s and despite improvements in dietary treatment mixed findings on growth outcome are still reported [73, 164-167]. Early studies [168, 169] demonstrated that children on higher doses

of protein substitute that exceeded the WHO/FAO/UNU 1973 [68] safe levels of protein intake, had improved growth. Smith [170] showed that even if amino acids are efficiently absorbed from the intestinal tract, compared to natural protein there was a higher loss of nitrogen as urea. McBurnie et al [171] and Holm et al [172] compared height, weight and head circumference in two prospective American collaborative studies, comparing 133 and 124 children with PKU over 8 and 4 years respectively. In both studies weight and height increased similar to control groups. In contrast, 3 European studies [173-175] found children with PKU had reduced height growth when compared to control subjects. Dhondt [173] reported normal height and weight was achieved after dietary relaxation at 8 years of age. Schaefer et al [176] reported negative weight and height in the first 2 years returning to normal at 3 years of age.

A recent systematic review and meta-analysis examining growth in subjects with PKU [165] reported normal growth at birth and during infancy, but children were significantly shorter and had lower weight for age than the reference populations during the first four years of life. Linear growth was reduced until the end of adolescence. These findings were not identified in patients with mild hyperphenylalaninemia following no dietary restriction. Accounting for the diverse heterogeneity of the study data when compared to reference groups, linear growth was reduced during the first three years of life, in puberty and on reaching adulthood.

However, optimal growth was noted in those studies where total protein intake was higher [177-180]. Nitrogen balance is regulated by urea production [181, 182], which is produced linearly in response to plasma amino acid concentrations. Ney et al [183] and van Calcar et al [118] suggested that CGMP may induce a slower and more sustained release of amino acids, leading to decreased urea and greater availability of amino acids for protein synthesis possibly leading to improved growth.

There are no long-term prospective studies or systematic/ meta-analysis on body composition in PKU. Of eleven studies completed in children (Table 1.6), comparison between the studies is difficult. This is due to a lack of national reference standards that would allow comparison of body composition data. The different techniques used to measure body composition, pubertal status and variable PKU phenotypes also makes comparison of data between studies difficult. Of six studies using healthy controls, four showed no statistical significance in body composition. One study showed a correlation with an increased blood phenylalanine concentration and higher fat mass in male subjects with PKU only [184]. Albersen et al [185] showed body fat was significantly higher with subjects with PKU, and higher in females >11 years. In a disorder where nitrogen for body composition and growth is from an artificial source, it is important to monitor lean and fat mass. Long term associated health problems such as type II diabetes and cardiometabolic health may be linked to an altered body composition due to the protein substitute [186, 187].

Table 1.6. Studies measuring body composition in children with PKU

Author/ year	Number/ age of subjects Body composition measurement technique	Parameters measured	Main outcome	Limitations
Allen 1996 Australia [188]	n=30 PKU (classical) Mean age; 9.6 y n=65 control Mean age; 11.2 y Skinfold thickness	• Body fat • Resting energy expenditure	• No differences in body fat compared to controls • No difference in resting energy expenditure	Skinfold measurements provide no information on lean mass.
Dobbelaere 2003 [167] France	n=20 PKU (classical) n=20 control Age and gender matched Mean age; 4.5 y Skinfold thickness Bioelectrical impedance	• Weight, height, body mass index (BMI,) head circumference • Skin folds triceps, biceps, subscapular and suprailiac measurement • Body density, body fat, lean mass • Blood tyrosine, and phenylalanine concentrations	• No differences in body composition compared with controls • Growth, significantly different from reference population, $p < 0.05$ • No correlation with phenylalanine biochemical bloods or calorie intake	Body mass index measures nutritional status not body composition Impedance associated with poor accuracy for individuals and groups

		• Zinc, selenium, thyroid, insulin, growth factor • Weighed 4 day dietary intake		
Huemer 2007 [189] Study over 12 months Austria	n=34 PKU (classical) n=34 control Age/ gender matched Mean age; 8.7 y Total body electrical conductivity (TOBEC)	• Weight, height, BMI • % fat, fat free mass • Blood phenylalanine concentrations	• No differences between groups for the measured parameters • Significant correlation between natural protein g/ kg/ d and fat free mass ($p < 0.0001$)	TOBEC rarely used and unknown accuracy compared to other body composition measurements
Albersen 2009 [185] Netherlands	n=20 PKU (classical) n=20 control Age/ gender matched Mean age; 10 y BodPod/ whole body air displacement plethysmograph	• Weight, height, BMI • % body fat • Blood phenylalanine concentrations	• No difference for weight, height, BMI • Body fat significantly higher in PKU despite similar BMI to controls $p=0.002$ • Body fat higher in girls > 11 y $p=0.027$ • Body fat increased with weight only in PKU • No correlation with blood phenylalanine	4/ 20 PKU children were from different ethnic background
Adamczyk 2011 [190] Poland	n=45 PKU (classical) Mean age; 13.8 y	• Weight, height, BMI	• Normal body fat, and lean body mass	No control group

	Group 1= 15 pre pubertal Group 2 =18 pubertal good control Group 3=12 pubertal poor control Dual X ray absorptiometry (DXA)	• Lean body mass • Fat mass • Total bone density • Bone mineral content • Ratio of bone mineral content/ lean body mass • Bone markers	• Statistically significant differences for ratio bone mineral content/ lean body mass between groups • Blood phenylalanine negatively affected bone status • Data compared with Polish DXA reference values	DXA radiation exposure, whole body bias dependent on size, gender and amount of fat
Douglas 2013 [191] USA	n=59 PKU (classical and mild) Mean age; 14.4 y BodPod/whole body air displacement plethysmograph Tricep, subscapular, suprailiac, thigh skinfold	• Weight, height, BMI • Body fat	• Normal body fat • Lean mass not evaluated • In the group found inverse relationship between age and body fat p=0.016 • Agreement between skinfold depends on equations used to convert measurement to body fat	Mixed PKU phenotype No control group
Rocha 2012 Rocha 2013 [192, 193] Portugal	n=89 PKU (classical, mild, hyperphenylalaninemia) Mean age; 14.4 y n=78 controls Mean age; 15.9 y	• Weight, height, BMI • Fat mass • Lean body mass • Body cell mass	• No differences in fat mass • No differences in lean body mass • No differences in body cell, muscular mass or phase angle	Impedance is associated with poor accuracy for individuals and groups Mixed PKU phenotype

	Bioelectrical impedance analysis (BIA)	• Muscular mass • Phase angle	• All classical PKU negative height z score • No differences in height compared to controls in children aged <19 y • In PKU group, aged >19y, height statistically significantly worse than controls p=0.017	
		• Blood pressure, amino acids • Glucose, insulin, total cholesterol high density cholesterol, triglycerides, C reactive protein, uric acid • Assessment of protein substitute and natural protein intake	• Anthropometric parameters no differences to controls • Higher triglycerides / high density cholesterol in PKU group • Metabolic syndrome no difference compared with controls • In PKU subjects those with central obesity had significantly higher triglycerides /high density cholesterol than those without central obesity	Mixed PKU phenotype
Doulgeraki 2014 [194] Greece	n=48 PKU (classical) Mean age; 10.9 y 32 HPA (mild hyperphenylalaninemia)	• Lean body mass • Fat mass • Bone mineral density	• No differences in body composition • Weight and BMI significantly different between mild PKU and classical PKU	Mixed PKU phenotype Control group not reported in study

	Mean age; 10.9 y n=57 control Age/gender matched Dual X ray absorptiometry (DXA)		• Bone mineral density lower in classical PKU compared to mild and controls • Fat mass significantly higher in PKU teenagers with poor phenylalanine control • Positive correlation between bone, muscle and fat mass in both groups and fat mass and phenylalanine concentrations	DXA radiation exposure, whole body bias dependent on size, gender and amount of fat
Mazzola 2016 [195] Brazil	n=27 PKU n=11 early diagnosed n=16 late diagnosed classical and mild n=27 control Age/gender matched Mean age; 12 y Bioelectrical impedance analysis (BIA)	• Weight, height, BMI • Fat mass • Lean body mass • Extracellular mass/ body cell mass ratio • Phase angle (PA)	• No differences in body fat • No differences in lean body mass • No effect on time of diagnosis or PKU phenotype	Age at diagnosis variable some early and late treated PKU Mixed PKU phenotype
Sailer2020 [184] USA	n=30 PKU n=30 control Age/gender matched	• Weight, height BMI • Fat mass • Lean body mass	• Male subjects with PKU had significantly lower lean body mass and more fat mass than controls p=0.024	Mixed PKU phenotype 13% on sapropterin

	4 subjects on sapropterin Mean age; 11.6y Dual X ray absorptiometry (DXA)	• 24h dietary recall	• No differences for females and controls when measuring same parameters • Age/ fat mass positively correlated with blood phenylalanine p=0.02 • Protein substitute negatively correlated with blood phenylalanine p=0.04 • Males with PKU had significantly lower height compared with controls $p < 0.05$ • No difference in energy intake between the groups	DXA radiation exposure, whole body bias dependent on size, gender and amount of fat

Legend: Kuvan (Sapropterin) drug treatment for PKU (BioMarin Pharmaceutical Inc); BMI, body mass index

Chapter 5A. *Growth and body composition in PKU children - a 3-year prospective study comparing the influence of L-amino acid to glycomacropeptide protein substitutes*

1.9 QUESTION 4. DOES A PROTEIN SUBSTITUTE BASED ON CGMP COMPARED TO L-AMINO ACIDS IMPROVE BONE MASS, DENSITY AND GEOMETRY IN PKU CHLDREN?

Bone mass is maintained by a complex and dynamic process involving resorption of bone by the osteoclast and formation of bone by the osteoblast. In children this is a dynamic continuous process of modelling and remodelling [196]. Peak bone mass, which programmes the future risk of osteoporosis, is established in childhood and adolescence [197, 198]. Factors that influence bone mass are varied including genetics, lean mass, adiposity, adipocytokines, physical activity and nutrition (Table 1.7). The relationship between fat and bone is contentious. Evidence [198] suggests that in early childhood, obesity confers a structural advantage, but with age this relationship is reversed and excessive fat is detrimental. Clark et al 2006 [199] reported a positive relationship between adiposity and bone mass accrual in 3082 healthy children. Others have reported opposite findings [200, 201]. Lean body mass is the strongest significant predictor of bone mineral content [202, 203] and strongly relates to bone mass and skeletal development in children.

Dietary protein promotes peripubertal bone growth and slows bone loss [204]. Protein is necessary for optimal bone metabolism during growth positively influencing bone mass, density and strength [204-206]. In children and adults with PKU, bone density is inconsistently reported [207-213]. Four systematic reviews and three meta-analysis

studies report mixed results. Enns et al [214] reported nine suboptimal bone health outcomes. The scope of this review was on general health problems in PKU and therefore failed to interpret the results on bone health in depth. Hansen et al [215], described a lower spine bone mineral density, but this review had methodological errors and assessment bias. Demirdas [216], reported bone mineral density (BMD) was within the normal range, and although lower than normal was not clinically important. There was no correlation with phenylalanine concentrations, vitamin D, parathyroid hormone and individual nutrients. Castro et al [217] supported the findings from Demirdas [216] showing BMD was lower than the reference groups but within the normal range. They also demonstrated an imbalance between bone formation and resorption, favouring bone removal.

Solverson et al [218] studied the effect of three different diets on bone strength in mice with or without PKU. They were given a low protein diet with a) CGMP b) amino acid or c) they were given a normal (casein) diet. The PKU mice fed either CGMP or amino acid had a lower BMD than non PKU mice. In PKU mice, who were fed the amino acids, the femur length independent of gender was significantly shorter compared to the PKU mice fed CGMP or normal diet. Skeletal fragility, brittle and weak femora was a consistent finding in the PKU mice regardless of gender or diet. The reduction of BMD and bone mineral content (BMC) of the femora measured by dual X ray absorptiometry (DXA) was more pronounced in the mice receiving amino acids compared with CGMP. This group concluded that the type of protein influenced bone outcome in mice, with CGMP giving better results compared to amino acids. However, careful consideration is needed to determine the impact of CGMP or amino acids on bone growth. In humans bone growth is

a slow multifaceted process affected by hormonal patterns, gender, obesity, dietary intake and physical activity.

No studies have examined the role of CGMP and amino acid protein substitute on long-term bone health in children with PKU.

Chapter 5B. *Evaluation of bone health in children with PKU: a three-year prospective study using two different protein substitutes bioactive glycomacropeptide compared to L-amino acids*

Table 1.7. Suggestive factors influencing bone mass

ADIPOCYTOKINE HORMONES		
Adiponectin	• Adiponectin regulates energy homeostasis, glucose and lipid metabolism and inflammatory pathways • Adiponectin levels were inversely related to bone mass accrual in childhood. Analysis using pQCT links periosteal growth, endosteal expansion and bone mineral content • Plasma adiponectin inversely related to visceral fat mass and body mass index (BMI)	[219] [220] [221, 222]
Leptin	• Leptin increases osteoblast proliferation and differentiation. It regulates osteoclast development • It promotes bone nodule formation • Leptin via the action on the central nervous system influences bone development • Leptin has a dose mediated effect; lower concentrations of leptin are osteoprotective, higher concentrations lead to bone loss	[198, 223-225] [198, 226]
Inflammatory cytokines	• Inter leukon 6, Tumour Necrosis Factor alpha may influence bone mass directly • Cytokines may influence appetite and weight indirectly affecting bone	[227, 228]
β CELL PRODUCTION		
Insulin/ IGF	• Insulin simulates osteoblast growth • Hyperinsulinemia increases fatty acids which inhibit bone resorption • Insulin growth factor (IGF1) and oestrogen influence growth	[229-232]

	• IGF 1 Stimulates calcium and phosphate metabolism increases calcitriol synthesis and enhances renal phosphate re-absorption	
NUTRITION		
Nutrients: protein/ calcium/ vitamin D/ fatty acids	• Osteoclast activity is related to food intake, and is decreased when energy intake is decreased • Glucose increases calcitonin secretions, decreasing parathyroid hormone • Food increases incretin hormones, glucagon like peptide (GLP1) and insulinotropic polypeptide (GIP), which increase insulin • Protein is a structural component of bone	[233] [223, 234, 235] [206, 236-238]
ACTIVITY		
Physical activity	• Exercise represents mechanical strain leading to bone deposition and improved bone mass • Bone turnover markers are not correlated with activity • Physical activity affects bone mineral density (BMD) and bone mineral content (BMC) of preadolescent girls. Greater activity having a positive influence on BMD • Girls 9-13y with higher activity have greater BMC and BMD than those with lower activity • Prepubescent children appear to have improved activity related osteogenic gains compared to adolescents	[239]

Legend: pQCT, peripheral quantitative computed tomography; BMI, body mass index; IGF1, insulin growth factor 1; GLP1, glucagon like peptide 1; GIP, gastric inhibitory polypeptide; BMD, bone mineral density; BMC, bone mineral content

1.10 QUESTION 5. DOES GLYCOMACROPEPTIDE IMPACT SATIETY AFFECTING THE ENERGY AND DIETARY INTAKE OF CHILDREN COMPARED TO L- AMINO ACID PROTEIN SUBSTITUTES?

There is general agreement that protein is the most satiating macronutrient increasing gastrointestinal hormones and diet induced thermogenesis [108, 240-242]. Gastrointestinal incretin hormones regulate food intake by inducing or suppressing hunger, ghrelin, an orexigenic hormone or satiety hormones which include (CCK (cholecystokinin), GLP 1 (glucagon like peptide 1) and peptide YY (PYY)) [243, 244]. Whey compared to casein is thought to have a greater impact on satiety by the action of CGMP and the release of cholecystokinin (CCK) [245, 246]. The evidence from various studies suggests there is only a tenuous relationship between CGMP and satiety [105, 241, 247-249] and there is no clear indication that one protein source is more satiating than others.

MacLeod et al [125] studied the impact of CGMP or amino acids on appetite in eleven subjects with PKU. In a cross over study energy intake was bespoke for each subject but remained the same over the study period. Bloods were collected fasting and postprandially (180 mins after breakfast) and analysed for total amino acids, plasma ghrelin and insulin concentrations. All subjects completed a motivation to eat visual analog scale (VAS) questionnaire. There were no differences between the two study diets for VAS. Postprandial total plasma amino acids and insulin concentrations were significantly higher with CGMP compared to amino acids. Postprandial ghrelin concentrations after the CGMP breakfast were significantly lower compared to the breakfast with amino acids. The authors proposed that CGMP had a positive effect on satiety due to lower ghrelin, and higher amino acid concentrations. In chapter 6 energy and dietary intake in children with PKU were studied systematically over a 3 year period. Two groups were studied one taking CGMP and a second

group taking amino acids as their protein substitute source. Any differences in appetite leading to a reduced energy intake were objectively examined.

Chapter 6. *The impact of the use of glycomacropeptide on satiety and dietary intake in phenylketonuria*

1.11 QUESTION 6. DO PROTEIN SUBSTITUTES LEAD TO BREATH MALODOUR AND ARE THEY IN PART RESPONSIBLE FOR NON-ADHERENCE IN CHILDREN WITH PKU?

In PKU there are no immediate clinical consequences to non-adherence, and this is a common problem from the start of adolescence [67, 250]. In clinical practice caregivers report their children have breath malodour, particularly after the consumption of a protein substitute. This may fuel non-adherence by lowering self-esteem, increasing bullying and affecting interpersonal communication. Social isolation and consequently lower adherence with protein substitutes may result. Other factors precipitating non-adherence are the relentless strict dietary regimen and lack of food choice spontaneity. Isolation and emotional insecurity particularly when socialising with peer groups is a common finding. CGMP has been shown to improve palatability [114, 117], which has been linked to improved adherence. No study has quantitatively measured breath odour in PKU children. In a cross over study using gas chromatography ion mobility spectrometry exhaled volatile organic compounds were measured in children taking CGMP or AA protein substitutes over the course of 10 hours.

Chapter 7. *Investigation of paediatric PKU breath malodour, comparing glycomacropeptide with phenylalanine free L- amino acid supplements.*

This thesis aims to draw together a series of questions centred around the efficacy of protein substitutes (CGMP and amino acids) essential in the management of PKU. The use of a bioactive protein substitute (CGMP) compared to conventional amino acids has not been extensively investigated. Over the course of 36 months, comparing CGMP with amino acids, we conducted eight studies reviewing phenylalanine tolerance, body composition, bone density, the effect on satiety and a preliminary investigation on amino acid absorption.

1.12 REFERENCES

1. Fitzpatrick, P.F., *Tetrahydropterin-dependent amino acid hydroxylases.* Annu Rev Biochem, 1999. **68**: p. 355-81.
2. Kaufman, S., *The phenylalanine hydroxylating system from mammalian liver.* Adv Enzymol Relat Areas Mol Biol, 1971. **35**: p. 245-319.
3. Hillert, A., et al., *The Genetic Landscape and Epidemiology of Phenylketonuria.* Am J Hum Genet, 2020. **107**(2): p. 234-250.
4. Sumaily, K.M. and A.H. Mujamammi, *Phenylketonuria: A new look at an old topic, advances in laboratory diagnosis, and therapeutic strategies.* Int J Health Sci (Qassim), 2017. **11**(5): p. 63-70.
5. Flydal, M.I. and A. Martinez, *Phenylalanine hydroxylase: function, structure, and regulation.* IUBMB Life, 2013. **65**(4): p. 341-9.
6. Ashe, K., et al., *Psychiatric and Cognitive Aspects of Phenylketonuria: The Limitations of Diet and Promise of New Treatments.* Front Psychiatry, 2019. **10**: p. 561.
7. Bickel, H., J. Gerrard, and E.M. Hickmans, *Influence of phenylalanine intake on phenylketonuria.* Lancet, 1953. **265**(6790): p. 812-3.
8. Weglage, J., et al., *Neurocognitive functioning in adults with phenylketonuria: results of a long term study.* Mol Genet Metab, 2013. **110 Suppl**: p. S44-8.
9. Bickel, H., *The effects of a phenylalanine-free and phenylalanine-poor diet in phenylpyruvic oligophrenis.* Exp Med Surg, 1954. **12**(1): p. 114-7.
10. Scriver, C.R., *Garrod's foresight; our hindsight.* J Inherit Metab Dis, 2001. **24**(2): p. 93-116.
11. DeRoche, K. and M. Welsh, *Twenty-five years of research on neurocognitive outcomes in early-treated phenylketonuria: intelligence and executive function.* Dev Neuropsychol, 2008. **33**(4): p. 474-504.
12. Huijbregts, S.C., et al., *The neuropsychological profile of early and continuously treated phenylketonuria: orienting, vigilance, and maintenance versus manipulation-functions of working memory.* Neurosci Biobehav Rev, 2002. **26**(6): p. 697-712.
13. Hanley, W.B., et al., *"Hypotyrosinemia" in phenylketonuria.* Mol Genet Metab, 2000. **69**(4): p. 286-94.
14. Blau, N., F.J. van Spronsen, and H.L. Levy, *Phenylketonuria.* Lancet, 2010. **376**(9750): p. 1417-27.
15. Blau, N., A. MacDonald, and F. van Spronsen, *There is no doubt that the early identification of PKU and prompt and continuous intervention prevents mental retardation in most patients.* Mol Genet Metab, 2011. **104 Suppl**: p. S1.
16. Pietz, J., et al., *Large neutral amino acids block phenylalanine transport into brain tissue in patients with phenylketonuria.* J Clin Invest, 1999. **103**(8): p. 1169-78.
17. Gonzalez, M.J., et al., *Impaired Neurotransmission in Early-treated Phenylketonuria Patients.* Semin Pediatr Neurol, 2016. **23**(4): p. 332-340.
18. Liang, L., et al., *Phenylketonuria-related synaptic changes in a BTBR-Pah(enu2) mouse model.* Neuroreport, 2011. **22**(12): p. 617-22.
19. Schlegel, G., et al., *Phenylketonuria: Direct and indirect effects of phenylalanine.* Exp Neurol, 2016. **281**: p. 28-36.
20. de Freitas, M.S., et al., *Ca(2+)-mediated phosphorylation and proteolysis activity associated with the cytoskeletal fraction from cerebral cortex of rats.* Neurochem Res, 1996. **21**(12): p. 1489-95.
21. Leuzzi, V., et al., *Biochemical, clinical and neuroradiological (MRI) correlations in late-detected PKU patients.* J Inherit Metab Dis, 1995. **18**(5): p. 624-34.

22. Malamud, N., *Neuropathology of phenylketonuria.* J Neuropathol Exp Neurol, 1966. **25**(2): p. 254-68.
23. Shah, S.N., N.A. Peterson, and C.M. McKean, *Lipid composition of human cerebral white matter and myelin in phenylketonuria.* J Neurochem, 1972. **19**(10): p. 2369-76.
24. Shefer, S., et al., *Is there a relationship between 3-hydroxy-3-methylglutaryl coenzyme a reductase activity and forebrain pathology in the PKU mouse?* J Neurosci Res, 2000. **61**(5): p. 549-63.
25. Dyer, C.A., et al., *Evidence for central nervous system glial cell plasticity in phenylketonuria.* J Neuropathol Exp Neurol, 1996. **55**(7): p. 795-814.
26. Reynolds, R., R. Burri, and N. Herschkowitz, *Retarded development of neurons and oligodendroglia in rat forebrain produced by hyperphenylalaninemia results in permanent deficits in myelin despite long recovery periods.* Exp Neurol, 1993. **124**(2): p. 357-67.
27. Thompson, A.J., et al., *Neurological deterioration in young adults with phenylketonuria.* Lancet, 1990. **336**(8715): p. 602-5.
28. Smith, I., J. Glossop, and M. Beasley, *Fetal damage due to maternal phenylketonuria: effects of dietary treatment and maternal phenylalanine concentrations around the time of conception (an interim report from the UK Phenylketonuria Register).* J Inherit Metab Dis, 1990. **13**(4): p. 651-7.
29. Bilder, D.A., et al., *Psychiatric symptoms in adults with phenylketonuria.* Mol Genet Metab, 2013. **108**(3): p. 155-60.
30. Surtees, R. and N. Blau, *The neurochemistry of phenylketonuria.* Eur J Pediatr, 2000. **159 Suppl 2**: p. S109-13.
31. Bone, A., A.K. Kuehl, and A.F. Angelino, *A neuropsychiatric perspective of phenylketonuria I: overview of phenylketonuria and its neuropsychiatric sequelae.* Psychosomatics, 2012. **53**(6): p. 517-23.
32. Mazur, A., et al., *Evaluation of somatic development in adult patients with previously undiagnosed and/or untreated phenylketonuria.* Med Princ Pract, 2010. **19**(1): p. 46-50.
33. Burton, B.K., et al., *A diversified approach for PKU treatment: routine screening yields high incidence of psychiatric distress in phenylketonuria clinics.* Mol Genet Metab, 2013. **108**(1): p. 8-12.
34. Ford, S., M. O'Driscoll, and A. MacDonald, *Living with Phenylketonuria: Lessons from the PKU community.* Mol Genet Metab Rep, 2018. **17**: p. 57-63.
35. Jahja, R., et al., *Cognitive profile and mental health in adult phenylketonuria: A PKU-COBESO study.* Neuropsychology, 2017. **31**(4): p. 437-447.
36. Didycz, B. and M. Bik-Multanowski, *Dynamics of hyperphenylalaninemia and intellectual outcome in teenagers with phenylketonuria.* Acta Biochim Pol, 2017. **64**(3): p. 527-531.
37. Waisbren, S.E., et al., *Psychosocial factors in maternal phenylketonuria: prevention of unplanned pregnancies.* Am J Public Health, 1991. **81**(3): p. 299-304.
38. Bilder, D.A., et al., *Neuropsychiatric comorbidities in adults with phenylketonuria: A retrospective cohort study.* Mol Genet Metab, 2017. **121**(1): p. 1-8.
39. Munro, T.A., *Phenylketonuria; data on 47 British families.* Ann Eugen, 1947. **14**(pt 1): p. 60-88.
40. Antshel, K.M. and S.E. Waisbren, *Developmental timing of exposure to elevated levels of phenylalanine is associated with ADHD symptom expression.* J Abnorm Child Psychol, 2003. **31**(6): p. 565-74.
41. Arnold, G.L., et al., *Prevalence of stimulant use for attentional dysfunction in children with phenylketonuria.* J Inherit Metab Dis, 2004. **27**(2): p. 137-43.

42. Burgard, P., *Development of intelligence in early treated phenylketonuria.* Eur J Pediatr, 2000. **159 Suppl 2**: p. S74-9.
43. Griffiths, P.V., et al., *Wechsler subscale IQ and subtest profile in early treated phenylketonuria.* Arch Dis Child, 2000. **82**(3): p. 209-15.
44. Huijbregts, S.C., et al., *Sustained attention and inhibition of cognitive interference in treated phenylketonuria: associations with concurrent and lifetime phenylalanine concentrations.* Neuropsychologia, 2002. **40**(1): p. 7-15.
45. Schmidt, H., et al., *Intelligence and professional career in young adults treated early for phenylketonuria.* Eur J Pediatr, 1996. **155 Suppl 1**: p. S97-100.
46. Channon, S., C. Mockler, and P. Lee, *Executive functioning and speed of processing in phenylketonuria.* Neuropsychology, 2005. **19**(5): p. 679-686.
47. White, D.A., et al., *Deficits in memory strategy use related to prefrontal dysfunction during early development: evidence from children with phenylketonuria.* Neuropsychology, 2001. **15**(2): p. 221-9.
48. Anderson, P.J., et al., *Are neuropsychological impairments in children with early-treated phenylketonuria (PKU) related to white matter abnormalities or elevated phenylalanine levels?* Dev Neuropsychol, 2007. **32**(2): p. 645-68.
49. Huijbregts, S.C., et al., *Motor function under lower and higher controlled processing demands in early and continuously treated phenylketonuria.* Neuropsychology, 2003. **17**(3): p. 369-79.
50. Leuzzi, V., et al., *Executive function impairment in early-treated PKU subjects with normal mental development.* J Inherit Metab Dis, 2004. **27**(2): p. 115-25.
51. Welsh, M.C., et al., *Neuropsychology of early-treated phenylketonuria: specific executive function deficits.* Child Dev, 1990. **61**(6): p. 1697-713.
52. Scriver, C.R., *The PAH gene, phenylketonuria, and a paradigm shift.* Hum Mutat, 2007. **28**(9): p. 831-45.
53. van Wegberg, A.M.J., et al., *The complete European guidelines on phenylketonuria: diagnosis and treatment.* Orphanet J Rare Dis, 2017. **12**(1): p. 162.
54. Guttler, F., *Hyperphenylalaninemia: diagnosis and classification of the various types of phenylalanine hydroxylase deficiency in childhood.* Acta Paediatr Scand Suppl, 1980. **280**: p. 1-80.
55. Blau, N., et al., *Diagnosis, classification, and genetics of phenylketonuria and tetrahydrobiopterin (BH4) deficiencies.* Mol Genet Metab, 2011. **104 Suppl**: p. S2-9.
56. Kwok, S.C., et al., *Nucleotide sequence of a full-length complementary DNA clone and amino acid sequence of human phenylalanine hydroxylase.* Biochemistry, 1985. **24**(3): p. 556-61.
57. Lidsky, A.S., et al., *Regional mapping of the phenylalanine hydroxylase gene and the phenylketonuria locus in the human genome.* Proc Natl Acad Sci U S A, 1985. **82**(18): p. 6221-5.
58. Blau, N., L. Bonafe, and B. Thony, *Tetrahydrobiopterin deficiencies without hyperphenylalaninemia: diagnosis and genetics of dopa-responsive dystonia and sepiapterin reductase deficiency.* Mol Genet Metab, 2001. **74**(1-2): p. 172-85.
59. Bonafe, L., et al., *Diagnosis of dopa-responsive dystonia and other tetrahydrobiopterin disorders by the study of biopterin metabolism in fibroblasts.* Clin Chem, 2001. **47**(3): p. 477-85.
60. Opladen, T., G.F. Hoffmann, and N. Blau, *An international survey of patients with tetrahydrobiopterin deficiencies presenting with hyperphenylalaninaemia.* J Inherit Metab Dis, 2012. **35**(6): p. 963-73.

61. Blau, N., et al., *A missense mutation in a patient with guanosine triphosphate cyclohydrolase I deficiency missed in the newborn screening program.* J Pediatr, 1995. **126**(3): p. 401-5.
62. Følliing, A., *Uber Ausscheidung von Phenylbrenztraubensäure in den Harn als Stoffwechselanomalie in Verbindung mit Imbezillität.* Ztschr Physio Chem, 1934b. **227**: p. 8.
63. Følling, A., *Utskillelse av fenylpyrodruesyre i urinen som stoffskifteanomali I forbindelse med imbecillitet.* Nord Med Tidskr, 1934a. **8**: p. 6.
64. Penrose, L.S., *Inheritance of phenylpyruvic amentia (phenylketonuria).* Lancet, 1935. **226**(5839): p. 192-4.
65. Penrose, L.S., *Phenylketonuria; a problem in eugenics.* Lancet, 1946. **1**(6409): p. 949-53.
66. Green.A, *Sheila- Unlocking the treatment for PKU*. 2020, Reddtich Worcester UK: Brewin Books. 172.
67. MacDonald, A., et al., *PKU dietary handbook to accompany PKU guidelines.* Orphanet J Rare Dis, 2020. **15**(1): p. 171.
68. World Health Organisation, *Protein and amino acid requirements in human nutition. Report of a joint WHO/FAO/UNU expert consultation (WHO Technical Report Series 935)*, in *United Nations University*. 2007. p. 1-265.
69. British Inherited Metabolic Diseases Group Dietitians, G., et al., *Development of national consensus statements on food labelling interpretation and protein allocation in a low phenylalanine diet for PKU.* Orphanet J Rare Dis, 2019. **14**(1): p. 2.
70. Evans, S., et al., *Uniformity of Food Protein Interpretation Amongst Dietitians for Patients with Phenylketonuria (PKU): 2020 UK National Consensus Statements.* Nutrients, 2020. **12**(8).
71. Jahja, R., et al., *Long-Term Follow-Up of Cognition and Mental Health in Adult Phenylketonuria: A PKU-COBESO Study.* Behav Genet, 2017. **47**(5): p. 486-497.
72. Schuck, P.F., et al., *Phenylketonuria Pathophysiology: on the Role of Metabolic Alterations.* Aging Dis, 2015. **6**(5): p. 390-9.
73. Thiele, A.G., et al., *Growth and Final Height Among Children With Phenylketonuria.* Pediatrics, 2017. **140**(5).
74. Wu, G., *Amino acids: metabolism, functions, and nutrition.* Amino Acids, 2009. **37**(1): p. 1-17.
75. van Spronsen, F.J., P.G. Smit, and R. Koch, *Phenylketonuria: tyrosine beyond the phenylalanine-restricted diet.* J Inherit Metab Dis, 2001. **24**(1): p. 1-4.
76. MacDonald, A., et al., *Administration of protein substitute and quality of control in phenylketonuria: a randomized study.* J Inherit Metab Dis, 2003. **26**(4): p. 319-26.
77. MacDonald, A., et al., *Factors affecting the variation in plasma phenylalanine in patients with phenylketonuria on diet.* Arch Dis Child, 1996. **74**(5): p. 412-7.
78. Ney, D.M., et al., *Nutritional management of PKU with glycomacropeptide from cheese whey.* J Inherit Metab Dis, 2009. **32**(1): p. 32-9.
79. Ney, D.M., et al., *Dietary glycomacropeptide supports growth and reduces the concentrations of phenylalanine in plasma and brain in a murine model of phenylketonuria.* J Nutr, 2008. **138**(2): p. 316-22.
80. Dalbeth, N., et al., *Effects of skim milk powder enriched with glycomacropeptide and G600 milk fat extract on frequency of gout flares: a proof-of-concept randomised controlled trial.* Ann Rheum Dis, 2012. **71**(6): p. 929-34.
81. Fox, G.B., et al., *Memory consolidation induces a transient and time-dependent increase in the frequency of neural cell adhesion molecule polysialylated cells in the adult rat hippocampus.* J Neurochem, 1995. **65**(6): p. 2796-9.

82. Wang, B. and J. Brand-Miller, *The role and potential of sialic acid in human nutrition.* Eur J Clin Nutr, 2003. **57**(11): p. 1351-69.
83. Brody, E.P., *Biological activities of bovine glycomacropeptide.* Br J Nutr, 2000. **84 Suppl 1**: p. S39-46.
84. Idota, T., Kawakami H and Nakajima., *Growth promoting effects of N-actylneuraminic acid containing substances on Bifidobacteria.* Biocscience, Biotechnology and Biochemistry, 1994. **58**: p. 413-416.
85. Neelima, et al., *Chemical and functional properties of glycomacropeptide (GMP) and its role in the detection of cheese whey adulteration in milk: a review.* Dairy Sci Technol, 2013. **93**(1): p. 21-43.
86. Nielsen P. and Tromholt N., *Method for production of a kappa-casein glycomacropeptide and use of a kappa-casein glycomacropeptide.* World Patent 9415952, 1994.
87. O'Riordan, N., et al., *Structural and functional characteristics of bovine milk protein glycosylation.* Glycobiology, 2014. **24**(3): p. 220-36.
88. Thomä-Worringer C., Søensen J., and López-Fandiño R., *Health effecs and technological features of caseingmacropeptide.* Int.Dairy J., 2006. **16**: p. 1324-1333.
89. Vance, B.A., et al., *Multiple dimeric forms of human CD69 result from differential addition of N-glycans to typical (Asn-X-Ser/Thr) and atypical (Asn-X-cys) glycosylation motifs.* J Biol Chem, 1997. **272**(37): p. 23117-22.
90. Vreeman, H.J., et al., *Characterization of bovine kappa-casein fractions and the kinetics of chymosin-induced macropeptide release from carbohydrate-free and carbohydrate-containing fractions determined by high-performance gel-permeation chromatography.* Biochem J, 1986. **240**(1): p. 87-97.
91. Dziuba J. and Minkiewicz P., *Influence of glycosylation on micelle-stabilizing ability and biological properties of C-terminal fragments of cow's kappa-casein.* Int Dairy J., 1996. **6**: p. 1017-1044.
92. Eigel WN., et al., *Nomenclature of proteins of cows milk: fifth revision.* Journal of Dairy Science, 1984. **67**: p. 1599-1631.
93. Holland, J.W., H.C. Deeth, and P.F. Alewood, *Analysis of O-glycosylation site occupancy in bovine kappa-casein glycoforms separated by two-dimensional gel electrophoresis.* Proteomics, 2005. **5**(4): p. 990-1002.
94. Otani, H., et al., *Inhibition of mitogen-induced proliferative responses of lymphocytes by bovine kappa-caseinoglycopeptides having different carbohydrate chains.* J Dairy Res, 1995. **62**(2): p. 349-57.
95. Mikkelsen, T.L., et al., *Immunogenicity of kappa-casein and glycomacropeptide.* J Dairy Sci, 2006. **89**(3): p. 824-30.
96. Ortega-Gonzalez, M., et al., *Validation of bovine glycomacropeptide as an intestinal anti-inflammatory nutraceutical in the lymphocyte-transfer model of colitis.* Br J Nutr, 2014. **111**(7): p. 1202-12.
97. Lopez-Posadas, R., et al., *Tissue-nonspecific alkaline phosphatase is activated in enterocytes by oxidative stress via changes in glycosylation.* Inflamm Bowel Dis, 2011. **17**(2): p. 543-56.
98. Ntemiri, A., et al., *Glycomacropeptide Sustains Microbiota Diversity and Promotes Specific Taxa in an Artificial Colon Model of Elderly Gut Microbiota.* J Agric Food Chem, 2017. **65**(8): p. 1836-1846.
99. Sawin, E.A., et al., *Glycomacropeptide is a prebiotic that reduces Desulfovibrio bacteria, increases cecal short-chain fatty acids, and is anti-inflammatory in mice.* Am J Physiol Gastrointest Liver Physiol, 2015. **309**(7): p. G590-601.

100. Zhang, X., et al., *Microbial production of sialic acid and sialylated human milk oligosaccharides: Advances and perspectives.* Biotechnol Adv, 2019. **37**(5): p. 787-800.
101. Yvon, M., et al., *Effects of caseinomacropeptide (CMP) on digestion regulation.* Reprod Nutr Dev, 1994. **34**(6): p. 527-37.
102. Cordova-Davalos, L.E., M. Jimenez, and E. Salinas, *Glycomacropeptide Bioactivity and Health: A Review Highlighting Action Mechanisms and Signaling Pathways.* Nutrients, 2019. **11**(3).
103. Hoefle, A.S., et al., *Postprandial metabolic responses to ingestion of bovine glycomacropeptide compared to a whey protein isolate in prediabetic volunteers.* Eur J Nutr, 2019. **58**(5): p. 2067-2077.
104. Keogh, J.B.C., P.M., *Weight loss using meal replacements high in glycomacropeptide-enriched whey powder.* Australian Journal of Dairy Technology,, 2009. **64**(1): p. 32-33.
105. Lam, S.M., et al., *The influence of whey protein and glycomacropeptide on satiety in adult humans.* Physiol Behav, 2009. **96**(1): p. 162-8.
106. Royle, P.J., G.H. McIntosh, and P.M. Clifton, *Whey protein isolate and glycomacropeptide decrease weight gain and alter body composition in male Wistar rats.* Br J Nutr, 2008. **100**(1): p. 88-93.
107. Beucher S., et al., *Effects of gastric digestive products from casein on CCK release by intestinal cells in rat.* J Nutr Biochem, 1994. **5**: p. 587-584.
108. Chunmuang, S., et al., *Effect of xylitol and fluoride on enamel erosion in vitro.* J Oral Sci, 2007. **49**(4): p. 293-7.
109. Guggenheim, B., et al., *Powdered milk micellar casein prevents oral colonization by Streptococcus sobrinus and dental caries in rats: a basis for the caries-protective effect of dairy products.* Caries Res, 1999. **33**(6): p. 446-54.
110. Nejad, A.K., A. Tatham, A., *The inhibitory effect of glycomacropeptide on dental erosion.* Dairy Science and Technology, 1998. **89**(3/4): p. 233-239.
111. Kawasaki, Y., et al., *Inhibition by lactoferrin and kappa-casein glycomacropeptide of binding of Cholera toxin to its receptor.* Biosci Biotechnol Biochem, 1992. **56**(2): p. 195-8.
112. Wang, B., et al., *Dietary sialic acid supplementation improves learning and memory in piglets.* Am J Clin Nutr, 2007. **85**(2): p. 561-9.
113. Wang, H.X., et al., *Sialylated milk oligosaccharides alter neurotransmitters and brain metabolites in piglets: an In vivo magnetic resonance spectroscopic (MRS) study.* Nutr Neurosci, 2019: p. 1-11.
114. van Calcar, S.C. and D.M. Ney, *Food products made with glycomacropeptide, a low-phenylalanine whey protein, provide a new alternative to amino Acid-based medical foods for nutrition management of phenylketonuria.* J Acad Nutr Diet, 2012. **112**(8): p. 1201-10.
115. Zeuner, B., et al., *Quantitative enzymatic production of sialylated galactooligosaccharides with an engineered sialidase from Trypanosoma rangeli.* Enzyme Microb Technol, 2016. **82**: p. 42-50.
116. Lis-Kuberka, J. and M. Orczyk-Pawilowicz, *Sialylated Oligosaccharides and Glycoconjugates of Human Milk. The Impact on Infant and Newborn Protection, Development and Well-Being.* Nutrients, 2019. **11**(2).
117. Lim, K., et al., *Acceptable low-phenylalanine foods and beverages can be made with glycomacropeptide from cheese whey for individuals with PKU.* Mol Genet Metab, 2007. **92**(1-2): p. 176-8.

118. van Calcar, S.C., et al., *Improved nutritional management of phenylketonuria by using a diet containing glycomacropeptide compared with amino acids.* Am J Clin Nutr, 2009. **89**(4): p. 1068-77.
119. Sawin, E.A., S.G. Murali, and D.M. Ney, *Differential effects of low-phenylalanine protein sources on brain neurotransmitters and behavior in C57Bl/6-Pah(enu2) mice.* Mol Genet Metab, 2014. **111**(4): p. 452-61.
120. Laclair, C.E., et al., *Purification and use of glycomacropeptide for nutritional management of phenylketonuria.* J Food Sci, 2009. **74**(4): p. E199-206.
121. Ahring, K.K., et al., *Comparison of Glycomacropeptide with Phenylalanine Free-Synthetic Amino Acids in Test Meals to PKU Patients: No Significant Differences in Biomarkers, Including Plasma Phe Levels.* J Nutr Metab, 2018. **2018**: p. 6352919.
122. Ney, D.M., et al., *Glycomacropeptide for nutritional management of phenylketonuria: a randomized, controlled, crossover trial.* Am J Clin Nutr, 2016. **104**(2): p. 334-45.
123. Pena, M.J., et al., *The Use of Glycomacropeptide in Patients with Phenylketonuria: A Systematic Review and Meta-Analysis.* Nutrients, 2018. **10**(11).
124. Zaki, O.K., et al., *The Use of Glycomacropeptide in Dietary Management of Phenylketonuria.* J Nutr Metab, 2016. **2016**: p. 2453027.
125. MacLeod, E.L., et al., *Breakfast with glycomacropeptide compared with amino acids suppresses plasma ghrelin levels in individuals with phenylketonuria.* Mol Genet Metab, 2010. **100**(4): p. 303-8.
126. Pinto, A., et al., *Nutritional status in patients with phenylketonuria using glycomacropeptide as their major protein source.* Eur J Clin Nutr, 2017.
127. Li, P., et al., *Amino acids and immune function.* Br J Nutr, 2007. **98**(2): p. 237-52.
128. *Dietary protein quality evaluation in human nutrition. Report of an FAQ Expert Consultation.* FAO Food Nutr Pap, 2013. **92**: p. 1-66.
129. Wu, G., *Dietary protein intake and human health.* Food Funct, 2016. **7**(3): p. 1251-65.
130. Patti, M.E. and C.R. Kahn, *The insulin receptor--a critical link in glucose homeostasis and insulin action.* J Basic Clin Physiol Pharmacol, 1998. **9**(2-4): p. 89-109.
131. Dillon, E.L., et al., *Muscle protein metabolism responds similarly to exogenous amino acids in healthy younger and older adults during NO-induced hyperemia.* Am J Physiol Regul Integr Comp Physiol, 2011. **301**(5): p. R1408-17.
132. Knopf, R.F., et al., *The normal endocrine response to ingestion of protein and infusions of amino acids. Sequential secretion of insulin and growth hormone.* Trans Assoc Am Physicians, 1966. **79**: p. 312-21.
133. Nair, K.S. and K.R. Short, *Hormonal and signaling role of branched-chain amino acids.* J Nutr, 2005. **135**(6 Suppl): p. 1547S-52S.
134. Nishitani, S., et al., *Branched-chain amino acids improve glucose metabolism in rats with liver cirrhosis.* Am J Physiol Gastrointest Liver Physiol, 2005. **288**(6): p. G1292-300.
135. White, P.J., et al., *Branched-chain amino acid restriction in Zucker-fatty rats improves muscle insulin sensitivity by enhancing efficiency of fatty acid oxidation and acyl-glycine export.* Mol Metab, 2016. **5**(7): p. 538-551.
136. Wang, Y., et al., *Amino acids regulate mTOR pathway and milk protein synthesis in a mouse mammary epithelial cell line is partly mediated by T1R1/T1R3.* Eur J Nutr, 2017. **56**(8): p. 2467-2474.
137. Anthony, J.C., et al., *Leucine stimulates translation initiation in skeletal muscle of postabsorptive rats via a rapamycin-sensitive pathway.* J Nutr, 2000. **130**(10): p. 2413-9.
138. Duan, Y., et al., *Key mediators of intracellular amino acids signaling to mTORC1 activation.* Amino Acids, 2015. **47**(5): p. 857-67.

139. Stumvoll, M., et al., *Effects of glucagon on renal and hepatic glutamine gluconeogenesis in normal postabsorptive humans.* Metabolism, 1998. **47**(10): p. 1227-32.
140. Burrin, D.G. and B. Stoll, *Metabolic fate and function of dietary glutamate in the gut.* Am J Clin Nutr, 2009. **90**(3): p. 850S-856S.
141. Kim, M.H. and H. Kim, *The Roles of Glutamine in the Intestine and Its Implication in Intestinal Diseases.* Int J Mol Sci, 2017. **18**(5).
142. Rezaei, R., et al., *Dietary supplementation with monosodium glutamate is safe and improves growth performance in postweaning pigs.* Amino Acids, 2013. **44**(3): p. 911-23.
143. San Gabriel, A. and H. Uneyama, *Amino acid sensing in the gastrointestinal tract.* Amino Acids, 2013. **45**(3): p. 451-61.
144. Bowery, N.G. and T.G. Smart, *GABA and glycine as neurotransmitters: a brief history.* Br J Pharmacol, 2006. **147 Suppl 1**: p. S109-19.
145. Fernstrom, J.D., *Effects and side effects associated with the non-nutritional use of tryptophan by humans.* J Nutr, 2012. **142**(12): p. 2236S-2244S.
146. Friedman, M. and C.E. Levin, *Nutritional and medicinal aspects of D-amino acids.* Amino Acids, 2012. **42**(5): p. 1553-82.
147. Wang, J., et al., *Nutrition, epigenetics, and metabolic syndrome.* Antioxid Redox Signal, 2012. **17**(2): p. 282-301.
148. Jobgen, W., et al., *Dietary L-arginine supplementation reduces white fat gain and enhances skeletal muscle and brown fat masses in diet-induced obese rats.* J Nutr, 2009. **139**(2): p. 230-7.
149. Geng, M., et al., *Reduced expression of intestinal N-acetylglutamate synthase in suckling piglets: a novel molecular mechanism for arginine as a nutritionally essential amino acid for neonates.* Amino Acids, 2011. **40**(5): p. 1513-22.
150. Morris, S.M., Jr., *Regulation of enzymes of the urea cycle and arginine metabolism.* Annu Rev Nutr, 2002. **22**: p. 87-105.
151. Flynn, N.E., et al., *The metabolic basis of arginine nutrition and pharmacotherapy.* Biomed Pharmacother, 2002. **56**(9): p. 427-38.
152. Meijer, A.J. and H.P. Sauerwein, *Amino acid-dependent signal transduction and insulin sensitivity.* Curr Opin Clin Nutr Metab Care, 1999. **2**(3): p. 207-11.
153. Ahmad, S., et al., *Protein oxidation: an overview of metabolism of sulphur containing amino acid, cysteine.* Front Biosci (Schol Ed), 2017. **9**: p. 71-87.
154. Brosnan, J.T. and M.E. Brosnan, *The sulfur-containing amino acids: an overview.* J Nutr, 2006. **136**(6 Suppl): p. 1636S-1640S.
155. Rigden, D.J., *The histidine phosphatase superfamily: structure and function.* Biochem J, 2008. **409**(2): p. 333-48.
156. Savelieva, K.V., et al., *Genetic disruption of both tryptophan hydroxylase genes dramatically reduces serotonin and affects behavior in models sensitive to antidepressants.* PLoS One, 2008. **3**(10): p. e3301.
157. Lou, H., *Large doses of tryptophan and tyrosine as potential therapeutic alternative to dietary phenylalanine restriction in phenylketonuria.* Lancet, 1985. **2**(8447): p. 150-1.
158. Weglage, J., et al., *Individual blood-brain barrier phenylalanine transport determines clinical outcome in phenylketonuria.* Ann Neurol, 2001. **50**(4): p. 463-7.
159. Gropper, S.S., D.M. Gropper, and P.B. Acosta, *Plasma amino acid response to ingestion of L-amino acids and whole protein.* J Pediatr Gastroenterol Nutr, 1993. **16**(2): p. 143-50.
160. Monch, E., et al., *Utilisation of amino acid mixtures in adolescents with phenylketonuria.* Eur J Pediatr, 1996. **155 Suppl 1**: p. S115-20.

161. Silk, D.B., et al., *Use of a peptide rather than free amino acid nitrogen source in chemically defined "elemental" diets.* JPEN J Parenter Enteral Nutr, 1980. **4**(6): p. 548-53.
162. Herrmann, M.E., et al., *Dependence of the utilization of a phenylalanine-free amino acid mixture on different amounts of single dose ingested. A case report.* Eur J Pediatr, 1994. **153**(7): p. 501-3.
163. Millward, D.J., et al., *Protein quality assessment: impact of expanding understanding of protein and amino acid needs for optimal health.* Am J Clin Nutr, 2008. **87**(5): p. 1576S-1581S.
164. Dokoupil, K., et al., *Optimising growth in phenylketonuria: current state of the clinical evidence base.* Clin Nutr, 2012. **31**(1): p. 16-21.
165. Ilgaz, F., et al., *Long-Term Growth in Phenylketonuria: A Systematic Review and Meta-Analysis.* Nutrients, 2019. **11**(9).
166. Zaffanello, M., G. Zamboni, and L. Tato, *Growth parameters in newborns with hyperphenylalaninaemia.* Paediatr Perinat Epidemiol, 2002. **16**(3): p. 274-7.
167. Dobbelaere, D., et al., *Evaluation of nutritional status and pathophysiology of growth retardation in patients with phenylketonuria.* J Inherit Metab Dis, 2003. **26**(1): p. 1-11.
168. Kindt, E., et al., *Fasting plasma amino acid concentrations in PKU children on two different levels of protein intake.* Acta Paediatr Scand, 1988. **77**(1): p. 60-6.
169. Kindt, E., et al., *Protein requirements in infants and children: a longitudinal study of children treated for phenylketonuria.* Am J Clin Nutr, 1983. **37**(5): p. 778-85.
170. Smith, J.L., C. Arteaga, and S.B. Heymsfield, *Increased Ureagenesis and Impaired Nitrogen Use during Infusion of a Synthetic Amino Acid Formula.* New England Journal of Medicine, 1982. **306**(17): p. 1013-1018.
171. Holm, V.A., et al., *Physical growth in phenylketonuria: II. Growth of treated children in the PKU collaborative study from birth to 4 years of age.* Pediatrics, 1979. **63**(5): p. 700-7.
172. McBurnie, M.A., et al., *Physical growth of children treated for phenylketonuria.* Ann Hum Biol, 1991. **18**(4): p. 357-68.
173. Dhondt, J.L., et al., *Physical growth in patients with phenylketonuria.* J Inherit Metab Dis, 1995. **18**(2): p. 135-7.
174. Verkerk, P.H., et al., *Impaired prenatal and postnatal growth in Dutch patients with phenylketonuria. The National PKU Steering Committee.* Arch Dis Child, 1994. **71**(2): p. 114-8.
175. Weglage, J., et al., *Growth in patients with phenylketonuria.* Eur J Pediatr, 1994. **153**(7): p. 537-8.
176. Schaefer, F., et al., *Growth and skeletal maturation in children with phenylketonuria.* Acta Paediatr, 1994. **83**(5): p. 534-41.
177. Belanger-Quintana, A. and M. Martinez-Pardo, *Physical development in patients with phenylketonuria on dietary treatment: a retrospective study.* Mol Genet Metab, 2011. **104**(4): p. 480-4.
178. Couce, M.L., et al., *New insights in growth of phenylketonuric patients.* Eur J Pediatr, 2015. **174**(5): p. 651-9.
179. Evans, M., H. Truby, and A. Boneh, *The relationship between dietary intake, growth and body composition in Phenylketonuria.* Mol Genet Metab, 2017. **122**(1-2): p. 36-42.
180. Hoeksma, M., et al., *Phenylketonuria: High plasma phenylalanine decreases cerebral protein synthesis.* Mol Genet Metab, 2009. **96**(4): p. 177-82.
181. Dangin, M., et al., *The digestion rate of protein is an independent regulating factor of postprandial protein retention.* Am J Physiol Endocrinol Metab, 2001. **280**(2): p. E340-8.

182. Young, V.R., et al., *Rates of urea production and hydrolysis and leucine oxidation change linearly over widely varying protein intakes in healthy adults.* J Nutr, 2000. **130**(4): p. 761-6.
183. Ney, D.M. and M.R. Etzel, *Designing medical foods for inherited metabolic disorders: why intact protein is superior to amino acids.* Curr Opin Biotechnol, 2017. **44**: p. 39-45.
184. Sailer, M., et al., *Nutrient intake, body composition, and blood phenylalanine control in children with phenylketonuria compared to healthy controls.* Mol Genet Metab Rep, 2020. **23**: p. 100599.
185. Albertson, A.M., et al., *Prospective associations among cereal intake in childhood and adiposity, lipid levels, and physical activity during late adolescence.* J Am Diet Assoc, 2009. **109**(10): p. 1775-80.
186. Despres, J.P. and I. Lemieux, *Abdominal obesity and metabolic syndrome.* Nature, 2006. **444**(7121): p. 881-7.
187. Forte, N., et al., *Obesity Affects the Microbiota-Gut-Brain Axis and the Regulation Thereof by Endocannabinoids and Related Mediators.* Int J Mol Sci, 2020. **21**(5).
188. Allen, J.R., et al., *Body protein in prepubertal children with phenylketonuria.* Eur J Clin Nutr, 1996. **50**(3): p. 178-86.
189. Huemer, M., et al., *Growth and body composition in children with classical phenylketonuria: results in 34 patients and review of the literature.* J Inherit Metab Dis, 2007. **30**(5): p. 694-9.
190. Adamczyk, P., et al., *Bone metabolism and the muscle-bone relationship in children, adolescents and young adults with phenylketonuria.* J Bone Miner Metab, 2011. **29**(2): p. 236-44.
191. Douglas, T.D., et al., *Accuracy of six anthropometric skinfold formulas versus air displacement plethysmography for estimating percent body fat in female adolescents with phenylketonuria.* JIMD Rep, 2013. **10**: p. 23-31.
192. Rocha, J.C., A. MacDonald, and F. Trefz, *Is overweight an issue in phenylketonuria?* Mol Genet Metab, 2013. **110 Suppl**: p. S18-24.
193. Rocha, J.C., et al., *Dietary treatment in phenylketonuria does not lead to increased risk of obesity or metabolic syndrome.* Mol Genet Metab, 2012. **107**(4): p. 659-63.
194. Doulgeraki, A., et al., *Body composition profile of young patients with phenylketonuria and mild hyperphenylalaninemia.* Int J Endocrinol Metab, 2014. **12**(3): p. e16061.
195. Mazzola, P.N., et al., *Analysis of body composition and nutritional status in Brazilian phenylketonuria patients.* Mol Genet Metab Rep, 2016. **6**: p. 16-20.
196. Frost, H.M., *Wolff's law: an 'MGS' derivation of Gamma in the Three-Way Rule for mechanically controlled lamellar bone modeling drifts.* Bone Miner, 1993. **22**(2): p. 117-27.
197. Bachrach, L.K., *Acquisition of optimal bone mass in childhood and adolescence.* Trends Endocrinol Metab, 2001. **12**(1): p. 22-8.
198. Dimitri, P., *Fat and bone in children - where are we now?* Ann Pediatr Endocrinol Metab, 2018. **23**(2): p. 62-69.
199. Clark, E.M., J.H. Tobias, and A.R. Ness, *Association between bone density and fractures in children: a systematic review and meta-analysis.* Pediatrics, 2006. **117**(2): p. e291-7.
200. Rauch, F., et al., *The 'muscle-bone unit' during the pubertal growth spurt.* Bone, 2004. **34**(5): p. 771-5.
201. Russell, M., et al., *Visceral fat is a negative predictor of bone density measures in obese adolescent girls.* J Clin Endocrinol Metab, 2010. **95**(3): p. 1247-55.

202. Carbone, J.W., J.P. McClung, and S.M. Pasiakos, *Recent Advances in the Characterization of Skeletal Muscle and Whole-Body Protein Responses to Dietary Protein and Exercise during Negative Energy Balance.* Adv Nutr, 2019. **10**(1): p. 70-79.
203. Crabtree, N.J., et al., *The relationship between lean body mass and bone mineral content in paediatric health and disease.* Bone, 2004. **35**(4): p. 965-72.
204. Rizzoli, R., et al., *Maximizing bone mineral mass gain during growth for the prevention of fractures in the adolescents and the elderly.* Bone, 2010. **46**(2): p. 294-305.
205. Bonjour, J.P., *Protein intake and bone health.* Int J Vitam Nutr Res, 2011. **81**(2-3): p. 134-42.
206. Bonjour, J.P., et al., *Protein intake and bone growth.* Can J Appl Physiol, 2001. **26 Suppl**: p. S153-66.
207. Al-Qadreh, A., et al., *Bone mineral status in children with phenylketonuria under treatment.* Acta Paediatr, 1998. **87**(11): p. 1162-6.
208. Ambroszkiewicz, J., et al., *[Concentration of osteoprotegerin, bone formation and resorption markers in patients with phenylketonuria].* Pol Merkur Lekarski, 2008. **25**(145): p. 57-60.
209. Barat, P., et al., *The impact of the control of serum phenylalanine levels on osteopenia in patients with phenylketonuria.* Eur J Pediatr, 2002. **161**(12): p. 687-8.
210. de Groot, M.J., et al., *Relationships between lumbar bone mineral density and biochemical parameters in phenylketonuria patients.* Mol Genet Metab, 2012. **105**(4): p. 566-70.
211. Greeves, L.G., et al., *Fractures and phenylketonuria.* Acta Paediatr, 1997. **86**(3): p. 242-4.
212. McMurry, M.P., et al., *Bone mineral status in children with phenylketonuria--relationship to nutritional intake and phenylalanine control.* Am J Clin Nutr, 1992. **55**(5): p. 997-1004.
213. Zeman, J., M. Bayer, and J. Stepan, *Bone mineral density in patients with phenylketonuria.* Acta Paediatr, 1999. **88**(12): p. 1348-51.
214. Enns, G.M., et al., *Suboptimal outcomes in patients with PKU treated early with diet alone: revisiting the evidence.* Mol Genet Metab, 2010. **101**(2-3): p. 99-109.
215. Hansen, S., et al., *Bone geometry, volumetric bone mineral density, microarchitecture and estimated bone strength in Caucasian females with systemic lupus erythematosus. A cross-sectional study using HR-pQCT.* Calcif Tissue Int, 2014. **95**(6): p. 530-9.
216. Demirdas, S., et al., *Bone health in phenylketonuria: a systematic review and meta-analysis.* Orphanet J Rare Dis, 2015. **10**: p. 17.
217. de Castro, M.J., et al., *Bone Status in Patients with Phenylketonuria: A Systematic Review.* Nutrients, 2020. **12**(7).
218. Solverson, P., et al., *Low bone strength is a manifestation of phenylketonuria in mice and is attenuated by a glycomacropeptide diet.* PLoS One, 2012. **7**(9): p. e45165.
219. Ouchi, N. and K. Walsh, *Adiponectin as an anti-inflammatory factor.* Clin Chim Acta, 2007. **380**(1-2): p. 24-30.
220. Sayers, A., et al., *The association between insulin levels and cortical bone: findings from a cross-sectional analysis of pQCT parameters in adolescents.* J Bone Miner Res, 2012. **27**(3): p. 610-8.
221. Arita, Y., et al., *Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity.* Biochem Biophys Res Commun, 1999. **257**(1): p. 79-83.
222. Diez, J.J. and P. Iglesias, *The role of the novel adipocyte-derived hormone adiponectin in human disease.* Eur J Endocrinol, 2003. **148**(3): p. 293-300.

223. Cornish, J., et al., *Leptin directly regulates bone cell function in vitro and reduces bone fragility in vivo.* J Endocrinol, 2002. **175**(2): p. 405-15.
224. Steppan, C.M. and M.A. Lazar, *Resistin and obesity-associated insulin resistance.* Trends Endocrinol Metab, 2002. **13**(1): p. 18-23.
225. Thomas, T., et al., *Leptin acts on human marrow stromal cells to enhance differentiation to osteoblasts and to inhibit differentiation to adipocytes.* Endocrinology, 1999. **140**(4): p. 1630-8.
226. Takeda, S., et al., *Leptin regulates bone formation via the sympathetic nervous system.* Cell, 2002. **111**(3): p. 305-17.
227. Magni, P., et al., *Molecular aspects of adipokine-bone interactions.* Curr Mol Med, 2010. **10**(6): p. 522-32.
228. Reid, I.R., *Fat and bone.* Arch Biochem Biophys, 2010. **503**(1): p. 20-7.
229. Caverzasio, J., C. Montessuit, and J.P. Bonjour, *Stimulatory effect of insulin-like growth factor-1 on renal Pi transport and plasma 1,25-dihydroxyvitamin D3.* Endocrinology, 1990. **127**(1): p. 453-9.
230. Cornish, J., et al., *Modulation of osteoclastogenesis by fatty acids.* Endocrinology, 2008. **149**(11): p. 5688-95.
231. Garnett, S.P., et al., *Relation between hormones and body composition, including bone, in prepubertal children.* Am J Clin Nutr, 2004. **80**(4): p. 966-72.
232. Palmer, G., J.P. Bonjour, and J. Caverzasio, *Stimulation of inorganic phosphate transport by insulin-like growth factor I and vanadate in opossum kidney cells is mediated by distinct protein tyrosine phosphorylation processes.* Endocrinology, 1996. **137**(11): p. 4699-705.
233. Ihle, R. and A.B. Loucks, *Dose-response relationships between energy availability and bone turnover in young exercising women.* J Bone Miner Res, 2004. **19**(8): p. 1231-40.
234. Care, A.D., et al., *Stimulatory effect of insulin on calcitonin secretion.* Horm Metab Res, 1998. **30**(4): p. 200-5.
235. Clowes, J.A., et al., *Effect of feeding on bone turnover markers and its impact on biological variability of measurements.* Bone, 2002. **30**(6): p. 886-90.
236. Ammann, P., et al., *Protein undernutrition-induced bone loss is associated with decreased IGF-I levels and estrogen deficiency.* J Bone Miner Res, 2000. **15**(4): p. 683-90.
237. Bourrin, S., et al., *Dietary protein restriction lowers plasma insulin-like growth factor I (IGF-I), impairs cortical bone formation, and induces osteoblastic resistance to IGF-I in adult female rats.* Endocrinology, 2000. **141**(9): p. 3149-55.
238. Bourrin, S., et al., *Dietary protein deficiency induces osteoporosis in aged male rats.* J Bone Miner Res, 2000. **15**(8): p. 1555-63.
239. Kambas, A., et al., *Physical activity may be a potent regulator of bone turnover biomarkers in healthy girls during preadolescence.* J Bone Miner Metab, 2017. **35**(6): p. 598-607.
240. Anderson, G.H., et al., *Protein source, quantity, and time of consumption determine the effect of proteins on short-term food intake in young men.* J Nutr, 2004. **134**(11): p. 3011-5.
241. Burton-Freeman, B.M., *Glycomacropeptide (GMP) is not critical to whey-induced satiety, but may have a unique role in energy intake regulation through cholecystokinin (CCK).* Physiol Behav, 2008. **93**(1-2): p. 379-87.
242. Chungchunlam, S.M., et al., *Effect of time of consumption of preloads on measures of satiety in healthy normal weight women.* Appetite, 2012. **59**(2): p. 281-8.
243. Holst, J.J., *Incretin hormones and the satiation signal.* Int J Obes (Lond), 2013. **37**(9): p. 1161-8.

244. Nauck M.A. and Meier J.J., *Incretin hormones: Their role in health and disease.* Diabetes Obes Metab, 2017. **Suppl 1**: p. 5-21.
245. Chungchunlam, S.M., et al., *Dietary whey protein influences plasma satiety-related hormones and plasma amino acids in normal-weight adult women.* Eur J Clin Nutr, 2015. **69**(2): p. 179-86.
246. Hall, W.L., et al., *Casein and whey exert different effects on plasma amino acid profiles, gastrointestinal hormone secretion and appetite.* Br J Nutr, 2003. **89**(2): p. 239-48.
247. Gustafson, D.R., et al., *Appetite is not influenced by a unique milk peptide: caseinomacropeptide (CMP).* Appetite, 2001. **36**(2): p. 157-63.
248. Keogh, J.B., et al., *Effect of glycomacropeptide fractions on cholecystokinin and food intake.* Br J Nutr, 2010. **104**(2): p. 286-90.
249. Veldhorst, M.A., et al., *Comparison of the effects of a high- and normal-casein breakfast on satiety, 'satiety' hormones, plasma amino acids and subsequent energy intake.* Br J Nutr, 2009. **101**(2): p. 295-303.
250. Cotugno, G., et al., *Adherence to diet and quality of life in patients with phenylketonuria.* Acta Paediatr, 2011. **100**(8): p. 1144-9.

CHAPTER 2

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PROTEIN SUBSTITUTES IN PKU: THEIR HISTORICAL EVOLUTION

nutrients

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Review

Protein Substitutes in PKU; Their Historical Evolution

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Abstract: Protein substitutes developed for phenylketonuria (PKU) are a synthetic source of protein commonly based on L-amino acids. They are essential in the treatment of phenylketonuria (PKU) and other amino acid disorders, allowing the antagonistic amino acid to be removed but with the safe provision of all other amino acids necessary for maintaining normal physiological function. They were first formulated by a chemist and used experimentally on a 2-year-old girl with PKU and their nutritional formulations and design have improved over time. Since 2008, a bioactive macropeptide has been used as a base for protein substitutes in PKU, with potential benefits of improved bone and gut health, nitrogen retention, and blood phenylalanine control. In 2018, animal studies showed that physiomimic technology coating the amino acids with a polymer allows a slow release of amino acids with an improved physiological profile. History has shown that in PKU, the protein substitute's efficacy is determined by its nutritional profile, amino acid composition, dose, timing, distribution, and an adequate energy intake. Protein substitutes are often given little importance, yet their pharmacological actions and clinical benefit are pivotal when managing PKU.

Keywords: phenylketonuria; protein substitute; amino acid; glycomacropeptide

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1. Introduction

Amino acids are unique substrates providing nitrogen, hydrocarbon skeletons and sulphur [1]. They are essential precursors for the synthesis of proteins, peptides, and low molecular weight substances such as glutathione, dopamine, nitric oxide, and serotonin [1]. In phenylketonuria (PKU), dietary treatment was made feasible with the introduction of low/free phenylalanine synthetic proteins (protein substitutes), that have gradually advanced with time. In the 1950s, these were originally derived from protein hydrolysates, but in the 1970s, phenylalanine-free amino acids were introduced. Protein substitutes provide the building blocks of tissue proteins and their amino acids are essential for the synthesis of hormones, enzymes, and other cellular processes. Therefore, their composition and nutritional profile is fundamental, helping prevent neurological devastation, allowing normal growth and biosynthetic functions. The original technology for making protein substitutes was crude and limited but now precision manufacturing has improved their quality.

Although Følling [2] first identified phenylpyruvic acid in the urine of untreated children with PKU, it was Penrose who recognised that it was a genetic recessive disorder and named it phenylketonuria (PKU) [3]. He was also the first to try a dietary treatment based on fruit, sugar, olive oil, and vitamins, but this protein-free diet lacked essential phenylalanine and all other amino acids, resulting in malnutrition and so the treatment was abandoned [4]. Twenty years later, protein substitutes were introduced, but their central role in the management of PKU remains undervalued.

2. Early Studies

Følling and Penrose [5] both demonstrated that giving phenylalanine to a PKU subject increased the excretion of phenylpyruvic acid. The type of phenylalanine ingested as

D or L isomers had different effects on phenylpyruvic excretion, with L phenylalanine leading to a greater production of phenylpyruvic acid. Similarly, in non-PKU subjects, L phenylalanine was the preferred metabolised substrate, with D and DL isomers leading to small amounts of phenylpyruvic acid but an absence when the L form was given due to its complete metabolism. From these studies, they concluded that phenylpyruvic acid excreted in PKU patients was due to an incomplete breakdown of phenylalanine. Tyrosine when administered had no effect on urine phenylpyruvic acid excretion, concluding this was metabolised normally. It was not until 1944 that Bernheim [6] demonstrated that the main metabolic pathway for phenylalanine was by parahydroxylation of phenylalanine to tyrosine. In 1953, Jarvis showed that it was the inability to perform this hydroxylation that resulted in phenylketonuria [7].

Penrose and Quastel [5] conducted a series of feeding studies where they found that by lowering the natural protein intake by >50% resulted in an immediate reduction in urinary phenylpyruvic acid in a patient with PKU. However, after the second day of treatment, urine phenylpyruvic acid re-appeared and increased over subsequent days. The authors noted a weight loss over the same time and hypothesised that catabolism led to the production of phenylpyruvic acid.

In 1951, a positive ferric chloride screening test in a symptomatic 2-year-old girl from Birmingham, UK, preceded the first successful dietary treatment in PKU. She was only the third child to be tested with the ferric chloride test at Birmingham Children's Hospital [8]. In PKU, phenylpyruvic acid present in urine causes the characteristic greenish-blue colour reaction when a few drops of ferric chloride are added [9]. On presentation, she was unable to talk, walk, or engage with her surroundings; her mother waited for the doctor every morning outside the hospital laboratory as she refused to accept that there was no treatment for her daughters' condition. Louis Woolf designed the first successful protein substitute formulation used in PKU. He was a chemist with a commercial background and had used hydrolysed casein to produce amino acids as a treatment for malnutrition after the Second World War. Cost was a priority in the post war years and protein hydrolysates were readily available and cheaper than pure amino acids. In 1949, he suggested that supplementation of carbon treated casein hydrolysate with appropriate amounts of missing amino acids (including a source of phenylalanine to prevent deficiency) could treat PKU. He was unable to convince his medical colleagues at Great Ormond Street (GOS) Children's Hospital, London to try his proposed treatment. He recalls: "*At GOS, the suggestion floated like a lead balloon, I was told not unkindly that I should be devising new diagnostic tests, not dreaming up crazy treatments for conditions that everybody knew were untreatable*" [10]. In collaboration with Drs Bickel, Hickman, and Gerrard from Birmingham, a modification of Louis Woolf's protein substitute was given to the 2-year-old child with PKU [11].

3. The First Protein Substitute

Casein was hydrolysed by boiling it for several hours with concentrated hydrochloric or sulphuric acid to produce a thick black amino acid liquid. This solution was neutralised with sodium hydroxide and then purified by the addition of activated carbon and finally filtered to produce a clear solution of amino acids. This solution contained phenylalanine, which was removed by a second filtration method using activated charcoal. This removed the aromatic amino acids: phenylalanine, tryptophan, and tyrosine (although a small residual amount of phenylalanine was detectable). To nutritionally improve the protein substitute carbohydrate, fat, vitamins and minerals were added, together with tryptophan and tyrosine [11]. This unpalatable solution was then mixed with sugar, wheat starch, double cream and water and given as a formula to infants. In older children, it was either flavoured with tomatoes and given as a soup [12], made into a blancmange with sugar, margarine and wheat starch [13] or mixed with vegetable oil, and sugar and flavoured with artificial flavourings [14].

The production of the original formula was difficult and time consuming and had to be done in a cold room or it would deteriorate. The black charcoal covered everything and

as the first formula was prepared, the sight of Dr. Bickel wrapped in layers of jumpers topped by a charcoal smudged lab coat became a common sight [8]. Woolf identified that a small amount of phenylalanine should be added to the formula as it was an essential amino acid [15]. He stressed the need for careful monitoring, and he was also the first to propose treatment for life in PKU [12,16,17].

Phenylalanine-free amino acids as a protein substitute for PKU were first tried in the USA in the 1950s [18], but had to be abandoned most likely due to the pure amino acid mixtures causing vomiting.

4. Amino Acid Requirements

In the early stages of making the protein substitute, the exact amino acid composition of the casein hydrolysate was unknown. Casein was low in sulphur containing amino acids and cysteine was also partly removed by hydrolysis and charcoal filtration. Bickel [19] suggested adding L cystine to the hydrolysate and Woolf proposed the addition of DL methionine [20].

The amounts of tryptophan and tyrosine added to the first protein substitutes were determined by amino acid requirements established in the early 1950s; this knowledge was pioneered by Rose [21–25], Holt and Snyderman [26,27]. It was estimated that an adult man required 1000 mg/day of L phenylalanine to maintain nitrogen equilibrium or 300 mg/day if tyrosine was provided [24]. Synderman [28] suggested that 90 mg per kg/day of L phenylalanine was needed by an infant, but this was reduced to 25 mg/kg per day if sufficient tyrosine was supplied. Other essential amino acid requirements were estimated from the work of Rose, Leverton [23,29] and Swendseid [30]. In Woolf's [12] original formula, 25 mg/kg/day of L tryptophan and 25 mg/kg/day of L methionine were given in addition to 50 mg/kg/day of L tyrosine, a surrogate essential amino acid in PKU (Table 1).

Table 1. The original composition of the first protein substitute designed by Louis Woolf (1958).

Product	Daily Intake
Casein hydrolysate	**24 g**
DL-tryptophan	**1 g**
L-tyrosine	**2 g**
DL-methionine	**1 g**
Sucrose	**90 g**
Cows milk	**0–200 mL**
Double cream	**85 mL**
Calcium hydrogen phosphate	**0.71 g**
Potassium chloride	**0.65 g**
Sodium chloride	**0.016 g**
Magnesium sulphate	**0.165 g**
Sodium citrate	**0.177 g**
Potassium iodide	**0.00013 g**
Citric acid	**0.08 g**
Water	**850 mL**

Table 1. *Cont.*

Product	Daily Intake
Vitamins and minerals	
Choline chloride	**100 mg**
Inositol	**216 mg**
Vitamin B12	**4 µg**
Aneurine hydrochloride (vitamin B1)	**0.5 mg**
Riboflavin	**0.5 mg**
Pyridoxine	**0.33 mg**
Nicotinamide	**3.33 mg**
Ascorbic acid	**40 mg**
α-Tocopherol	**0.33 mg**
Acetomenaphthone (vitamin K)	**0.5 mg**
Biotin	**0.17 mg**
Folic acid	**0.35 mg**
Vitamin A	**3000 iu**
Vitamin D	**500 iu**
Zinc sulphate	**0.0014 g**
Ferrous sulphate	**0.15 g**
Manganous sulphate	**0.0008 g**
Cupric sulphate	**0.003 g**

There were challenges when administering the artificial diet in PKU [11]. The first child to start treatment was admitted to hospital for 6 weeks. The musty smell associated with PKU disappeared, plasma and urinary phenylalanine concentrations returned to normal, and there was a negative ferric chloride test when the diet was commenced. However, the child lost weight and within 5 days of treatment, plasma tyrosine concentrations were un-recordable (with a change in hair pigmentation), and plasma phenylalanine was raised. Tyrosine (1.5 g/daily) was added, correcting the low plasma tyrosine concentrations and temporarily arrested weight loss. However, after a further 3 weeks, aminoaciduria was noted, and in the fifth week, blood phenylalanine increased and phenylpyruvic acid reappeared in the urine; this was associated with weight loss, vomiting, and the child was described as unwell. These observations were important, highlighting that tyrosine became an essential amino acid in PKU as a consequence of the biochemical block in converting phenylalanine to tyrosine. Aminoaciduria, a result of weight loss and catabolism due to phenylalanine deficiency, led to an increase in catabolism and a subsequent increase in blood phenylalanine. Adding a measured amount of phenylalanine back into the diet (typically 250–500 mg or equivalent to around 5–10 g protein/day) increased plasma and urine concentrations, but to levels significantly below pre-treatment concentrations. Laboratory analysis was laborious, each blood test was analysed in duplicate and the production of a chromatogram took 3 days of intensive labour [8].

After 6 months of treatment, this child made remarkable progress; followed by a cascade of successful case studies. Woolf [17] reported 3 cases, with a further publication of 10 cases in 1958 [12], in which children were treated from the age of three weeks to 5 years of age. Armstrong and Tyler [31] reported the treatment of five children, and Armstrong and Binkley in 1956 [32] followed the progress of an infant starting treatment at 40 days of age. All reported that a low phenylalanine diet, supplemented with a low phenylalanine protein hydrolysate corrected the major biochemical abnormalities.

It was also established that sufficient carbohydrate and fat (including a source of linoleic acid) was necessary to prevent protein catabolism [20,33–35]. Woolf reported that

the daily intake of hydrolysate should be high correcting for the inefficient utilisation of the amino acids [12].

5. Commercial Protein Hydrolysate Preparations

Production of the hydrolysate moved from hospital laboratories to commercial production in late 1953/early 1954. In Europe, Cymogran 1954/5 (Allen and Hanbury, London, UK), XP Albumaid (1960) (Scientific Hospital Supplies, Liverpool, UK) and Minafen (designed for infants in 1955), (Trufood Ltd., Guildford, UK) were developed and the US produced Lofenalac (Mead Johnson, Chicago, IL, USA) in 1958. In the spirit of commercial interest, Trufood and Allen and Hanbury agreed to share production with one company making an infant substitute Minafen (Trufood)and the other (Allen and Hanbury) a preparation for older children Cymogran. Limited practical instructions were provided on how to reconstitute these formulas and families had to weigh the prescribed powder. The main difference between hospital and factory production was the use of ion exchange resins to separate phenylalanine, dispensing with the sodium hydroxide and carbon filtration. These synthetic filters consisted of microbeads from resin or polymers, allowing the separation and purification of the hydrolysed casein. These products were supplemented with variable amounts of vitamins, minerals, carbohydrate and fat.

6. The First UK PKU Guidelines

In 1960, the UK Ministry of Health [9] provided guidelines on screening and early detection of PKU, together with recommendations on optimal blood phenylalanine concentrations and provision of protein substitutes. They proposed screening by the ferric chloride test at 4–6 weeks of age (which was later replaced by the Guthrie method in 1969 [36]). To prevent phenylalanine deficiency, a target blood phenylalanine concentration slightly above normal was recommended (90–120 mmol/L), with blood phenylalanine monitoring done twice weekly until stability was achieved, and then weekly or monthly monitoring was required.

In infancy, a protein substitute, formulated and reconstituted similar to regular milk-based infant formula was recommended. A second protein substitute with a lower energy content was advocated for older children.

7. Nutritional Deficiencies with Early Protein Substitutes

In the early history of treating PKU by diet, there were concerns about 'over- treating' patients and maintaining very low phenylalanine blood concentrations. Nutritional deficiencies, malnutrition, and even death were linked to dietary treatment [37]. In the 1960s, severe skin rashes in babies on Minafen (Allen and Hanbury Ltd., London, UK) were reported [38–40]. Woolf [12] described a child with faltering growth and hair loss when acetyl DL tryptophan was accidentally given instead of DL tryptophan; stopping the acetyl derivative immediately reversed the symptoms. Studies in animals fed synthetic low phenylalanine diets [41] led to the addition of choline, riboflavin, folic acid, and vitamin E to the hydrolysate preparations. Two reports of folic acid deficiency were described [42,43], one child had megaloblastic anaemia due to folic acid deficiency exacerbated by vomiting and poor feeding and subsequently died. Hypoglycaemia was also reported in two cases [44].

8. Amino Acid Preparations

In the late 1960s, commercial amino acid formulas were made from pure crystalline amino acids by fermentation of bacteria. They were manufactured by the Japanese at an affordable cost [45]. The first product for PKU was Aminogram Food Supplement [46,47], which had several advantages, compared to hydrolysed formulas including improved taste and a lower daily volume, with an amino acid composition that could be easily adapted for the treatment of other aminoacidopathies such as maple syrup urine disease, homocystinuria, and tyrosinaemia type 1.

Manz [48] reported anorexia and vomiting in some infants given amino acid preparations. Metabolic acidosis was observed when the preparations contained amino acids in the form of hydrochloride salts or when the ratio of sulphur containing amino acids was too high, leading to higher urinary pH and increased renal net acid excretion. Modifications in the amino acid preparations normalised the renal net acid excretion and acidosis.

The early commercial preparations of L amino acid substitutes required separate supplementation with vitamins and minerals and careful monitoring of nutritional status was essential. These vitamin and mineral supplements were commonly deficient in molybdenum, chromium, selenium, and pantothenic acid [49,50].

L-amino acid substitutes nutritionally complete: In 1980, the first UK amino acid preparation supplemented with carbohydrate, vitamins, mineral, and trace elements and designed for children over 1 year of age with PKU was manufactured. It was flavoured for improved taste and palatability. In 1988, a similar product (but also with added taurine and carnitine), but formulated for children over the age of 8 years and suitable for maternal PKU, was introduced [51]. From the 1990s, further advances were made in the nutritional formulations, taste, and presentation of protein substitutes (Table 2). Although selenium supplementation was added to protein substitutes from the late 1980s, many countries were wary about adding selenium to protein substitutes due to concerns about its toxicity which had been responsible for deaths in man and animals and was referred to as the 'essential poison' [52]. Consequently, this led to reports of many cases of biochemical selenium deficiency [53,54].

Table 2. Introduction of protein substitutes.

Laboratory produced preparation	
1952	**Hydrolysed casein, powdered preparation, nutritionally incomplete, low phenylalanine** Addition of tyrosine, tryptophan and methionine, carbohydrate, fat, vitamins and minerals
Commercial produced preparations	
1954	**Hydrolysed casein, powdered preparation, nutritionally incomplete, low phenylalanine** Trufood (infant product), Allen and Handbury/Mead Johnson (older children)
1960	**L amino acid, powdered preparation, nutritionally incomplete phenylalanine free** Powell and Scholfield powdered preparation taken as a drink
1980	**L amino acid, powdered preparation with added carbohydrate and fat nutritionally complete, phenylalanine free** Powdered flavoured preparation taken as a drink
1988	**L amino acid, powdered preparation with added carbohydrate and fat nutritionally complete, phenylalanine free** Powdered preparation taken as a drink for >8 years and adults
1988	**L amino acid, powdered preparation, with added carbohydrate and fat, nutritionally complete designed for infants, phenylalanine free** Powdered preparation used for infants 0–12 months
2001	**L amino acid, powdered preparation with added carbohydrate and essential fatty acids, nutritionally complete, phenylalanine free** Powdered flavoured preparation taken as a drink
2001	**L amino acid powdered preparation, spoonable paste added carbohydrate, fat free, nutritionally complete, phenylalanine free** Powdered flavoured preparation taken as a spoonable paste
2002	**L amino acid tablets, nutritionally incomplete, phenylalanine free** Amino acid tablets
2003	**L amino acid powdered preparation, low carbohydrate, no fat, nutritionally complete, phenylalanine free** Powdered flavoured preparation taken as low volume drink or spoonable paste for children >8 years

Table 2. *Cont.*

2006	**L amino acid ready to drink preparation, low carbohydrate no fat, nutritionally complete, phenylalanine free** Ready to drink flavoured liquid
2008	**Casein macropeptide with L amino acid nutritionally complete with essential fatty acids, low phenylalanine.** Powdered preparation make into a low volume drink
2018	**Slow release L amino acid preparation, carbohydrate and fat free, nutritionally complete, phenylalanine free** Micro tablets, made from L amino acids coated with ethyl cellulose and alginate which slowly release the L amino acids

It was also established that the fat intake of children with PKU was low [55] and n-3 long chain polyunsaturated fatty acid status was sub-optimal [56]. This led to the addition of essential fatty acids to protein substitute powders designed for children [57]. Around the same time, long chain polyunsaturated fatty acids were added to infant protein substitutes in 2000; in a double blind randomised study, infants received either a formula with or without a supplemented fat blend of long chain polyunsaturated fatty acids (LC-PUFA). The results clearly showed the benefit of supplementation [58] and led to the addition of LC-PUFA to other products designed for older children.

Over the years, there has been much endeavor to ensure that protein substitutes meet changing nutritional trends and accommodate nutritional requirements according to life stage. In 2011, the first phenylalanine-free infant formula containing a specific mixture of prebiotic oligosaccharides was introduced [59]. This helped maintain levels of bifidobacteria and lower stool pH in infants with PKU. There is concern about increasing obesity rates in the PKU and non PKU population, so many recent protein substitutes introduced for children, teenage, and adults with PKU have a lower carbohydrate and energy composition [60]. Impact on lowering obesity rates has not yet been proven.

The nutritional adaptation of protein substitutes in order to gain clinical benefit is an area likely to grow in the future. Recently, specific nutrient combinations (containing uridine monophosphate, docosahexaenoic acid, eicosapentaenoic acid, choline, phospholipids, folic acid, vitamins B12, B6, C, and E, and selenium) have been studied in PKU mice to examine the impact on synaptic deficits in PKU [61]. The specific nutrients are precursors and cofactors for the synthesis of phospholipids thought to be beneficial in improving the neurotransmitter/synaptic changes in PKU. This combination of nutrients has been shown to have a benefit on synapse formation, morphology, and function in mouse models of Alzheimer's disease so it may be an important nutritional adaptation of protein substitutes for older patients [62].

9. Choice of Protein Substitutes

The choice, composition, and presentation of protein substitutes have expanded at fast moving rates since the turn of the century. This time was associated with the evolution of pre-packaged and premeasured products, which has not only improved convenience but also accuracy, adherence, and ease of protein substitute prescription for clinicians.

Spoonable low volume protein substitutes: An innovative substitute designed for young children with PKU was produced in 2000, based on phenylalanine free amino acids and starch to which a small amount of water was added, forming a gel/paste that was similar in consistency to a weaning food [60]. This low volume, fat free, lower calorie, more concentrated amino acid substitute had the advantage of allowing transition onto a second stage product from the age of 6 months, in line with complementary feeding. It was presented in premeasured sachets (dispensing with the need for large tins of formula),

was easy to prepare, with a good consistency and acceptable taste. Normal infant feeding behaviour, teething, and intercurrent infection can lead to its rejection in late infancy so perseverance and a consistent approach is needed by parents [63,64].

Ready to drink liquid protein substitutes: In 2005, 'ready to drink' flavoured phenylalanine free amino acid pouches were introduced. These small volume, lower energy protein substitutes were convenient and compact, allowing greater independence for children and teenagers. Patients were less self-conscious taking a liquid drink compared to a powdered preparation [65]. The pharmacological efficacy of these lower volume substitutes did not compromise nutritional biochemistry or phenylalanine concentrations, which remained the same or improved [60]. One potential problem was abdominal discomfort (constipation/diarrhoea), attributed to the hyperosmolar concentration of the lower volume protein substitutes [66,67], and like all concentrated amino acid products, they should be administered with additional water.

Protein substitute tablets: Amino acid tablets and modular systems were also introduced around 2000. Modular systems are when a combination of amino acid tablets, capsules, liquids, powder, or bars of amino acids are used to provide daily protein substitute requirements, allowing flexibility of choice. In a randomised crossover study, it was shown that subjects with PKU successfully took at least 40% of their protein substitute as tablets, with an improvement in adherence and significantly lower blood phenylalanine concentration [68]. The quantity of tablets to meet protein requirements was around 70/day, and they were not nutritionally complete, requiring extra supplementation with vitamins and minerals. They provide an alternative for older children and adults who struggle taking conventional protein substitute. Micro-tablets of amino acids have since been introduced.

Caseinglycomacropeptide with amino acids (CGMP-AA): CGMP-AA was introduced in the UK in 2017, although first used in the USA as a protein substitute for PKU in 2008 [69]. CGMP is purified from whey by anion exchange chromatography, but the final product does contain residual amounts of aromatic amino acids including phenylalanine [70]. CGMP-AA is different from amino acid substitutes; approximately 40% of the product is composed of amino acids, with the rest as a bioactive peptide; based on a macropeptide, they are associated with improved taste and palatability [71].

Slow-release protein substitute: A prolonged release product was first developed in 2014 [72] but there was little supporting published data demonstrating its effectiveness. In 2018, a slow release preparation containing amino acids coated with ethyl cellulose and alginate was introduced. Based on physiomimic technology, the bitter taste and smell of amino acids was improved, and as this product is not mixed with fluid, it does not have an osmolality. Most importantly, the technology prolongs the release of amino acids into the systemic circulation. Animal and human kinetic studies demonstrate a reduced peak concentration of amino acids. This new technology suggests a physiological absorption of amino acids similar to natural protein [73,74]. In a short-term observational study using prolonged amino acids in subjects with PKU, it was well tolerated, with fewer gastrointestinal symptoms and no change in blood phenylalanine concentrations [75].

10. Pharmacological Importance of Protein Substitutes

The amount of protein equivalent (g/kg) from protein substitutes affects blood phenylalanine control [76–79]. As early as 1961, an observational study performed by O'Daly [80] showed protein substitutes significantly lowered blood phenylalanine concentrations. Further studies have shown that phenylalanine tolerance is increased when total protein intake from a protein substitute is increased [79,81].

Protein substitutes have an important function at the blood brain barrier. Large neutral amino acids including phenylalanine compete for LAT1, a large neutral amino acid transporter allowing entry of amino acids into the brain [82]. Phenylalanine has a particularly high affinity for LAT1 and protein substitutes are the only source of competitive large neutral amino acids necessary to prevent excess phenylalanine entering the brain.

These pharmacological effects of ingesting an amino acid rich formula are frequently neglected and given little scientific credence, and yet they have a significant impact on phenylalanine metabolism and long-term physical and neurological outcome. The gut also controls the absorption of amino acids across the epithelial membrane. Phenylalanine is transported as a carrier mediated sodium dependent process which requires energy. Similar to the blood brain barrier, large neural amino acids are transported in the gut by LAT1, also known as SLC5A7, for which phenylalanine has a high affinity [83,84].

11. Protein Substitute Requirements

Human requirements for each amino acid are specific to age, metabolic demands (immune/neuromuscular), and growth rate (protein deposition) [76,85,86]. For protein synthesis to occur, all the amino acids should be available; absence of one leads to the cessation of synthesis [1]. Snyderman [87] reported that the complete withdrawal of phenylalanine from the diet in a normal infant led to the depression in several other amino acids, the most prominent being tyrosine, hence the importance of tyrosine supplementation. Woolf showed that the nitrogen content of the artificial substitute was not an exchange for natural protein; the hydrolysate contained less nitrogen, was rapidly absorbed from the gut with greater oxidation and urinary amino acid losses [88,89], therefore sufficient product was needed to meet nitrogen requirements.

A protein substitute intake that just meets minimum WHO requirements (WHO/FAO/UNU 2007) [90] may result in 'latent' catabolism, leading to body tissue breakdown, increasing phenylalanine concentrations. Protein utilisation is enhanced by a supply of carbohydrate and fat [91,92] further illustrated in a randomised controlled study in PKU subjects by MacDonald [93] and supported by Illsinger [94].

The European PKU Guidelines recommend that the total protein intake should supply 40% more than the FAO/WHO/UNU safe levels of protein intake [95]. However, this amount is arbitrary and unconfirmed by research [67]. A collaborative study [96] involving 63 European and Turkish IMD centres concluded that the amount of total protein prescribed by different European countries was not uniform. All centres gave higher protein equivalents than the recommended 2007 WHO/FAO/UNU [90] safe levels of protein intake with Western European centres prescribing less total protein then other European regions.

To maximise the utilisation of amino acids and minimise the variation in phenylalanine concentrations, protein substitutes should be taken frequently, a minimum of three times a day. MacDonald [97] demonstrated that the greater the amount of protein substitute consumed between waking and 4 p.m., the greater the decrease in phenylalanine concentrations. Likewise, when protein substitute was given 4 hourly for 24 h, there was a marked stablisation in phenylalanine concentrations, reducing phenylalanine variability [98].

Tyrosine, a precursor of catecholamine neurotransmitters (dopamine, norepinephrine, and epinephrine), thyroxine, and melanin, is an essential amino acid in PKU due to the limited or absent hydroxylation of phenylalanine. It is hydrophobic and the absolute quantities added to protein substitutes are not defined. Indicator amino acid oxidation studies [99] suggest tyrosine should provide 19 mg/kg/day, although current protein substitutes provide approximately 5 times above current recommendations. The importance of tyrosine was recognised by the Report of the Medical Research Council working party on PKU [100], which recommended that protein substitutes should be nutritionally complete and contain 100–120 mg/kg/day of tyrosine.

12. Protein Substitute Administration

In the early history, the practicalities of administering an acid based hydrolysed unflavoured product were particularly challenging. Bentovim [46] described the struggles families faced trying to persuade children to take the acid tasting formula: the large daily volume that needed to be consumed, regular vomiting, refusal to eat permitted food due to negative associations with the substitute, the bad smell, and lack of palatability. Furthermore, children experienced isolation and psychological difficulties particularly

in the school years. This was one of the factors leading to diet cessation as early as 6 years [101–103]. An extract from the *Cork Examiner* describes the struggles faced by one family adapting to the news that their two children had been diagnosed with PKU and the dietary changes and challenges made to improve their neurological outcome [104].

Despite the advances in technology, almost all protein substitutes have a strong taste and odour and are associated with poor palatability and breath odour. They are a burden to patients as they must be consumed a minimum of three times daily and spread evenly throughout the day. Ford [66] reported 293 of 631 participants with PKU (39% of adults, 11% of children) either did not take protein substitute or took less than their prescribed amounts.

> Verbatim extract from study: *Our greatest struggle is getting our son taking his protein substitute. He refuses to take it and it can take up to 45 min for him to finish one with a lot of upset*.

Evans [105], in a case control study in PKU children of weaning age, highlighted the stress, anxiety, and struggles associated with protein substitute administration. Maternal anxiety regarding child rejection of protein substitute increased with time peaking at 12–24 months. Similarly, in 2016, MacDonald [106] reported in 114 children with PKU, dietary management was associated with a considerable time and financial burden for caregivers, with much time spent supervising protein substitute intake.

13. Conclusions

In PKU, the early pioneers understood the physiological importance of protein substitutes. They stressed the need for a balanced amino acid profile, for even administration throughout the day, together with an adequate energy intake and dietary treatment for life. Although these principals remain unchanged 70 years later, each decade has witnessed improvements in the delivery and nutritional composition of protein substitutes, which remain of fundamental importance in the treatment of PKU. Further changes are needed, to deliver improved taste and odour-free products, with the properties of natural protein delivering a stable chemical environment associated with optimal physiological function and patient tolerance.

An extract from the *Irish Cork Examiner* describes the struggles of a family diagnosed with PKU in 1959, the son aged 4 and the daughter $2\frac{1}{2}$ years old. This extract describes the determination, sacrifice, hardship, and success against the better judgement of expert advice.

25 October 1962. Phenylketonuria: A story of heartbreak and hope.

> *"Treatment might help your daughter" he said "but for your son detection has come too late." He would deteriorate so much that at a later stage institutional care was inevitable. No one had attempted treatment on a child over 2 years. But the specialist was willing to give my little girl a trial. I pleaded for both of them not knowing the terrible struggle this entailed.*
>
> *The boy was difficult, backward and had no speech while his sister could neither walk or talk and was unable to sit up alone and was extremely difficult to manage. Those first months of 1959 were a nightmare from which there was no awakening. The introduction of the unpalatable diet and the cessation of stews, broths and chocolate sundaes brought tears and tantrums. How I dreaded the ice cream vendors that first summer and the laughing lolly licking youngsters who stood on our corner. The synthetic protein (Minafen) was unpleasant to take, but I have found it can be disguised reasonably well in savouries and cookies.*
>
> *Each child is allowed approximately 270 mg of phenylalanine per day according to body weight. If the child were to have 1oz of porridge oats this would cover 241.5 mg of their daily allowance, whereas 1oz of tomatoes would only represent 5 mg, so planning meals for them was at one time a highly complicated business. Now I possess a simplified chart of foods with a low phenylalanine content and by drawing on almost every cook book in print for ideas I have complied my own Cook Book for Phenylketonurias. Special gluten free flour must be used for bread making and Kosher margarine replaces butter, bread*

making with wheat starch was a different matter "Neolite or just plain leather "was my husband's query at my first attempt. Meals for ourselves present a real problem. It is so difficult to take a hearty T bone steak or peach meringue while two pair of eyes watch with longing. Meals out are impossible as is home entertainment, but it has been so worthwhile, the little girl unable to talk or walk takes some chasing and her speech is coming slowly. Responsiveness and alertness have taken place in slow but sure degrees. My son now 7 has made remarkable progress benefiting from a normal education.'

Although detection of PKU and treatment soon after birth is essential for complete recovery we have proved beyond all doubt that much can still be done.

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References

1. Wu, G. Dietary protein intake and human health. *Food Funct.* **2016**, *7*, 1251–1265. [CrossRef] [PubMed]
2. Følliing, A. Uber Ausscheidung von Phenylbrenztraubensäure in den Harn als Stoffwechselanomalie in Verbindung mit Imbezillität. *Z. Physiol. Chem.* **1934**, *227*, 169–176. [CrossRef]
3. Penrose, L. Inheritance of phenylpyruvic amentia (phenylketonuria). *Lancet* **1935**, *226*, 192–194. [CrossRef]
4. Laxova, R. Lionel Sharples Penrose, 1898–1972: A personal memoir in celebration of the centenary of his birth. *Genetics* **1998**, *150*, 1333–1340.
5. Penrose, L.; Quastel, J.H. Metabolic studies in phenylketonuria. *Biochem. J.* **1937**, *31*, 266–274. [CrossRef]
6. Bernheim, M.L.; Bernheim, F. The production of a hydroxyphenyl compound from l-phenylalanine incubated with liver slices. *J. Biol. Chem.* **1944**, *152*, 481. [CrossRef]
7. Jervis, G. Phenylpyruvic oligophrenia deficiency of phenylalanine-oxidizing system. *Proc. Soc. Exp. Biol. Med.* **1953**, *82*, 514–515.
8. Green, A. *Sheila—Unlocking the Treatment for PKU*; Brewin Books: Reddtich, UK, 2020; p. 172.
9. Medical, Research, and Council. Treatment of Phenylketonuria. *Br. Med. J.* **1963**, *1*, 1691–1697. [CrossRef]
10. Woolf, L.I.; Adams, J. The Early History of PKU. *Int. J. Neonatal Screen.* **2020**, *6*, 59. [CrossRef]
11. Bickel, H.; Gerrard, J.; Hickmans, E.M. Influence of phenylalanine intake on phenylketonuria. *Lancet* **1953**, *265*, 812–813. [CrossRef]
12. Woolf, L.I.; Griffiths, R.; Moncrieff, A.; Coates, S.; Dillistone, F. The Dietary Treatment of Phenylketonuria. *Arch. Dis. Child.* **1958**, *33*, 31–45. [CrossRef] [PubMed]
13. Blainey, J.D.; Gulliford, R. Phenylalanine-restricted Diets in the Treatment of Phenylketonuria. *Arch. Dis. Child.* **1956**, *31*, 452–466. [CrossRef] [PubMed]
14. Hsia, D.Y.; Knox, W.; Quinn, K.V.; Paine, R.S. A one-year, controlled study of the effect of low-phenylalanine diet on phenylketonuria. *Pediatrics* **1958**, *21*, 178–202. [PubMed]
15. Alonso-Fernández, J.R.; Colón, C. Article Commentary: The contributions of Louis I Woolf to the treatment, early diagnosis and understanding of phenylketonuria. *J. Med. Screen.* **2009**, *16*, 205–211. [CrossRef] [PubMed]
16. Woolf, L.I. The Dietary Treatment of Phenylketonuria: Not Proven. *Dev. Med. Child Neurol.* **2008**, *9*, 244–245. [CrossRef]
17. Woolf, L.I.; Griffiths, R.; Moncrieff, A. Treatment of Phenylketonuria with a Diet Low in Phenylalanine. *BMJ* **1955**, *1*, 57–64. [CrossRef]

18. Armstrong, M.D.; Shaw, K.N. Studies on phenylketonuria. III. The metabolism of o-tyrosine. *J. Biol. Chem.* **1955**, *213*, 805–811. [CrossRef]
19. Bickel, H. The effects of a phenylalanine-free and phenylalanine-poor diet in phenylpyruvic oligophrenis. *Exp. Med. Surg.* **1954**, *12*, 114–117.
20. Woolf, L.I. Nutrition in relation to phenylketonuria. *Proc. Nutr. Soc.* **1962**, *21*, 21–29. [CrossRef]
21. Rose, W.C. Amino acid requirements of man. *Nutr. Rev.* **2009**, *34*, 307–309. [CrossRef]
22. Rose, W.C.; Coon, M.J.; Lambert, G.F.; Howe, E. The amino acid requirements of man. VIII. The metabolic availability of the optical isomers of acetyltryptophan. *J. Biol. Chem.* **1955**, *212*, 201–205. [CrossRef]
23. Rose, W.C.; Leach, B.; Coon, M.J.; Lambert, G.F. The amino acid requirements of man. IX. The phenylalanine requirement. *J. Biol. Chem.* **1955**, *213*, 913–922. [CrossRef]
24. Rose, W.C.; Wixom, R.L. The amino acid requirements of man. XIV. The sparing effect of tyrosine on the phenylalanine requirement. *J. Biol. Chem.* **1955**, *217*, 95–101. [CrossRef]
25. Rose, W.C.; Wixom, R.L. The amino acid requirements of man. XIII. The sparing effect of cystine on the methionine requirement. *J. Biol. Chem.* **1955**, *216*, 753–773.
26. Holt, L.E.; Snyderman, S.E. Report to the Council the Amino Acid Requirements of Infants. *JAMA* **1961**, *175*, 100–103. [CrossRef] [PubMed]
27. Holt, L.; Snyderman, S. Protein and amino acid requirements of infants and children. *Nutr. Abstr. Rev.* **1965**, *35*, 1–13.
28. Snyderman, S.E.; Holt, L.E.; Smellie, F.; Boyer, A.; Westall, R.G. The Essential Amino Acid Requirements of Infants: Valine. *Arch. Pediatr. Adolesc. Med.* **1959**, *97*, 186–191. [CrossRef]
29. Leverton, R.M.; Johnson, N.; Ellison, J.; Geschwender, D.; Schmidt, F. The quantitative amino acid requirements of young women. IV. Phenylalanine, with and without tyrosine. *J. Nutr.* **1956**, *58*, 341–353. [CrossRef]
30. Swendseid, M.E.; Williams, I.; Dunn, M.S. Amino Acid Requirements of Young Women Based on Nitrogen Balance Data. *J. Nutr.* **1956**, *58*, 495–505. [CrossRef]
31. Armstrong, M.D.; Tyler, F.H. Studies on phenylketonuria. i. restricted phenylalanine intake in phenylketonuria. *J. Clin. Investig.* **1955**, *34*, 565–580. [CrossRef]
32. Armstrong, M.D.; Binkley, E.L., Jr. Studies on phenylketonuria. V. Observations on a newborn infant with phenylketonuria. *Proc. Soc. Exp. Biol. Med.* **1956**, *93*, 418–420. [CrossRef] [PubMed]
33. Brimblecombe, F.; Stoneman, M.; Maliphant, R. Dietary treatment of an infant with phenylketonuria. *Lancet* **1959**, *273*, 609–611. [CrossRef]
34. Munro, H.N.; Naismith, D.J. The influence of energy intake on protein metabolism. *Biochem. J.* **1953**, *54*, 191–197. [CrossRef] [PubMed]
35. Munro, H.N.; Thomson, W.S. Influence of glucose on amino acid metabolism. *Metabolism* **1953**, *2*, 354–361.
36. Guthrie, R.; Susi, A. A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants. *Pediatrics* **1963**, *32*, 338–343.
37. Holt, K.S. Difficulties and Dangers in the Management of Phenylketonuria. *Acta Paediatr.* **1963**, *52*, 417–423. [CrossRef]
38. Moncrieff, A.; Wilkinson, R.H. Further Experiences in the Treatment of Phenylketonuria. *BMJ* **1961**, *1*, 763–767. [CrossRef]
39. Umbarger, B. Phenylketonuria—Treating the Disease and Feeding the Child. *Arch. Pediatr. Adolesc. Med.* **1960**, *100*, 908. [CrossRef]
40. Brimblecombe, F.S.W.; Blainey, J.D.; Stoneman, M.E.R.; Wood, B.S.B. Dietary and Biochemical Control of Phenylketonuria. *BMJ* **1961**, *2*, 793–798. [CrossRef]
41. Wilson, K.M.; Clayton, B.E. Importance of Choline during Growth, with Particular Reference to Synthetic Diets in Phenylketonuria. *Arch. Dis. Child.* **1962**, *37*, 565–577. [CrossRef]
42. Arakawa, T.; Hirata, K.; Ohara, K.; Fujii, M.; Takahashi, Y.; Kato, H. Folic Acid Deficiency Due to a Low-phenylalanine Diet. *Tohoku J. Exp. Med.* **1965**, *87*, 296–300. [CrossRef] [PubMed]
43. Royston, N.J.W.; Parry, T.E. Megaloblastic Anaemia Complicating Dietary Treatment of Phenylketonuria in Infancy. *Arch. Dis. Child.* **1962**, *37*, 430–435. [CrossRef] [PubMed]
44. Dodge, P.R.; Mangall, E.L.; Crawford, J.D.; Knapp, J.; Paine, R.S. Hypoglycemia complicating treatment of phenylketonuria with a phenylalanine-deficient diet; report of two cases. *N. Engl. J. Med.* **1959**, *260*, 1104–1111. [CrossRef] [PubMed]
45. Kumagai, H. Microbial Production of Amino Acids in Japan. In *History of Modern Biotechnology I*; Fiechter, A., Ed.; Springer: Berlin/Heidelberg, Germany, 2000; pp. 71–85.
46. Bentovim, A.; Clayton, B.E.; Francis, D.E.M.; Shepherd, J.; Wolff, O.H. Use of an Amino Acid Mixture in Treatment of Phenylketonuria. *Arch. Dis. Child.* **1970**, *45*, 640–650. [CrossRef]
47. Smith, I.; Clayton, B.; Wolff, O. New variant of phenylketonuria with progressive neurological illness unresponsive to phenylalanine restriction. *Lancet* **1975**, *305*, 1108–1111. [CrossRef]
48. Manz, F.; Schmidt, H.; Schärer, K.; Bickel, H. Acid-Base Status in Dietary Treatment of Phenylketonuria. *Pediatr. Res.* **1977**, *11*, 1084–1087. [CrossRef]
49. Alexander, F.W.; Clayton, B.E.; Delves, H.T. Mineral and Trace-metal Balances in Children Receiving Normal and Synthetic Diets. *Qjm: Int. J. Med.* **1974**, *43*, 89–111. [CrossRef]
50. Lawson, M.S.; Clayton, B.; Delves, H.T.; Mitchell, J.D. Evaluation of a new mineral and trace metal supplement for use with synthetic diets. *Arch. Dis. Child.* **1977**, *52*, 62–67. [CrossRef]

51. Wardley, B.L.; Taitz, L.S. Clinical trial of a concentrated amino acid formula for older patients with phenylketonuria (Maxamum XP). *Eur. J. Clin. Nutr.* **1988**, *42*, 81–86.
52. Jukes, T.H. Selenium, an "essential poison". *J. Appl. Biochem.* **1983**, *5*, 233–234.
53. Greeves, L.G.; Carson, D.J.; Craig, B.G.; McMaster, D. Potentially Life-Threatening Cardiac Dysrhythmia in a Child with Selenium Deficiency and Phenylketonuria. *Acta Paediatr.* **2008**, *79*, 1259–1262. [CrossRef] [PubMed]
54. Lombeck, I.; Kasperek, K.; Feinendegen, L.; Bremer, H. Serum-selenium concentrations in patients with maple-syrup-urine disease and phenylketonuria under dieto-therapy. *Clin. Chim. Acta* **1975**, *64*, 57–61. [CrossRef]
55. Rose, H.J.; White, F.; Macdonald, A.; Rutherford, P.J.; Favre, E. Fat intakes of children with PKU on low phenylalanine diets. *J. Hum. Nutr. Diet.* **2005**, *18*, 395–400. [CrossRef] [PubMed]
56. Lohner, S.; Fekete, K.; Decsi, T. Lower n-3 long-chain polyunsaturated fatty acid values in patients with phenylketonuria: A systematic review and meta-analysis. *Nutr. Res.* **2013**, *33*, 513–520. [CrossRef]
57. Cleary, M.; Feillet, F.; White, F.; Vidailhet, M.; Macdonald, A.; Grimsley, A.; Maurin, N.; De Baulny, H.O.; Rutherford, P.J. Randomised controlled trial of essential fatty acid supplementation in phenylketonuria. *Eur. J. Clin. Nutr.* **2006**, *60*, 915–920. [CrossRef]
58. Agostoni, C.; Harvie, A.; McCulloch, D.L.; Demellweek, C.; Cockburn, F.; Giovannini, M.; Murray, G.; Harkness, R.A.; Riva, E. A randomized trial of long-chain polyunsaturated fatty acid supplementation in infants with phenylketonuria. *Dev. Med. Child Neurol.* **2006**, *48*, 207–212. [CrossRef]
59. Macdonald, A.; Cochrane, B.; Wopereis, H.; Loveridge, N. Specific prebiotics in a formula for infants with Phenylketonuria. *Mol. Genet. Metab.* **2011**, *104*, S55–S59. [CrossRef]
60. Macdonald, A.; Lilburn, M.; Cochrane, B.; Davies, P.; Daly, A.; Asplin, D.; Hall, S.K.; Cousins, A.; Chakrapani, A.; Robinson, P.; et al. A new, low-volume protein substitute for teenagers and adults with phenylketonuria. *J. Inherit. Metab. Dis.* **2004**, *27*, 127–135. [CrossRef]
61. Bruinenberg, V.M.; Van Vliet, D.; Van Der Goot, E.; Counotte, D.S.; Kuhn, M.; Van Spronsen, F.J.; Van Der Zee, E.A. Long-term dietary intervention with low Phe and/or a specific nutrient combination improve certain aspects of brain functioning in phenylketonuria (PKU). *PLoS ONE* **2019**, *14*, e0213391. [CrossRef]
62. De Wilde, M.C.; Penke, B.; van der Beek, E.M.; Kuipers, A.A.; Kamphuis, P.J.; Broersen, L.M. Neuroprotective effects of a specific multi-nutrient intervention against Abeta42-induced toxicity in rats. *J. Alzheimers Dis.* **2011**, *27*, 327–339. [CrossRef]
63. Evans, S.; Daly, A.; Chahal, S.; Macdonald, J. Food acceptance and neophobia in children with phenylketonuria: A prospective controlled study. *J. Hum. Nutr. Diet.* **2016**, *29*, 427–433. [CrossRef] [PubMed]
64. Evans, S.; Daly, A.; Macdonald, J.; Pinto, A.; Macdonald, A. Fifteen years of using a second stage protein substitute for weaning in phenylketonuria: A retrospective study. *J. Hum. Nutr. Diet.* **2017**, *31*, 349–356. [CrossRef] [PubMed]
65. Macdonald, A.; Lilburn, M.M.; Davies, P.; Evans, S.; Daly, A.; Hall, S.K.; Hendriksz, C.; Chakrapani, A.; Lee, P. 'Ready to drink' protein substitute is easier is for people with phenylketonuria. *J. Inherit. Metab. Dis.* **2006**, *29*, 526–531. [CrossRef] [PubMed]
66. Ford, S.; O'Driscoll, M.; Macdonald, A. Living with Phenylketonuria: Lessons from the PKU community. *Mol. Genet. Metab. Rep.* **2018**, *17*, 57–63. [CrossRef]
67. MacDonald, A.; Van Wegberg, A.M.J.; Ahring, K.; Beblo, S.; Bélanger-Quintana, A.; Burlina, A.; Campistol, J.; Coşkun, T.; Feillet, F.; Giżewska, M.; et al. PKU dietary handbook to accompany PKU guidelines. *Orphanet J. Rare Dis.* **2020**, *15*, 171. [CrossRef]
68. Macdonald, A.; Ferguson, C.; Rylance, G.; Morris, A.A.M.; Asplin, D.; Hall, S.K.; Booth, I.W. Are tablets a practical source of protein substitute in phenylketonuria? *Arch. Dis. Child.* **2003**, *88*, 327–329. [CrossRef]
69. Ney, D.M.; Hull, A.K.; Van Calcar, S.C.; Liu, X.; Etzel, M.R. Dietary Glycomacropeptide Supports Growth and Reduces the Concentrations of Phenylalanine in Plasma and Brain in a Murine Model of Phenylketonuria. *J. Nutr.* **2008**, *138*, 316–322. [CrossRef]
70. Nakano, T.; Silva-Hernandez, E.; Ikawa, N.; Ozimek, L. Purification of kappa-casien glycomacropeptide from sweet whey with undetectable level of phenylalanine. *Biotechnol. Prog.* **2002**, *18*, 409–412. [CrossRef]
71. Lim, K.; Van Calcar, S.C.; Nelson, K.L.; Gleason, S.T.; Ney, D.M. Acceptable low-phenylalanine foods and beverages can be made with glycomacropeptide from cheese whey for individuals with PKU. *Mol. Genet. Metab.* **2007**, *92*, 176–178. [CrossRef]
72. Giovannini, M.; Riva, E.; Salvatici, E.; Cefalo, G.; Radaelli, G. Randomized Controlled Trial of a Protein Substitute with Prolonged Release on the Protein Status of Children with Phenylketonuria. *J. Am. Coll. Nutr.* **2014**, *33*, 103–110. [CrossRef]
73. Scheinin, M.; Barassi, A.; Junnila, J.J.T.; Lovró, Z.; Reiner, G.; Sarkkinen, E.; Macdonald, A. Amino Acid Plasma Profiles from a Prolonged-Release Protein Substitute for Phenylketonuria: A Randomized, Single-Dose, Four-Way Crossover Trial in Healthy Volunteers. *Nutrients* **2020**, *12*, 1653. [CrossRef] [PubMed]
74. Giarratana, N.; Gallina, G.; Panzeri, V.; Frangi, A.; Canobbio, A.; Reiner, G. A New Phe-Free Protein Substitute Engineered to Allow a Physiological Absorption of Free Amino Acids for Phenylketonuria. *J. Inborn Errors Metab. Screen.* **2018**, *6*, 1–9. [CrossRef]
75. Macdonald, A.; Ashmore, C.; Daly, A.; Pinto, A.; Evans, S. An Observational Study Evaluating the Introduction of a Prolonged-Release Protein Substitute to the Dietary Management of Children with Phenylketonuria. *Nutrients* **2020**, *12*, 2686. [CrossRef] [PubMed]
76. Acosta, P.B.; Yannicelli, S. Protein intake affects phenylalanine requirements and growth of infants with phenylketonuria. *Acta Paediatr.* **1994**, *83*, 66–67. [CrossRef] [PubMed]

77. Duran, G.P.; Rohr, F.J.; Slonim, A.; Güttler, F.; Levy, H.L. Necessity of Complete Intake of Phenylalanine-free Amino Acid Mixture for Metabolic Control of Phenylketonuria. *J. Am. Diet. Assoc.* **1999**, *99*, 1559–1563. [CrossRef]
78. Kindt, E.; Lunde, H.A.; Gjessing, L.R.; Halvorsen, S.; Lie, S.O. Fasting Plasma Amino Acid Concentrations in PKU Children on Two Different Levels of Protein Intake. *Acta Paediatr.* **1988**, *77*, 60–66. [CrossRef] [PubMed]
79. Macdonald, A.; Chakrapani, A.; Hendriksz, C.; Daly, A.; Davies, P.; Asplin, D.; Hall, K.; Booth, I.W. Protein substitute dosage in PKU: How much do young patients need? *Arch. Dis. Child.* **2005**, *91*, 588–593. [CrossRef]
80. O'Daly, S. Phenylketonuria treated with a high phenylalanine intake and casein-hydrolysate/aminoacid mixtures. *Lancet* **1961**, *277*, 1379–1383. [CrossRef]
81. Acosta, P.B. Recommendations for protein and energy intakes by patients with phenylketonuria. *Eur. J. Nucl. Med. Mol. Imaging* **1996**, *155*, S121–S124. [CrossRef]
82. Van Vliet, D.; Ruinenberg, V.M.; Mazzola, P.N.; van Faassen, M.H.; de Blaauw, P.; Kema, I.P.; Heiner-Fokkema, M.R.; van Anholt, R.D.; van der Zee, E.A.; Spronsen, F.J. Large Neutral Amino Acid Supplementation Exerts Its Effect through Three Synergistic Mechanisms: Proof of Principle in Phenylketonuria Mice. *PLoS ONE* **2015**, *10*, e0143833. [CrossRef]
83. Adibi, S.A.; Morse, E.L. Intestinal transport of dipeptides in man: Relative importance of hydrolysis and intact absorption. *J. Clin. Investig.* **1971**, *50*, 2266–2275. [CrossRef] [PubMed]
84. Hidalgo, I.J.; Borchardt, R.T. Transport of a large neutral amino acid (phenylalanine) in a human intestinal epithelial cell line: Caco-2. *Biochim. Biophys. Acta Biomembr.* **1990**, *1028*, 25–30. [CrossRef]
85. Kindt, E.; Motzfeldt, K.; Halvorsen, S.; Lie, S. Protein requirements in infants and children: A longitudinal study of children treated for phenylketonuria. *Am. J. Clin. Nutr.* **1983**, *37*, 778–785. [CrossRef] [PubMed]
86. Przyrembel, H. Recommendations for protein and amino acid intake in phenylketonuria patients. *Eur. J. Nucl. Med. Mol. Imaging* **1996**, *155*, S130–S131. [CrossRef]
87. Snyderman, S.E.; Pratt, E.L.; Cheung, M.W.; Norton, P.; Holt, L.E.; Hansen, A.E.; Panos, T.C. The Phenylalanine Requirement of the Normal Infant. *J. Nutr.* **1955**, *56*, 253–263. [CrossRef]
88. Gropper, S.S.; Gropper, D.M.; Acosta, P.B. Plasma Amino Acid Response to Ingestion of L-Amino Acids and Whole Protein. *J. Pediatr. Gastroenterol. Nutr.* **1993**, *16*, 143–150. [CrossRef]
89. Jones, B.J.; Lees, R.; Andrews, J.; Frost, P.; Silk, D.B. Comparison of an elemental and polymeric enteral diet in patients with normal gastrointestinal function. *Gut* **1983**, *24*, 78–84. [CrossRef]
90. World Health Organisation. *Protein and Amino Acid Requirements in Human Nutition*; Report of a Joint WHO/FAO/UNU Expert Consultation (WHO Technical Report Series 935), in United Nations University; WHO: Geneva, Switzerland, 2007; pp. 1–265.
91. Reeds, P.J.; Garlick, P.J. Protein and Amino Acid Requirements and the Composition of Complementary Foods. *J. Nutr.* **2003**, *133*, 2953S–2961S. [CrossRef]
92. Viteri, F.E. INCAP Studies of Energy, Amino Acids, and Protein. *Food Nutr. Bull.* **2010**, *31*, 42–53. [CrossRef]
93. Macdonald, A.; Rylance, G.; Hall, S.K.; Asplin, D.; Booth, I.W. Factors affecting the variation in plasma phenylalanine in patients with phenylketonuria on diet. *Arch. Dis. Child.* **1996**, *74*, 412–417. [CrossRef]
94. Illsinger, S.; Meyer, U.; Vaske, B.; Das, A.M.; Lücke, T. Branched chain amino acids as a parameter for catabolism in treated phenylketonuria. *Amino Acids* **2004**, *28*, 45–50. [CrossRef] [PubMed]
95. Van Wegberg, A.M.J.; Macdonald, A.; Ahring, K.; Bélanger-Quintana, A.; Blau, N.; Bosch, A.M.; Burlina, A.; Campistol, J.; Feillet, F.; Giżewska, M.; et al. The complete European guidelines on phenylketonuria: Diagnosis and treatment. *Orphanet J. Rare Dis.* **2017**, *12*, 1–56. [CrossRef] [PubMed]
96. Aguiar, A.; Ahring, K.; Almeida, M.; Assoun, M.; Quintana, A.B.; Bigot, S.; Bihet, G.; Malmberg, K.B.; Burlina, A.; Bushueva, T.; et al. Practices in prescribing protein substitutes for PKU in Europe: No uniformity of approach. *Mol. Genet. Metab.* **2015**, *115*, 17–22. [CrossRef] [PubMed]
97. Macdonald, A.; Rylance, G.W.; Asplin, D.; Hall, S.K.; Booth, I.W. Does a single plasma phenylalanine predict quality of control in phenylketonuria? *Arch. Dis. Child.* **1998**, *78*, 122–126. [CrossRef] [PubMed]
98. Macdonald, A.; Rylance, G.; Davies, P.; Asplin, D.; Hall, S.K.; Booth, I.W. Administration of protein substitute and quality of control in phenylketonuria: A randomized study. *J. Inherit. Metab. Dis.* **2003**, *26*, 319–326. [CrossRef] [PubMed]
99. Elango, R.; Ball, R.O.; Pencharz, P.B. Amino acid requirements in humans: With a special emphasis on the metabolic availability of amino acids. *Amino Acids* **2009**, *37*, 19–27. [CrossRef]
100. Medical, Research, and Council. Phenylketonuria due to phenylalanine hydroxylase deficiency: An unfolding story. Medical Research Council Working Party on Phenylketonuria. *BMJ* **1993**, *306*, 115–119. [CrossRef]
101. Blaskovics, M.; Nelson, T.L. Phenylketonuria and its variations. A review of recent developments. *Calif. Med.* **1971**, *115*, 42–57.
102. Fisch, R.O.; Torres, F.; Gravem, H.J.; Greenwood, C.S.; Anderson, J.A. Twelve years of clinical experience with phenylketonuria: A statistical evaluation of symptoms, growth, mental development, electroencephalographic records, serum phenylalanine levels, and results of dietary management. *Neurology* **1969**, *19*, 659. [CrossRef]
103. Koff, E.; Kammerer, B.; Boyle, P.; Pueschel, S.M. Intelligence and phenylketonuria: Effects of diet termination. *J. Pediatr.* **1979**, *94*, 534–537. [CrossRef]
104. Cowan, M. *Phenylketonuria: A Story of Heartbreak and Hope*; Cork Examiner: Cork, Ireland, 1962.

105. Evans, S.; Daly, A.; Wildgoose, J.; Cochrane, B.; Chahal, S.; Ashmore, C.; Loveridge, N.; Macdonald, A. How Does Feeding Development and Progression onto Solid Foods in PKU Compare with Non-PKU Children During Weaning? *Nutrients* **2019**, *11*, 529. [CrossRef] [PubMed]
106. Macdonald, A.; Smith, T.A.; De Silva, S.; Alam, V.; Van Loon, J.M. The personal burden for caregivers of children with phenylketonuria: A cross-sectional study investigating time burden and costs in the UK. *Mol. Genet. Metab. Rep.* **2016**, *9*, 1–5. [CrossRef] [PubMed]

CHAPTER 3A

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GLYCOMACROPEPIDE IN CHILDREN WITH PHENYLKETONURIA: DOES ITS PHENYLALANINE CONTENT AFFECT BLOOD PHENYLALANINE CONTROL?

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RESEARCH PAPER

Glycomacropeptide in children with phenylketonuria: does its phenylalanine content affect blood phenylalanine control?

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[Correction added on 21 February 2017, after first online publication: The article has been edited to correct error in terminology used. 'Plasma phenylalanine' has been changed to 'blood phenylalanine' all throughout the article.]

Abstract

Background: In phenylketonuria (PKU), there are no data available for children with respect to evaluating casein glycomacropeptide (CGMP) as an alternative to phenylalanine-free protein substitutes [Phe-free L-amino acid (AA)]. CGMP contains a residual amount of phenylalanine, which may alter blood phenylalanine control.
Methods: In a prospective 6-month pilot study, we investigated the effect on blood phenylalanine control of CGMP-amino acid (CGMP-AA) protein substitute in 22 PKU subjects (13 boys, nine girls), median age (range) 11 years (6–16 years). Twelve received CGMP-AA and nine received Phe-free L-AA, (1 CGMP-AA withdrawal). Subjects partially or wholly replaced Phe-free L-AA with CGMP-AA. If blood phenylalanine exceeded the target range, the CGMP-AA dose was reduced and replaced with Phe-free L-amino acids. The control group remained on Phe-free L-AAs. Phenylalanine, tyrosine and Phe : Tyr ratio concentrations were compared with the results for the previous year.
Results: In the CGMP-AA group, there was a significant increase in blood phenylalanine concentrations (pre-study, 275 μmol L^{-1}; CGMP-AA, 317 μmol L^{-1}; $P = 0.02$), a decrease in tyrosine concentrations (pre-study, 50 μmol L^{-1}; CGMP-AA, 40 μmol L^{-1}; $P = 0.03$) and an increase in Phe : Tyr ratios (pre-study, Phe : Tyr 4.9:1; CGMP-AA, Phe : Tyr 8:1; $P = 0.02$). In the control group there was a non-significant fall in phenylalanine concentrations (pre-study 325μmol/L: study 280μmol/L [p = 0.9], and no significant changes for tyrosine or phe/tyr ratios [p = 0.9]. Children taking the CGMP-AA found it more acceptable to L-AA.
Conclusions: Blood phenylalanine control declined with CGMP-AA but, by titrating the dose of CGMP-AA, blood phenylalanine control remained within target range. The additional intake of phenylalanine may have contributed to the change in blood phenylalanine concentration. CGMP-AA use requires careful monitoring in children.

Introduction

In phenylketonuria (PKU), a protein substitute free of phenylalanine, but nutritionally complete in all other amino acids, vitamins and minerals, is an essential part of dietary management. The original protein substitutes were based on casein hydrolysates [(1)] and, during the 1970s, they were gradually replaced by L-amino acid supplements (L-amino acids) [(2,3)]. Although considerably improved in palatability and nutritional composition, the amino acid composition (g protein equivalent^{-1}) of protein substitutes has changed little. It is established that their dosage and timing [(4,5)] may have an impact on blood phenylalanine control.

Casein glycomacropeptide (CGMP) is a 64 amino acid phosphorylated glycopeptide released from κ casein by the

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action of chymosin (rennin) during the cheese-making process; the end product has been adapted for use as a protein substitute in PKU. CGMP was first given to a 29-year-old PKU male after safety studies in PKU mice showed improvement in metabolic control [6–8]. Although its use is not well established within Europe [9], it is clear that CGMP-amino acid (AA) supplements offer several theoretical advantages compared to conventional Phe-free L-amino acid supplements. They have been reported to have a better taste [10,11], increase the efficacy of protein utilisation [12,13], improve nitrogen retention [7,14], potentially improve long-term bone health [15] and have intestinal anti-inflammatory activity that may be important in long term health in PKU [16]. There is a suggestion that they reduce post-prandial phenylalanine concentrations [10].

Unmodified CGMP contains very low levels of arginine, cysteine, histidine, tryptophan, leucine and tyrosine, and the content of the large neutral amino acids, threonine and isoleucine, is two to three times the amount of other protein substitutes based on L-amino acid supplements. Commercial preparations of CGMP-AA, used in the treatment of PKU, require supplementation with limiting amino acids. [17] Although the optimal profile of L-amino acids added to CGMP-AA is yet to be determined, van Calcar (2009) [10] suggested they provide 130% more histidine, methionine, leucine and tryptophan and 150% more tyrosine than the American (2002) dietary reference intakes. Commercial CGMP-AA protein substitutes contain residual phenylalanine (1.5–1.8 mg g^{-1} protein) and there are no published long-term human studies reporting the safety, both in terms of growth and blood phenylalanine control, of CGMP-AA in children. Considering CGMP-AA protein substitutes contain phenylalanine, it is important to establish via longitudinal, long-term studies whether CGMP-AA based protein substitutes have any adverse effect on blood phenylalanine concentrations in children with PKU.

In the first of a series of studies, we investigated the impact on blood phenylalanine control of a CGMP-AA based protein substitute with an amino acid profile that aimed to provide the World Health Organization/Food and Agriculture Organization/United Nations University (WHO/FAO/UNU) (2007) requirements in children aged 5–16 years with PKU.

Materials and methods

Subjects

Twenty-two (13 boys, nine girls) children with PKU were recruited, with a median age (range) of 11 years (6–16 years). Nineteen children were European and three were of Pakistani origin. Inclusion criteria included known adherence with protein substitute and low phenylalanine diet, aged 5–16 years achieving 70% of blood phenylalanine concentrations within the age-related phenylalanine target range (120–360 μmol L^{-1} for children 5–10 years, 120–600 μmol L^{-1} for patients 11–16 years), diagnosed by new-born screening, and not treated with sapropterin dihydrochloride.

The South Birmingham Research Ethics committee granted a favourable ethical opinion. Written consent was obtained for all subjects from at least one caregiver with parental responsibility and written assent was obtained from the subject if appropriate for their age and level of understanding.

Selection into control or CGMP-AA group

Children chose which product they preferred to have as their protein substitute: CGMP-AA or Phe-free L-amino acid (L-AA). To ensure adherence with CGMP-AA at 5 weeks before the start of the study, each child was asked to take 20 g of protein equivalent from CGMP-AA for 7 days. If they preferred the CGMP-AA, they entered the CGMP-AA arm of the study at week 0 (baseline); otherwise, they remained on Phe-free L-amino acids and so entered the control study arm at week 0.

CGMP-AA protein substitute

The CGMP-AA was supplemented with amino acids to meet WHO/FAO/UNU (2007) requirements, with added carbohydrate, fat and a comprehensive range of micronutrients. Each 20 g of protein equivalent from the CGMP-AA based protein substitute contained 30 mg of phenylalanine (Table 1).

Intake of CGMP-AA and conventional phenylalanine-free L-amino acid

The amount of CGMP-AA was individually prescribed for each child, with the dose titrated against blood phenylalanine concentrations. Subjects partially or wholly replaced their conventional L-amino acid supplement with CGMP-AA. If blood phenylalanine concentrations were above target range for 3 consecutive weeks, the dose of CGMP-AA was reduced by 20 g day^{-1} protein equivalent and replaced with 20 g of protein equivalent from Phe-free L-amino acids. Subjects in the control group remained on their usual Phe-free L-amino acid supplement throughout the study period (Table 2).

Anthropometric measurements

At baseline (week 0) and at the end of the study (week 26), weight, height and body mass index were recorded.

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Table 1 Nutritional and amino acid profiles for 20 g of protein equivalent of study CGMP product

	Study product CGMP-AA Per 35 g
Energy MJ kJ^{-1} (kcal kJ^{-1})	0.502 (120)
Protein equivalent (g)	20
Total carbohydrate (g)	6.5
Sugars (g)	2.2
Total fat (g)	1.5
Saturates (g)	0.2
Monounsaturated (g)	0.44
Polyunsaturates (g)	0.12
Docosahexaenoic acid (mg)	84
Arachidonic acid (AA) (mg)	0
Eicosapentaenoic acid (mg)	0
Fibre (g)	0.1
Salt (g)	0.53
Vitamin A (μg RE)	283
Vitamin D (μg)	4.5
Vitamin E (mg)	6.5
Vitamin C (mg)	38
Vitamin K (μg)	35
Vitamin B_1 (thiamine) (mg)	0.68
Vitamin B_2 (riboflavin) (mg)	0.78
Vitamin B_3 (niacin) (mg)	8.4
Vitamin B_5 (pantothenic acid) (mg)	2.7
Vitamin B_6 (pyridoxine) (mg)	1.0
Vitamin B_7 (biotin) (μg)	63.9
Vitamin B_9 (folic acid) (μg)	136
Vitamin B_{12} (cyanocobalamin) (μg)	1.6
Choline (mg)	204
Sodium (mmol)	9
Potassium (mmol)	6.8
Chloride (mg)	7
Calcium (mg)	407
Phosphorous (mg)	395
Magnesium (mg)	128
Iron (mg)	7.3
Copper (μg)	748
Zinc (mg)	7.3
Manganese (mg)	1.1
Iodine (μg)	85.7
Molybdenum (μg)	49
Selenium (μg)	29.9
Chromium (μg)	29.9
Amino acids (g 20 g^{-1} PE)	
L-alanine	0.76
L-arginine	1
L-aspartic acid	2.04
L-cysteine	0.01
Glycine	2.77
L-histidine	0.42
L-isoleucine	1.37
L-leucine	1.3
L- lysine	1.07
L-methionine	0.54
L-phenylalanine	**0.03**
L-proline	1.51
L-serine	0.98
L-threonine	2.17
L-tryptophan	0.17
L-tyrosine	1.01
L-valine	1.31
L-glutamine	1.73
Total amino acids	**20.19**
L-carnitine (mg)	1.01
Taurine (mg)	1.13

CGMP-AA, casein glycomacropeptide amino acid; RE, retinol equivalent.

The same investigator measured weight and height. Height was measured on a Harpenden stadiometer (Holtain Ltd, Crymych, UK) and weight was measured on calibrated digital scales. Both were measured to the nearest 0.1 unit decimal point.

Dietary assessments

Each subject/caregiver completed a 3-day weighed food diary. Monthly home visits were conducted to assess adherence with protein substitute and low phenylalanine diet. The 3-day weighed food intake was calculated using the MICRODIET (Microdiet Downlee Systems Ltd, Chapel-en-le-Frith, UK), analysing phenylalanine, protein and energy intake from all natural food sources (e.g. 50 mg of phenylalanine exchange foods, low protein prescribed product, fruit and vegetables).

Blood phenylalanine/tyrosine monitoring

From baseline to study end, weekly overnight fasting morning blood spots were collected by trained caregivers at home. Blood specimens were sent via the postal service to the laboratory at Birmingham Children's Hospital. Blood samples were collected on filter cards [Perkin Elmer 226 (UK Standard NBS); Perkin Elmer, Waltham, MA, USA]. All of the cards had a standard thickness and the blood phenylalanine and tyrosine concentrations were calculated on a 3.2-mm punch by tandem mass spectrometry.

The median of the sum of all the weekly blood phenylalanine, tyrosine and phenylalanine to tyrosine ratios (Phe : Tyr) during the 6-month study period was calculated and compared with median of the sum of all the weekly/fortnightly blood concentrations from the previous 12 months prior to entering the study.

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Table 2 Comparison of the median change in blood phenylalanine concentrations (range) from the previous 12 months (before start of study) to week 26 (end of study) for the CGMP-AA and L-AA groups and, for the CGMP-AA group, the percentage contribution of protein equivalent from CGMP-AA from baseline to end of study and median CGMP-AA protein equivalent intake at 6 months

CGMP-AA group							Control group			
		Median phenylalanine μmol L^{-1} (range)		Median % protein equivalent from CGMP-AA		Median protein equivalent from CGMP-AA at 6 months			Median phenylalanine μmol L^{-1} (range)	
Subjects	Age (years)	pre- CGMP-AA	6 month CGMP-AA	Baseline	6 month		Subjects	Age (years)	12 month pre-study	6 month study
1	15	255 (160–440)	345 (290–420)	100	100	60	1	12	360 (100–560)	455 (250–910)
2	11	350 (210–490)	390 (280–510)	100	100	80	2	10	470 (80–570)	245 (110–700)
3	11	550 (240–1120)	610 (320–990)	100	100	60	3	14	350 (180–710)	350 (130–1080)
4	10	315 (70–550)	430 (280–760)	100	30	50	4	9	325 (90–670)	280 (60–450)
5	6	270 (60–510)	420 (160–680)	100	30	20	5	14	540 (100–740)	540 (240–610)
6	6	320 (180–510)	470 (110–710)	100	30	20	6	7	235 (100–500)	180 (80–610)
7	7	220 (90–520)	265 (100–980)	100	70	40	7	12	210 (70–580)	280 (100–520)
8	8	170 (90–660)	250 (110–610)	80	75	60	8	14	240 (70–880)	180 (60–850)
9	7	225 (80–400)	290 (130–700)	75	75	75	9	6	190 (80–480)	270 (110–690)
10	8	225 (100–520)	220 (80–490)	30	30	30				
11	7	280 (80–690)	270 (50–410)	30	30	30				
12	11	290 (80–630)	200 (70–600)	30	30	30				
Median		275	317	100	50	45			325	280
$P = 0.02$							P = ns			

CGMP-AA, casein glycomacropeptide amino acid.

Acceptability

After 7 days of taking CGMP-AA, caregivers and patients completed acceptability questionnaires. They were asked questions about taste, smell, texture, mouthfeel and overall acceptability of the CGMP-AA.

Statistical analysis

Statistical analysis was performed using nonparametric Wilcoxon matched pairs signed rank testing and the Pearson correlation coefficient. $P < 0.05$ was considered statistically significant.

Results

Subjects

Twelve children (aged 6–16 years) were in the CGMP-AA group and nine (aged 6–14 years) were in the L-AA group. At baseline in both the CGMP-AA and L-AA groups, the median protein equivalent from protein substitute was 60 g day^{-1} (60–80 g). Prior to starting the CGMP-AA protein substitute, one subject took a powdered preparation (XP Maxamum; Nutricia Ltd, Trowbridge, UK); 11 liquid pouches [Lophlex PKU, $n = 2$ (Nutricia Ltd) and PKU Cooler, $n = 9$ (Vitaflo Ltd, Liverpool, UK)]. In the L-AA group, the entire protein substitute was from Phe-free L-AA supplements. They either received liquid pouches (LQ Lophlex PKU, $n = 1$; PKU Cooler, $n = 7$) or powdered preparations (PKU gel, $n = 1$; Vitaflo Ltd).

In the CGMP-AA group, CGMP-AA was titrated with their usual L-AA product, the median intake of protein equivalent was 50% from CGMP-AA and 50% from Phe-free L-AA at 6 months. In both groups, the median amount of prescribed natural protein from food sources was 5 g day^{-1} (3–30 g). One 12-year-old boy was excluded from the CGMP-AA group because he failed to adhere to the inclusion criteria of blood phenylalanine control within 70% of target range as a result of poor dietary adherence.

Blood phenylalanine concentrations

At the end of the study period, there was a significant increase in blood phenylalanine concentrations in the CGMP-AA group (pre-CGMP-AA, 275 μmol L^{-1}; with CGMP-AA, 317 μmol L^{-1}; $P = 0.02$), although the median blood phenylalanine control remained within target range. Overall, 75% of the subjects had higher median phenylalanine concentrations compared to the retrospective results for the previous year. In seven subjects, the amount of protein equivalent from CGMP-AA was reduced to avoid median phenylalanine concentrations

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above target concentrations (Fig. 1 and Table 2). Three subjects remaining on only 20 g of protein equivalent from CGMP-AA had no increase in blood phenylalanine concentrations. However, there was no significant correlation between the dose of CGMP-AA and change in blood phenylalanine concentrations ($r = 0.6$) (Fig. 1). In the control group, there was a nonsignificant fall in median phenylalanine concentrations (pre-study, 325 μmol L^{-1}; study, 280 μmol L^{-1}; $P = 0.9$) compared to the previous year's median results.

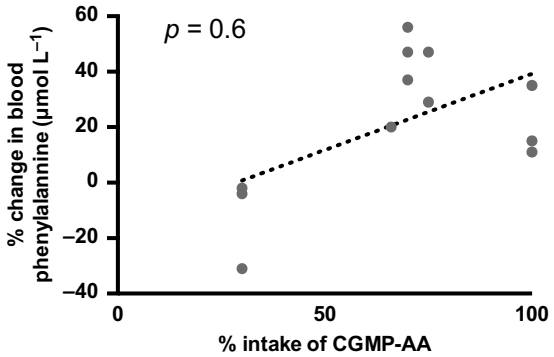


Figure 1 Spearman correlation natural protein intake (g day^{-1}) compared to percentage change in blood phenylalanine concentrations. [Correction added on 21 February 2017, after first online publication: Figure 1 was previously captured with incorrect x-axis and has been corrected in this current version.]

Tyrosine concentrations (table 3)

In the CGMP-AA group, there was a significant decrease in tyrosine concentrations (pre-study, 50 μmol L^{-1}; with CGMP-AA, 40 μmol L^{-1}; $P = 0.03$), although this was still above the lower reference range. In the control group, tyrosine concentrations remained unchanged (pre and post study 40μmol/L [p = 1]).

Phenylalanine and tyrosine ratio (table 4)

There was a significant difference in the Phe/Tyr ratio in the CGMP-AA group (Phe : Tyr ratio pre-study, 4.9:1, with CGMP-AA, 8:1; $P = 0.02$). There was no significant difference in the Phe : Tyr ratio in the control group (pre-study phe/tyr 5.8:1, with study 6.7:1).

Three-day weighed food intake (table 5)

The total protein intake from all foods consumed (natural protein from phenylalanine exchanges, low protein prescribed foods, fruits and vegetables) was similar for the CGMP-AA (week 0, 10.8 g day^{-1}; week 26, 9.8 g day^{-1}) and control groups (week 0, 9 g day^{-1}; week 26, 10 g day^{-1}). Both groups consumed 50% more natural protein compared to the prescribed amounts (additional amounts of natural protein: CGMP-AA group, 5 g day^{-1}; control group, 4.5 g day^{-1}). Although there was no

Table 3 Comparison of the median change in blood tyrosine concentrations (range) from the previous 12 months (before start of study) to week 26 (end of study) for CGMP-AA and L-AA groups and, for the CGMP-AA group, the percentage contribution of protein equivalent from CGMP-AA from baseline to end of study

CGMP-AA group						Control group			
		Median tyrosine μmol L^{-1} (range)		Median protein equivalent from CGMP-AA at 6 months				Median tyrosine μmol L^{-1} (range)	
Subjects	Age (years)	pre- CGMP-AA	6 month CGMP-AA	Baseline	6 month	Subjects	Age (years)	12 month pre-study	6 month study
1	15	50 (30–60)	40 (30–60)	100	100	1	12	40 (30–70)	40 (30–50)
2	11	60 (40–140)	50 (40–70)	100	100	2	10	60 (40–250)	70 (40–140)
3	11	50 (40–140)	40 (30–40)	100	100	3	14	75 (40–270)	70 (40–110)
4	10	35 (30–60)	40 (30–90)	100	30	4	9	40 (30–120)	40 (30–50)
5	6	100 (40–240)	45 (30–190)	100	30	5	14	50 (30–90)	60 (30–130)
6	6	80 (40–290)	40 (30–190)	100	30	6	7	40 (30–70)	40 (30–70)
7	7	70 (40–240)	40 (30–80)	100	70	7	12	50 (30–70)	40 (40–60)
8	8	45 (20–170)	30 (20–50)	80	75	8	14	45 (30–80)	40 (20–100)
9	7	40 (30–60)	40 (20–170)	75	75	9	6	40 (30–70)	40 (30–60)
10	8	50 (30–140)	50 (30–130)	30	30				
11	7	60 (30–90)	60 (50–80)	30	30				
12	11	40 (30–130)	50 (30–60)	30	30				
Median		50	40	100	50			40	40
$P = 0.03$						P = ns			

CGMP-AA, casein glycomacropeptide amino acid.

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Table 4 Comparison of median phenylalanine and tyrosine ratios with the previous 12 months (before start of study) with week 26 (end of study) for each subject in the CGMP-AA and control groups

CGMP-AA group				Control group			
	Age	Median phe/tyr ratio			Age	Median phe/tyr ratio	
Subjects	Year	12 month pre-study	6 month CGMP-AA	Subjects	Year	12 month pre-study	6 month study
1	15	5:1	8.6:1	1	12	8.5:1	11.1:1
2	11	5.8:1	7.8:1	2	10	7.8:1	3.5:1
3	11	11:1	15.5:1	3	14	4.7:1	5:1
4	10	9:1	10.8:1	4	9	8.1:1	7:1
5	6	2.7:1	9.3:1	5	14	10.8:1	9:1
6	6	4:1	11.8:1	6	7	5.8:1	4.5:1
7	7	3.1:1	6.6:1	7	12	4.2:1	7:1
8	8	3.8:1	8.3:1	8	14	5.3:1	4.5:1
9	7	5.6:1	7.3:1	9	6	4.7:1	6.7:1
10	8	4.5:1	4.4:1				
11	7	4.7:1	4.5:1				
12	11	7.3:1	4:1				
Median Phe:try		4.9:1	8:1			5.8:1	6.7:1
$P = 0.02$				$P =$ ns			

CGMP-AA, casein glycomacropeptide amino acid.
[Correction added on 21 February 2017, after first online publication: All ratio listed in this table were previously interchanged and incorrect and have been corrected in this current version.]

Table 5 Comparison of daily dietary intake for energy [MJ day^{-1} (kcal day^{-1})], natural protein, fat and carbohydrate (g day^{-1}) for both CGMP-AA and control groups at the start and end of the study period

	CGMP-AA group				Control group			
	Energy MJ (kcal)	Natural protein g	Fat g	Carbohydrate g	Energy MJ (kcal)	Natural protein g	Fat g	Carbohydrate g
Baseline (week 0)	7.067 (1689)	10.8	52	300	6.795 (1624)	9	53	292
End (week 26)	7.071 (1690)	9.8*	59	300	6.820 (1630)	10	45	300

*includes a median intake of 1.2 g of natural protein from casein glycomacropeptide amino acid (CGMP-AA).
[Correction added on 21 February 2017, after first online publication: The values in column "Energy MJ (kcal)" under the Control group had the wrong decimal place and have been corrected in this current version.]

significant difference in natural protein intake between the two groups, in the CGMP-AA group, an additional median intake of 60 mg of phenylalanine (equal to 12% of the dietary protein) was derived from the CGMP-AA protein substitute. Energy intake was similar in both groups (Table 5).

Anthropometric measurements

No significant changes were recorded for weight, height and BMI both at the start and end of the study between the control and study group (Table 6).

Acceptability

All of the subjects in the CGMP-AA group described the protein substitute as acceptable; with improved taste, mouth feel, texture and smell compared to their conventional Phe-free L-AA substitute.

Discussion

Regarding PKU, this is the first controlled, longitudinal study reporting the impact of CGMP-AA protein substitute compared to traditional Phe-free L-amino acid supplements on blood phenylalanine, tyrosine and Phe/Tyr concentrations in children over a 6-month period. We have identified a significant increase in blood phenylalanine, Phe/Tyr ratios and a significant decrease in tyrosine concentrations over the study period. Overall, median blood phenylalanine control was maintained within the target range, although only with adjustment to the amount of total protein substitute provided by CGMP-AA and the Phe-free L-amino acid supplements. Energy and natural protein intake were similar in both study groups. However, the overall change in blood phenylalanine concentration that we observed was still less than that reported in a 3 week randomised cross-over study in 30 adults with PKU who took all their protein substitute

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Table 6 Median (range) of weight and height *Z*-scores for CGMP-AA and control groups from start to end of the study period

	CGMP-AA group		Control group	
	Weight *Z*-score	Height *Z*-score	Weight *Z*-score	Height *Z*-score
Baseline (week 0)	0.660 (−0.610 to 1.130)	0.170 (−0.61 to 1.130)	0.752 (−1.082 to 2.09)	0.465 (−1.164 to 1.842)
End (week 26)	0.910 (−1.0 to 2.460)	0.145 (−0.670 to 1.3)	0.869 (−1.164 to 1.842)	0.4290 (−1.184 to 1.902)

CGMP-AA, casein glycomacropeptide amino acid.

as CGMP-AA, although the differences were not statistically significant in the present study [18].

The impact of CGMP-AA on blood phenylalanine control appeared to vary according to the total amount of protein equivalent the CGMP-AA provided. When 40 g or more of protein equivalent (providing an extra 60–90 mg day^{-1} of phenylalanine) replaced the entire Phe-free L-amino acid supplement, all subjects showed an increase in blood phenylalanine concentrations; when 20 g of protein equivalent from CGMP-AA (providing an extra 30 mg day^{-1} of phenylalanine) replaced Phe-free L-amino acids, blood phenylalanine remained unchanged. Although, overall, we did not find a significant correlation between the intake of CGMP-AA and the change in blood phenylalanine concentrations, we decreased the daily amount of CGMP-AA when blood phenylalanine concentrations increased consistently. Zaki *et al.* [19] in a two phase, nonrandomised study, gave 50% of the protein substitute from unmodified GMP for 9 weeks, followed by conventional amino acid for 9 weeks in a group of 10 children with a median age 6 years. There were no significant differences in blood phenylalanine or Phe/Tyr ratio between the phases, and it was concluded that a 50% replacement of L-AA with GMP was viable. Neither the amount of protein equivalent from protein substitute, nor phenylalanine tolerance was reported.

There are several possible explanations for the significant increase in blood phenylalanine concentrations in the CGMP-AA group. First, the impact of the residual amount of phenylalanine in CGMP-AA is likely to vary according to the phenylalanine tolerance of the individual; a median intake of 60 g of protein equivalent provides an extra 90 mg of phenylalanine. If a child is taking 60 g of protein equivalent from CGMP-AA but is only prescribed 4 g day^{-1} of natural protein, they will receive an extra 45% of phenylalanine from CGMP-AA; in contrast, if a child is taking 30 g day^{-1} natural protein, they would only receive an extra 6% of phenylalanine from CGMP-AA. However, it was interesting to observe in the present study that the same increase in blood phenylalanine concentrations occurred regardless of phenylalanine tolerance.

Secondly, blood phenylalanine and tyrosine concentrations may be influenced by the composition and ratio of amino acids provided in this CGMP-AA product. All CGMP-AA protein substitutes are developed to modify the overall amino acid profile to ensure that no indispensable and conditionally indispensable amino acids are deficient. However, the CGMP-AA product studied had lower amounts of the large neutral amino acids (LNAA) isoleucine, leucine, tyrosine, histidine, and tryptophan than conventional Phe-free L-amino acid supplements, which may have influenced our findings. Both large neutral and the cationic amino acids share a common L-type amino acid transporter enzyme (LAT1) allowing entry of amino acids across the blood–brain barrier. LNAA supplementation in a mouse model reduced blood and brain phenylalanine by 33% and 26%, respectively [20]. Furthermore, leucine is important in stimulating muscle protein synthesis; thus, higher amounts in CGMP-AA may improve blood Phe control. Developing a new formulation of CGMP-AA with increased concentrations of some LNAA (isoleucine, leucine, histidine, tyrosine and tryptophan) should theoretically lower the blood phenylalanine.

In the present study, children taking CGMP-AA had significantly lower tyrosine concentrations with a higher Phe/Tyr ratio. Other studies have described lower tyrosine concentrations with CGMP. van Calcar *et al.* [10] reported tyrosine concentrations below the normal range after a short-term study in 11 subjects taking CGMP; similarly, in one case report when CGMP was given for 10 weeks, an additional 1000 mg of tyrosine was supplemented to achieve normal tyrosine concentrations [8]. Tyrosine is a conditionally essential amino acid in PKU and it appears that is it difficult to increase plasma and even brain concentrations of tyrosine in PKU mouse models [20]. It has been suggested that supplemented tyrosine is used to restore the profound brain norepinephrine deficiency [20].

Other factors that may have influenced phenylalanine concentrations include: failure to complete the prescribed dose of protein substitute, additional natural protein intake in excess of daily allowance, severity of PKU, weight loss, illness or non-adherence to diet, particularly in older children, which can influence blood phenylalanine concentrations. Throughout this pilot study, all of the subjects were visited monthly at home to check adherence and diet histories, as well as to assess stocks of protein substitute used and monitor growth. No differences in dietary patterns or growth were observed. Although, in both groups, blood

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phenylalanine control remained within target ranges, in the CGMP-AA, it increased significantly. Although the older subjects were less adherent to dietary management, this applied equally to both groups.

There are limitations to the present study. First, completion of a randomised blind study was not possible. The protein substitutes CGMP-AA and Phe-free L-AA have different tastes, textures, smell and appearances, making a randomised blind study difficult. Second, children were resistant to transitioning between protein substitutes, which would be necessary to conduct a long-term randomised controlled trial. A further limitation was the need to reduce the dosage of CGMP-AA once phenylalanine concentrations increased beyond target ranges. This arguably lessened the significance of our findings, although the ethical priority was to maintain phenylalanine concentrations within target guidelines. Finally, gaining adherence in the older age groups in both the CGMP-AA and control group was challenging.

CGMP is associated with taste advantages and potential valuable nutraceutical properties, although caution is warranted and further research is needed before this product can be fully utilised as a replacement for traditional Phe-free L-AA products in children. It does contain residual phenylalanine and its potential destabilising effect on blood phenylalanine concentrations has to be assessed and monitored carefully in children. It is essential that we fully understand the impact of additional phenylalanine intake and the amino acid profile of this protein substitute in children with PKU who are expected to maintain blood phenylalanine within uncompromising and low target ranges.

Transparency declaration

The lead author affirms that this manuscript is an honest, accurate and transparent account of the study being reported, that no important aspects of the study have been omitted and that any discrepancies from the study as lanned (and registered with) have been explained. The reporting of this work is compliant with STROBE guidelines.

Conflict of interests, source of funding and authorship

Professor Anita MacDonald, Anne Daly, Sharon Evans and Satnam Chalal declare that they have undertaken evaluation work for the nutritional companies: Vitaflo Ltd, Nutricia Ltd and Firstplay Dietary Foods. Si Santra has no conflicts of interest to declare in this project.

Funding was received by Vitaflo International to carry out this piece of original work, and we declare that the work is in agreement with the Transparency Declaration. AD was the main researcher for the paper, collected and assimilated data, and wrote the paper. SE, SC, SS and AMcD helped with the collection of data, the interpretation of the results, and made critical reviews and changes to the text as appropriate. All authors critically reviewed the manuscript and approved the final version submitted for publication.

References

1. Woolf LI, Griffiths R & Moncrieff A (1955) Treatment of phenylketonuria with a diet low in phenylalanine. *Br Med J* **1**, 57–64.
2. Bentovim A, Clayton BE, Francis DE *et al.* (1970) Use of an amino acid mixture in treatment of phenylketonuria. *Arch Dis Child* **45**, 640–650.
3. Smith I, Francis DE, Clayton BE *et al.* (1975) Comparison of an amino acid mixture and protein hydrolysates in treatment of infants with phenylketonuria. *Arch Dis Child* **50**, 864–870.
4. MacDonald A, Rylance G, Davies P *et al.* (2003) Administration of protein substitute and quality of control in phenylketonuria: a randomized study. *J Inherit Metab Dis* **26**, 319–326.
5. MacDonald A, Rylance G, Hall SK *et al.* (1996) Factors affecting the variation in plasma phenylalanine in patients with phenylketonuria on diet. *Arch Dis Child* **74**, 412–417.
6. Hennermann JB, Roloff S, Gellermann J *et al.* (2013) Chronic kidney disease in adolescent and adult patients with phenylketonuria. *J Inherit Metab Dis* **36**, 747–756.
7. Ney DM, Gleason ST, van Calcar SC *et al.* (2009) Nutritional management of PKU with glycomacropeptide from cheese whey. *J Inherit Metab Dis* **32**, 32–39.
8. Ney DM, Hull AK, van Calcar SC *et al.* (2008) Dietary glycomacropeptide supports growth and reduces the concentrations of phenylalanine in plasma and brain in a murine model of phenylketonuria. *J Nutr* **138**, 316–322.
9. Aguiar A, Ahring K, Almeida MF *et al.* (2015) Practices in prescribing protein substitutes for PKU in Europe: no uniformity of approach. *Mol Genet Metab* **115**, 17–22.
10. van Calcar SC, MacLeod EL, Gleason ST *et al.* , *et al.* (2009) Improved nutritional management of phenylketonuria by using a diet containing glycomacropeptide compared with amino acids. *Am J Clin Nutr* **89**, 1068–1077.
11. Lim K, van Calcar SC, Nelson KL *et al.* (2007) Acceptable low-phenylalanine foods and beverages can be made with glycomacropeptide from cheese whey for individuals with PKU. *Mol Genet Metab* **92**, 176–178.

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12. Gropper SS & Acosta PB (1991) Effect of simultaneous ingestion of L-amino acids and whole protein on plasma amino acid and urea nitrogen concentrations in humans. *JPEN J Parenter Enteral Nutr* **15**, 48–53.
13. Metges CC & Barth CA (2000) Metabolic consequences of a high dietary-protein intake in adulthood: assessment of the available evidence. *J Nutr* **130**, 886–889.
14. van Calcar SC & Ney DM (2012) Food products made with glycomacropeptide, a low-phenylalanine whey protein, provide a new alternative to amino Acid-based medical foods for nutrition management of phenylketonuria. *J Acad Nutr Diet* **112**, 1201–1210.
15. Solverson P, Murali SG, Litscher SJ *et al.* (2012) Low bone strength is a manifestation of phenylketonuria in mice and is attenuated by a glycomacropeptide diet. *PLoS ONE* **7**, e45165.
16. Brody EP (2000) Biological activities of bovine glycomacropeptide. *Br J Nutr* **84**(Suppl 1), S39–S46.
17. Laclair CE, Ney DM, MacLeod EL *et al.* (2009) Purification and use of glycomacropeptide for nutritional management of phenylketonuria. *J Food Sci* **74**, E199–E206.
18. Ney DM, Stroup BM, Clayton MK *et al.* (2016) Glycomacropeptide for nutritional management of phenylketonuria: a randomized, controlled, crossover trial. *Am J Clin Nutr* **104**, 334–345.
19. Zaki OK, El-Wakeel L, Ebeid Y *et al.* (2016) The use of glycomacropeptide in dietary management of phenylketonuria. *J Nutr Metab* **2016**, 2453027.
20. van Vliet D, van Dam E, van Rijn M *et al.* (2015) Infants with Tyrosinemia Type 1: should phenylalanine be supplemented? *JIMD Rep* **18**, 117–124.

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CHAPTER 3B

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GLYCOMACROPEPTIDE: LONG TERM USE AND IMPACT ON BLOOD PHENYLALANINE, GROWTH AND NUTRITIONAL STATUS IN CHILDREN WITH PKU

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Glycomacropeptide: long-term use and impact on blood phenylalanine, growth and nutritional status in children with PKU

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Abstract: In phenylketonuria, casein glycomacropeptide (CGMP) requires modification with the addition of some essential and semi essential amino acids to ensure suitability as a protein substitute. The optimal amount and ratio of additional amino acids is undefined.

Aim: A longitudinal, parallel, controlled study over 12 months evaluating a CGMP (CGMP-AA2) formulation compared with phenylalanine-free L-amino acid supplements (L-AA) on blood Phe, Tyr, Phe:Tyr ratio, biochemical nutritional status and growth in children with PKU. The CGMP-AA2 contained 36 mg Phe per 20 g protein equivalent.

Methods: Children with PKU, with a median age of 9.2 y (5-16y) were divided into 2 groups: 29 were given CGMP-AA2, 19 remained on Phe-free L-AA. The CGMP-AA2 formula gradually replaced L-AA, providing blood Phe concentrations were maintained within target range. Median blood Phe, Tyr, Phe:Tyr ratio and anthropometry, were compared within and between the two groups at baseline, 26 and 52 weeks. Nutritional biochemistry was studied at baseline and 26 weeks only.

Results: At the end of 52 weeks only 48% of subjects were able to completely use CGMP-AA2 as their single source of protein substitute. At 52 weeks CGMP-AA2 provided a median of 75% (30–100) of the total protein substitute with the remainder being given as L-AA. Within the CGMP-AA2 group, blood Phe increased significantly between baseline and 52 weeks: [baseline to 26 weeks; baseline Phe 270 μmol/L (170–430); 26 weeks, Phe 300 μmol/L (125–485) $p = 0.06$; baseline to 52 weeks: baseline, Phe 270 μmol/L (170–430), 52 weeks Phe 300 μmol/L (200–490), $p < 0.001$)]. However, there were no differences between the CGMP-AA2 and L-AA group for Phe, Tyr, Phe:Tyr ratio or anthropometry at any of the three measured time points. Within the CGMP-AA2 group only weight ($p = 0.0001$) and BMI z scores ($p = 0.0001$) increased significantly between baseline to 52 weeks. Whole blood and plasma selenium were significantly higher (whole blood selenium [$p = 0.0002$]; plasma selenium [$p = 0.0007$]) at 26 weeks in the CGMP-AA2 group compared L-AA. No differences were observed within the L-AA group for any of the nutritional markers.

Conclusions: CGMP-AA increases blood Phe concentrations and so it can only be used partly to contribute to protein substitute in some children with PKU. CGMP-AA should be carefully introduced in children with PKU and close monitoring of blood Phe control is essential.

Keywords: Glycomacropeptide, Phenylalanine, Large neutral amino acids, Protein substitute, Phenylketonuria

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Introduction

The composition, balance and ratio of amino acids in protein substitutes for phenylketonuria (PKU) requires further consideration and study. Protein substitutes not only provide 50–80% of the total nitrogen supply for growth; they also have a number of physiological properties potentially influencing phenylalanine (Phe) concentrations and anabolism [1, 2]. The delivery of amino acids (AA) into the systemic circulation alters their plasma ratio affecting the uptake of AA at both brain [3–5] and gut membranes [6]. Protein substitutes also contain higher amounts of leucine compared with cow's milk protein (mean amount per 10 g protein equivalent: L-AA, 127 mg ± 23 mg; cow's milk, 100 ± 10 mg). Leucine stimulates muscle protein synthesis and anabolism by activation of a complex pathway involving mTOR (mammalian target of rapamycin), which stimulates insulin secretion [7]. However, there is evidence that L-AA are absorbed more rapidly than intact protein sources that requires digestion leading to less AA retention [8, 9] whereas low Phe glycomacropeptide (CGMP) containing a peptide component may be more efficacious [10]. CGMP is a 'residue' peptide found in the extracted whey component of cheese. Although it is high in some large neutral amino acids (LNAA) such as threonine and isoleucine, it is low in several essential AA and tyrosine, and so requires supplementation to ensure suitability for use in PKU.

Protein substitutes are essential in the management of PKU, but the majority are based on mono amino acids. Some children struggle accepting the taste, smell and prescribed daily volume, while others complain of abdominal discomfort and bad breath. CGMP a peptide-based substitute with added amino acids offers an alternative choice to the traditional mono amino acid substitutes. Identifying suitable alternative protein substitutes with acceptable palatability and improved biological efficacy is important in the treatment of PKU.

Commercial CGMP protein substitutes (supplemented with L-amino acids) (CGMP-AA) contain at least 36 mg of Phe per 20 g protein equivalent (Vitaflo International Ltd.). We have previously reported the impact of CGMP-AA1 on blood Phe concentrations in a group of children aged 5–16 years of age using a formula containing 30 mg of Phe per 20 g protein equivalent [11]. Although, the blood Phe concentrations remained within the target reference range, they increased significantly compared with a control group who remained on L-AA. Therefore, we concluded that CGMP-AA1 could only partially replace the L-AA in most children. We also considered that the blood Phe control might be improved by using a CGMP-AA with a higher concentration of some essential and conditionally essential AA comparable to conventional L-AA supplements. Therefore, we made small adjustments to the AA formulation of the CGMP-AA, so they were similar in profile to conventional L-AA supplements.

In this paper, we explain the changes made to the AA profile of a CGMP-AA formula and the impact this had on blood Phe, Tyr, and Phe:Tyr ratio. As CGMP has not been extensively used as a protein substitute in children, we also investigated changes in the biochemical nutritional status and growth in children using modified CGMP-AA compared to conventional protein substitutes. Therefore, in a 12-month longitudinal, prospective study, we describe the impact of using this modified CGMP-AA formulation (CGMP-AA2) compared with a control group of children taking conventional Phe-free L-AA supplements only.

Methods

Subjects

Fifty children (28 boys, 22 girls) with PKU were recruited. Their median age at recruitment was 9.2 years (range 5-16y). Forty-seven children were European and 3 were of Pakistani origin. Inclusion criteria included: diagnosed by newborn screening, aged 5 to 16 years, not treated with sapropterin dihydrochloride, known adherence with protein substitute, and 70% of blood Phe concentrations within Phe target range for 6 months prior to study entry. Target blood Phe ranges for children aged 5 to 12 years were < 360 μmol/L and for 12 years and older < 600 μmol/L according to the European guideline recommendations [12]. Based on untreated blood Phe levels at newborn screening and dietary Phe tolerance, two children in the CGMP-AA2, and one child in the L-AA group had mild PKU with the majority having classical PKU.

The study was registered by the Health Research Authority and was given a favourable ethical opinion by the South Birmingham Research Ethical Committee. Written informed consent was given for all subjects by at least one caregiver with parental responsibility and written assent was obtained from the subjects if appropriate for their age and level of understanding.

CGMP-AA formulations (Table 1)

CGMP-AA2 is a berry or vanilla flavoured powdered protein substitute containing 20 g of protein equivalent, and 36 mg of Phe per 35 g sachet. Each sachet was mixed with 120 ml of water or phenylalanine-free milk replacement.

CGMP-AA2 was modified following the findings of a pilot study in which CGMP-AA1 was used. Additional Tyr, tryptophan, leucine and histidine (but less methionine and lysine) were added to provide a similar AA profile to conventional Phe-free L-AA supplements. For each 1 g of protein equivalent, it contained 112 mg Tyr, 20 mg tryptophan, 150 mg leucine, and 35 mg histidine. Lysine and methionine were reduced compared with

Table 1 The nutrient composition of CGMP-AA1 and CGMP-AA2 compared with conventional Phe-free L-AA

Nutrients	Units	CGMP-AA1		CGMP-AA2		Phe-free L-AA*	
		Pilot study Week 0–26		Week 0–52		Week 0–52	
		Per 100 g	Per 20 g PE sachet	Per 100 g	Per 20 g PE sachet	Per 100 ml	Per 20 g PE pouch
Calories	Kcal	342	120	342	120	71	124
Protein equivalent	g	57.1	20	57.1	20	11.5	20
Total Carbohydrate	g	18.6	6.5	18.6	6.5	5.4	9.4
Sugars	g	6.3	2.2	6.3	2.2	4.5	7.8
Total Fat	g	4.3	1.5	4.3	1.5	0.4	0.7
DHA	mg	240	84	240	84	77	134
AA	mg	–	–	–	–	–	–
Fibre	g	0	0	0.3	0.1	–	–
Salt	g	1.5	0.53	1.5	0.53	0.25	0.43
Vitamins and minerals							
Vitamin A	μg RE	809	283	809	283	160	278
Vitamin D	μg	12.9	4.5	12.9	4.5	5.8	10
Vitamin E	mg αTE	18.6	6.5	18.6	6.5	3	5.2
Vitamin C	mg	109	38	109	38	21	36
Vitamin K	μg	100	35	100	35	19	34
Thiamine	mg	1.9	0.68	1.9	0.68	0.4	0.7
Riboflavin	mg	2.2	0.78	2.2	0.78	0.44	0.77
Niacin	mg	24	8.4	24	8.4	4.8	8.4
Vitamin B6	mg	2.9	1.0	2.9	1.0	0.5	0.9
Folic Acid	μg	389	136	389	136	77	134
Vitamin B12	μg	4.6	1.6	4.6	1.6	0.9	1.6
Biotin	μg	183	63.9	183	63.9	36	63
Pantothenic acid	mg	7.7	2.7	7.7	2.7	1.5	2.6
Choline	mg	583	204	583	204	115	201
Sodium	mmol	25.8	9.0	25.8	9.0	4.2	7.3
Potassium	mmol	19.3	6.8	19.3	6.8	4.5	7.9
Chloride	mmol	0.6	0.2	0.6	0.2	3.9	6.8
Calcium	mg	1163	407	1163	407	230	400
Phosphorus	mmol	34.2	12	34.2	12	6.6	11.4
Magnesium	mg	366	128	366	128	72	125
Iron	mg	20.9	7.3	20.9	7.3	4.2	7.3
Copper	μg	2137	748	2137	748	420	730
Zinc	mg	20.9	7.3	20.9	7.3	4.2	7.3
Manganese	mg	3.1	1.1	3.1	1.1	0.6	1
Iodine	μg	245	85.7	245	85.7	49	84
Molybdenum	μg	140	49	140	49	28	48
Selenium	μg	85.4	29.9	85.4	29.9	17	29
Chromium	μg	85.4	29.9	85.4	29.9	17	29
L-amino acids							
L-Alanine	g	2.17	0.76	2.23	0.78	0.53	0.92
L-Arginine	g	2.86	1.00	2.71	0.95	0.86	1.5
L-Aspartic Acid	g	5.83	2.04	3.20	1.12	1.36	2.37

Table 1 The nutrient composition of CGMP-AA1 and CGMP-AA2 compared with conventional Phe-free L-AA *(Continued)*

Nutrients	Units	CGMP-AA1		CGMP-AA2		Phe-free L-AA*	
		Pilot study Week 0–26		Week 0–52		Week 0–52	
		Per 100 g	Per 20 g PE sachet	Per 100 g	Per 20 g PE sachet	Per 100 ml	Per 20 g PE pouch
L-Cystine	g	0.03	0.01	0.03	0.01	0.35	0.61
L-Glutamine	g	7.11	2.49	7.34	2.57		
Glycine	g	7.91	2.77	3.43	1.2	1.35	2.35
L-Histidine	g	1.20	0.42	2.00	0.7	0.53	0.92
L-Isoleucine	g	3.91	1.37	3.86	1.35	0.93	1.62
L-Leucine	g	3.71	1.30	8.57	3.00	1.46	2.54
L-Lysine	g	3.06	1.07	2.29	0.80	0.96	1.67
L-Methionine	g	1.54	0.54	0.80	0.28	0.26	0.45
L-Phenylalanine	g	0.09	0.03	0.09	0.03	–	–
L-Proline	g	4.31	1.51	4.34	1.52	0.97	1.69
L-Serine	g	2.80	0.98	2.74	0.96	0.6	1.04
L-Threonine	g	6.20	2.17	6.29	2.2	0.93	1.62
L-Tryptophan	g	0.49	0.17	1.14	0.40	0.29	0.5
L-Tyrosine	g	2.89	1.01	6.40	2.24	1.37	2.38
L-Valine	g	3.23	1.13	3.11	1.09	1.07	1.86

*protein substitute based on 20 g PE of PKU Cooler 20 (Vitaflo International Ltd)
§ L- Phenylalanine, CGMP-AA1 0.086g /100g; 0.030g/20g PE (30mg /sachet) CGMP-AA2 0.100g/100g; 0.036g/20g PE (36mg/sachet)

CGMP-AA1 but still provided minimum amino acid requirements suggested by the WHO/FAO/UNU 2007 [13]. A further difference in the formulation of CGMP-AA2, was the residual Phe content. Due to the manufacturing process of CGMP, there was a 17% increase in residual Phe and CGMP-AA2 contained 36 mg Phe compared to CGMP-AA1 containing 30 mg Phe for every 20 g protein equivalent. No changes were made to the composition of carbohydrate, fat and micronutrients of the CGMP-AA2 throughout the study. Vitaflo International Ltd. produced the CGMP-AA2 study formulation.

Control group

Children in the control group remained on their usual Phe-free L-AA, during the study period, with no changes to the AA formulations throughout the study.

Selection into control or CGMP-AA2 group

Children chose the product they preferred: CGMP-AA2 or Phe-free L-AA. They remained on this formula and in these groups for the duration of the study.

Study design (Fig. 1)

After completion of the pilot study, and following the changes made to the CGMP formulation, a total of 50 children were recruited and followed up for 12 months. This paper reports the prospective results of Phe and Tyr blood concentrations, Phe:Tyr ratio, nutritional biochemistry and growth (weight, height and BMI z scores) over 12 months, using modified CGMP-AA2 compared to Phe-free L-AA protein substitutes. At baseline and 26 weeks, morning fasted pre-prandial venous samples were collected for nutritional markers. Anthropometry, height and weight together with a stock check of protein substitute usage, diet history, and food frequency questionnaire were collected monthly. All baseline measurements were collected when the children were on Phe-free L-AA. Baseline data for Phe and Tyr blood concentrations, and Phe:Tyr ratio were calculated as a median value from the previous 12 months before study commencement. Data for anthropometry and nutritional biochemistry were collected at the start of the study.

Titration of CGMP-AA2 in the diet

Taking into consideration that CGMP-AA2 contained Phe (36 mg for each 20 g protein equivalent), the dose of CGMP-AA2 was individually prescribed and titrated with the blood Phe concentrations. In the CGMP-AA2 group all the children started with a minimum of 20 g of protein equivalent from CGMP-AA2, with Phe-free L-AA providing the remainder of protein substitute intake. If blood concentrations were stable or decreased, CGMP-AA2 was increased by 20 g per day protein equivalent reducing the Phe-free L-AA by the same amount of protein. If there was any suggestion that blood Phe control deteriorated (criteria blood Phe ≥ target range for 3 consecutive weeks), the dose of CGMP–AA2 was reduced, with a concomitant increase in Phe-free L-AA providing the remaining total protein

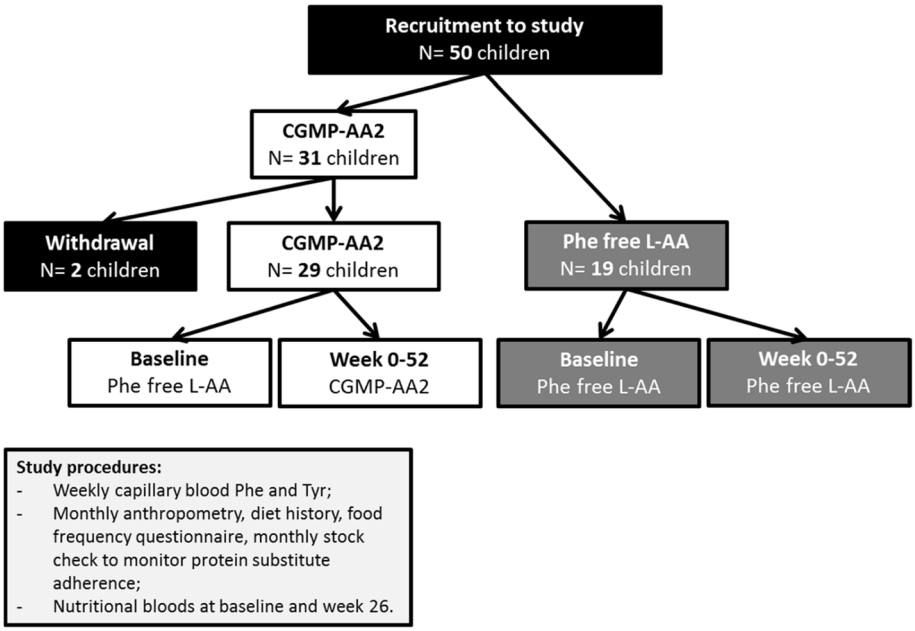


Fig. 1 Diagrammatic scheme showing recruitment and introduction of CGMP-AA2 and Phe-free L-AA

equivalent requirement. No change or reduction was made to the dietary Phe intake of subjects on the CGMP-AA2.

Nutritional markers

At baseline and 26 weeks, a fasting morning venous blood sample was collected and analysed for zinc, selenium (plasma and whole blood), calcium, magnesium, phosphate, C-reactive protein (CRP) haemoglobin, MCV (mean cell volume), ferritin, vitamin B12 and 25-hydroxy vitamin D. The laboratory received all samples within 30 min of collection preventing any deterioration in sample quality. All the samples were stored under appropriate conditions and then measured at the same time point, minimising laboratory variations in measurement techniques.

Zinc and selenium (whole blood and plasma) were collected in a lithium heparin 'trace metal' tube and analysed using inductively coupled plasma mass spectrometry. Vitamin D was measured as a clotted sample via mass spectrometry. Calcium, magnesium, phosphate, and CRP were all measured in zinc free lithium heparin tubes and measured on a Roche photometric analyser. Ferritin and vitamin B12 were collected in Vacuette Z serum clotted activator tubes and analysed by an Access analyser using electroluminescence. Haemoglobin and MCV were collected into EDTA tubes and measured on a Sysmex XN 2000 analyser.

Blood Phe/Tyr monitoring

Throughout the study, trained caregivers collected weekly early morning fasted blood spots at home. Blood specimens were sent via first class post to the laboratory at Birmingham Children's Hospital. Blood samples were collected on filter cards, Perkin Elmer 226 (UK Standard NBS). All the cards had a standard thickness and the blood Phe and Tyr concentrations were calculated on a 3.2 mm punch by MS/MS tandem mass spectrometry.

Anthropometry, dietary recall and monitoring

Monthly weight and height measurements were collected using portable Seca scales and stadiometer. A mean of three readings were taken and measured to one decimal point. Home visits were completed monthly, collecting a dietary history and food frequency questionnaire, together with adherence monitoring of protein substitute usage by performing a stock check of the amount of protein substitute used. Three trained dietitians measured and assessed the children each month.

Statistics

Comparisons of Phe data across time were carried out using a linear mixed models approach, which account for both within and between subject levels of variance. Models are constructed which include terms for 'time' (Baseline, 26 weeks and 52 weeks), treatment effect (CGMP-AA2 and L-AA) and their interaction. The subject identifier is included as a random effect. Phe is included on the square root scale to ensure normality of model residuals. Results are presented in terms of the mean effect at each time point with associated 95% confidence intervals. Differences between time points and

groups are obtained using analysis of deviance tables. All analyses are carried out using programme R (Version 3).

Statistical analysis for nutritional biochemistry and anthropometrics was performed within the groups using non-parametric Wilcoxon matched pairs signed rank test. When comparisons were made between the groups non-parametric Mann Whitney test was performed. Nutritional parameters were measured at baseline and 26 weeks only, anthropometry at baseline, 26 and 52 weeks.

Results

Subjects

Of the 50 recruited children, 31 were in the CGMP-AA2 group and 19 in the L-AA control group. Prior to the study start: 6 subjects took powdered protein substitutes [XP Maxamum (Nutricia Ltd.), $n = 1$; PKU Anamix first spoon (Nutricia Ltd.), $n = 3$; PKU gel (Vitaflo International Ltd.), $n = 2$]; and 44 subjects took liquid pouches [PKU Lophlex LQ (Nutricia Ltd.), n = 3; PKU Cooler (Vitaflo International Ltd.), $n = 41$]. In the L-AA group, they either received liquid pouches [PKU Lophlex LQ (Nutricia Ltd.), n = 2; PKU Cooler (Vitaflo International Ltd.), $n = 14$] or powdered preparations [PKU gel (Vitaflo International Ltd.), n = 3]. At the start of the study, the median age was 8.4 years (5–16) in the CGMP-AA2 group, and 11.1 years (5–15) in the L-AA group. Median Phe concentrations at baseline for CGMP-AA2 were 270 μmol/L (170–430) and for L-AA, 315 μmol/L (140–600).

The total median daily dose of protein equivalent before and throughout the study in both groups for protein substitute was 60 g/day (range, 40-60 g). The median number of prescribed protein exchanges was 5 g protein/day (range 3-30 g) or 250 mg Phe (range, 150-1500 mg).

Subject withdrawal

One boy and one girl (aged 12 years) in the CGMP-AA2 group were withdrawn from the study, both failed to comply with the study protocol, one failing to return blood Phe samples and both had poor adherence with a low Phe diet. A total of 48 children completed the study; 29 in the CGMP-AA2 and 19 in the Phe-free L-AA group.

Comparison of blood Phe, Tyr, Phe:Tyr ratio between CGMP-AA2 and Phe-free L-AA groups and within groups at: baseline, week 26 and 52 (Tables 2, 3, 4**)** [Additional file 1. This file shows four additional diagrams, Figures S1A, B, C and S2. Supplementary figures show a mixed linear model for phe, tyr and phe: tyr ratio at baseline, 26 and 52 weeks for all subjects, and Fig. 2 shows phe for subjects< 12 y].

For each group the median Phe and Tyr values were calculated for each subject over a time period from baseline to week 26, and from week 27 to 52. The median of the collective medians has been reported.

Blood Phe levels

Phe levels are consistently higher in the L-AA group compared with the CGMP-AA2 group. Both groups observed a rise in Phe levels over time, but this rise was more pronounced in the CGMP-AA2 group with a statistical difference between baseline and 52 weeks ($p < 0.001$).

Blood Tyr levels

Whilst Tyr levels were consistently higher in the CGMP-AA2 groups, there were no significant differences between the CGMP-AA2 and L-AA groups and no differences in the change in Tyr over time.

Phe: Tyr ratio

For the CGMP-AA2 group there was a small but consistent rise in the Phe:Tyr ratio with the difference between baseline and 26 weeks ($p = 0.010$) and baseline and 52 weeks ($p < 0.001$) both statistically significant. Changes in the Phe:Tyr ratio in the L-AA group were not statistically significant.

Sub- group analysis for children < 12 years of age (Table 5) [Additional file 1]

We compared blood Phe concentrations over the same time period in children aged < 12 years in both groups. There were 25 children in the CGMP-AA2 group (median age 8.9y), and 9 children in the Phe-free L-AA group (median age 9.2y). There were no significant differences between the groups for median blood Phe at baseline, 26 or 52 weeks. However, a significant difference

Table 2 Changes in the median (range) blood Phe concentrations (μmol/L) within and between the CGMP-AA2 and Phe-free L-AA group at baseline, 26 and 52 weeks

Group	Number of subjects	Baseline μmol/L (range)	Wk 26 μmol/L (range)	Wk 52 μmol/L (range)	*P* value
CGMP-AA2	29	270 $^{\S *}$ (170–430)	300§ (125–485)	300* (200–490)	$^{\S}p = 0.06$ $^{*}p = < 0.0011$
Phe-free L-AA	19	315 $^{\S\S **}$ (140–600)	325§§ (180–580)	340** (190–600)	$^{\S\S}P = 0.687$ $^{**}p = 0.236$

Wilcoxon test § GMP baseline to 26 wk., * GMP baseline to 52 wk
Wilcoxon test §§ L-AA baseline to 26 wk., ** L-AA baseline to 52 wk
ABREVIATIONS: *CGMP-AA* low phenylalanine glycomacropeptide protein substitute supplemented with L-amino acids, *Phe-free L-AA* Phenylalanine free L-amino acid supplement

Table 3 Changes in the median (range) blood Tyr concentrations (μmol/L) within and between the CGMP-AA2 and Phe-free L-AA group at baseline, 26 and 52 weeks

Group	Number of subjects	Baseline μmol/L (range)	Wk 26 μmol/L (range)	Wk 52 μmol/L (range)	P value
CGMP-AA2	29	$50^{\S *}$ (30–180)	$50^{\S}$ (30–210)	50^{*} (30–170)	$^{\S}p = 0.916$ $^{*}p = 0.239$
Phe-free L-AA	19	$40^{\S\S **}$ (40–100)	$40^{\S\S}$ (30–105)	40^{**} (30–80)	$^{\S\S}p = 0.672$ $^{**}p = 0.111$

Wilcoxon test § GMP baseline to 26 wk., * GMP baseline to 52 wk
Wilcoxon test §§ L-AA baseline to 26 wk., ** L-AA baseline to 52 wk
ABREVIATIONS: *CGMP-AA* low phenylalanine glycomacropeptide protein substitute supplemented with L-amino acids, *Phe-free L-AA* Phenylalanine free L-amino acid supplement

was observed for median blood Phe in the CGMP-AA2 group between baseline and week 26 ($p = 0.022$) and baseline and week 52 (p = 0.010). No differences were observed at any time point in the Phe-free L-AA group.

Anthropometry (Fig. 3)

Comparison of weight, height and BMI z scores between the CGMP-AA 2 and Phe-free L-AA groups and within groups at: Baseline, week 26 and 52

There was no statistical significance for median weight, height or BMI between the CGMP-AA2 and L-AA group at each of the measured time points. However, in the CGMP-AA2 group a significant increase was observed for median (range), weight z score from baseline (0.55, − 1.93-2.34) to week 52 (0.77, − 1.75-2.60), (p = < 0.0001) and BMI z score from baseline (0.58, range − 0.88-2.83) to week 52 (0.92 range, − 0.67-2.98), (p = < 0.0001). A significant increase was also observed from week 26 (0.53 range, − 1.8-2.47) to 52 (0.77, range − 1.75-2.60) for weight z score (p = < 0.0001), and BMI z score between week 26 (0.61, range − 1.9-2.81) and week 52 (0.92 range, − 0.67-2.98) (p = < 0.0001).

In the Phe-free L-AA group there was no significant differences for median weight or BMI z scores: baseline to week 52 (weight z score: [$p = 0.11$]; BMI z score, [$p = 0.14$]) or from 26 to 52 weeks: (weight z score, [$p = 0.80$]; BMI z score, [$p = 0.32$]). However, at baseline the control group had a median weight, height and BMI that were nearly double that of the CGMP-AA2 group.

Nutritional biochemistry (Table 6)

Comparison of nutritional biochemistry between the CGMP-AA2 and Phe-free L-AA groups and within groups at baseline and 26 weeks

The median values for all nutritional parameters measured at baseline and 26 weeks were all within the reference ranges. One exception, in both groups was vitamin B12, which was higher than the reference range at 26 weeks. No differences were found between any of the parameters at baseline when all the children were taking Phe-free L-AA.

Whole blood and plasma selenium were significantly higher (whole blood selenium [$p = 0.0002$]; plasma selenium [$p = 0.0007$]) at 26 weeks in the CGMP-AA2 group compared L-AA.

Within the CGMP-AA2 group between baseline and week 26, whole blood and plasma selenium significantly increased (whole blood selenium [p = < 0.0001]; plasma selenium [$p = 0.0005$]) and ferritin decreased ($p = 0.0006$). Median values all remained within the reference ranges.

Discussion

This is the first longitudinal, comparative prospective study over 12 months reporting the use of CGMP-AA2 compared to conventional protein substitute L-AA in children with PKU. After 12 months of using the modified CGMP-AA2 there were no differences for Phe, Tyr, Phe:Tyr ratio and anthropometry compared to the control group using L-AA. However, in the same period within the CGMP-AA2 group, blood Phe concentrations significantly increased although this was only by 30 μmol/L. When comparing children < 12 years of age,

Table 4 Changes in the median (range) blood Phe:Tyr ratio within and between the CGMP-AA2 and Phe-free L-AA group at baseline, 26 and 52 weeks

Group	Number of subjects	Baseline (range)	Wk 26 (range)	Wk 52 (range)	P value
CGMP-AA2	29	$5.7^{\S *}$ (1.5–11)	$6.4^{\S}$ (1.3–11.7)	6.4^{*} (1.7–11.5)	$^{\S}p = 0.010$ $^{*}p < 0.010$
Phe-free L-AA	19	$7.2^{\S\S **}$ (3.1–15)	$6.8^{\S\S}$ (3–11.7)	7.4^{**} (4–15.1)	$^{\S\S}p = 0.555$ $^{**}p = 0.246$

Wilcoxon test § GMP baseline to 26 wk., * GMP baseline to 52 wk
Wilcoxon test §§ L-AA baseline to 26 wk., ** L-AA baseline to 52 wk
ABREVIATIONS: *CGMP-AA* low phenylalanine glycomacropeptide protein substitute supplemented with L-amino acids, *Phe-free L-AA* Phenylalanine free L-amino acid supplement

Table 5 Changes in the median (range) blood Phe concentrations (μmol/L) within and between the CGMP-AA2 and Phe-free L-AA group at baseline, 26 and 52 weeks for children < 12 years

Group	Number of subjects	Baseline μmol/L (range)	Wk 26 μmol/L (range)	Wk 52 μmol/L (range)	P value
CGMP-AA2	25	270 §* (170–360)	300§ (125–415)	290* (200–450)	$^{§}p = 0.04$ $^{*}p = 0.04$
Phe-free L-AA	9	288 §§** (140–470)	287§§ (180–348)	291* (190–344)	$^{§}P = 0.90$ $^{**}p = 0.41$

Wilcoxon test § GMP baseline to 26 wk., * GMP baseline to 52 wk
Wilcoxon test §§ L-AA baseline to 26 wk., ** L-AA baseline to 52 wk
ABABBREVIATIONS: *CGMP-AA* low phenylalanine glycomacropeptide protein substitute supplemented with L-amino acids, *Phe-free L-AA* Phenylalanine free L-amino acid supplement

within the CGMP-AA2 group, the same small but consistent significant increase for blood Phe concentrations were evident. Weight and BMI z scores significantly increased within the CGMP-AA2 group. Plasma and whole blood selenium improved although ferritin decreased but all nutritional measurements remained within the reference ranges. Identifying the reason for these physical and biochemical changes over 12 months in the CGMP-AA2 group is important to assess the suitability of using CGMP-AA2 as a protein substitute in children with PKU.

We have previously reported a small but significant increase in blood Phe concentrations in the first group of children recruited to the pilot study using CGMP-AA1 [11]. CGMP-AA1 was based on an AA profile that met the minimum safe levels of amino acid intake (WHO/FAO/UNU 2007) [13] for Tyr, tryptophan, leucine, and histidine. CGMP-AA1 contained 30 mg of Phe for every 20 g protein equivalent. A modified CGMP-AA formula (CGMP-AA2) was produced by making small adjustments to the AA composition of CGMP-AA1, increasing some of the LNAA (Tyr, tryptophan, leucine and histidine) in amounts similar to conventional L-AA supplements. Although in this study, we examined a different cohort of children, after using CGMP-AA2 for 12 months, blood Phe concentrations, were lower than those in the pilot study using CGMP-AA1. Median Phe concentrations at the end of the pilot study 317 μmol/L compared to CGMP-AA2 after 12 months Phe 300 μmol/L. We were also able to increase the amount of protein equivalent supplied from CGMP-AA2 to 75% of total protein equivalent, without any reduction in dietary Phe intake. However, at the end of this study period, only 14 of 29 children (48%) were able to fully transition to CGMP-AA2 as their only protein substitute, which suggests that the residual Phe concentration present in CGMP-AA2 still increases blood Phe concentrations in children.

Identifying the optimal amino acid profile for protein substitutes is challenging, although, the ratio and amount of LNAA appears important. It is suggested that LNAA supplementation competes with Phe uptake at the gut and blood brain barrier (BBB): LNAA cross the intestinal mucosa using a carrier protein similar to that at the BBB [14–16]. In vitro studies investigating intestinal epithelial transport of amino acids, indicate that lysine, histidine, leucine and Tyr significantly reduce Phe uptake [6]. High concentrations of LNAA compete with the transport of Phe at

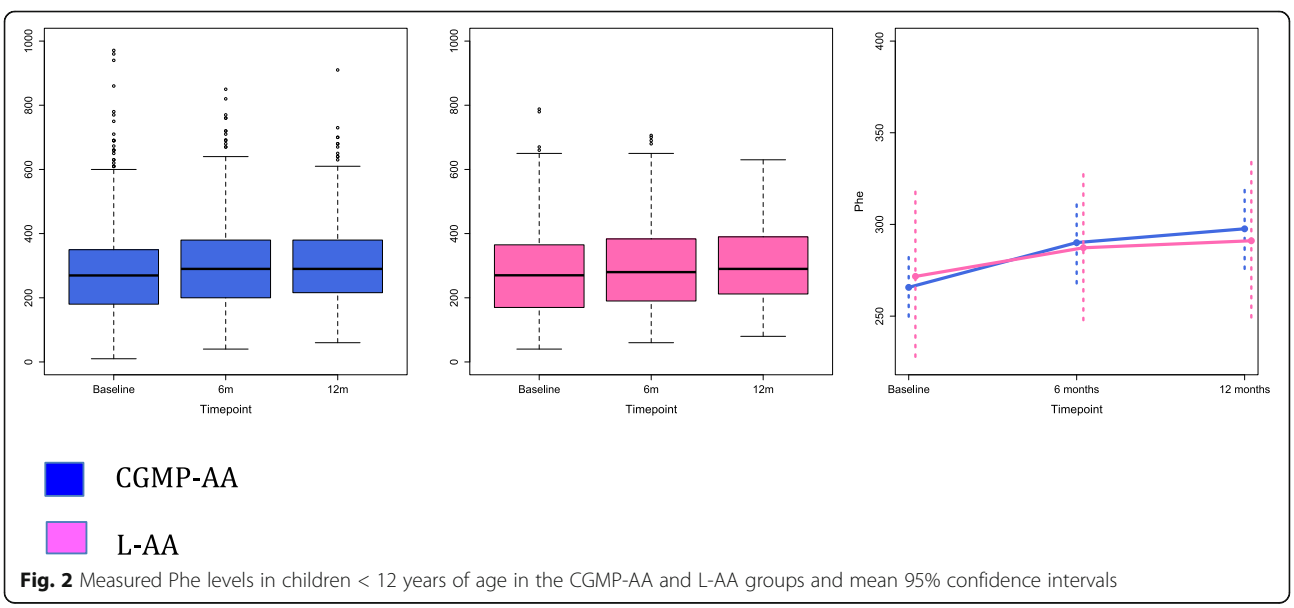


Fig. 2 Measured Phe levels in children < 12 years of age in the CGMP-AA and L-AA groups and mean 95% confidence intervals

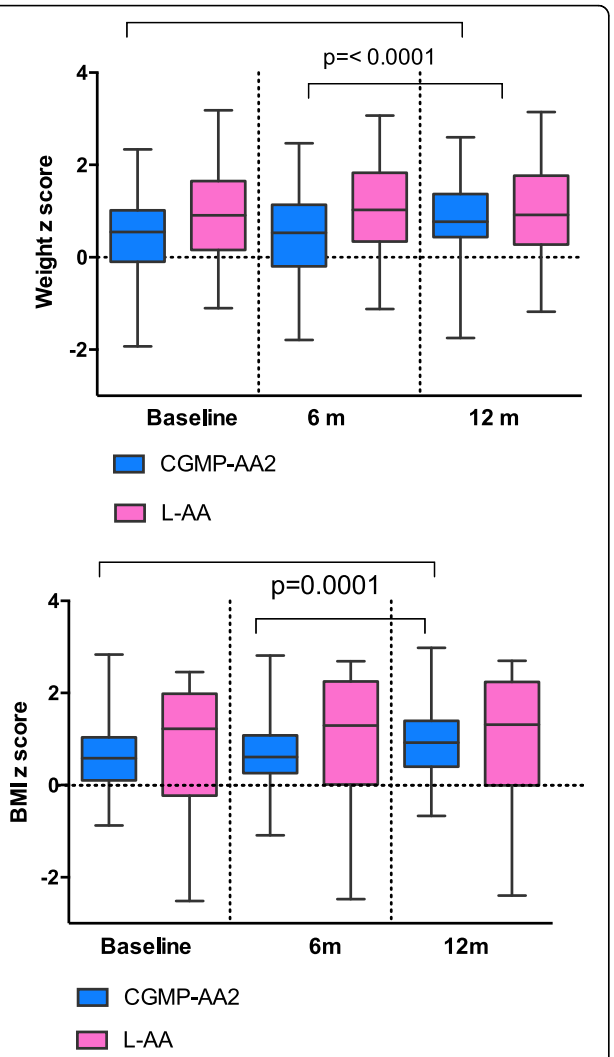


Fig. 3 Weight and BMI z scores for CGMP-AA2 and L-AA at baseline, 26 and 52 weeks

the gut cellular membrane and may decrease blood Phe concentrations. Additionally LNAA supplementation has been shown to reduce blood and brain Phe concentrations [17, 18] and restore some of the disturbed Phe transport across the BBB by altering monoaminergic neurotransmitter concentrations. In mice studies, mice chow with added LNAA, improved brain tryptophan, serotonin and norepinephrine concentrations [4]. There are other functional effects of protein substitutes, which indirectly affect blood Phe concentrations. These include the rate of delivery of L-AA into the systemic circulation and muscle protein anabolism. Amino acids play an important, but as yet not fully understood role in nutritional signalling and regulation of multiple cellular processes [19]. Leucine, is a potential insulin secretagogue when administered with carbohydrate and protein, acting as a pharmaconutrient AA improving muscle protein synthesis, by stimulating mRNA changes via insulin independent and dependent pathways [7, 20]. Van Loon et al. maximised endogenous insulin secretion by the combined ingestion of carbohydrate and wheat protein hydrolysate, with added leucine and Phe [21]. In vitro studies, using incubated β cells of the pancreas, have shown that arginine, leucine and phenylalanine have a strong insulinotropic effect [22]. Further studies are needed to maximize our understanding of the physiological absorption process of AA protein substitutes, with the goal, to achieve a normal absorption pattern comparable to natural intact protein.

In contrast, many L-AA are bitter tasting and unpleasant to take, particularly leucine, tryptophan and histidine [23, 24]. By adding more of these AA to CGMP-AA formulations, it potentially decreases their acceptability and palatability. It has been suggested by Van Calcar [25] that the AA profile in CGMP-AA should provide 130 to 150% of the 2002 Institute of Medicine American dietary reference intake for histidine, leucine, methionine, tryptophan and Tyr to compensate for faster absorption and degradation of AA [26].

In our study, 52% of children in the CGMP-AA2 group were prescribed a combination of CGMP-AA2 and separate Phe-free L-AA supplements, as they could not fully transition onto CGMP-AA2 in order to maintain blood Phe concentrations within the target range of 120 to 360 μmol/L. The median amount of protein substitute provided by CGMP-AA2 that could be tolerated without affecting blood Phe control was 75% of the total amount. This contrasts with other researchers findings that have reported that the Phe content of CGMP-AA has little effect on blood Phe concentration. In an uncontrolled, short term study reported in 10 children (aged 4 to 16 years) with PKU, when 50% of their total protein requirements were supplied by 'GMP cheese' for 9 weeks, blood Phe concentrations decreased by a median of 114 μmol/L although this was not statistically significant [27]. In a short-term randomized cross over trial in 30 patients aged 15 years or over, comparing CGMP-AA with L-amino acid supplements only, CGMP-AA was associated with a non-significant increase in Phe of 62 ± 40 μmol/L, although 10 of the 30 patients were prescribed sapropterin (likely to have improved Phe tolerance), 6 patients appeared less adherent with CGMP-AA and overall subjects were only studied over a short time and had a higher baseline blood Phe levels in comparison to our study group [28]. Furthermore, increases of between 60 to 102 μmol/L may be unacceptable in children particularly as evidence is accumulating to suggest that optimal blood Phe may be below 240 μmol/L [29].

Median nutritional blood parameters in both groups at baseline and 26 weeks were all within reference ranges,

Table 6 Median nutritional biochemistry comparing CGMP-AA2 with Phe-free L-AA at baseline and week 26

Parameters	Reference range	Base-line (range)		Significance Base-line	26wk (range)		Significance at 26wk
		Phe-free L-AA	CGMP-AA2		Phe-free L-AA	CGMP-AA2	
Zinc μmol/L	11–24	14.3 (12.9–17.4)	14.4 (11.3–18.2)	p = 0.3	14.55 (12.3–17.5)	14.0 (10.5–16.4)	$p = 0.24$
C reactive protein mg/L	< 10	1.0 (1–4)	1.0 (1–11)	$p = 0.27$	1.0 (1–3)	1.0 (1–6)	$p = 0.69$
Whole blood Selenium	ns	0.94 (0.76–1.21)	0.99[§] (0.48–1.38)	$p = 0.57$	0.98[*] (0.75–1.23)	1.22[*§] (0.86–1.56)	**[§]p = < 0.0001 [*]p = 0.0002**
Selenium μmol/L	**4-7y** 0.7–1.7 **7-18y** 0.62–1.3	0.86 (0.53–1.1)	0.91[§] (0.38–1.4)	p = 0.57	0.85[*] (0.58–1.14)	1.12[*§] (0.58–1.35)	**[§]p = 0.0005 [*]p = 0.0007**
Calcium mmol/L	2.2–2.7	2.46 (2.33–2.56)	2.49 (2.32–2.63)	$p = 0.56$	2.44 (2.31–2.59)	2.45 (2.25–2.68)	$p = 0.77$
Magnesium mmol/L	0.7–1	0.84 (0.75–0.92)	0.85 (0.72–1.0)	p = 0.68	0.82 (0.72–0.92)	0.84 (0.76–0.92)	p = 0.14
Phosphate mmol/L	0.9–1.8	1.30 (0.94–1.53)	1.38 (1.0–1.59)	$p = 0.08$	1.33 (1.0–1.58)	1.39 (1.0–1.71)	$p = 0.20$
25 hydroxy vitamin D nmol/L	> 50	93.9 (38.8–182.3)	95.3 (60–162)	p = 0.27	84.2 (47.6–139)	87.6 (47.9–158)	$p = 0.49$
Haemoglobin g/L	120–140	137 (119–164)	136 (101–156)	p = 0.55	141 (120–164)	135 (109–155)	p = 0.23
MCV fl	78–93	82 (76–90)	82 (75–91)	$p = 0.48$	84 (77–93)	82.5 (76–88)	$p = 0.50$
Ferritin μg/L	11–60	32.8 (20.9–63.1)	27.4[§§] (17.9–116.5)	$p = 0.16$	30.80 (17.6–75.6)	25.2[§§] (15.9–91.6)	**[§§]p = 0.0006**
Vitamin B12ng/L	245–798	798 (390–1500)	1088 (333–1500)	p = 0.1	745 (308–1500)	922 (437–1500)	$p = 0.23$

ns not specified
Wilcoxon test (paired and unpaired t test)
[§]Whole blood and plasma selenium CGMP-AA2 baseline compared with CGMP-AA2 at 26 weeks
*Whole blood and plasma selenium CGMP-AA2 26 weeks compared with L-AA at 26 weeks
[§§] Ferritin CGMP-AA2 baseline compared with CGMP-AA2 at 26 weeks

with the exception on vitamin B12 being higher than the reference range at 26 weeks in both groups. There were no biochemical signs of vitamin or mineral deficiencies in line with what was retrospectively reported in adult PKU patients [30]. There was a significant increase in whole blood and plasma selenium between the groups at 26 weeks, and within the CGMP-AA2 group from baseline to 26 weeks. The selenium content of both products was similar, a median intake of 60 g protein equivalent from Phe-free L-AA provided 87 mg of selenium compared with CGMP-AA2 providing 90 mg. It is only conjecture, but absorption of selenium in the CGMP-AA2 group may be enhanced based on its bioactive properties. It is also possible that CGMP may modulate microbiota resulting in a different absorption or bioavailability. Furthermore, whey protein is rich in the sulfhydryl amino acid cysteine, which is a precursor of glutathione, and may in part explain the higher selenium concentrations in the CGMP-AA2 group. Muniz-Naveiro [31] reported the largest percentage of selenium in cow's milk was found in the whey phase, although unmodified CGMP is not high in cysteine or selenium. Peptides containing isoleucine, proline, lysine, glutamine, aspartic and glutamic acid have been shown to have antioxidant properties; hence peptide structure and amino acid sequencing influence biological function. The bioactive antioxidant properties of CGMP and absorption in the gut may have a selenium sparing effect compared to protein substitutes without a peptide base [32, 33].

Weight and BMI increased significantly in the CGMP-AA2 group with an increase in weight and BMI first becoming evident from 26 weeks, this may be related to some children using a protein free milk replacement to make up their powdered CGMP-AA2 protein substitute. A mean intake of 400 ml/day of protein-free milk replacement would increase energy intake by 270 kcal/day. Only 9 children were routinely adding additional milk substitute to their CGMP-AA2 but there were no significant differences evident between children using protein free milk or water to prepare the CGMP-AA 2, but this may not be apparent due to the small numbers in the group. We changed this practice when it was observed that children were gaining extra weight. Another consideration influencing growth is age; the Phe-free L-AA group were an older patient cohort and therefore, over the 12-month period it is difficult to quantify how many were actively reaching puberty and the effect this has on weight, height and BMI.

A further consideration is that CGMP based on a protein source may be more efficiently utilized increasing muscle mass compared to L-AA. Longer-term observations of both study groups will hopefully answer this question with data describing both fat free and fat mass index.

There are several limitations to this study that need to be considered. We were unable to conduct a randomised controlled blind trial. CGMP-AA2 and conventional L-AA supplements are very different in taste, texture and appearance rendering any blind or randomised trial very challenging in children who may not easily accept changes to their protein substitutes [34]. In this study, 40% of children preferred to stay on their usual Phe-free L-amino acid supplement (control group) and so they were a self-selected, not age-matched group and children in the control group were older than the study group. Also in teenage children in both study groups, ensuring dietary adherence was difficult although patients were monitored closely with monthly home visits to check stock levels of protein substitute and dietary intake. A further limitation was the use of low protein milk substitute to make up the protein substitute, in addition to increasing energy intake it possibly altered CGMP-AA2 absorption, affecting amino acid kinetics.

Conclusions

It is clear that the biological functions of CGMP may potentially offer many health benefits in PKU [35–40] and it is likely to play a significant future role in provision of low Phe protein substitute. However, it is important that the manufacturers of CGMP seek to reduce its Phe content and the formulation of CGMP-AA2 requires further research and development in order to ensure optimal amino acid profiling.

Additional file

Additional file 1: Supplementary data using mixed linear models to express measured Phe, Tyr and Phe: Tyr ratio in the CGMP-AA2 and L-AA groups. **Figure S1A, B and C** showing mixed linear models for measured Phe, Tyr, Phe:Tyr ratio for CGMP-AA2 and L-AA groups and mean 95% confidence intervals. **Figure S2.** showing Mixed linear model for measured Phe levels in children < 12 years of age in the CGMP-AA2 and L-AA groups and mean 95% confidence intervals. (DOCX 823 kb)

Abbreviations

CGMP-AA: Casein glycomacropeptide – amino acids;
GMP: Glycomacropeptide; L-AA: L-amino acids; PKU: Phenylketonuria

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Availability of data and materials

All data and material is available for review and additional data as Additional file 1.

Authors' contributions

All authors have actively participated in contributing to this publication. All authors read and approved the final manuscript.

Authors' information

Authors information email and full names given on first page.

Ethics approval and consent to participate

We have gained Ethical approval and participant consent. The study was registered by the Health Research Authority and was given a favourable ethical opinion by the South Birmingham Research Ethical Committee. Ref nos 13/WM/0435.

Consent for publication

All subjects and authors have given informed consent for publication.

Competing interests

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References

1. MacDonald A, et al. Administration of protein substitute and quality of control in phenylketonuria: a randomized study. J Inherit Metab Dis. 2003;26(4):319–26.
2. Weigel C, et al. Effects of various dietary amino acid preparations for phenylketonuric patients on the metabolic profiles along with postprandial insulin and ghrelin responses. Ann Nutr Metab. 2007;51(4):352–8.
3. van Spronsen FJ, et al. Large neutral amino acids in the treatment of PKU: from theory to practice. J Inherit Metab Dis. 2010;33(6):671–6.
4. van Vliet D, et al. Large neutral amino acid supplementation exerts its effect through three synergistic mechanisms: proof of principle in phenylketonuria mice. PLoS One. 2015;10(12):e0143833.
5. Pardridge WM. Blood-brain barrier carrier-mediated transport and brain metabolism of amino acids. Neurochem Res. 1998;23(5):635–44.
6. Hidalgo IJ, Borchardt RT. Transport of a large neutral amino acid (phenylalanine) in a human intestinal epithelial cell line: Caco-2. Biochim Biophys Acta. 1990;1028(1):25–30.
7. Leenders M, van Loon LJ. Leucine as a pharmaconutrient to prevent and treat sarcopenia and type 2 diabetes. Nutr Rev. 2011;69(11):675–89.
8. Gropper SS, Acosta PB. Effect of simultaneous ingestion of L-amino acids and whole protein on plasma amino acid and urea nitrogen concentrations in humans. JPEN J Parenter Enteral Nutr. 1991;15(1):48–53.
9. Metges CC, et al. Kinetics of L-[1-(13)C] leucine when ingested with free amino acids, unlabeled or intrinsically labeled casein. Am J Physiol Endocrinol Metab. 2000;278(6):E1000–9.

10. van Calcar SC, et al. Improved nutritional management of phenylketonuria by using a diet containing glycomacropeptide compared with amino acids. Am J Clin Nutr. 2009;89(4):1068–77.
11. Daly A, et al. Glycomacropeptide in children with phenylketonuria: does its phenylalanine content affect blood phenylalanine control? J Hum Nutr Diet. 2017;30(4):515–23.
12. van Spronsen FJ, et al. Key European guidelines for the diagnosis and management of patients with phenylketonuria. In: Lancet diabetes Endocrinol; 2017.
13. Protein and amino acid requirements in human nutrition. World Health Organ Tech Rep Ser, vol. 935; 2007. p. 1–265. back cover
14. Matalon R, et al. Large neutral amino acids in the treatment of phenylketonuria (PKU). J Inherit Metab Dis. 2006;29(6):732–8.
15. van Vliet D, et al. Infants with Tyrosinemia type 1: should phenylalanine be supplemented? JIMD Rep. 2015;18:117–24.
16. Matalon R, et al. Future role of large neutral amino acids in transport of phenylalanine into the brain. Pediatrics. 2003;112(6 Pt 2):1570–4.
17. Pietz J, et al. Large neutral amino acids block phenylalanine transport into brain tissue in patients with phenylketonuria. J Clin Invest. 1999;103(8):1169–78.
18. Concolino D, et al. Long-term treatment of phenylketonuria with a new medical food containing large neutral amino acids. Eur J Clin Nutr. 2016;71(1):51–5.
19. Kimball SR, Jefferson LS. Regulation of protein synthesis by branched-chain amino acids. Curr Opin Clin Nutr Metab Care. 2001;4(1):39–43.
20. Sener A, Malaisse WJ. The stimulus-secretion coupling of amino acid-induced insulin release: insulinotropic action of branched-chain amino acids at physiological concentrations of glucose and glutamine. Eur J Clin Investig. 1981;11(6):455–60.
21. van Loon LJ, et al. Amino acid ingestion strongly enhances insulin secretion in patients with long-term type 2 diabetes. Diabetes Care. 2003;26(3):625–30.
22. Xu G, et al. Metabolic regulation by leucine of translation initiation through the mTOR-signaling pathway by pancreatic beta-cells. Diabetes. 2001;50(2):353–60.
23. Kawai M, Hayakawa Y. Complex taste--taste of D-amino acids. Chem Senses. 2005;30(Suppl 1):i240–1.
24. Solms J. The taste of amino acids, peptides and proteins. Int Z Vitaminforsch. 1969;39(3):320–2.
25. van Calcar SC, Ney DM. Food products made with glycomacropeptide, a low-phenylalanine whey protein, provide a new alternative to amino acid-based medical foods for nutrition management of phenylketonuria. J Acad Nutr Diet. 2012;112(8):1201–10.
26. Institute of Medicine. Food and Nutrition Board Dietary Reference Intakes for Energy, Carbohydrates, Fiber, Fat, Protein and amino acids. Washington DC: National Academy Press; 2002.
27. Zaki OK, et al. The use of Glycomacropeptide in dietary Management of Phenylketonuria. J Nutr Metab. 2016;2016:2453027.
28. Ney DM, et al. Glycomacropeptide for nutritional management of phenylketonuria: a randomized, controlled, crossover trial. Am J Clin Nutr. 2016;104(2):334–45.
29. Jahja R, et al. Neurocognitive evidence for revision of treatment targets and guidelines for phenylketonuria. J Pediatr. 2014;164(4):895–899 e2.
30. Pinto A, et al. Nutritional status in patients with phenylketonuria using glycomacropeptide as their major protein source. Eur J Clin Nutr. 2017;71(10):1230–4.
31. Muniz-Naveiro O, et al. Selenium content and distribution in cow's milk supplemented with two dietary selenium sources. J Agric Food Chem. 2005;53(25):9817–22.
32. Li T, et al. Casein Glycomacropeptide hydrolysates exert Cytoprotective effect against cellular oxidative stress by up-regulating HO-1 expression in HepG2 cells. Nutrients. 2017;9(1):31.
33. Li T, et al. Upregulation of heme oxygenase-1 mediates the anti-inflammatory activity of casein glycomacropeptide (GMP) hydrolysates in LPS-stimulated macrophages. Food Funct. 2017;8(7):2475–84.
34. Evans S, et al. Food acceptance and neophobia in children with phenylketonuria: a prospective controlled study. J Hum Nutr Diet. 2016;29(4):427–33.
35. Brody EP. Biological activities of bovine glycomacropeptide. Br J Nutr. 2000;84(Suppl 1):S39–46.
36. Keogh JB, Clifton P. The effect of meal replacements high in glycomacropeptide on weight loss and markers of cardiovascular disease risk. Am J Clin Nutr. 2008;87(6):1602–5.
37. Ney DM, et al. Dietary glycomacropeptide supports growth and reduces the concentrations of phenylalanine in plasma and brain in a murine model of phenylketonuria. J Nutr. 2008;138(2):316–22.
38. Solverson P, et al. Glycomacropeptide, a low-phenylalanine protein isolated from cheese whey, supports growth and attenuates metabolic stress in the murine model of phenylketonuria. Am J Physiol Endocrinol Metab. 2012;302(7):E885–95.
39. Solverson P, et al. Low bone strength is a manifestation of phenylketonuria in mice and is attenuated by a glycomacropeptide diet. PLoS One. 2012;7(9):e45165.
40. Wang B, et al. Dietary sialic acid supplementation improves learning and memory in piglets. Am J Clin Nutr. 2007;85(2):561–9.

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CHAPTER 4A

PUBLICATION NUMBER 4

THE EFFECT OF GLYCOMACROPEPTIDE VERSUS AMINO ACIDS ON PHENYLALANINE AND TYROSINE VARIABILITY OVER 24 HOURS IN CHILDREN WITH PKU: A RANDOMISED CONTROLLED TRIAL

nutrients

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Article

The Effect of Glycomacropeptide versus Amino Acids on Phenylalanine and Tyrosine Variability over 24 Hours in Children with PKU: A Randomized Controlled Trial

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Abstract: Introduction: In phenylketonuria (PKU), evidence suggests that casein glycomacropeptide supplemented with rate-limiting amino acids (CGMP-AA) is associated with better protein utilisation and less blood phenylalanine (Phe) variability. **Aim:** To study the impact of CGMP-AA on blood Phe variability using 3 different dietary regimens in children with PKU. **Methods:** This was a 6-week randomised controlled cross-over study comparing CGMP-AA vs. Phe-free L-amino acids (L-AA) assessing blood Phe and tyrosine (Tyr) variability over 24 h in 19 children (7 boys) with PKU, with a median age of 10 years (6–16). Subjects were randomised to 3 dietary regimens: (1) R1, CGMP-AA and usual dietary Phe (CGMP + Phe); (2) R2, CGMP-AA − Phe content of CGMP-AA from usual diet (CGMP − Phe); and (3) R3, L-AA and usual dietary Phe. Each regimen was administered for 14 days. Over the last 48 h on days 13 and 14, blood spots were collected every 4 h at 08 h, 12 h, 16 h, 20 h, 24 h, and 04 h. Isocaloric intake and the same meal plan and protein substitute dosage at standardised times were maintained when blood spots were collected. **Results:** Eighteen children completed the study. Median Phe concentrations over 24 h for each group were (range) R1, 290 (30–580), R2, 220 (10–670), R3, 165 (10–640) µmol/L. R1 vs. R2 and R1 vs. R3 $p < 0.0001$; R2 vs. R3 $p = 0.0009$. There was a significant difference in median Phe at each time point between R1 vs. R2, $p = 0.0027$ and R1 vs. R3, $p < 0.0001$, but not between any time points for R2 vs. R3. Tyr was significantly higher in both R1 and R2 [70 (20–240 µmol/L] compared to R3 [60 (10–200) µmol/L]. In children < 12 years, blood Phe remained in the target range (120–360 µmol/L), over 24 h, for 75% of the time in R1, 72% in R2 and 64% in R3; for children aged ≥ 12 years, blood Phe was in target range (120–600 µmol/L) in R1 and R2 for 100% of the time, but 64% in R3. **Conclusions:** The residual Phe in CGMP-AA increased blood Phe concentration in children. CGMP-AA appears to give less blood Phe variability compared to L-AA, but this effect may be masked by the increased blood Phe concentrations associated with its Phe contribution. Reducing dietary Phe intake to compensate for CGMP-AA Phe content may help.

Keywords: glycomacropeptide; phenylalanine; phenylketonuria; phenylalanine variability; amino acids; tyrosine

1. Introduction

In phenylketonuria (PKU), there is an inability to metabolise dietary phenylalanine (Phe) into tyrosine (Tyr) due to a deficiency of Phe hydroxylase. It is treated by a low Phe diet, and the major source of protein equivalent is provided by a Phe-free or low Phe protein substitute, in the form of either L-amino acids (L-AA) or casein glycomacropeptide (CGMP-AA). CGMP is a 64-amino acid peptide, found in the whey residue produced as a by-product of cheese production from κ casein. This protein is naturally low in Phe as well as other indispensable, large neutral amino acids (histidine, leucine, tryptophan and Tyr). Supplementation with rate-limiting amino acids provides a suitable protein substitute, allowing CGMP to be administered as a protein replacement in PKU (CGMP-AA).

A disadvantage of CGMP-AA is that it contains residual Phe (typically 36 mg per 20 g protein equivalent). For patients prescribed 60 g/day protein equivalent from this source, it will supply 108 mg/day Phe, which may affect blood Phe control, especially when Phe intake by natural protein intake is low. Most children with classical PKU have a daily Phe intake varying from 3 to 8 g of natural protein. Studies examining the effect of residual Phe in CGMP-AA are largely adult-focused and limited by small subject numbers over short study time periods. In a single-dose cross-over intervention study, the metabolic effect of peptide-bound AA in CGMP-AA was examined in 8 older (aged 15 to 48 years) patients who consumed four different drinks containing either pure AA, CGMP-AA or a mixture of CGMP-AA and AA. After 240 min, no significant changes in blood Phe concentrations were reported, and they concluded that the small amounts of Phe in CGMP-AA did not increase blood Phe concentrations, although patients had high baseline blood Phe concentrations and were only studied for 4 h [1]. Ney et al. [2] examined the efficacy of CGMP-AA compared to L-AA in a randomised cross-over study, in which subjects took a low Phe diet with L-AA vs. CGMP-AA for three weeks. To account for the residual Phe in CGMP-AA, subjects were instructed to lower their dietary Phe intake. Blood Phe tended to increase (62 μmol/L) when subjects took CGMP-AA compared to a decrease when taking L-AA (85 μmol/L), although this difference did not reach statistical significance. The increased Phe concentration was associated with subjects not following protocol or failing to lower their dietary Phe intake when on CGMP-AA. Although Phe was not significantly increased, there is a suggestion that CGMP-AA plus usual low Phe diet influences Phe control. Van Calcar and coworkers [3] studied 11 subjects and reported lower Phe concentrations after an overnight fast using CGMP-AA compared to L-AA supplements, suggesting a slower release of AA in CGMP-AA. Other studies have also shown that protein synthesis and nitrogen retention are improved with 'complete' protein compared with individual L-amino acids [4,5]. However, further kinetic studies are required to examine if CGMP-AA is associated with a slower rate of amino acid absorption than conventional L-AA, particularly as CGMP-AA is supplemented with amino acids.

It is established that protein substitutes consisting of synthetic mono amino acids have a lower biological and functional efficacy compared to intact protein sources [6,7]. After the ingestion of Phe-free L-AA there is a rapid rise followed by a rapid decline in plasma amino acid concentrations compared with whole protein. Non-physiological 24 h blood Phe variability is also associated with Phe-free L-AA supplements [8–10].

In PKU, the residual Phe in CGMP-AA may mask any potential benefit associated with protein assimilation and reduced blood Phe variability. So far, no studies have examined the impact of CGMP-AA on 24-h blood Phe or Tyr variability when using CGMP-AA as a protein substitute in PKU; also, no studies have compared 24-h blood Phe variability with and without adjustment for the Phe content of the CGMP-AA.

The findings from a recent systematic review [11] suggest there is little evidence to support the impact of residual Phe in CGMP-AA on Phe control. However, studies to date have not been specific in addressing this question in children. Does residual Phe in CGMP-AA have a clinical impact on metabolic control? The aim of this randomised controlled cross-over study was to assess the blood Phe variability over 24 h in children with PKU, comparing conventional Phe-free L-amino supplements with CGMP-AA. In order to examine the impact of the Phe content of CGMP-AA on blood Phe variability we studied CGMP-AA under two conditions: a) no dietary adjustment for the Phe content of CGMP-AA, and b) dietary adjustment for the Phe content of CGMP-AA by lowering dietary Phe intake.

2. Materials and Methods

2.1. Subjects

We recruited 19 children (7 boys) diagnosed by newborn screening with PKU, with a median age of 10 years (range 6–16 years) from two treatment centres. Seventeen were European and two were of Pakistani origin. Inclusion criteria included being diagnosed by newborn screening, aged 6–16 years, not treated with sapropterin, known adherence with protein substitute, 70% of blood Phe concentrations within the Phe target range for age, for 6 months before starting the study and the ability to take both CGMP-AA and Phe-free L-AA supplements. Exclusion criteria were children taking sapropterin, those with co-morbidity, an inability to take CGMP-AA and Phe-free L-AA or poor blood Phe control failing to attain 70% of the routine blood Phe concentrations within the target range for 6 months prior to the study. Target blood Phe concentrations were according to the European PKU Guidelines for age [12]; for children < 12 years, 120–360 μmol/L, and for patients ≥ 12 years, 120–600 μmol/L.

The study was registered by the Health Research Authority and given a favourable ethical opinion by The West Midlands Research Ethical Committee. Written consent was obtained for all subjects from at least one caregiver with parental responsibility and written assent from the subjects if appropriate for their age and level of understanding.

2.2. CGMP-AA and Phe-Free L-AA Formulation

The CGMP-AA formula contained approximately 65% CGMP and 35% L-AA. It was supplemented with Tyr, tryptophan, leucine, arginine, methionine, cysteine and histidine, vitamins and minerals. One 35 g sachet of CGMP-AA (Vitaflo International Ltd., Liverpool, UK) contained 20 g of protein equivalent, and 36 mg of Phe, 120 kcal, 6.3 g carbohydrate and 1.6 g of fat, supplying 110 mg of DHA. Each sachet was mixed with 120 mL of water.

Conventional Phe-free L-AA, contained 100% mono amino acids, vitamins and minerals to meet dietary reference values. Children took their usual prescribed L-AA supplements taken from liquid pouches or as a powder taken in a semi-solid format. The Phe-free L-AA had a similar energy profile to CGMP-AA. The same dose of protein equivalent for each individual subject was taken throughout the study, and was administered at the same time (3 or 4 times daily) from both CGMP-AA and Phe-free L-AA (Table 1).

Table 1. Nutritional composition of casein glycomacropeptide supplemented with rate-limiting amino acids (CGMP-AA) compared with the conventional Phe-free L-AAs used in the study.

		CGMP-AA 20 g PE	**L-AA Protein Substitutes 20 g PE**				
Nutrients	Units	CGMP-AA 1 sachet 35 g Powder Flavoured	PKU Cooler 20 1 pouch = 174 mL	LQ Lophlex 20 1 pouch = 125 mL	XP Maxamum 1 × 50 g sachet Powder Unflavoured	First spoon 50 g powder Unflavoured	PKU Express 1 × 34 g sachet Powder Flavoured
Calories	Kcal	120	130	120	149	164	101
Protein equivalent	G	20	20	20	19.5	20	20
Total carbohydrate	G	6.3	8.9	8.8	17	19.2	4.7
Sugars	G	2.2	5.9	8.8	1.6	12.8	0.3
Total fat	G	1.6	1.6	0.44	<0.25	0.6	0.07
DHA	Mg	110	134	150	0	104	0
AA	Mg	-	-	-	-	-	-
Salt	G	0.55	0.26	0	0	0	0.43
Vitamin A	µg RE	259	261	285	355	600	283
Vitamin D	µg	5.0	10	8	3.9	13.2	4.5
Vitamin E	mg αTE	5.30	5.2	3.2	2.6	7.1	5.3
Vitamin C	Mg	26	37	17.8	90	75	36.7
Vitamin K	µg	23	24	24.9	35	57	34
Thiamine	Mg	0.60	0.7	0.43	0.7	0.75	0.68
Riboflavin	Mg	0.60	0.77	0.5	0.7	0.75	0.78
Niacin	Mg	3.20	3.5	7.1	6.8	3.4	8.4
Vitamin B6	Mg	0.60	0.87	0.58	1.1	0.75	1
Folic Acid	µg	102	101	120	250	84	136
Vitamin B12	µg	1.6	1.6	1.8	1.8	1.9	1.6
Biotin	µg	13	13	53.4	70	27.8	63.9
Pantothenic acid	Mg	2.0	1.9	1.8	2.5	4.2	2.7
Choline	Mg	204	200	153	161	139	204
Sodium	Mmol	12	4.5	<10	12.1	9.1	7.5
Potassium	Mmol	5.9	6.1	<25	9	13.5	8
Chloride	Mmol	0.20	3.9	<25	7.9	9	6.9
Calcium	Mg	399	400	356	335	626	407
Phosphorus	Mmol	13	11	8.9	10.8	14.8	11.4
Magnesium	Mg	115	110	107	143	88	128
Iron	Mg	7.4	7.3	5.3	11.8	12.4	7.3
Copper	Mg	0.60	0.73	0.53	0.7	0.63	0.75
Zinc	Mg	7.4	5.6	3.9	6.8	8.8	7.3
Manganese	Mg	0.40	0.5	0.53	1.1	0.65	1.1
Iodine	µg	84	85	58.4	53.3	127	85.7
Molybdenum	µg	20	23	25	53.5	18.4	49
Selenium	µg	30	26	26.8	25	23.8	29.9
Chromium	µg	12	14	10.6	25	21	29.9

Table 1. *Cont.*

		CGMP-AA 20 g PE		L-AA Protein Substitutes 20 g PE			
Amino acid profile							
L-Alanine	G	0.83	0.92	1.16	0.85	0.95	0.87
L-Arginine	G	0.96	1.5	2	1.6	1.65	1.41
L-Aspartic acid	G	1.31	2.37	1.75	1.5	1.55	2.23
L-Cystine	G	0.24	0.61	0.51	0.6	0.62	0.57
L-Glutamine	G	2.70		0	2.6	0	1.73
Glycine	G	0.71	2.35	1.88	1.5	1.47	2.22
L-Histidine	G	0.70	0.92	0.79	0.9	0.95	0.87
L-Isoleucine	G	1.42	1.62	1.24	1.4	1.47	1.52
L-Leucine	G	3.02	2.54	2.13	2.4	2.48	2.39
L-Lysine	G	0.95	1.67	1.63	1.9	1.71	1.58
L-Methionine	G	0.28	0.45	0.34	0.4	0.39	0.42
L-Phenylalanine	G	0.036	-	0	0	0	0
L-Proline	G	1.60	1.69	2	1.7	1.79	1.59
L-Serine	G	1.01	1.04	1.09	1.1	1.09	0.99
L-Threonine	G	2.29	1.62	1.04	1.2	1.23	1.54
L-Tryptophan	G	0.40	0.5	0.41	0.45	0.48	0.48
L-Tyrosine	G	2.25	2.38	1.88	2.1	2.2	2.24
L-Valine	G	1.14	1.86	1.38	1.6	1.58	1.76

Abbreviations: PE: Protein equivalent; Phe: Phenylalanine; CGMP-AA: casein glycomacropeptide supplemented with limiting amino acids; L-AA: L-amino acid (protein substitute based on 20 g PE); DHA: Docosahexaenoic acid; AA: Arachidonic acid.

2.3. Study Design

In a 6-week randomised controlled cross-over study, each subject was randomised to take three different regimens at home for 14 days (Figure 1):

- R1: Subjects took CGMP-AA only as their protein substitute and their usual prescribed Phe allowance from food (CGMP-AA + Phe);
- R2: Subjects took CGMP-AA only, but the amount of Phe contained in the CGMP was deducted from their dietary Phe allowance (CGMP-AA − Phe);
- R3: Subjects took Phe-free L-AA with their usual dietary Phe allowance (Phe-free L-AA).

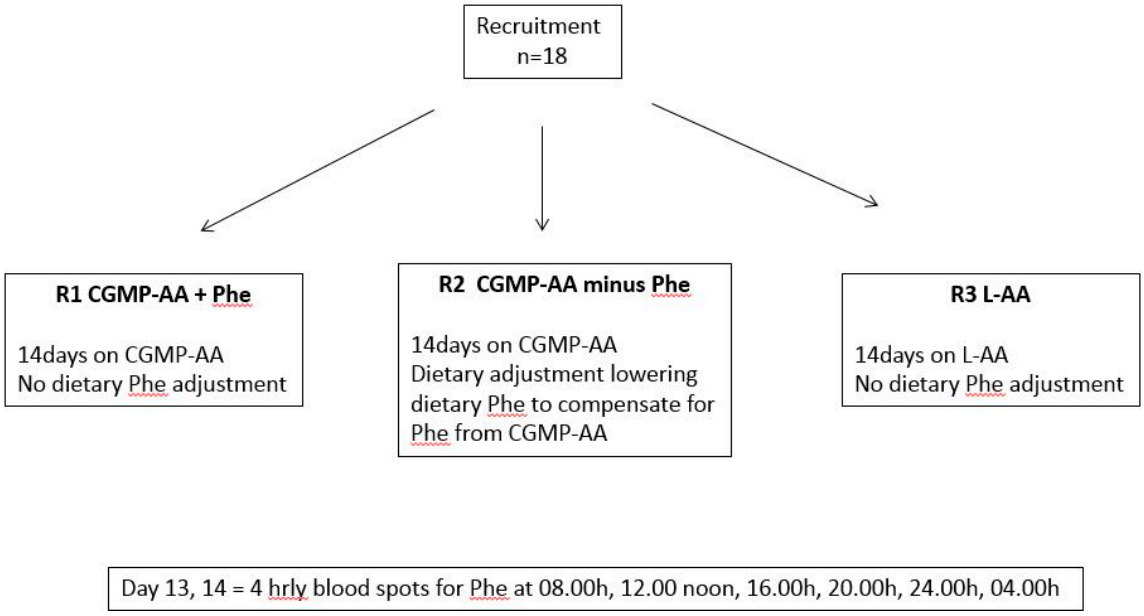


Figure 1. Diagram showing overview of study methodology. Each subject was randomised to R1, R2 and R3 for 14 days, on day 13 and 14, 4 hourly blood spots were collected for Phe and Tyr concentrations.

During the last 48 h of each study period (days 13 and 14), four hourly Phe and Tyr blood spot tests were taken day and night (08.00 am, 12.00 noon, 16.00 pm, 20.00 pm, 24.00 midnight, and 04.00 am). Twelve blood spots were collected during each study regimen, with a total of 36 blood spots over the entire study for each subject. A mean blood value for each time point over 2 days was calculated for each subject. Using these values, a median value for the whole subject group was determined representing a 24 h profile.

All the children had their first blood spots taken at 8.00 am (baseline) and they were completed at 04.00 am on the third day.

Randomisation was generated by a block randomisation system and the random order was kept within a sealed envelope.

2.4. Standardisation of Meals

On day 12, 13 and 14 of each study period, subjects followed the same meal plan and took their protein substitute at the same time each day. This was to achieve isocaloric intake and minimise variability due to differences in daily routines and dietary patterns. In R2, when dietary Phe was withdrawn, to compensate for the Phe in CGMP-AA, the energy content was replaced using low-protein food sources or glucose polymer solutions. Parents/caregivers weighed and recorded all food on day 12, 13 and 14 during each study period.

2.5. Blood Spots for Phe and Tyr

Throughout the study, trained caregivers collected four hourly blood spots at home. Blood spots for Phe and Tyr were collected on filter cards, Perkin Elmer 226 (UK Standard NBS, Public Health England, London, UK). All parents/caregivers had received previous training on blood spot collection and their technique was reviewed prior to study commencement. Blood spots were returned to the laboratory at Birmingham Women's and Children's Hospital. All the cards had a standard thickness and the blood Phe and Tyr concentrations were calculated on a 3.2 mm punch by MS/MS tandem mass spectrometry.

All subjects received careful supervision on blood collection days with daily home visits from the lead researcher to ensure blood spots were taken at correct times and were of adequate size. Additional support was provided by telephone calls made by the lead researcher throughout the evening and night-time to ensure all blood spots were collected.

2.6. Statistics

Sample size calculations determined that 18 subjects were required to demonstrate a difference of 30 μmol/L variation in Phe over 48 h based on a two-sided alpha level 0.05, a power of 80% and a standard deviation of 40 μmol/L.

Data are summarised as median (range) for continuous data and frequencies of counts and percentages for categorical data. Longitudinal regression techniques were used to evaluate the change in Phe and Tyr over time. Models were constructed which included time as a categorical factor and the randomisation identifier. Patient identifiers are included as a random effect to evaluate both between and within levels of variability. Further models were constructed which included time as a continuous covariate to give the average change in Phe levels over the study period. Of secondary interest was the stability in Phe levels, and this was assessed by measuring the difference between the Phe levels at each time point and the daily median.

3. Results

Regarding subject withdrawal, one girl (aged 12 years) withdrew from the study on day 1 of the first regimen after she decided not to continue with the blood spot sampling.

A total of 18 children with PKU completed the study. The median natural protein intake from food was 5 g/day (range 3–30 g). A median daily dose of 60 g (40–80 g) of protein equivalent from protein substitute was consumed between three evenly divided daytime doses. The median intake of Phe supplied by the CGMP-AA was 108 mg/day (range 70–140).

At enrolment, 4 subjects had powdered Phe-free L-AA, [XP Maxamum (Nutricia Ltd., Trowbridge, UK), $n = 1$; PKU First spoon (Nutricia Ltd.) $n = 2$; PKU Express (Vitaflo International Ltd., Liverpool, UK), $n = 1$], and 15 subjects had Phe-free liquid L-AA pouches [LQ Lophlex (Nutricia Ltd.) $n = 2$, PKU Cooler (Vitaflo International Ltd.) $n = 13$]. When taking the CGMP-AA formula, all children mixed this with water.

Regarding the comparison between regimens 1, 2 and 3 for median Phe concentrations over 24 h (Table 2), there was a difference in blood Phe variability depending on the regimen that was followed. The median Phe was 290 (range 30–580 μmol/L) in R1, 220, (range 10–670 μmol/L) in R2 and 165 (range 10–640 μmol/L) in R3. There was a significant difference between the three regimens, [R1 vs. R2 $p < 0.0001$, R1 vs. R3 $p < 0.0001$, R2 vs. R3 $p = 0.0009$]. All median Phe levels were within the European PKU target guidelines (12).

Regarding the comparison between regimens 1, 2 and 3 for median Phe concentrations for each time point (Table 3, Figure 2), there was a significant difference in median Phe at each time point (i.e., 08.00 h, 12.00 h, 16.00 h, 20.00 h, 24.00 h, and 04.00 h) between R1 vs. R2, [median value $p = 0.0027$] and R1 vs. R3 [$p < 0.0001$]. No significant difference was observed between any of the time points for R2 vs. R3. The Phe concentrations were consistently higher at all time points in R1 and lowest in R3.

Regarding the comparison of the variation in Phe concentrations between regimens 1, 2 and 3 during each 8 hourly time period (08.00 h–16.00 h), (16.00 h to 24.00 h), (24.00 h to 08.00 h) (Table 4, Figure 3), measuring the Phe over 8 hourly time periods showed there was less variation with R1 and R2 compared with R3. In all three regimens, the largest change in blood Phe occurred overnight between 24.00 h and 08.00 h, R1: +35 µmol/L, R2: +20 µmol/L, R3: +70 µmol/L. The largest change in Phe at all three measured 8 hourly intervals was in R3. In all three regimens, Phe decreased between 08.00–16.00 h and from16.00 h-24.00 h, the least change was in R1, then R2 and the greatest change always in R3.

For subjects up to 12 years of age, the European blood Phe target range was maintained at 75% at each time point over 24 h in R1, 72% in R2 and 64% in R3. For subjects ≥ 12 years (n = 3) blood Phe target range was maintained 100% of the time in R1 and R2 and 64% in R3.

Median Phe Concentrations for Each Subject Compared with Recommended Target Reference Ranges for R1, R2 and R3

Comparing Phe concentrations with European PKU age related guidelines for children <12 years (120–360 µmol/L), the number of subjects with concentrations < 120 µmol/L was lowest in R1 (7%), then R2 (22%) and highest in R3 (35%). Phe concentrations > 360 µmol/L were highest in R1 (18%), then R2 (7%) and no concentrations > 360 µmol/L were recorded in R3. For older children ≥12 years (120–600 µmol/L), in R1 and R2 all Phe concentrations were within reference range, but in R3, one subject had concentrations < 120 µmol/L, and one had concentrations > 600 µmol/L (Figure 4).

For children < 12 years, the mean percentage of time over 24 h that Phe concentrations were < 120 µmol/L was 7% (SD 6) for R1 (n = 4), 12% (SD 8) for R2 (n = 7), and 15% (SD 9) for R3 (n = 9). The mean percentage time > 360 µmol/L was 16% (SD 4) in R1 (n = 4), and 25% in R2 (n = 1). No member of the R3 group had levels > 360 µmol/L.

In the older age group of ≥ 12 years, over a 24 h period, R1 and R2 had no Phe concentrations outside the reference range. In R3, the percentage time over 24 h with Phe concentrations < 120 µmol/L was 25% in one child, and in another child, Phe concentrations were > 600 µmol/L for 4% of the time.

Regarding the comparison between regimens 1, 2 and 3 for median Tyr concentrations over 24 h (Table 2), the median Tyr concentrations were highest in R1 and R2 (Tyr 70, range 20–240 µmol/L) and lowest in R3 (60, range 10–200 µmol/L). When median Tyr concentrations were compared over 24 h, there was a significant difference between regimens R1 vs. R3 (p = 0.0028) and R2 vs. R3 (p = 0.0016). There was no difference between R1 vs. R2 (p = 0.96).

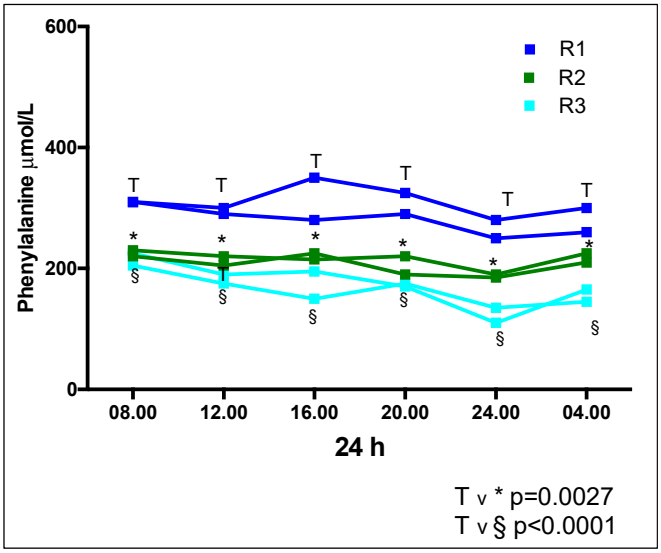


Figure 2. Comparison of median Phe concentrations (µmol/L) at each time point for regimens 1, 2 and 3.

Table 2. Comparison of median Phe (μmol/L) and Tyr (μmol/L) concentrations (range) over 24 h for regimens 1, 2 and 3.

	R1	R2	R3
Phe μmol/L	290 (30–580) *	220 (10–670) ^{T}	165 (10–640) ^{TT}
Tyr μmol/L	70 (20–240) **	70 (20–220) §	60 (10–200) §§

Wilcoxon test Phe * v^{T}, * v ^{TT} $p < 0.0001$, ^{T} v^{TT} $p = 0.009$. Wilcoxon test Tyr ** v §§, § v §§, $p = 0.002$. Subtext symbols ^{*,TT,§,§§} show comparisons between the groups R1, R2 and R3.

Table 3. Comparison of median Phe (μmol/L) and Tyr (μmol/L) concentrations (range) at each time point for regimens 1, 2 and 3.

Time	R1		R2		R3	
	Phe μmol/L	Tyr μmol/L	Phe μmol/L	Tyr μmol/L	Phe μmol/L	Tyr μmol/L
08.00	310 (150–490) ^{T}	40 (30–120)	230 (115–560) ^{1}	50 (30–120)	220 (80–440) ^{TT}	40 (20–200)
12.00	290 (80–450) ^{T}	90 (40–150)	220 (40–600) ^{2}	90 (20–170)	185 (30–480) ^{TT}	80 (20–200)
16.00	280 (80–500) ^{T}	90 (30–240) §	225 (70–670) ^{3}	90 (30–220) **	190 (20–640) ^{TT}	70 (30–160) §§**
20.00	300 (80–580) ^{T}	80 (20–190)	210 (50–570) ^{4}	75 (20–150)	170 (20–480) ^{TT}	80 (10–180)
24.00	275 (30–500) ^{T}	70 (30–150)	185 (10–490) ^{5}	85 (30–180) ***	125 (10–440) ^{TT}	60 (30–190) ***
04.00	275 (50–550) ^{T}	60 (30–110) §	210 (70–610) ^{6}	60 (30–280) ****	150 (40–440) ^{TT}	50 (30–100) §§****

Wilcoxon ^{T}v^{TT} p < 0.0001, ^{T}v^{1} $p = 0.0015$, ^{T}v^{2} $p = 0.0028$, ^{T}v^{3} $p = 0.0016$, ^{T}v^{4} $p = 0.0039$, ^{T}v^{5} $p = 0.0552$, ^{T}v^{6} $p = 0.0073$, Wilcoxon test § v §§ $p = 0.03$, ** v** $p = 0.01$, *** v*** $p = 0.02$, **** v**** $p = 0.007$. Subtext symbols e.g., ^{T,TT,} **,***,**** show comparisons between the groups R1, R2 and R3.

Table 4. Comparison of the variation in Phe (μmol/L) concentrations between regimens 1, 2 and 3 over 8 hourly time periods.

Time	R1 Phe μmol/L	R2 Phe μmol/L	R3 Phe μmol/L
08.00–16.00	−30	−5	−35
16.00–24.00	−5	−40	−65
24.00–08.00	+35	+20	+70

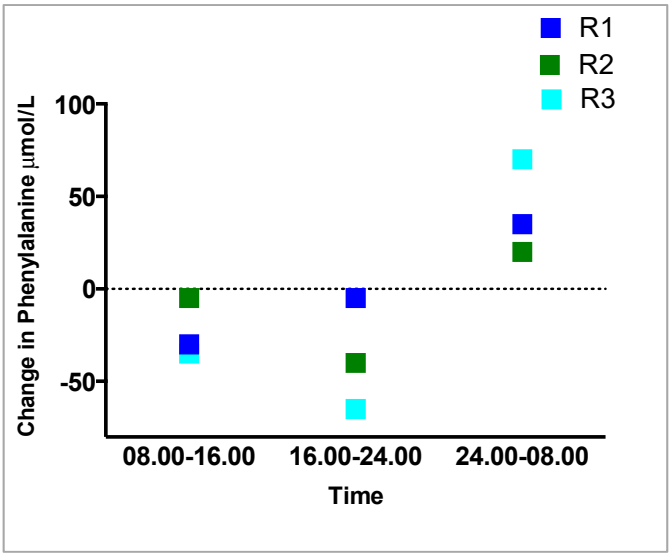
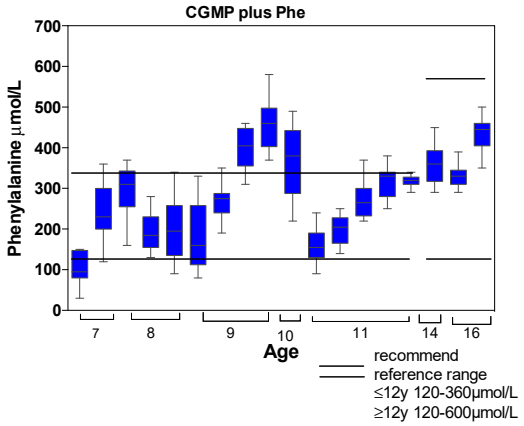
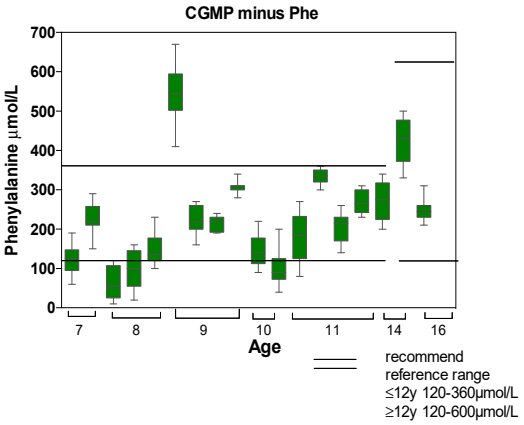


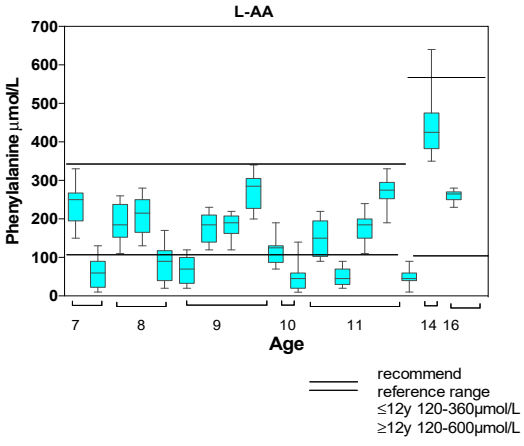
Figure 3. Comparison in the variation in Phe (μmol/L) concentrations between regimens 1, 2 and 3 over 8 hourly time periods.



A



B



C

Figure 4. (**A**) Median Phe (µmol/L) concentrations and ranges for each subject in R1 (CGMP + Phe) compared with recommended age target ranges (target Phe range 120 to 360 µmol/L aged < 12 years; 120 to 600 µmol/L aged ≥ 12 years). (**B**) Median Phe (µmol/L) concentrations and ranges for each subject in R2 (CGMP-Phe) compared with recommended age target ranges (target Phe range 120 to 360 µmol/L aged < 12 years; 120 to 600 µmol/L aged ≥ 12 years). (**C**) Median Phe (µmol/L) concentrations and ranges for each subject in R3 (L-AA) compared with recommended age target ranges (target Phe range 120 to 360 µmol/L aged < 12 years; 120 to 600 µmol/L aged ≥ 12 years).

4. Discussion

In PKU, this is the first randomised controlled study to review the 24-h blood Phe and Tyr variability using CGMP-AA compared with conventional L-AA. The impact of the residual Phe content of CGMP-AA on 24-h blood Phe and Tyr profiles under controlled conditions was also studied. When comparing each of the three regimens for the percentage of Phe concentrations within the target range for the respective ages, it was clear that the CGMP-AA minus the dietary Phe gave the most consistent results. However, this study clearly demonstrated that the Phe supplied by CGMP-AA adversely affected blood Phe control in children. In the R1 group, 18% had Phe concentrations greater than the reference range compared to none in the R3 group, suggesting that residual Phe in CGMP impacts metabolic control. However, the median Phe concentrations remained within the current targets for all three test regimens [13].

It has been well established that timing of protein substitution affects diurnal Phe variation [8]. In a study in which 16 children with PKU received a Phe-free AA every 4 h during the day and night, the median differences in blood Phe concentrations were 40 μmol/L per day. When the protein substitute was given in 3 divided doses over 10 h, the median difference in blood Phe was 140 μmol/L per day. In the current study, the protein substitute was given three times a day over the course of 12 h. The median difference in Phe, measured between the highest and lowest values, over 24 h were lowest in R1 at 35 μmol/L, followed by R2 at 45 μmol/L, and highest in R3 at 95 μmol/L. This may suggest that CGMP-AA has some influence on Phe stability compared to the L-AA group.

There is evidence to suggest that the digestion, absorption and metabolism of CGMP-AA and L-AA may vary. Amino acid availability depends directly on the quantity and quality of the dietary source of nitrogen. Protein utilization is affected by factors such as the rate of protein digestion, amino acid absorption, together with the presence and amount of other essential and non-essential amino acids and macronutrients [7,14]. The speed of protein digestion and amino acid absorption from the gut has a major effect on whole body protein anabolism. Studies measuring protein utilization have shown that a 'slowly' digested protein, e.g., casein, is better utilized compared with a 'fast' one such as milk-soluble protein isolate [15,16]. The amino acid profile of dietary proteins may also increase the secretion of insulin, which has a positive correlation with plasma leucine, phenylalanine and arginine concentrations [17,18]. Postprandial protein metabolism is a complex biochemical process influenced by the type of protein ingested, macronutrients, hormonal responses and timing of dietary intake. An even delivery of Phe into the systemic system may enable a steady state of protein utilization improving absorption kinetics increasing whole body protein balance and skeletal muscle protein synthesis.

Some studies [19–22] suggest that fluctuations in blood Phe concentrations may influence intellectual outcome, although the exact mechanism of how is unknown. In non-PKU adults, Phe fluctuates by no more than 50% over 24 h. It has been shown that the daily fluctuations in plasma-free amino acid levels are significantly affected by dietary conditions, with more frequent feeding leading to less fluctuation [23,24]. The stabilization of blood Phe concentrations would lead to less diurnal fluctuation, and this may have a positive benefit neurologically and physiologically. However, there is no agreed definition of Phe fluctuation. A review of the literature suggests that it is the cumulative effect of day-to-day fluctuations over months and years that may affect cognition impacting on intelligence quotients [25,26]. A recent short study over 26 weeks by Feldmann concluded that Phe fluctuations were negatively correlated with IQ in children with mild PKU [27]. Although no study has definitively shown that fluctuation in Phe affects neurocognitive outcome, evidence suggests there is a correlation, and therefore the stabilization of Phe concentrations should be the aim of treatment. In R2 and R3 with controlled Phe intake, the only influence on outcomes is the type of protein substitute consumed, and the evidence in this study suggests that CGMP-AA may have a positive physiological impact on reducing variability.

Tyr concentrations were highest for all three regimens at 12.00 midday and progressively declined over 24 h, with the lowest concentrations at 08.00 am. Tyr concentrations showed less stability than Phe.

Only R1 showed a steady fall over 24 h. Tyr concentrations in R2 and R3, showed greater variations (rising and falling) within the 24 h period. The impact of this pattern has not been studied in detail. Tyr is an important functional amino acid; as a large neutral amino acid, it competes at the blood–brain barrier with Phe, and it is closely associated with the monoamine neurotransmitter dopamine. There is also some suggestion that Tyr from L-AA protein substitutes are less bioavailable because of altered gut bacteria compared to CGMP-AA protein substitutes [28]. More work is needed to explore the association of Tyr and bioavailability on long-term neurocognitive outcomes using CGMP-AA and L-AA protein substitutes.

There are limitations to this study; for instance, we only enrolled a limited number of children. However, because the study design was a randomised cross-over study with each child acting as their own control, it increased the power of the study. We have not accounted for energy expenditure over the three days, which could have influenced our results, although the children were at school for some of the study period and the rest of the time was closely supervised. Although protein substitute was given at the same time point each day for each child, this was not a standardised time for the group of children, but they took it according to their individual, usual daily routine.

5. Conclusions

This study clearly demonstrates that the residual Phe in CGMP-AA, when used as the only source of protein substitute in children, has a significant impact on blood Phe control. In its present format, the formulation of CGMP-AA should be used with caution in children < 12 years of age, as there is a risk of Phe concentrations increasing beyond target recommendations. However, the glycomacropeptide structure appears to lead to the stabilization of Phe concentrations with less fluctuation over a 24-h period compared to conventional amino acid formula. Further studies and research are necessary to refine the CGMP-AA product providing a lower Phe or Phe-free CGMP-AA, allowing greater use of glycomacopeptide, and possibly leading to better stabilization of Phe concentrations over the day.

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References

1. Ahring, K.K.; Lund, A.M.; Jensen, E.; Jensen, T.G.; Brøndum-Nielsen, K.; Pedersen, M.; Bardow, A.; Holst, J.J.; Rehfeld, J.F.; Møller, L.B. Comparison of Glycomacropeptide with Phenylalanine Free-Synthetic Amino Acids in Test Meals to PKU Patients: No Significant Differences in Biomarkers, Including Plasma Phe Levels. *J. Nutr. Metab.* **2018**, *2018*, 6352919. [CrossRef] [PubMed]

2. Ney, D.M.; Stroup, B.M.; Clayton, M.K.; Murali, S.G.; Rice, G.M.; Rohr, F.; Levy, H.L. Glycomacropeptide for nutritional management of phenylketonuria: A randomized, controlled, crossover trial. *Am. J. Clin. Nutr.* **2016**, *104*, 334–345. [CrossRef] [PubMed]
3. van Calcar, S.C.; MacLeod, E.L.; Gleason, S.T.; Etzel, M.R.; Clayton, M.K.; Wolff, J.A.; Ney, D.M. Improved nutritional management of phenylketonuria by using a diet containing glycomacropeptide compared with amino acids. *Am. J. Clin. Nutr.* **2009**, *89*, 1068–1077. [CrossRef] [PubMed]
4. Gropper, S.S.; Gropper, D.M.; Acosta, P.B. Plasma amino acid response to ingestion of L-amino acids and whole protein. *J. Pediatr. Gastroenterol. Nutr.* **1993**, *16*, 143–150. [CrossRef] [PubMed]
5. Monch, E.; Herrmann, M.E.; Brösicke, H.; Schöffer, A.; Keller, M. Utilisation of amino acid mixtures in adolescents with phenylketonuria. *Eur. J. Pediatr.* **1996**, *155*, S115–S120. [CrossRef] [PubMed]
6. Bujko, J.; Schreurs, V.V.A.M.; Nolles, J.A.; Verreijen, A.M.; Koopmanschap, R.E.; Verstegen, M.W.A. Application of a [$^{13}CO_2$] breath test to study short-term amino acid catabolism during the postprandial phase of a meal. *Br. J. Nutr.* **2007**, *97*, 891–897. [CrossRef] [PubMed]
7. Metges, C.C.; Barth, C.A. Metabolic consequences of a high dietary-protein intake in adulthood: Assessment of the available evidence. *J. Nutr.* **2000**, *130*, 886–889. [CrossRef] [PubMed]
8. MacDonald, A.; Rylance, G.; Davies, P.; Asplin, D.; Hall, S.K.; Booth, I.W. Administration of protein substitute and quality of control in phenylketonuria: A randomized study. *J. Inherit. Metab. Dis.* **2003**, *26*, 319–326. [CrossRef] [PubMed]
9. MacDonald, A.; Rylance, G.; Hall, S.K.; Asplin, D.; Booth, I.W. Factors affecting the variation in plasma phenylalanine in patients with phenylketonuria on diet. *Arch. Dis. Child.* **1996**, *74*, 412–417. [CrossRef] [PubMed]
10. MacDonald, A.; Rylance, G.W.; Asplin, D.; Hall, S.K.; Booth, I.W. Does a single plasma phenylalanine predict quality of control in phenylketonuria? *Arch. Dis. Child.* **1998**, *78*, 122–126. [CrossRef] [PubMed]
11. Pena, M.J.; Pinto, A.; Daly, A.; MacDonald, A.; Azevedo, L.; Rocha, J.C.; Borges, N. The Use of Glycomacropeptide in Patients with Phenylketonuria: A Systematic Review and Meta-Analysis. *Nutrients* **2018**, *10*, 1794. [CrossRef] [PubMed]
12. van Wegberg, A.M.J.; MacDonald, A.; Ahring, K.; Bélanger-Quintana, A.; Blau, N.; Bosch, A.M.; Burlina, A.; Campistol, J.; Feillet, F.; Giżewska, M.; et al. The complete European guidelines on phenylketonuria: Diagnosis and treatment. *Orphanet. J. Rare Dis.* **2017**, *12*, 162. [CrossRef] [PubMed]
13. van Spronsen, F.J.; van Wegberg, A.M.J.; Ahring, K.; Bélanger-Quintana, A.; Blau, N.; Bosch, A.M.; Burlina, A.; Campistol, J.; Feillet, F.; Giżewska, M.; et al. Key European guidelines for the diagnosis and management of patients with phenylketonuria. *Lancet Diabetes Endocrinol.* **2017**, *5*, 743–756. [CrossRef]
14. Dangin, M.; Boirie, Y.; Garcia-Rodenas, C.; Gachon, P.; Fauquant, J.; Callier, P.; Ballèvre, O.; Beaufrère, B. The digestion rate of protein is an independent regulating factor of postprandial protein retention. *Am. J. Physiol. Endocrinol. Metab.* **2001**, *280*, E340–E348. [CrossRef] [PubMed]
15. Boirie, Y.; Dangin, M.; Gachon, P.; Vasson, M.-P.; Maubois, J.-L.; Beaufrère, B. Slow and fast dietary proteins differently modulate postprandial protein accretion. *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 14930–14935. [CrossRef] [PubMed]
16. Lacroix, M.; Bos, C.; Léonil, J.; Airinei, G.; Luengo, C.; Daré, S.; Benamouzig, R.; Fouillet, H.; Fauquant, J.; Tomé, D.; et al. Compared with casein or total milk protein, digestion of milk soluble proteins is too rapid to sustain the anabolic postprandial amino acid requirement. *Am. J. Clin. Nutr.* **2006**, *84*, 1070–1079. [CrossRef] [PubMed]
17. Calbet, J.A.; MacLean, D.A. Plasma glucagon and insulin responses depend on the rate of appearance of amino acids after ingestion of different protein solutions in humans. *J. Nutr.* **2002**, *132*, 2174–2182. [CrossRef] [PubMed]
18. van Loon, L.J.; Saris, W.H.M.; Verhagen, H.; Wagenmakers, A.J.M. Plasma insulin responses after ingestion of different amino acid or protein mixtures with carbohydrate. *Am. J. Clin. Nutr.* **2000**, *72*, 96–105. [CrossRef] [PubMed]
19. Huijbregts, S.C.; De Sonneville, L.M.J.; Licht, R.; Van Spronsen, F.J.; Sergeant, J.A. Short-term dietary interventions in children and adolescents with treated phenylketonuria: Effects on neuropsychological outcome of a well-controlled population. *J. Inherit. Metab. Dis.* **2002**, *25*, 419–430. [CrossRef] [PubMed]

20. Huijbregts, S.C.; de Sonneville, L.M.J.; Licht, R.; van Spronsen, F.J.; Verkerk, P.H.; Sergeant, J.A. Sustained attention and inhibition of cognitive interference in treated phenylketonuria: Associations with concurrent and lifetime phenylalanine concentrations. *Neuropsychologia* **2002**, *40*, 7–15. [CrossRef]
21. Anastasoaie, V.; Kurzius, L.; Forbes, P.; Waisbren, S. Stability of blood phenylalanine levels and IQ in children with phenylketonuria. *Mol. Genet. Metab.* **2008**, *95*, 17–20. [CrossRef] [PubMed]
22. Arnold, G.L.; Kramer, B.M.; Kirby, R.S.; Plumeau, P.B.; Blakely, E.M.; Sanger Cregan, L.S.; Davidson, P.W. Factors affecting cognitive, motor, behavioral and executive functioning in children with phenylketonuria. *Acta Paediatr.* **1998**, *87*, 565–570. [CrossRef] [PubMed]
23. Hussein, M.A.; Young, V.R.; Murray, E.; Scrimshaw, N.S. Daily fluctuation of plasma amino acid levels in adult men: Effect of dietary tryptophan intake and distribution of meals. *J. Nutr.* **1971**, *101*, 61–69. [CrossRef] [PubMed]
24. Scriver, C.R.; Gregory, D.M.; Sovetts, D.; Tissenbaum, G. Normal plasma free amino acid values in adults: The influence of some common physiological variables. *Metabolism* **1985**, *34*, 868–873. [CrossRef]
25. Vilaseca, M.A.; Lambruschini, N.; Gómez-López, L.; Gutiérrez, A.; Fusté, E.; Gassió, R.; Artuch, R.; Campistol, J. Quality of dietary control in phenylketonuric patients and its relationship with general intelligence. *Nutr. Hosp.* **2010**, *25*, 60–66. [PubMed]
26. Waisbren, S.E.; Noel, K.; Fahrbach, K.; Cella, C.; Frame, D.; Dorenbaum, A.; Levya, H. Phenylalanine blood levels and clinical outcomes in phenylketonuria: A systematic literature review and meta-analysis. *Mol. Genet. Metab.* **2007**, *92*, 63–70. [CrossRef] [PubMed]
27. Feldmann, R.; Schallert, M.; Nguyen, T.; Och, U.; Rutsch, F.; Weglage, J. Children and adolescents with phenylketonuria display fluctuations in their blood phenylalanine levels. *Acta Paediatr.* **2018**, *108*, 541–543. [CrossRef] [PubMed]
28. Ney, D.M.; Murali, S.G.; Stroup, B.M.; Nair, N.; Sawin, E.A.; Rohr, F.; Levy, H.L. Metabolomic changes demonstrate reduced bioavailability of tyrosine and altered metabolism of tryptophan via the kynurenine pathway with ingestion of medical foods in phenylketonuria. *Mol. Genet. Metab.* **2017**, *121*, 96–103. [CrossRef] [PubMed]



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CHAPTER 4B

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PRELIMINARY INVESTIGATION TO REVIEW IF A GLYCOMACROPEPTIDE COMPARED TO L-AMINO ACID PROTEIN SUBSTITUTE ALTERS THE PRE AND POST AMINO ACID PROFILE IN CHILDREN WITH PHENYLKETONURIA

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Preliminary Investigation to Review If a Glycomacropeptide Compared to L-Amino Acid Protein Substitute Alters the Pre- and Postprandial Amino Acid Profile in Children with Phenylketonuria

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Abstract: In Phenylketonuria (PKU), the peptide structure of the protein substitute (PS), casein glycomacropeptide (CGMP), is supplemented with amino acids (CGMP-AA). CGMP may slow the rate of amino acid (AA) absorption compared with traditional phenylalanine-free amino acids (Phe-free AA), which may improve nitrogen utilization, decrease urea production, and alter insulin response. Aim: In children with PKU, to compare pre and postprandial AA concentrations when taking one of three PS's: Phe-free AA, CGMP-AA 1 or 2. Methods: 43 children (24 boys, 19 girls), median age 9 years (range 5–16 years) were studied; 11 took CGMP-AA1, 18 CGMP-AA2, and 14 Phe-free AA. Early morning fasting pre and 2 h postprandial blood samples were collected for quantitative AA on one occasion. A breakfast with allocated 20 g protein equivalent from PS was given post fasting blood sample. Results: There was a significant increase in postprandial AA for all individual AAs with all three PS. Postprandial AA histidine ($p < 0.001$), leucine ($p < 0.001$), and tyrosine ($p < 0.001$) were higher in CGMP-AA2 than CGMP-AA1, and leucine ($p < 0.001$), threonine ($p < 0.001$), and tyrosine ($p = 0.003$) higher in GCMP-AA2 than Phe-free AA. This was reflective of the AA composition of the three different PS's. Conclusions: In PKU, the AA composition of CGMP-AA influences 2 h postprandial AA composition, suggesting that a PS derived from CGMP-AA may be absorbed similarly to Phe-free AA, but this requires further investigation.

Keywords: phenylketonuria; PKU; glycomacropeptide; amino acid; absorption

1. Introduction

Protein substitutes are an essential source of synthetic protein in the dietary treatment of classical phenylketonuria (PKU). Protein is the second major constituent in the body, critical for growth and supporting a wide range of metabolic and cellular functions. Amino acids (AA) are engaged in a dynamic process of protein synthesis and degradation. In PKU, it is critical that the AA profile of protein substitutes are carefully developed, with a balance of AAs that meet WHO 2007 [1] minimal AA requirements [2,3]. Furthermore, there is evidence that modification of the large neutral amino

acid (LNAA) profile (including tyrosine, leucine, isoleucine, methionine, valine, histidine, threonine and tryptophan) will enhance brain AA concentrations. In mice fed LNAA, an improvement in brain neurophysiology with increased brain serotonin and norepinephrine, and lower phenylalanine concentrations was observed [4]. It is also important that the composition and protein source of protein substitutes help support physiological AA absorption [5].

In PKU, protein substitutes are either manufactured using artificial AA without phenylalanine or based on caseinglycomacropeptide (CGMP), a by-product of the cheese making process [6]. Artificial AAs are generated from plant-based materials converted into sugars, fermented, and purified [7]. In contrast, CGMP modified for use in PKU is a mix of bioactive glycopeptides containing residual phenylalanine with the addition of essential and semi essential artificial AAs (CGMP-AA). Some authors [8,9] describe CGMP as an intact protein. However, proteins are large macromolecules made up of one or more polypeptide chains, whereas CGMP is a 64 macropeptide and therefore classification as an intact protein source is a misrepresentation [10]. Although there is knowledge about the absorption of AAs and intact protein, little is known about the absorption properties of CGMP-AA.

It is well recognized that the kinetic and biochemical properties of natural proteins change depending on the quality of protein, which is determined by its AA pattern, the speed of digestion, absorption and release of AAs into the circulation [11]. Protein metabolism has been extensively studied [12,13]. It is established that whey protein is rapidly absorbed, and the release of some individual AAs (leucine, isoleucine) influences anabolic and hormonal pathways [11,14,15]. In contrast, the appearance of plasma AAs following a meal with casein is slower, with protein synthesis increased and breakdown inhibited [11,16,17]. AAs are directly available for absorption by the small intestine, and so are quickly absorbed, potentially resulting in their transient imbalance, and altering their bioavailability. AA antagonism, the presence of high concentrations of specific AAs, may alter the AA equilibrium, impairing the absorption of other AAs and limiting absorption and metabolism [18]

In PKU, several authors [19–22] have demonstrated improved utilization of AAs when protein substitutes are taken in divided doses throughout the day. However, this still does not produce a normal physiological response; protein substitutes are usually taken as an addition and not as an integral part of a meal. In an animal study, the metabolic and biological influences of CGMP were compared with Phe-free AA, with CGMP giving a more physiological response decreasing metabolic stress and immunity [23]. In subjects with PKU, van Calcar et al. [24] suggested CGMP improved protein synthesis and nitrogen retention compared to Phe-free AA. However, peptides may be absorbed more rapidly than AA [14,25], thereby questioning the rate of delivery of AAs from CGMP-AA.

In this pilot, parallel study in children with PKU, we aimed to investigate if there were any differences following an overnight fast in the pre and postprandial AA absorption after taking one breakfast dose of Phe-free AA compared with two different CGMP-AA formulations with varying AA compositions.

2. Ethical Permission

The South Birmingham Research Ethics committee granted a favorable ethical opinion. The study was registered 13/WM/0435 IRAS (integrated research application system) 129497. Written informed consent was obtained for all subjects from at least one caregiver with parental responsibility and written assent obtained from the subject if appropriate for their age and level of understanding.

3. Materials and Methods

3.1. Inclusion Criteria

Entry into the study included: children diagnosed with PKU by newborn screening, aged 5–16 years and treated with diet only. Children had to be adherent with diet and protein substitute intake, with 70% of routine blood phenylalanine concentrations within European PKU Guideline target range [26] for

six months before study enrolment. Target blood phenylalanine ranges were 120 to 360 μmol/L for children aged 5 up to 12 years and 120 to 600 μmol/L for 12 years and older [26].

3.2. Study Design

Pre and postprandial AA absorption was measured on one occasion after six months of taking either Phe-free AA, or one of two CGMP formulations (CGM-AA1 or CGMP-AA2). Children attended the hospital after an overnight fast (minimal fasting time 10 h). CGMP-AA1 had been taken for six months by 11 children as part of a pilot study the results have previously been published [27]. Following the results of the pilot study, a further 18 children were recruited and took CGMP-AA2, which was a modification of CGMP-AA1. Nineteen children remained on Phe-free AA. All children had fasting capillary finger pricks (0.5 mL), for quantitative plasma AAs. Children then took 20 g protein equivalent from Phe-free AA, CGMP-AA1 or CGMP-AA2, followed by a breakfast providing less than one third of their phenylalanine/natural protein allowance (median 2 g natural protein (100 mg phenylalanine), range 1–6). After 120 min post protein substitute and breakfast, a second capillary sample was taken for AAs.

3.3. Protein Substitutes (PHE-FREE AA and CGMP1, CGMP2)

The AA profile and nutritional composition of the three different protein substitutes (provided by Vitaflo International) are given in Table 1. All the children in the Phe-free AA group took the same liquid pouch (PKU Cooler 20). For each 20 g protein equivalent, Phe-free AA provided 124 kcal, 9.4 g carbohydrate, and 0.7 g fat, and CGMP-AA1 and CGMP-AA2, 120 kcal, 6.5 g carbohydrate, and 1.5 g fat. CGMP-AA2 had increased amounts of tyrosine, leucine, histidine, and tryptophan, and less methionine, lysine, glycine, and aspartic acid than CGMP-AA1. Except for threonine (higher in CGMP-AA1 and 2), glycine and methionine (higher in CGMP-AA1), and leucine (higher in CGMP-AA2), all the other AAs were slightly but not significantly higher in the Phe-free AA. Glutamine was naturally present in CGMP-AA, but not added to Phe-free AA. The energy content of the three products was similar, although the carbohydrate content was 30% higher in the Phe-free AA, and fat content 53% higher in the two CGMP-AA products, but overall fat intake was low from all three protein substitutes.

The single dose of Phe-free AA, CGMP-AA1, and CGMP-AA2 given in this study provided 20 g protein equivalent. The CGMP-AA1 and CGMP-AA2 also provided an additional 36 mg phenylalanine for each 20 g protein equivalent. The children chose either Phe-free AA or CGMP-AA, depending on their taste preference.

Table 1. Nutritional composition of CGMP-AA 1, CGMP-AA2, and PHE-FREE AA protein substitutes.

Protein Substitute		**CGMP-AA1**	**CGMP-AA2**	**PHE-FREE AA**
Nutrients	**Units**	**Per 20 g PE Sachet**	**Per 20 g PE Sachet**	**Per 20 g PE Pouch**
Calories	Kcal	120	120	124
Protein equivalent	g	20	20	20
Total Carbohydrate	g	6.5	6.5	9.4
Sugars	g	2.2	2.2	7.8
Total Fat	g	1.5	1.5	0.7
Docosahexaenoic Acid	mg	84	84	134
Arachidonic Acid	mg	-	-	-
Fiber	g	0.1	0.1	-
Comprehensive amino acid profile				
L-amino acids		**CGMP-AA1**	**CGMP-AA2**	**PHE-FREE AA**
		20 g PE	**20 g PE**	**20 g PE**

Table 1. *Cont.*

L-Alanine	g	0.76	0.83	0.92
L-Arginine	g	1.00	0.96	1.5
L-Aspartic Acid	g	2.04	1.31	2.37
L-Cystine	g	0.01	0.24	0.61
L-Glutamine	g	2.49	2.70	-
Glycine	g	2.77	1.20	2.35
L-Histidine	g	0.42	0.70	0.92
L-Isoleucine	g	1.37	1.35	1.62
L-Leucine	g	1.30	3.00	2.54
L-Lysine	g	1.07	0.80	1.67
L-Methionine	g	0.54	0.28	0.45
L-Phenylalanine	g	0.03	0.03	-
L-Proline	g	1.51	1.52	1.69
L-Serine	g	0.98	0.96	1.04
L-Threonine	g	2.17	2.20	1.62
L-Tryptophan	g	0.17	0.40	0.5
L-Tyrosine	g	1.01	2.24	2.38
Taurine	g	-	-	0.04
L-Valine	g	1.13	1.09	1.86

CGMP-AA 1/CGMP-AA 2: casein glycomacropeptide formula 1 and 2; PHE-FREE AA: Phenylalanine-free L-amino acid (PKU Cooler 20, Vitaflo International).

3.4. Measurement of Quantitative Plasma Amino Acids

Capillary blood samples were collected into a Sarstedt tube and analyzed by ion exchange HPLC with postcolumn derivatization and spectrophotometric detection (Biochrom, Harvard Bioscience, Holliston, MA, USA). Prior to analysis, separated lithium-heparinized plasma was deproteinized 1:1 with 8% sulphosalicylic acid containing an internal standard, S-2-amino-ethyl-L-cysteine hydrochloride (Sigma, Merck, St. Louis, MO, USA). Quantitative amino acids (QAA) were analyzed except tryptophan and asparagine, which are not reported by our laboratory. Nonproteinogenic AA ornithine, citrulline, and taurine were included in the analysis. The individual pre- and postprandial AAs were quantitated (QAA) and the total AAs, total large neutral amino acids (LNAA), total essential amino acids (EAA), and total branched chain amino acids (BCAA) were calculated from these results. We report total AAs, LNAAs, BCAA, and EAAs, together with individual AAs.

3.5. Statistics

Descriptive statistics are reported as medians with associated interquartile ranges. Differences in AAs at baseline and follow-up are assessed using a paired *t*-test. Differences between the three treatment groups are performed using linear regression with differences at follow-up adjusting for baseline covariate values. All analyses are performed in the statistical package R (Version 3.3).

4. Results

4.1. Subjects

Forty-three (41 European and 2 Asian origin) children with PKU, with a median age of 9 years (range 5–16) were recruited and participated in the study. The number of children in each group was: Phe-free AA, n = 14 (8 boys and 6 girls), CGMP-AA1, n = 11 (5 boys and 6 girls), and CGMP-AA2, n = 18 (11 boys and 7 girls). The median age (range) in the groups were: CGMP-AA1, 8.3 years (6–16), CGMP-AA2, 8.4 years (5–14) and Phe-free AA, 12.9 years (5–15). There was significant difference in age between CGMP-AA2 and the Phe-free AA group ($p = 0.001$). The median phenylalanine concentration for 12 months pre-study (all the children were taking Phe-free-AA) was 288 μmol/L (140–600).

In all three groups, the median daily dose of protein equivalent from protein substitute was 60 g/day (range 40–80 g), and the median amount of prescribed natural protein was 5.5 g/day (range

3–30 g) or 275 mg phenylalanine (range 150–1500 mg). The majority had classical PKU, except two children who were mild according to their untreated blood phenylalanine levels at diagnosis and dietary phenylalanine tolerance.

4.2. Quantitative Plasma Amino Acid Results

Individual Amino Acids

Significant pre and postprandial differences for most individual AA were observed within each group (Table 2).

Preprandial valine was significantly lower with CGMP-AA2 than CGMP-AA1 ($p = 0.031$) and CGMP-AA2 vs. Phe-free AA ($p < 0.001$). For CGMP-AA1 vs. Phe-free AA, there were no significant preprandial changes.

Postprandial CGMP-AA1 vs. CGMP-AA2: histidine ($p < 0.001$), leucine ($p < 0.001$) and tyrosine ($p < 0.001$) were significantly higher for CGMP-AA2, while methionine ($p < 0.001$) significantly lower compared with CGMP-AA1.

Postprandial CGMP-AA1 vs. Phe-free AA: histidine ($p = 0.005$) and tyrosine ($p = 0.005$) were significantly higher in Phe-free AA, but isoleucine ($p = 0.008$), methionine ($p < 0.001$), threonine ($p = 0.001$) significantly lower. For CGMP-AA2 v Phe-free AA, leucine ($p < 0.001$), threonine ($p < 0.001$), and tyrosine ($p = 0.003$) were all significantly higher in CGMP-AA2.

Changes in the pre and postprandial AA concentrations between the groups appeared to be mainly a reflection of the different amino acid compositions of the three protein substitutes, being most evident between CGMP-AA1 and CGMP-AA2. CGMP-AA2 had higher amounts of histidine, leucine, and tyrosine, and lower methionine and valine compared to CGMP-AA1.

4.3. Total Amino Acids, LNAA, BCAA, and EAA

There were similar significant pre and postprandial changes within the groups for total AAs, LNAA, BCAA and EAA (Table 3(a–d), Figure 1a–d). No significant pre or postprandial changes were observed between any of the three groups when comparing total AA, LNAA, BCAA, or EAAs.

Table 2. Median (range) fasting pre and postprandial individual amino acids for CGPM-AA1, CGMP-AA2, and PHE-FREE AA protein substitutes.

Median L-Amino Acids µmol/L (range)	CGMP-AA1 (*n* = 11)		CGMP-AA 2 (*n* = 18)		PHE-FREE AA (*n* = 14)		*p* Value
	Pre-Prandial	Post-Prandial	Pre-Prandial	Post-Prandial	Pre-Prandial	Post-Prandial	
Alanine	320 ^ (171–425)	482 ^,** (266–685)	277 ^^,§ (182–566)	457 ^^ (304–701)	356 ^^^,§ (153–463)	412 ^^^,** (319–612)	^ 0.007, ^^ <0.0001, ^^^ <0.002 § 0.012, ** 0.036
Arginine	44 ^ (27–56)	77 ^ (28–138)	38 ^^ (14–56)	94 ^^ (23–130)	38 ^^^ (16–57)	74 ^^^ (33–168)	^ 0.003, ^^ <0.0001, ^^^ 0.0001
Aspartic acid	21 T (12–35)	19 (12–36)	17 T (11–28)	15 (10–31)	21 (6–31)	18 (11–42)	T 0.03
Citrulline	39 T (30–51)	39 (28–55)	29 T (23–46)	33 (24–49)	37 (16–48)	35 (17–47)	T 0.02
Cystine	36 ^,T (18–45)	28 ^ (12–44)	16 ^^,T,§ (4–35)	21 ^^,§§ (5–35)	30 § (19–57)	31 §§ (20–171)	^ 0.02, ^^ 0.001 T 0.003, § <0.001, §§ <0.001
Glutamine	495 ^ (406–542)	476 ^,TT (258–578)	472 ^^ (350–606)	513 ^^,TT (396–675)	461 (339–583)	455 (345–613)	^ 0.001, ^^ 0.001 TT 0.035
Glutamic acid	62 ^ (40–79)	71 ^ (45–104)	63 ^^ (31–133)	70 ^^ (41–155)	75 (22–110)	78 (33–111)	^ 0.04, ^^ 0.02
Glycine	323 ^ (227–429)	476 ^,TT,** (291–767)	306 ^^ (195–446)	342 ^^,TT (227–526)	303 ^^^ (189–380)	368 ^^^,** (199–642)	^ 0.003, ^^ <0.0001, ^^^ 0.004 TT <0.001, ** 0.001
Histidine	66 (56–83)	62 TT,** (39–83)	75 ^^ (62–119)	107 ^^,TT (70–151)	81 ^^^ (46–99)	99 ^^^,** (53–181)	^^ <0.0001, ^^^ 0.003 TT <0.001, ** 0.005
Isoleucine	51^ (39–71)	172 ^,** (97–270)	43 ^^ (34–62)	168 ^^ (96–230)	55 ^^^ (30–72)	130 ^^^,** (68–311)	^ 0.001, ^^ <0.0001, ^^^ 0.0001 ** 0.008
Leucine	92 ^ (73–116)	195 ^,TT (99–296)	87 ^^ (72–130)	356 ^^,TT,§§ (227–440)	108 ^^^ (66–127)	199 ^^^,§§ (108–474)	^ 0.001, ^^ <0.0001, ^^^ 0.0001 TT <0.001, §§ <0.001
Lysine	147 ^ (102–179)	210 ^ (128–314)	130 ^^,§ (94–172)	188 ^^ (96–332)	155 ^^^,§ (109–193)	216 ^^^ (148–385)	^ 0.002, ^^ <0.0001, ^^^ 0.0001 § 0.04
Methionine	20 ^ (13–23)	48 ^,TT,** (28–82)	18 ^^ (15–27)	30 ^^,TT (15–42)	19 ^^^ (14–27)	29 ^^^,** (19–52)	^ 0.001, ^^ <0.0001, ^^^ 0.002 TT <0.001, ** <0.001
Ornithine	66 ^ (53–114)	87 ^ (55–175)	72 ^^ (37–96)	91 ^^ (69–136)	83 ^^^ (37–99)	99 ^^^ (51–141)	^ 0.004, ^^ 0.0004, ^^^ 0.004

Table 2. *Cont.*

Median L-Amino Acids µmol/L (range)	CGMP-AA1 (*n* = 11)		CGMP-AA 2 (*n* = 18)		PHE-FREE AA (*n* = 14)		*p* Value
	Pre-Prandial	Post-Prandial	Pre-Prandial	Post-Prandial	Pre-Prandial	Post-Prandial	
Proline	116 $^{\wedge}$ (93–183)	282 $^{\wedge}$ (174–522)	106 $^{\wedge\wedge}$ (80–284)	275 $^{\wedge\wedge}$ (198–530)	141 $^{\wedge\wedge\wedge}$ (83–322)	258 $^{\wedge\wedge\wedge}$ (161–447)	$^{\wedge}$0.002, $^{\wedge\wedge}$ <0.0001, $^{\wedge\wedge\wedge}$ 0.0001
Serine	156 $^{\wedge}$ (123–227)	202 $^{\wedge}$ (131–321)	146 $^{\wedge\wedge}$ (119–196)	193 $^{\wedge\wedge}$ (119–251)	155 (71–228)	169 (132–325)	$^{\wedge}$ 0.008, $^{\wedge\wedge}$ <0.0001
Taurine	54 (35–73)	53 (36–326)	55 (26–92)	47 (33–74)	56 (39–98)	56 (47–100)	
Threonine	161 $^{\wedge}$ (90–241)	307 $^{\wedge,**}$ (200–614)	141 $^{\wedge\wedge}$ (80–215)	331 $^{\wedge\wedge,\S\S}$ (240–440)	129 $^{\wedge\wedge\wedge}$ (67–193)	214 $^{\wedge\wedge\wedge,**,\S\S}$ (122–358)	$^{\wedge}$ 0.002, $^{\wedge\wedge}$ <0.0001, $^{\wedge\wedge\wedge}$ 0.0001 ** 0.001, §§ <0.001
Tyrosine	37 $^{\wedge}$ (29–68)	80 $^{\wedge,TT,**}$ (34–149)	49 $^{\wedge\wedge}$ (36–84)	181 $^{\wedge\wedge,TT,\S\S}$ (126–327)	47 $^{\wedge\wedge\wedge}$ (31–73)	136 $^{\wedge\wedge\wedge,**,\S\S}$ (52–206)	$^{\wedge}$ 0.002, $^{\wedge\wedge}$ <0.0001, $^{\wedge\wedge\wedge}$ 0.0001 ^{TT} <0.001,** 0.005, §§ 0.003
Valine	214 $^{\wedge,T}$ (157–278)	345 $^{\wedge}$ (206–580)	168 $^{\wedge\wedge,\S,T}$ (131–243)	363 $^{\wedge\wedge}$ (248–538)	227 $^{\wedge\wedge\wedge,\S}$ (136–345)	407 $^{\wedge\wedge\wedge}$ (240–628)	$^{\wedge}$ 0.002, $^{\wedge\wedge}$ <0.0001,$^{\wedge\wedge\wedge}$ 0.0001 ^{T} 0.031, § <0.001

Key: $^{\wedge}$, $^{\wedge\wedge}$, $^{\wedge\wedge\wedge}$ significant differences within CGMP1,2 and Phe-free AA. Preprandial changes: T = CGMP1 v2, § = CGMP2 vPhe-free AA. Postprandial changes TT = CGMP1v2, ** = GGMP1 vs. Phe-free AA, §§ = CGMP2 vs. Phe free AA. *p* = significant value

Table 3. (**a**) Median pre and postprandial total amino acids results for CGMP-AA1, CGMP-AA2, and PHE-FREE AA protein substitutes. (**b**) Median pre and postprandial EAA for CGMP-AA1, CGMP-AA2, and PHE-FREE AA protein substitutes. (**c**) Median pre and postprandial BCAA for CGMP-AA1, CGMP-AA2, and PHE-FREE AA protein substitutes. (**d**) Median pre and postprandial LNAA for CGMP-AA1, CGMP-AA2, and PHE-FREE AA protein substitutes.

(**a**)							
	CGMP-AA1 *n*= 11		**CGMP-AA2 *n* = 18**		**PHE-FREE AA *n* = 14**		***p* Value**
Total AA µmol/L (range)	**Pre-Prandial**	**Post-Prandial**	**PRE-PRANDIAL**	**Pre-Prandial**	**Pre-Prandial**	**Pre-Prandial**	
	753 * (219–5257)	1473 * (237–5659)	1375 ** (317–8344)	3249 ** (291–9139)	1067 *** (336–8513)	1922 *** (396–9064)	* 0.0003, ** <0.0001, *** <0.0001
Amino acids: alanine, arginine, aspartic acid, cystine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, proline, serine, threonine, tyrosine, valine (excluding phenylalanine, tryptophan, citrulline)							
(**b**)							
EAA µmol/L (range)	**Pre-Prandial**	**Pre-Prandial**	**Pre-Prandial**	**Pre-Prandial**	**Pre-Prandial**	**Pre-Prandial**	
	92 * (20–214)	195 * (48–345)	87 ** (18–168)	188 ** (30–363)	108 *** (17–223)	199 *** (30–415)	* 0.03 ** 0.02 *** 0.02
Amino acids: histidine, isoleucine, leucine, lysine, methionine, threonine, valine (excluding phenylalanine, tryptophan)							
(**c**)							
BCAA µmol/L (range)	**Pre-Prandial**	**Pre-Prandial**	**Pre-Prandial**	**Pre-Prandial**	**Pre-Prandial**	**Pre-Prandial**	
	92 (51–214)	195 (172–345)	87 (43–168)	356 (168–363)	98 (46–223)	214 (131–415)	ns
Amino acids: isoleucine, leucine, valine							
(**d**)							
LNAA µmol/L (range)	**Pre-Prandial**	**Pre-Prandial**	**Pre-Prandial**	**Pre-Prandial**	**Pre-Prandial**	**Pre-Prandial**	
	66 * (20–214)	172 * (48–345)	75 ** (18–167)	180 ** (30–363)	67 *** (17–223)	138 *** (30–415)	* 0.03, ** 0.02, *** 0.02
Amino acids: histidine, isoleucine, leucine, methionine, threonine, tyrosine, valine							

CGMP-AA 1, CGMP-AA 2: caseinglycomacropeptide formula 1 and 2, PHE-FREE AA: Phenylalanine-free L-amino acids.*, **, *** *p* = significant value, ns not significant.

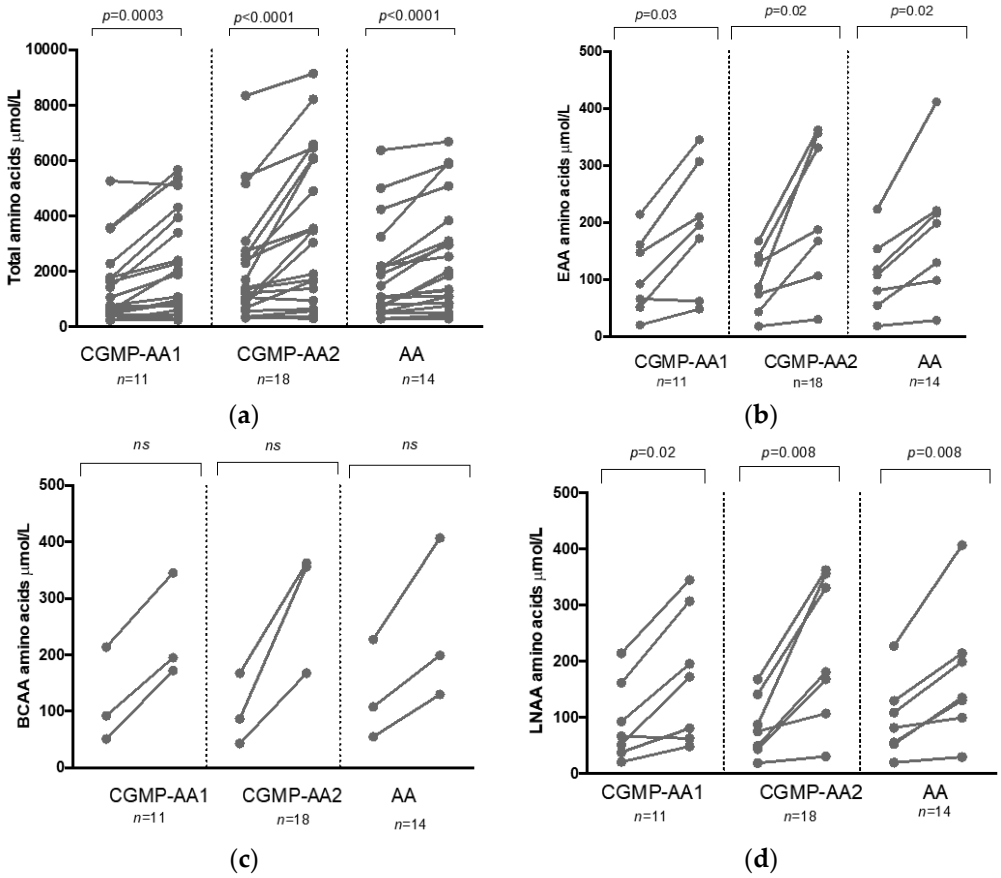


Figure 1. (**a**) Total median pre and postprandial amino acid concentrations for total amino acids (n = 17) for CGMP-AA1, CGMP-AA2, and PHE-FREE AA protein substitutes. (**b**). Total median pre and postprandial amino acid concentrations for EAA (n = 7) for CGMP-AA1, CGMP-AA 2, and PHE-FREE AA protein substitutes. (**c**) Total median pre and postprandial amino acid concentrations for BCAA (n = 3) for CGMP-AA1, CGMP-AA2, and PHE-FREE AA protein substitutes. (**d**) Total median pre and postprandial amino acid concentrations for LNAA (n = 8) for CGMP-AA1, CGMP-AA2, and PHE-FREE AA protein substitutes.

5. Discussion

This pilot study showed that that the postprandial AA concentrations largely reflected the AA profile of each of the protein substitutes used. CGMP-AA2 contained higher amounts of tyrosine, histidine, and leucine, and lower amounts of methionine and valine compared to CGMP-AA1. These changes were mirrored in the higher postprandial peaks of tyrosine, histidine, and leucine observed between CGMP-AA1 vs. CGMP-AA2. Although there was no postprandial change between the groups for valine, preprandial levels were lower between CGMP-AA1 vs. CGMP-AA2 and CGMP-AA2 vs. Phe-free AA. This is not easily explained physiologically, but may reflect a chance finding, or changes as a result of the competition between the AAs.

It is interesting to speculate on the postprandial changes, as it seems the more AA added to the protein substitute, the higher the AA concentrations were when measured at 120 min. The physiological consequence of these higher AAs is unknown. Postprandial tyrosine was significantly higher in CGMP-AA2 compared with CGMP-AA1 as a direct response of adding extra tyrosine. Norepinephrine is derived from tyrosine and is a principal brain neurotransmitter and so the provision of adequate tyrosine is essential to produce this monaminergic neurotransmitter, which is of clinical significance. Ney et al. [28] measured fasting tyrosine and tryptophan concentrations in subjects taking Phe-free AA compared to CGMP-AA and found their concentrations were 50% higher with Phe-free AA. Gut serotonin levels and microbiome-derived compounds made from tyrosine and tryptophan,

although not significantly different, were higher in the CGMP-AA group, suggesting an improved bioavailability of tyrosine and tryptophan [29].

In our study, although individual AAs changed significantly within groups, no significant differences were observed between groups for total AAs, LNAAs, BCAAs, and EAAs. After 120 min, AA concentrations had increased significantly above fasting levels with a 56% increase in CGMP-AA1, 73% increase in CGMP-AA2 and a 42% increase in the Phe-free AA group. The total AA concentration per 20 g protein equivalent for CGMP-AA1 was 21 g, CGMP-AA2, 22 g, and Phe-free AA 24 g. It seems unlikely that the peptide-based CGMP-AA offered any advantage in minimizing the kinetic release of AA, although postprandial bloods were not measured in the first hour post consumption.

This exploratory investigation was a crude assessment to explore if there were any kinetic differences between AAs and a peptide based CGMP-AA with a different AA profile. In a crossover study in eleven adults with PKU, MacLeod et al. [30] measured postprandial AAs after 180 min following a breakfast with Phe-free AA or CGMP-AA. CGMP-AA was consumed as GMP foods rather than drinks. In the CGMP-AA group, postprandial threonine and isoleucine were significantly higher, and total AA concentrations just reached a significant difference compared to the Phe-free AA group. The authors suggested that based on the higher concentrations of insulin and total plasma AAs in the GMP group, CGMP-AA had an improved AA absorption profile compared to Phe-free AA. Although the preprandial breakfast was isocaloric, the AA composition of both products was not stated and the protein substitute in the form of a food versus a liquid may alter the absorption of AAs. Similarly, a non-physiological response causing a rapid rise in insulin concentrations may not be ideal. What remains unknown both in this and our own study is at what point maximum and nadir concentrations for AAs were reached, and neither study measured concentrations over 240 min or used a whole protein source as a comparison from which maximum and minimum AA concentrations could be compared.

Ahring et al. [31] compared two groups of protein substitutes: group 1, CGMP only versus Phe-free AA (different protein sources but the same AA composition) and group 2, CGMP-AA (CGMP with added AAs) versus Phe-free AA, (different protein sources and the same AA composition). The AA profile was different between group 1 and 2. Measurements were made over 240 min. They reported no differences in the absorption of total AAs between the two groups, suggesting that CGMP made no impact on the absorption rate of AAs. However, some individual AAs changed significantly between the groups. For example, the tyrosine amounts in the different product consumed were markedly different; group 1, 0.05 g and group 2, 10.81 g. At 30 min post ingestion, plasma tyrosine concentrations in group 2 were double those in group 1, reflecting changes in the AA profile of the protein substitute rather than the source of protein.

Gropper et al. [32] and others [33–35] have demonstrated that the type and quality of protein influences kinetic absorption. Healthy volunteers ingested one of three protein sources: AAs, a mixture of 75% AA with 25% natural protein, or whole protein. After 150 min, the only AA profile significantly higher than baseline was the group ingesting whole protein. In the two other groups, peak AA concentrations were reached before this time point. These studies showed that peak AA concentrations from a Phe-free AA or Phe-free AA combined with an intact protein source were more rapidly absorbed compared with an intact protein source only.

Both the time of arrival and pattern of AAs in the systemic circulation are important for effective protein synthesis. For this to occur efficiently all essential AAs must be presented to the tissues in appropriate amounts simultaneously. In PKU, the delivery of AAs to tissues is accelerated compared to a diet based on mixed proteins [12]. Glutamine is the most abundant free AA in the body, with a wide range of diverse molecular actions [36]. Its primary source is skeletal muscle. Both BCAA and lysine, by different mechanisms, act as precursors for glutamine synthesis, and leucine can stimulate the release of glutamine and alanine from muscles. Threonine in high concentrations can decrease glutamine formation [37]. The importance of understanding the delivery of AAs from protein substitutes and

their effect on molecular pathways is crucial to long-term health outcomes for patients reliant on protein substitutes for their main source of nitrogen.

There are limitations to our findings. This was a pilot study with the aim to explore AA absorption from protein substitutes with different AA compositions. The liquid Phe-free AA preparation contained 30% more carbohydrate and 50% less fat than the CGMP-AA preparations. Breakfast provided similar food choices and the protein content was controlled, providing no more than 30% of their natural/phenylalanine daily allowance; however, we did not standardize the breakfast for all subjects, nor did we measure the AA concentrations every 30 min or over the course of 240 min as recommended by others [11,37], thereby missing the peak and baseline values. Similarly, we did not collect any other supporting data such as insulin and glucose concentrations to review the effect of insulinotropic AAs between the different protein sources. Our AA analysis did not measure tryptophan. We did not compare children with PKU with a control group taking a standard breakfast only without protein substitute.

6. Conclusions

In conclusion, the delivery, timing, and ratios of AAs are essential to maximize nitrogen utilization and biochemical functions. This pilot investigation compared three protein substitutes with different AA compositions and two different protein sources. It appeared that the AA composition rather than protein source was more important in determining postprandial plasma AAs. Further detailed work is needed to understand the kinetic and functional roles of protein substitute based on different protein sources and their metabolic impact

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References

1. World Health Organisation; Food and Agriculture Organization of the United Nations; United Nations University. Protein and Amino Acid Requirements in Human Nutition. In *Report of a Joint WHO/FAO/UNU Expert Consultation*; WHO Technical Report Series 935; WHO: Geneva, Switzerland, 2007; pp. 1–265.
2. Macdonald, A.; Daly, A.; Davies, P.; Asplin, D.; Hall, K.; Rylance, G.; Chakrapani, A. Protein substitutes for PKU: What's new? *J. Inherit. Metab. Dis.* **2004**, *27*, 363–371. [CrossRef] [PubMed]
3. Woolf, L.I.; Griffiths, R.; Moncrieff, A. Treatment of phenylketonuria with a diet low in phenylalanine. *Br. Med. J.* **1995**, *1*, 57–64. [CrossRef] [PubMed]
4. van Vliet, D.; Bruinenberg, V.M.; Mazzola, P.N.; van Faassen, M.H.; de Blaauw, P.; Kema, I.P.; Fokkema, M.R.H.; van Anholt, R.D.; van der Zee, E.A.; van Spronsen, F.J. Large Neutral Amino Acid Supplementation Exerts Its Effect through Three Synergistic Mechanisms: Proof of Principle in Phenylketonuria Mice. *PLoS ONE* **2015**, *10*, e0143833. [CrossRef] [PubMed]

5. MacDonald, A.; Singh, R.H.; Rocha, J.C.; van Spronsen, F.J. Optimising amino acid absorption: Essential to improve nitrogen balance and metabolic control in phenylketonuria. *Nutr. Res. Rev.* **2019**, *32*, 70–78. [CrossRef]
6. Neelima, S.R.; Rajput, Y.S.; Mann, B. Chemical and functional properties of glycomacropeptide (GMP) and its role in the detection of cheese whey adulteration in milk: A review. *Dairy Sci. Technol.* **2013**, *93*, 21–43. [CrossRef]
7. Usuda, Y.; Hara, Y.; Kojima, H. Toward Sustainable Amino Acid Production. *Adv. Biochem. Eng. Biotechnol.* **2017**, *159*, 289–304.
8. Macleod, E.L.; Ney, D.M. Nutritional Management of Phenylketonuria. *Ann. Nestle Eng.* **2010**, *68*, 58–69. [CrossRef] [PubMed]
9. Ney, D.M.; Gleason, S.T.; van Calcar, S.C.; MacLeod, E.L.; Nelson, K.L.; Etzel, M.R.; Rice, G.M.; Wolff, J.A. Nutritional management of PKU with glycomacropeptide from cheese whey. *J. Inherit. Metab. Dis.* **2009**, *32*, 32–39. [CrossRef]
10. O'Riordan, N.; Kane, M.; Joshi, L.; Hickey, R.M. Structural and functional characteristics of bovine milk protein glycosylation. *Glycobiology* **2014**, *24*, 220–236. [CrossRef]
11. Boirie, Y.; Dangin, M.; Gachon, P.; Vasson, M.P.; Maubois, J.L.; Beaufrere, B. Slow and fast dietary proteins differently modulate postprandial protein accretion. *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 14930–14935. [CrossRef]
12. Dangin, M.; Boirie, Y.; Garcia-Rodenas, C.; Gachon, P.; Fauquant, J.; Callier, P.; Ballevre, O.; Beufrere, B. The digestion rate of protein is an independent regulating factor of postprandial protein retention. *Am. J. Physiol. Endocrinol. Metab.* **2001**, *280*, E340–348. [CrossRef] [PubMed]
13. Fouillet, H.; Mariotti, F.; Gaudichon, C.; Bos, C.; Tome, D. Peripheral and splanchnic metabolism of dietary nitrogen are differently affected by the protein source in humans as assessed by compartmental modeling. *J. Nutr.* **2002**, *132*, 125–133. [CrossRef] [PubMed]
14. Gorissen, S.H.M.; Trommelen, J.; Kouw, I.W.K.; Holwerda, A.M.; Pennings, B.; Groen, B.B.L.; Wall, B.T.; Churchward, T.A.-V.; Horstman, A.M.H.; Koopman, R.; et al. Protein Type, Protein Dose, and Age Modulate Dietary Protein Digestion and Phenylalanine Absorption Kinetics and Plasma Phenylalanine Availability in Humans. *J. Nutr.* **2020**, *150*, 2041–2050. [CrossRef] [PubMed]
15. Park, S.; Church, D.D.; Azhar, G.; Schutzler, S.E.; Ferrando, A.A.; Wolfe, R.R. Anabolic response to essential amino acid plus whey protein composition is greater than whey protein alone in young healthy adults. *J. Int. Soc. Sports Nutr.* **2020**, *17*, 9. [CrossRef] [PubMed]
16. Luiking, Y.C.; Deutz, N.E.; Jakel, M.; Soeters, P.B. Casein and soy protein meals differentially affect whole-body and splanchnic protein metabolism in healthy humans. *J. Nutr.* **2005**, *135*, 1080–1087. [CrossRef] [PubMed]
17. Mulet-Cabero, A.I.; Torcello-Gomez, A.; Saha, S.; Mackie, A.R.; Wilde, P.J.; Brodkorb, A. Impact of caseins and whey proteins ratio and lipid content on in vitro digestion and ex vivo absorption. *Food Chem.* **2020**, *319*, 126514. [CrossRef]
18. Roth, E.; Druml, W. Plasma amino acid imbalance: Dangerous in chronic diseases? *Curr. Opin. Clin. Nutr. Metab. Care* **2011**, *14*, 67–74. [CrossRef]
19. Macdonald, A.; Rylance, G.; Davies, P.; Asplin, D.; Hall, S.K.; Booth, I.W. Administration of protein substitute and quality of control in phenylketonuria: A randomized study. *J. Inherit. Metab. Dis.* **2003**, *26*, 319–326. [CrossRef]
20. Mönch, E.; Herrmann, M.E.; Brösicke, H.; Schöffer, A.; Keller, M. Utilisation of amino acid mixtures in adolescents with phenylketonuria. *Eur. J. Nucl. Med. Mol. Imaging* **1996**, *155*, S115–S120. [CrossRef]
21. Weigel, C.; Rauh, M.; Kiener, C.; Rascher, W.; Knerr, I. Effects of Various Dietary Amino Acid Preparations for Phenylketonuric Patients on the Metabolic Profiles along with Postprandial Insulin and Ghrelin Responses. *Ann. Nutr. Metab.* **2007**, *51*, 352–358. [CrossRef]
22. Macdonald, A.; Rylance, G.; Hall, S.K.; Asplin, D.; Booth, I.W. Factors affecting the variation in plasma phenylalanine in patients with phenylketonuria on diet. *Arch. Dis. Child.* **1996**, *74*, 412–417. [CrossRef] [PubMed]
23. Solverson, P.; Murali, S.G.; Brinkman, A.S.; Nelson, D.W.; Clayton, M.K.; Yen, C.-L.E.; Ney, D.M. Glycomacropeptide, a low-phenylalanine protein isolated from cheese whey, supports growth and attenuates metabolic stress in the murine model of phenylketonuria. *Am. J. Physiol. Metab.* **2012**, *302*, E885–E895. [CrossRef] [PubMed]

24. Van Calcar, S.C.; MacLeod, E.L.; Gleason, S.T.; Etzel, M.R.; Clayton, M.K.; Wolff, J.; Ney, D.M. Improved nutritional management of phenylketonuria by using a diet containing glycomacropeptide compared with amino acids. *Am. J. Clin. Nutr.* **2009**, *89*, 1068–1077. [CrossRef] [PubMed]
25. Silk, D.B.A.; Fairclough, P.D.; Clark, M.L.; Hegarty, J.E.; Addison, J.M.; Burston, D.; Clegg, K.M.; Matthews, D.M. Use of a Peptide Rather Than Free Amino Acid Nitrogen Source in Chemically Defined "Elemental" Diets. *J. Parenter. Enter. Nutr.* **1980**, *4*, 548–553. [CrossRef] [PubMed]
26. Van Wegberg, A.M.J.; Macdonald, A.; Ahring, K.; Bélanger-Quintana, A.; Blau, N.; Bosch, A.M.; Burlina, A.B.; Campistol, J.; Feillet, F.; Gizewska, M.; et al. The complete European guidelines on phenylketonuria: Diagnosis and treatment. *Orphanet J. Rare Dis.* **2017**, *12*, 162. [CrossRef]
27. Daly, A.; Evans, S.; Chahal, S.; Santra, S.; Macdonald, A. Glycomacropeptide in children with phenylketonuria: Does its phenylalanine content affect blood phenylalanine control? *J. Hum. Nutr. Diet.* **2017**, *30*, 515–523. [CrossRef]
28. Ney, D.M.; Murali, S.G.; Stroup, B.M.; Nair, N.; Sawin, E.A.; Rohr, F.; Levy, H.L. Metabolomic changes demonstrate reduced bioavailability of tyrosine and altered metabolism of tryptophan via the kynurenine pathway with ingestion of medical foods in phenylketonuria. *Mol. Genet. Metab.* **2017**, *121*, 96–103. [CrossRef]
29. Pinto, A.; Almeida, M.F.; Ramos, P.C.; Rocha, S.; Guimas, A.; Ribeiro, R.; Martins, E.; Bandeira, A.; Macdonald, A.; Rocha, J.C. Nutritional status in patients with phenylketonuria using glycomacropeptide as their major protein source. *Eur. J. Clin. Nutr.* **2017**, *71*, 1230–1234. [CrossRef]
30. MacLeod, E.L.; Clayton, M.K.; Van Calcar, S.C.; Ney, D.M. Breakfast with glycomacropeptide compared with amino acids suppresses plasma ghrelin levels in individuals with phenylketonuria. *Mol. Genet. Metab.* **2010**, *100*, 303–308. [CrossRef]
31. Ahring, K.K.; Lund, A.M.; Jensen, E.; Jensen, T.G.; Brøndum-Nielsen, K.; Pedersen, M.; Bardow, A.; Holst, J.J.; Rehfeld, J.F.; Møller, L.B. Comparison of Glycomacropeptide with Phenylalanine Free-Synthetic Amino Acids in Test Meals to PKU Patients: No Significant Differences in Biomarkers, Including Plasma Phe Levels. *J. Nutr. Metab.* **2018**, *2018*, 6352919. [CrossRef]
32. Gropper, S.S.; Gropper, D.M.; Acosta, P.B. Plasma Amino Acid Response to Ingestion of L-Amino Acids and Whole Protein. *J. Pediatr. Gastroenterol. Nutr.* **1993**, *16*, 143–150. [CrossRef] [PubMed]
33. Adibi, S.A.; Morse, E.L. Intestinal transport of dipeptides in man: Relative importance of hydrolysis and intact absorption. *J. Clin. Investig.* **1971**, *50*, 2266–2275. [CrossRef] [PubMed]
34. Adibi, S.A.; Allen, E.R. Impaired Jejunal Absorption Rates of Essential Amino Acids Induced by Either Dietary Caloric or Protein Deprivation in Man. *Gastroenterol.* **1970**, *59*, 404–413. [CrossRef]
35. Marrs, T.C.; Addison, J.M.; Burston, D.; Matthews, D.M. Changes in plasma amino acid concentrations in man after ingestion of an amino acid mixture simulating casein, and a tryptic hydrolysate of casein. *Br. J. Nutr.* **1975**, *34*, 259–265. [CrossRef]
36. Curi, R.; Lagranha, C.; Doi, S.; Sellitti, D.; Procopio, J.; Pithon-Curi, T.; Corless, M.; Newsholme, P. Molecular mechanisms of glutamine action. *J. Cell. Physiol.* **2005**, *204*, 392–401. [CrossRef]
37. Nilsson, M.; Holst, J.J.; Björck, I.M. Metabolic effects of amino acid mixtures and whey protein in healthy subjects: Studies using glucose-equivalent drinks. *Am. J. Clin. Nutr.* **2007**, *85*, 996–1004. [CrossRef]



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CHAPTER 5A

PUBLICATION NUMBER 6

GROWTH AND BODY COMPOSITION IN PKU CHLDREN A 3 YEAR PROSPECTIVE STUDY COMPARING THE EFFECTS OF L-AMINO ACID TO GLYCOMACROPEPTIDE PROTEIN SUBSTITUTES

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Growth and Body Composition in PKU Children—A Three-Year Prospective Study Comparing the Effects of L-Amino Acid to Glycomacropeptide Protein Substitutes

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Abstract: Protein quality and quantity are important factors in determining lean body (muscle) mass (LBM). In phenylketonuria (PKU), protein substitutes provide most of the nitrogen, either as amino acids (AA) or glycomacropeptide with supplementary amino acids (CGMP-AA). Body composition and growth are important indicators of long-term health. In a 3-year prospective study comparing the impact of AA and CGMP-AA on body composition and growth in PKU, 48 children were recruited. *N* = 19 (median age 11.1 years, range 5–15 years) took AA only, *n* = 16 (median age 7.3 years, range 5–15 years) took a combination of CGMP-AA and AA, (CGMP50) and 13 children (median age 9.2 years, range 5–16 years) took CGMP-AA only (CGMP100). A dual energy X-ray absorptiometry (DXA) scan at enrolment and 36 months measured LBM, % body fat (%BF) and fat mass (FM). Height was measured at enrolment, 12, 24 and 36 months. No correlation or statistically significant differences (after adjusting for age, gender, puberty and phenylalanine blood concentrations) were found between the three groups for LBM, %BF, FM and height. The change in height z scores, (AA 0, CGMP50 +0.4 and CGMP100 +0.7) showed a trend that children in the CGMP100 group were taller, had improved LBM with decreased FM and % BF but this was not statistically significant. There appeared to be no advantage of CGMP-AA compared to AA on body composition after 3-years of follow-up. Although statistically significant differences were not reached, a trend towards improved body composition was observed with CGMP-AA when it provided the entire protein substitute requirement.

Keywords: phenylketonuria; body composition; glycomacropeptide; protein substitute

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1. Introduction

There are concerns about increasing obesity and consequential long-term comorbidities in both the general and phenylketonuria (PKU) populations [1–3]. A reliance on an

"artificial" diet may alter normal physiological processes such as the distribution of fat and lean mass, adversely affecting long-term health outcomes [4]. Body composition is a key component of health, and it typically refers to the quantification of body fat and muscle mass—changes that cannot be adequately assessed by body weight or body mass index (BMI) [5,6]. In PKU, reports of body composition are few and there are no long-term prospective studies or systematic/meta-analyses reviewing body composition. Therefore, it is difficult to extrapolate any association between body composition and other factors such as muscular fitness, adiposity and longer-term health outcomes.

In classical PKU, a low-phenylalanine diet requires substantial modification of usual dietary patterns. For most patients with PKU, high-biological-value proteins are excluded (e.g., meat, fish, eggs and dairy products), with low-phenylalanine/phenylalanine-free protein substitutes providing the principle source of obligatory nitrogen, which is essential to maintain metabolic control and enable optimal growth and lean body mass [7,8]. Muscle contributes up to 40–45% of body weight, and skeletal muscle is the largest store of peptides and free amino acids [9]. Reliance on "synthetic" nitrogen sources may compromise body composition; lean mass is dependent on amino acid availability and, compared to natural protein, delivery and utilisation of amino acids from protein substitutes is sub-optimal [10–12].

A consistent finding from a systematic meta-analysis in healthy subjects [13] is that lean mass is a strong predictor of bone mass. Lean mass, and therefore bone mass, particularly in teenagers with PKU, may be compromised as it coincides with a time when adherence with diet and protein substitutes is being challenged [14,15]. Additionally, the physiological increase in lean body mass and fat mass differs in adolescent boys and girls, further exacerbating the difficulties in body composition interpretation [16].

The synthetic protein given in PKU is usually derived from amino acids without phenylalanine (AA). More recently, casein glycomacropeptide (CGMP), a bioactive phosphoglycopeptide, has been used as an alternative low-phenylalanine protein substitute (CGMP-AA). It is associated with better palatability, so adherence is improved [17], but it is unknown if this bioactive macropeptide will alter body composition in PKU. The type of protein, its absorption and amino acid composition alters insulin and glucagon responses. Insulin stimulates protein synthesis [18,19], while glucagon increases amino acid catabolism. Some non-PKU studies have shown that nitrogen retention is improved when protein is in the form of oligopeptides compared to whole protein or amino acids [20–22]. It is possible that CGMP, a whey-derived macropeptide, may promote nitrogen retention, improving lean body mass synthesis [23] and growth potential in children with PKU.

This 3-year, prospective, longitudinal study in children with PKU aimed to compare the impact of two different sources of protein substitute, AA and low-phenylalanine CGMP-AA, on growth and body composition by comparing height, lean body mass and fat mass.

2. Materials and Methods

2.1. Methods

Children were included in the study if they were diagnosed with PKU by newborn screening, aged 5–16 years of age, on dietary treatment only and adherent with protein substitute, with 70% of routine blood phenylalanine concentrations within phenylalanine target range for 6 months before study enrolment. Target blood phenylalanine range for children aged 5–12 years was 120 to $\leq$ 360 µmol/L and for 13 years and older was 120 to < 600 µmol/L, as recommended by the European PKU guidelines [24].

CGMP-AA and AA Protein Substitutes

Two types of protein substitute were studied: AA and CGMP-AA. AA were either powders made up with water to a semi-solid consistency or ready-to-drink liquids providing 10, 15 or 20 g of protein equivalent, tailored to a child's protein requirements. The CGMP-AA powdered protein substitute (a test product via Vitaflo International Ltd. Liverpool, UK) contained 36 mg of phenylalanine for each 20 g protein equivalent and

was reconstituted by adding 120 mL of water. Both products had a similar energy profile per 20 g protein equivalent; CGMP-AA, 120 Kcal, 6.5 g carbohydrate and 1.5 g fat; AA, 124 Kcal, 9.4 g carbohydrate and 0.7 g fat. Threonine and leucine were higher in the CGMP-AA product.

2.2. Study Design

In this prospective, longitudinal, 3-year study, home visits were conducted 3 monthly collecting dietary information, weight and height. Dual energy X-ray absorptiometry scans (DXA) measured body composition at enrolment and at 36 months. At enrolment, all the children were on AA protein substitute and had a Tanner pubertal assessment. Following the DXA scan, the patients were divided into 3 subgroups:

(1) AA: protein substitute given as AA only;
(2) CGMP50: patients tolerating a combination of CGMP-AA and AA;
(3) CGMP100: patients tolerating all their protein substitute as CGMP-AA.

Due to the negative impact on blood phenylalanine control, only some children were able to meet their protein requirements using only CGMP-AA [25]. Therefore, in addition to the AA group, a third group (CGMP50) was introduced where a combination of CGMP-AA and AA provided approximately 50% of the protein equivalent intake.

2.2.1. Selection into CGMP-AA or AA Group

The children chose CGMP-AA or AA, depending on their taste preference. Those in the CGMP-AA group entered CGMP50 or CGMP100 groups depending on their phenylalanine blood concentrations.

2.2.2. Dual X-ray Absorptiometry (DXA)

A DXA scan of the total body to assess body composition (fat and lean body mass) was carried out by two trained operators, using a GE Lunar iDXA and Encore ^{TM} software version 13.1 (GE Healthcare, Wisconsin, MD, USA). Trunk thickness and body weight were utilised to ensure that each child was scanned in the most appropriate acquisition mode. Children lay supine on a bed, while the DXA scan was completed. At baseline and 36 months, the following parameters were measured: lean body mass (LBM) g, fat mass (FM) g, % body fat (%BF), weight (kg), height (cm) and body mass index (BMI) (kg/m^2). Daily quality assurance tests were performed according to the manufacturer's instructions. The precision of the instrument was calculated as 1.0% for fat and 0.5% for lean in normal-weight subjects.

2.2.3. Anthropometric Measurements

Weight and height were measured by one of two metabolic dietitians. Height was measured using a Harpenden stadiometer (Holtain Ltd., Crymych, UK) and weight on calibrated digital scales (Seca, Medical Measuring Systems and Scales, Birmingham, UK. Model 875); weight was measured to the nearest 0.1 g and height to the nearest 0.1 cm. Weight, height and BMI were analysed over four time points; baseline, 12, 24 and 36 months.

2.2.4. Blood Phenylalanine Levels

Throughout the study, trained caregivers collected weekly early morning fasted blood spots on filter cards, Perkin Elmer 226 (UK Standard NBS) at home. Blood specimens were sent via first class post to the laboratory at Birmingham Children's Hospital. All the cards had a standard thickness, and the blood phenylalanine concentrations were calculated on a 3.2 mm punch by MS/MS tandem mass spectrometry.

2.2.5. Pubertal Status

A general medical examination was conducted and pubertal status was measured at enrolment using the Tanner picture index [26]. Stage 1 and 2 were classified as pre-pubertal and stage 3, 4 and 5 as pubertal.

2.3. Statistical Analysis of Anthropometry and Body Composition

Continuous data are presented as medians with associated inter-quartile ranges (IQR); categorical data are presented as frequencies of counts with associated percentages. Outcome data were divided into anthropometric data, weight (kg), height (cm) and body mass index (BMI) (kg/m^2), which were measured as standardised scores, and body composition was measured as lean body mass g, % body fat (%BF) and fat mass g. Anthropometric and body composition data were compared with blood phenylalanine concentrations. Standardised height was represented as the change in height and height z scores at each time point relative to baseline. Given the number of patients and the difference in ages between the groups, analysis was performed using longitudinal regression, which adjusts for patient age. Although standardised measures implicitly account for patient age, it was retained as a covariate in the analysis to avoid any confounding due to age when comparing treatment groups.

Correlations between the outcome data were calculated using Pearson's correlation coefficient. Anthropometric data were analysed using longitudinal modelling techniques, including main effects for time and age and evaluating the effect of treatment within each time-point. Models were further adjusted for patient age, accounting for the differences in the enrolment age between the treatment groups. Body composition outcomes were measured at two time points; data were analysed using analysis of covariance (ANCOVA) techniques, analysing the 36-month data as the outcome and adjusting for the enrolment data, including patient age at enrolment as well as their pre-pubescent status and gender.

Power Calculation

This prospective intervention study took as a primary outcome measure a conservative difference between CGMP and AA groups using day-to-day blood phenylalanine concentrations from a previous study. Twenty children maintaining blood phenylalanine concentrations between 100 and 400 μmol/L would detect a 5% reduction in blood phenylalanine concentrations outside the expected target range, at a power of detection of 88% and at a significance level of $p = 0.05$. A minimum of 45 children was the target recruitment aim.

2.4. Ethical Permission

The South Birmingham Research Ethics committee granted a favourable ethical opinion, reference 13/WM/0435 and IRAS (integrated research application system) number 129497. Written informed consent was obtained for all subjects from at least one caregiver with parental responsibility and written assent obtained from the subject if appropriate for their age and level of understanding.

3. Results

3.1. Subjects

Fifty children (28 boys, 22 girls) with PKU were recruited. Forty-seven children were European and three were of Asian origin. Forty-eight completed the study: 29 in the CGMP-AA group and 19 in the AA group. A significant difference in age was noted between the AA and CGMP50 groups ($p = 0.005$) and between the CGMP50 and CGMP100 groups ($p = 0.04$) (Table 1).

Table 1. Subject characteristics at recruitment.

	AA	CGMP50	CGMP100
Number recruited	*n* = 19	*n* = 16	*n* = 13
Girls	*n* = 8	*n* = 8	*n* = 5
Boys	*n* = 11	*n* = 8	*n* = 8
Median age y (range)	11.1 (5–15)	7.3 (5–15)	9.2 (5–16)
% of children prepubertal (stage 1 and 2)	32%	69%	62%
Girls	*n* = 2	*n* = 6	*n* = 5
Boys	*n* = 4	*n* = 5	*n* = 3
% of children pubertal (stage 3 to 5)	68%	31%	38%
Girls	*n* = 6	*n* = 2	*n* = 0
Boys	*n* = 7	*n* = 3	*n* = 5

AA, amino acid; CGMP, glycomacropeptide; CGMP50, protein substitute based on combination of CGMP and AA; CGMP100, protein substitute based on CGMP only.

3.2. Pubertal Status

Pre-pubertal status (stage 1 and 2): 32% (*n* = 6/19) were pre-pubertal in the AA group, 69% (*n* = 11/16) in the CGMP50 group and 62% (*n* = 8/13) in the CGMP100 group.

Late puberty (stage 3 to 5): 68% (*n* = 13/19) in the AA group, 31% (*n* = 5/16) in the CGMP50 group and 38% (*n* = 5/13) in the CGMP100 group.

All had classical PKU, except two with mild PKU based on untreated blood phenylalanine levels at diagnosis and dietary phenylalanine tolerance.

3.3. Subject Withdrawal

One boy and one girl (aged 12 and 11 years, respectively) in the CGMP-AA group were excluded from the study, as both were unable to adhere to the study protocol. One failed to return blood phenylalanine samples and both had poor adherence to their phenylalanine-restricted diet.

3.4. Protein Substitutes and Phenylalanine Concentrations

We have previously reported the types/manufacturers of protein substitutes taken by the AA, CGMP50 and CGMP100 groups. Similarly, the median phenylalanine concentrations have been reported at baseline and year 3 [27]. Median phenylalanine concentrations were within recommended target reference ranges for children aged $\leq$ 11 and $\geq$ 12 years old [24].

The median daily dose of protein equivalent from protein substitute was 60 g/day (range 40–80 g), and the median amount of prescribed natural protein was 5.5 g protein/day (range 3–30 g) or 275 mg/day of phenylalanine (range 150–1500 mg) in all groups.

3.5. Body Composition Lean Mass, Fat Mass and % Body Fat

Body composition was analysed using ANCOVA, adjusting for patient age, gender, phenylalanine concentration and pre-pubescent status (Table 2). No statistically significant differences were found between the three treatment groups for lean body mass, %BF or fat mass. All parameters increased over the 3-year study period.

3.6. Lean Body Mass, % Body Fat and Fat Mass

ANCOVA showed no significant differences in lean body mass, fat mass or % body fat between the treatment groups, although a trend for improved lean body mass, fat mass and % body fat was observed in the CGMP100 group.

Table 2. Median (range) lean mass, fat mass and % body fat in the AA, CGMP50 and CGMP 100 groups at enrolment and 36 months.

Body Composition	Time of Assessment	AA (Range) *n* = 19	GMP50 (Range) *n* = 13	GMP100 (Range) *n* = 16
Lean mass (g)	Enrolment	26,702 (16,920–34,209)	16,334 (14,280–17,686)	20,060 (16,451–21,947)
	36 m	32,560 (25,893–40,511)	23,921 (22,725–26,477)	31,268 (25,561–35,875)
Delta		5858 (8973–6302)	7587 (8445–8791)	11,208 (9110–13,928)
Fat mass (g)	Enrolment	9528 (6961–15,018)	5764 (4504–6758)	6688 (5057–8811)
	36 m	17,216 (10,930–20,687)	12,945 (10,678–16,519)	12,220 (8347–13,101)
Delta		7688 (3969–5669)	7181 (6174–9761)	5532 (3290–4290)
% body fat	Enrolment	29 (23–36)	24 (22–28)	25 (19–30)
	36 m	35 (25–39)	33 (30–36)	28 (20–33)
Delta		6	9	3

AA, amino acid; CGMP, glycomacropeptide; CGMP50, protein substitute based on combination of CGMP and AA; CGMP100, protein substitute based on CGMP only; g, grams; kg, kilograms.

3.7. Changes in Height Z Scores

Accounting for the age and gender differences, there were no statistically significant differences for height within or between the groups. At the end of the 3-year study, all groups had a positive height z score. We have previously reported weight and BMI z scores over the 3 year period [27], showing no statistical differences between the groups (Tables 3 and 4, Figure 1).

Table 3. Median z scores (range) for height in AA, CGMP50 and CGMP100 groups measured annually from enrolment to 36 months in PKU children.

Time (Months)	AA Height z Score *n* = 19	CGMP50 Height z Score *n* = 16	CGMP100 Height z Score *n* = 13
Enrolment (range)	0.2 (−0.2 to 0.8)	−0.1 (−0.6 to 0.6)	−0.1 (−0.4 to 0.3)
12 months (range)	0.2 (−0.2 to 0.6)	0.1 (−0.4 to 0.5)	0.1 (−0.1 to 0.3)
24 months (range)	0.2 (−0.1 to 0.5)	0.2 (−0.2 to 0.5)	0.4 (0.0 to 0.7)
36 months (range)	0.2 (0.0 to 0.5)	0.3 (−0.1 to 0.7)	0.6 (0.1 to 0.7)
Delta height z score	0	+0.4	+0.7

AA, amino acid; CGMP, glycomacropeptide; CGMP50, protein substitute based on combined CGMP and AA; CGMP100, protein substitute based on CGMP only.

Statistical modelling showed a trend in the CGMP100 group towards improved growth and a reduction in total body fat percentage and improved lean body mass. Analysis of the delta change in height was divided by age for those $\leq$ 10 and $\geq$ 11 years.

Table 4. ANOVA regression model showing values for age, treatment (AA, CGMP50, CGMP100) and treatment/time.

	Df	Sum Sq	Mean Sq	*F* Value	Pr (>F)
Age	1	0.015	0.015	0.020	0.887
Treatment	2	1.225	0.613	0.843	0.432
Treatment/time	3	2.081	0.694	0.954	0.415
Residuals	185	134.418	0.727		

1 = AA, 2 = CGMP50, 3 = CGMP100.

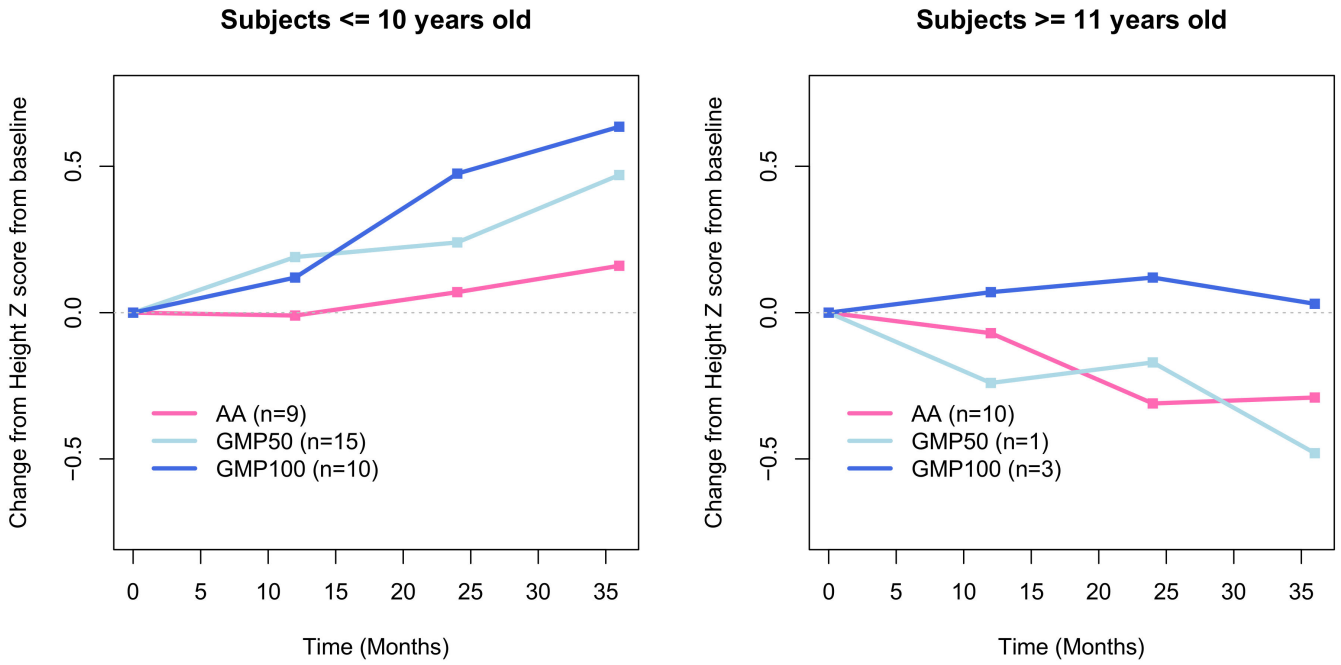


Figure 1. Change in median height z score from baseline to 36 months in the amino acids (AA), GCMP100 and CGMP 50 groups. Legend: AA, amino acid; CGMP, glycomacropeptide; CGMP50, protein substitutes based on combined CGMP and AA; CGMP100, protein substitute based on CGMP only.

4. Discussion

Body composition and height over 36 months in a group of children with PKU taking CGMP or AA protein substitutes showed no statistically significant changes in any of the measured parameters. However, in the CGMP100 group, statistical modelling indicated a trend ($p = 0.42$) towards improved longitudinal growth, a reduction in fat mass and % body fat and improved lean body mass. When growth was represented as a median change from baseline over time, it showed that the CGMP100 group had the greatest change in height. However, age modified this trend and, although the CGMP100 group continued to show improved height growth, it did not reach significance.

We can only speculate about a suggested trend in improved body composition when taking CGMP as the complete source of protein substitute. One possible explanation is the bioactivity of CGMP; it is rich in the branched chain amino acids isoleucine and leucine, which are potent modulators of protein turnover and have been shown to have a significant effect on insulin and glucose metabolism [28,29]. If CGMP, by the action of these amino acids, improves insulin sensitivity, it is possible that growth may be improved. However, we did not collect any information on insulin resistance in this group of subjects, so it is difficult to draw any firm conclusions.

Huemer et al. [30] measured growth and body composition over 12 months in 34 children with classical PKU. Total protein intake was 124% of the German recommended daily allowance. A significant correlation was found between lean body mass and intake of natural protein, suggesting that improved natural protein intake was beneficial. Evans et al. [31] also reported a similar significant relationship between a lower % fat mass and a higher total, natural and protein substitute intake, with natural protein > 0.5 g/kg/day associated with an improved body composition; no relationship was found between natural protein intake and improved height z scores. Evans, similarly, to Hoeksma et al. [32], observed that neither natural protein nor energy intake correlated with linear growth, as reported by Aldamiz Echevarria et al. [33]. The effect of a low-protein diet on energy balance and postprandial fat oxidation has received little attention in subjects with PKU. A study by Alfheeaid et al. [34] reported a lower thermal effect of feeding and fat oxidation after healthy subjects had taken a meal containing special low-protein foods and protein substitutes, possibly leading to a higher fat mass and altered body composition. Patients with milder PKU, responsive to sapropterin dihydrochloride (BH4), have a higher natural protein tolerance but there appeared to be no advantage in height, weight, body mass index or growth velocity when BH4 was compared to conventional PKU therapy [35]. There are no studies reporting body composition in BH4-responsive patients who use fewer special low-protein food products and have a wider range of natural protein sources compared to the classically treated patients with PKU.

The importance of adequate protein intake from protein substitutes (both quality and quantity) in patients with PKU has been documented by many authors [36–41]. No studies have identified the protein digestibility score or absorption kinetics of CGMP protein substitutes; this is important to ascertain protein efficiency. In healthy adults, protein-containing meals taken at regular intervals improve skeletal muscle protein by 25%, reinforcing the need to consume protein substitutes in divided doses [42–44]. The optimal amount of protein substitute based on free amino acids or CGMP remains undefined, but any factor leading to protein inefficiency may compromise body composition and optimal height and increase the incidence of overweight. Another confounding factor that may affect body composition is the effect of a long-term low-phenylalanine diet higher in carbohydrates, which may be associated with a higher risk of adiposity and insulin resistance [45,46]. All of these factors may lead to under achievement of optimal growth potential in children with PKU [47].

Comparison of body composition by gender, regardless of group, showed that lean body mass was statistically significantly higher in males than females, consistent with reports in the literature ($p = 0.013$) [48]. Fat mass and lean body mass vary with age, gender and pubertal status. Various authors have reported an age-related increase in lean body mass index being more rapid in males compared with females, particularly between the ages of 11 and 16 years, which is in line with the rapid accrual of lean body mass during male puberty [49]. Children gain lean mass disproportionately to height and this is more pronounced in boys compared to girls [48].

A multitude of methods exist for assessing body composition, including DXA, bioelectrical impedance (BIA) and whole-body air displacement plethysmograph (Bodpod), each having their own assumptions, advantages and inadequacies [50]. Unfortunately, there is a lack of standardised reference data, making interpretation and comparison of results challenging. Sensitive and accurate measurements are needed to detect differences in visceral, compared to central, fat accumulation, as ponderal and body mass index alone are unable to detect subtle differences. The gold standard for measuring body composition is a four-compartment (4C) model [51]. DXA has been evaluated against 4C models in children, and although it overestimates body fat by 1–4% depending on age, sex and body size, the correlation compared to a 4C model is good despite the small error [52,53]. Reference data for comparison of body composition parameters are limited; a recent publication by Ofenheimer [54] has produced age and gender specific reference percentiles of body composition parameters for European children and adolescents. Comparison of our data

would indicate appropriate body composition for fat and lean body mass when calculated as a median between baseline and 36 months.

There are limitations to this study: we did not have a healthy reference control group or UK-based reference data to compare body composition parameters, an inherent problem when using the DXA for body composition analysis. Endocrine parameters such as bone age or growth hormone were not measured, but they may have explained differences in linear growth. Until kinetic studies are conducted, it is unknown if a peptide compared to amino acids alters the delivery and assimilation of amino acids, leading to improved lean body mass and growth. We did not collect parental height data, which may have been useful as a comparison within the groups. Not all the children were able to completely replace their full amino acid requirement with CGMP-AA, which may have reduced the strength of our findings. There were small numbers of children in each study group. More older children chose to stay on their AA supplement compared to the younger age group, who were more willing to try an alternative protein substitute, which may have led to some bias.

5. Conclusions

In this 3-year longitudinal study, we found no noticeable differences in body composition between the groups taking CGMP-AA and AA. However, there was a trend towards improved body composition in the group taking all of their protein substitute as CGMP-AA. This may suggest that CGMP does confer some biological benefit. Proof of concept will only be possible via larger controlled studies and over a longer duration throughout childhood.

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Informed Consent Statement: Informed consent was obtained for all subjects involved in the study.

Data Availability Statement: Data available at the request of the corresponding author.

Conflicts of Interest: A.D., research funding from Vitaflo International, financial support from Nutricia, Mevalia & Vitaflo International to attend study days and conferences. S.E., research funding from Nutricia, financial support from Nutricia & Vitaflo International to attend study days and conferences. A.P. has received an educational grant from Cambrooke Therapeutics and grants from Vitaflo International, Nutricia, Merck Serono, Biomarin and Mevalia to attend scientific meetings. C.A. received honoraria from Nutricia and Vitaflo International to attend study days and conferences. J.C.R. is a member of the European Nutritionist Expert Panel (Biomarin), the Advisory Board for Applied Pharma Research and Nutricia, and received honoraria as a speaker from APR, Merck Serono, Biomarin, Nutricia, Vitaflo, Cambrooke, PIAM and Lifediet. A.M. received research funding and honoraria from Nutricia, Vitaflo International & Merck Serono, and is a member of the European Nutrition Expert Panel (Merck Serono International), the Sapropterin Advisory Board (Merck Serono international) and the Advisory Board Element (Danone-Nutricia). The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Abdullah, A.; Peeters, A.; de Courten, M.; Stoelwinder, J. The magnitude of association between overweight and obesity and the risk of diabetes: A meta-analysis of prospective cohort studies. *Diabetes Res. Clin. Pract.* **2010**, *89*, 309–319. [CrossRef] [PubMed]
2. Moreno, L.A.; Pineda, I.; Rodriguez, G.; Fleta, J.; Giner, A.; Juste, M.G.; Sarria, A.; Bueno, M. Leptin and metabolic syndrome in obese and non-obese children. *Horm. Metab. Res.* **2002**, *34*, 394–399. [CrossRef]
3. Sarria, A.; Moreno, L.A.; Garcí-LIop, L.A.; Fleta, J.; Morellon, M.P.; Bueno, M. Body mass index, triceps skinfold and waist circumference in screening for adiposity in male children and adolescents. *Acta Paediatr.* **2001**, *90*, 387–392. [CrossRef]
4. Kelly, D.A. Nutritional factors affecting growth before and after liver transplantation. *Pediatr. Transpl.* **1997**, *1*, 80–84.
5. Cole, T.J.; Freeman, J.V.; Preece, M.A. Body mass index reference curves for the UK, 1990. *Arch. Dis. Child.* **1995**, *73*, 25–29. [CrossRef]
6. Javed, A.; Jumean, M.; Murad, M.H.; Okorodudu, D.; Kumar, S.; Somers, V.K.; Sochor, O.; Lopez-Jimenez, F. Diagnostic performance of body mass index to identify obesity as defined by body adiposity in children and adolescents: A systematic review and meta-analysis. *Pediatr. Obes.* **2015**, *10*, 234–244. [CrossRef]
7. Dokoupil, K.; Gokmen-Ozel, H.; Lammardo, A.M.; Motzfeldt, K.; Robert, M.; Rocha, J.C.; van Rijn, M.; Ahring, K.; Bélanger-Quintana, A.; MacDonald, A.; et al. Optimising growth in phenylketonuria: Current state of the clinical evidence base. *Clin. Nutr.* **2012**, *31*, 16–21. [CrossRef]
8. MacDonald, A.; Chakrapani, A.; Hendriksz, C.; Daly, A.; Davies, P.; Asplin, D.; Hall, K.; Booth, I.W. Protein substitute dosage in PKU: How much do young patients need? *Arch. Dis. Child.* **2006**, *91*, 588–593. [CrossRef]
9. Wu, G. Dietary protein intake and human health. *Food Funct.* **2016**, *7*, 1251–1265. [CrossRef]
10. Gropper, S.S.; Acosta, P.B. Effect of simultaneous ingestion of L-amino acids and whole protein on plasma amino acid and urea nitrogen concentrations in humans. *J. Parenter. Enteral Nutr.* **1991**, *15*, 48–53. [CrossRef]
11. Gropper, S.S.; Gropper, D.M.; Acosta, P.B. Plasma amino acid response to ingestion of L-amino acids and whole protein. *J. Pediatr. Gastroenterol. Nutr.* **1993**, *16*, 143–150. [CrossRef]
12. Monch, E.; Herrmann, M.E.; Brösicke, H.; Schöffer, A.; Keller, M. Utilisation of amino acid mixtures in adolescents with phenylketonuria. *Eur. J. Pediatr.* **1996**, *155*, S115–S120. [CrossRef]
13. Smith, J.J.; Eather, N.; Morgan, P.J.; Plotnikoff, R.C.; Faigenbaum, A.D.; Lubans, D.R. The health benefits of muscular fitness for children and adolescents: A systematic review and meta-analysis. *Sports Med.* **2014**, *44*, 1209–1223. [CrossRef] [PubMed]
14. MacDonald, A.; Van Rijn, M.; Feillet, F.; Lund, A.M.; Bernstein, L.; Bosch, A.M.; Gizewska, M.; Van Spronsen, F.J. Adherence issues in inherited metabolic disorders treated by low natural protein diets. *Ann. Nutr. Metab.* **2012**, *61*, 289–295. [CrossRef] [PubMed]
15. Walter, J.H.; White, F.J.; Hall, S.K.; MacDonald, A.; Rylance, G.; Boneh, A.; Francis, D.E.; Shortland, G.J.; Schmidt, M.; Vail, A.; et al. How practical are recommendations for dietary control in phenylketonuria? *Lancet* **2002**, *360*, 55–57. [CrossRef]
16. Kleinman, R.E.; Greer, F.R. *Pediatric Nutrition*; American Academy of Pediatrics: Elk Grove Village, IL, USA, 2019; p. 1731.
17. Lim, K.; van Calcar, S.C.; Nelson, K.L.; Gleason, S.T.; Ney, D.M. Acceptable low-phenylalanine foods and beverages can be made with glycomacropeptide from cheese whey for individuals with PKU. *Mol. Genet. Metab.* **2007**, *92*, 176–178. [CrossRef] [PubMed]
18. Boucher, J.; Softic, S.; El Ouaamari, A.; Krumpoch, M.T.; Kleinridders, A.; Kulkarni, R.N.; O'Neill, B.T.; Kahn, C.R. Differential Roles of Insulin and IGF-1 Receptors in Adipose Tissue Development and Function. *Diabetes.* **2016**, *65*, 2201–2213. [CrossRef]
19. James, H.A.; O'Neill, B.T.; Nair, K.S. Insulin Regulation of Proteostasis and Clinical Implications. *Cell Metab.* **2017**, *26*, 310–323. [CrossRef] [PubMed]
20. Adibi, S.A. Intestinal transport of dipeptides in man: Relative importance of hydrolysis and intact absorption. *J. Clin. Investig.* **1971**, *50*, 2266–2275. [CrossRef]
21. Akhavan, T.; Luhovyy, B.L.; Brown, P.H.; Cho, C.E.; Anderson, G.H. Effect of premeal consumption of whey protein and its hydrolysate on food intake and postmeal glycemia and insulin responses in young adults. *Am. J. Clin. Nutr.* **2010**, *91*, 966–975. [CrossRef]
22. Silk, D.B.; Fairclough, P.D.; Clark, M.L.; Hegarty, J.E.; Addison, J.M.; Burston, D.; Clegg, K.M.; Matthews, D.M. Use of a peptide rather than free amino acid nitrogen source in chemically defined "elemental" diets. *JPEN J. Parenter Enteral Nutr.* **1980**, *4*, 548–553. [CrossRef]
23. Van Calcar, S.C.; MacLeod, E.L.; Gleason, S.T.; Etzel, M.R.; Clayton, M.K.; Wolff, J.A.; Ney, D.M. Improved nutritional management of phenylketonuria by using a diet containing glycomacropeptide compared with amino acids. *Am. J. Clin. Nutr.* **2009**, *89*, 1068–1077. [CrossRef] [PubMed]
24. Van Wegberg, A.M.J.; MacDonald, A.; Ahring, K.; Bélanger-Quintana, A.; Blau, N.; Bosch, A.M.; Burlina, A.; Campistol, J.; Feillet, F.; Giżewska, M.; et al. The complete European guidelines on phenylketonuria: Diagnosis and treatment. *Orphanet J. Rare Dis.* **2017**, *12*, 1–56. [CrossRef] [PubMed]
25. Daly, A.; Evans, S.; Chahal, S.; Santra, S.; Macdonald, A. Glycomacropeptide in children with phenylketonuria: Does its phenylalanine content affect blood phenylalanine control? *J. Hum. Nutr. Diet.* **2017**, *30*, 515–523. [CrossRef] [PubMed]
26. Tanner, J.M.; Whitehouse, R.H. Clinical longitudinal standards for height, weight, height velocity, weight velocity, and stages of puberty. *Arch. Dis. Child.* **1976**, *51*, 170–179. [CrossRef] [PubMed]
27. Daly, A.; Evans, S.; Pinto, A.; Jackson, R.; Ashmore, C.; Rocha, J.C.; Macdonald, A. The Impact of the Use of Glycomacropeptide on Satiety and Dietary Intake in Phenylketonuria. *Nutrients* **2020**, *12*, 2704. [CrossRef] [PubMed]

28. Bifari, F.; Nisoli, E. Branched-chain amino acids differently modulate catabolic and anabolic states in mammals: A pharmacological point of view. *Br. J. Pharmacol.* **2017**, *174*, 1366–1377. [CrossRef] [PubMed]
29. Lynch, C.J.; Adams, S.H. Branched-chain amino acids in metabolic signalling and insulin resistance. *Nat. Rev. Endocrinol.* **2014**, *10*, 723–736. [CrossRef]
30. Huemer, M.; Huemer, C.; Möslinger, D.; Huter, D.; Stöckler-Ipsiroglu, S. Growth and body composition in children with classical phenylketonuria: Results in 34 patients and review of the literature. *J. Inherit. Metab. Dis.* **2007**, *30*, 694–699. [CrossRef]
31. Evans, M.; Truby, H.; Boneh, A. The relationship between dietary intake, growth and body composition in Phenylketonuria. *Mol. Genet. Metab.* **2017**, *122*, 36–42. [CrossRef] [PubMed]
32. Hoeksma, M.; Van Rijn, M.; Verkerk, P.H.; Bosch, A.M.; Mulder, M.F.; De Klerk, J.B.C.; De Koning, T.J.; Rubio-Gozalbo, E.; De Vries, M.; Sauer, P.J.J.; et al. The intake of total protein, natural protein and protein substitute and growth of height and head circumference in Dutch infants with phenylketonuria. *J. Inherit. Metab. Dis.* **2005**, *28*, 845–854. [CrossRef]
33. Aldámiz-Echevarría, L.; Bueno, M.A.; Couce, M.L.; Lage, S.; Dalmau, J.; Vitoria, I.; Andrade, F.; Blasco-Alonso, J.; Alcalde, C.; Gil, D.; et al. Anthropometric characteristics and nutrition in a cohort of PAH-deficient patients. *Clin. Nutr.* **2014**, *33*, 702–717. [CrossRef]
34. Alfheeaid, H.; Gerasimidis, K.; Năstase, A.-M.; Elhauge, M.; Cochrane, B.; Malkova, D.; Gerasimidis, K. Impact of phenylketonuria type meal on appetite, thermic effect of feeding and postprandial fat oxidation. *Clin. Nutr.* **2018**, *37*, 851–857. [CrossRef] [PubMed]
35. Aldámiz-Echevarría, L.; Bueno, M.A.; Couce, M.L.; Lage, S.; Dalmau, J.; Vitoria, I.; Andrade, F.; Llarena, M.; Blasco-Alonso, J.; Alcalde, C.; et al. Tetrahydrobiopterin therapy vs phenylalanine-restricted diet: Impact on growth in PKU. *Mol. Genet. Metab.* **2013**, *109*, 331–338. [CrossRef]
36. Acosta, P.B.; Yannicelli, S. Protein intake affects phenylalanine requirements and growth of infants with phenylketonuria. *Acta Paediatr.* **1994**, *407*, 66–67. [CrossRef] [PubMed]
37. Acosta, P.B.; Yannicelli, S.; Singh, R.; Mofidi, S.; Steiner, R.; DeVincentis, E.; Jurecki, E.; Bernstein, L.; Gleason, S.; Chetty, M.; et al. Nutrient intakes and physical growth of children with phenylketonuria undergoing nutrition therapy. *J. Am. Diet. Assoc.* **2003**, *103*, 1167–1173. [CrossRef]
38. Macdonald, A.; Rylance, G.; Davies, P.; Asplin, D.; Hall, S.K.; Booth, I.W. Administration of protein substitute and quality of control in phenylketonuria: A randomized study. *J. Inherit. Metab. Dis.* **2003**, *26*, 319–326. [CrossRef] [PubMed]
39. Rocha, J.C.; Van Rijn, M.; Van Dam, E.; Ahring, K.; Bélanger-Quintana, A.; Dokoupil, K.; Ozel, H.G.; Lammardo, A.M.; Robert, M.; Heidenborg, C.; et al. Weight Management in Phenylketonuria: What Should Be Monitored? *Ann. Nutr. Metab.* **2015**, *68*, 60–65. [CrossRef] [PubMed]
40. Weglage, J.; Brämswig, J.H.; Koch, H.G.; Karassalidou, S.; Ullrich, K. Growth in patients with phenylketonuria. *Eur. J. Nucl. Med. Mol. Imaging* **1994**, *153*, 537–538. [CrossRef]
41. Rocha, J.C.; Macdonald, A. Dietary intervention in the management of phenylketonuria: Current perspectives. *Pediatr. Heal. Med. Ther.* **2016**, *ume 7*, 155–163. [CrossRef]
42. Herrmann, M.E.; Brosicke, H.G.; Keller, M.; Monch, E.; Helge, H. Dependence of the utilization of a phenylalanine-free amino acid mixture on different amounts of single dose ingested. A case report. *Eur. J. Pediatr.* **1994**, *153*, 501–503. [CrossRef]
43. Macdonald, A.; Rylance, G.; Hall, S.K.; Asplin, D.; Booth, I.W. Factors affecting the variation in plasma phenylalanine in patients with phenylketonuria on diet. *Arch. Dis. Child.* **1996**, *74*, 412–417. [CrossRef]
44. Macdonald, A.; Rylance, G.W.; Asplin, D.; Hall, S.K.; Booth, I.W. Does a single plasma phenylalanine predict quality of control in phenylketonuria? *Arch. Dis. Child.* **1998**, *78*, 122–126. [CrossRef] [PubMed]
45. Couce, M.L.; Guler, I.; Anca-Couce, A.; Lojo, M.; Mirás, A.; Leis, R.; Pérez-Muñuzuri, A.; Fraga, J.M.; Gude, F. New insights in growth of phenylketonuric patients. *Eur. J. Nucl. Med. Mol. Imaging* **2014**, *174*, 651–659. [CrossRef]
46. Moretti, F.; Pellegrini, N.; Salvatici, E.; Rovelli, V.; Banderali, G.; Radaelli, G.; Scazzina, F.; Giovannini, M.; Verduci, E. Dietary glycemic index, glycemic load and metabolic profile in children with phenylketonuria. *Nutr. Metab. Cardiovasc. Dis.* **2017**, *27*, 176–182. [CrossRef]
47. Ilgaz, F.; Pinto, A.; Gökmen-Özel, H.; Rocha, J.C.; Van Dam, E.; Ahring, K.; Bélanger-Quintana, A.; Dokoupil, K.; Karabulut, E.; Macdonald, A. Long-Term Growth in Phenylketonuria: A Systematic Review and Meta-Analysis. *Nutrients* **2019**, *11*, 2070. [CrossRef] [PubMed]
48. Wells, J.C.K.; E Williams, J.; Chomtho, S.; Darch, T.; Grijalva-Eternod, C.; Kennedy, K.; Haroun, D.; Wilson, C.; Cole, T.J.; Fewtrell, M.S. Body-composition reference data for simple and reference techniques and a 4-component model: A new UK reference child. *Am. J. Clin. Nutr.* **2012**, *96*, 1316–1326. [CrossRef] [PubMed]
49. Weber, D.R.; Moore, R.H.; Leonard, M.B.; Zemel, B.S. Fat and lean BMI reference curves in children and adolescents and their utility in identifying excess adiposity compared with BMI and percentage body fat. *Am. J. Clin. Nutr.* **2013**, *98*, 49–56. [CrossRef] [PubMed]
50. Wells, J.C.K.; Fewtrell, M.S.; E Williams, J.; Haroun, D.; Lawson, M.S.; Cole, T.J. Body composition in normal weight, overweight and obese children: Matched case–control analyses of total and regional tissue masses, and body composition trends in relation to relative weight. *Int. J. Obes.* **2006**, *30*, 1506–1513. [CrossRef]
51. Roemmich, J.N.; Clark, P.A.; Weltman, A.; Rogol, A.D. Alterations in growth and body composition during puberty. I. Comparing multicompartment body composition models. *J. Appl. Physiol.* **1997**, *83*, 927–935. [CrossRef]

52. Sopher, A.B.; Thornton, J.C.; Wang, J.; Pierson, R.N.; Heymsfield, S.B.; Horlick, M. Measurement of Percentage of Body Fat in 411 Children and Adolescents: A Comparison of Dual-Energy X-Ray Absorptiometry With a Four-Compartment Model. *Pediatrics* **2004**, *113*, 1285–1290. [CrossRef] [PubMed]
53. Williams, J.E.; Wells, J.C.K.; Wilson, C.M.; Haroun, D.; Lucas, A.; Fewtrell, M.S. Evaluation of Lunar Prodigy dual-energy X-ray absorptiometry for assessing body composition in healthy persons and patients by comparison with the criterion 4-component model. *Am. J. Clin. Nutr.* **2006**, *83*, 1047–1054. [CrossRef] [PubMed]
54. Ofenheimer, A.; Breyer-Kohansal, R.; Hartl, S.; Burghuber, O.C.; Krach, F.; Schrott, A.; Franssen, F.M.E.; Wouters, E.F.M.; Breyer, M. Reference charts for body composition parameters by dual-energy X-ray absorptiometry in European children and adolescents aged 6 to 18 years—Results from the Austrian LEAD (Lung, hEart, sociAl, boDy) cohort. *Pediatr. Obes.* **2021**, *16*, e12695. [CrossRef] [PubMed]

CHAPTER 5B

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A THREE YEAR LONGITUDINAL STUDY COMPARING BONE MASS, DENSITY AND GEOMETRY MEASURED BY DXA, pQCT AND BONE TURNOVER MARKERS IN PKU CHILDREN TAKING EITHER L-AMINO ACIDS OR GLYCOMACROPEPTIDE PROTEIN SUBSTITUES

nutrients

Research

A three-year longitudinal study comparing bone mass, density and geometry measured by DXA, pQCT and bone turnover markers in children with PKU taking either L-amino acid or glycomacropeptide protein substitutes.

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Abstract

In patients with phenylketonuria (PKU), treated by diet therapy only, evidence suggests that areal bone mineral density (BMDa) is within the normal clinical range but is below the population norm. **Aims**: To study longitudinal bone density, mass and geometry over 36 months in children with PKU taking either amino acids (AA) or casein glycomacropeptide (CGMP-AA) as their main protein source. **Methodology**: 48 subjects completed the study: 19 in the AA group (median age 11.1y, range 5-15y) and 29 in the CGMP-AA group. The CGMP-AA was further divided into 2 groups, CGMP100 (n=13), children taking CGMP-AA only and CGMP50 (n=16), children taking a combination of CGMP-AA and AA. Dual X-ray absorptiometry (DXA) was measured at enrolment and 36 months, peripheral quantitative computer tomography (pQCT) at 36 months only, and serum blood and urine bone turnover markers (BTM) and blood bone biochemistry at enrolment, 6, 12 and 36 months. **Results:** No statistical significant differences were found between the three groups for DXA outcome parameters: BMDa (L2-L4 BMDa g/cm^2), bone mineral apparent density (L2-L4 BMAD g/cm^3) and total body less head BMDa (TBLH g/cm^2). All blood biochemistry markers were within the reference ranges, and BTM showed active bone turnover with a trend for BTM to decrease with increasing age. **Conclusions:** Bone density was clinically normal, although the median z scores were below the population mean. BTM showed active bone turn over and blood biochemistry was within the reference ranges. There appeared no advantage of a macropeptide based protein substitute compared to one based on AA on bone density,mass and geometry.

Key words: PKU, bone mineral density, bone turnover markers, osteoporosis, blood biochemistry, casein glycomacropeptide, amino acid, protein substiute

1. Introduction

Optimal bone mass is key to preventing the risk of fractures later in life, and many factors influence peak bone mass accretion including genetics, physical activity, body composition and quality of diet. Severe dietary restriction may be problematic in conditions such as phenylketonuria (PKU) which requires rigorous exclusion of many natural foods [1]. In children with classical PKU, the majority of protein is provided by a low phenylalanine, semi synthetic protein (protein substitute), with some limited dietary phenylalanine given from natural foods according to individual metabolic tolerance and disorder severity. Dependency on a synthetic protein may compromise both peak bone mass attainment and bone geometry [2, 3].

Protein substitutes fundamental in the treatment of classical PKU are traditionally derived from essential and non-essential amino acids and usually supplemented with added vitamins, minerals and trace minerals aiming to achieve optimal growth, bone mass and body composition. Protein substitutes are necessary life-long but long-term adherence is difficult to sustain particularly during adolescence [4, 5]. This is a particulary vulnerable time for maximising bone mass, density, mineralization and growth potential. Amino acids contribute to the structural components of bone in addition to those of growth and tissue maintenance [2, 6, 7].

Protein has a positive effect on bone [8, 9], dietary protein intake promotes peripubertal bone growth and delays bone loss [10, 11]. Several long-term prospective observational studies [7, 12] have shown a significant positive association between protein intake and bone mineral content, periosteal circumference, cortical area and an index of strength strain. These studies reinforce that a moderate to high protein diet promotes bone accretion. Converesly, the acid ash theory suggests that a high protein intake (with protein substitutes based on amino acids) is detrimental to bone accretion [10, 13]. High protein diets and protein substitues contain sulphur rich amino acids and together with anions such as phosphate produce sulphuric and phosphoric acid. To buffer this acidic environment, it is suggested that calcium stored in bones is excreted in the urine as bicarbonate [14-18], and this process slowly demineralises bone. However, a systematic and meta analysis reviewing calcium balance studies in response to changes in phosphate supplementation dismiss this theory [19, 20]. Although the urine pH is lower when taking a protein rich diet, the pH of the extracellular fluid is undisturbed due to the regulatory control performed by the kidneys [10].

The use of casein glycomacropeptide supplemented with amino acids (CGMP-AA) is associated with improved bone mass in PKU animal models [21], but the use of CGMP (a bioactive peptide) compared to AA protein substitutes and their influence on bone mass, density and geometry has not been studied in children with PKU.

In this longitudinal prospective controlled study over 36 months we investigated the efficacy of CGMP–AA compared to AA protein substitute on bone mass, density, geometry and turnover markers in children with PKU.

1.1 Ethical approval

The study was registered by the Health Research Authority and was given a favourable ethical opinion by the South Birmingham Research Ethical Committee (referenced 13/WM/0435 and IRAS (integrated research application system) number 129497. Written informed consent was given by at least one caregiver with parental responsibility and written assent obtained from the subjects if appropriate for their age and level of understanding.

2. Materials and Methods

2.1 Methods

Inclusion criteria included: children with PKU diagnosed by newborn screening, aged 5-16 years, not treated with sapropterin dihydrochloride, known adherence with protein substitutes, and maintainance of 70% of blood phenylalanine concentrations within European PKU target therapeutic range for 6 months prior to study enrolment [22]. Target blood phenylalanine ranges for children aged 5-12 years were 120 to ≤ 360 μmol/L and for 12 years and older 120 to ≤600 μmol/L.

2.1.1 CGMP-AA and AA protein substitutes

The CGMP-AA (a test product by Vitaflo International Ltd. Liverpool, UK) was a flavoured powder. Each 35g sachet contained 20g protein equivalent, and 36 mg phenylalanine, mixed with 120mL of water. The flavoured AA's were either powders mixed with water or ready prepared liquids providing 10, 15 or 20g of protein equivalent. Both CGMP-AA and AA products had a similar nutritional and AA profile; except CGMP-AA contained residual phenylalanine, and higher amounts of threonine and leucine.

2.1.2 Selection into CGMP or AA group.

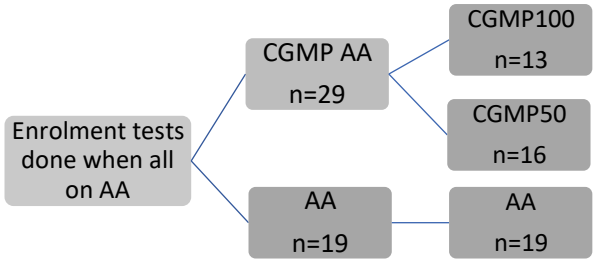
The children chose the product they preferred, depending on their taste preference: CGMP-AA or AA. They remained on this formula for the duration of the study.

2.2 Study design

The primary aim of this 3-year longitudinal study was to compare bone mass, density and geometry of children with PKU taking CGMP-AA or AA as their primary protein source. The following examinations were conducted: dual energy X ray absorptiometry (DXA), together with blood bone biochemistry, bone serum and urine turnover markers. Peripheral quantitative computed tomography (pQCT) of the forearm was performed at 36 months only (Figure 1).

A previous pilot study [23] demonstrated that the residual phenylalanine in the CGMP-AA led to compromised phenylalanine control in some children. Therefore the CGMP-AA group was subdivided into: 1) CGMP100 group where the children took the entire protein substitute as CGMP-AA; 2) CGMP50, where children took a combination of AA and CGMP-AA. There was also a third group of children who remained on their usual AA protein substitute (AA group).

Figure 1 Diagramatic scheme for study methodology, enrolment to 36 months



Enrolment	6m	12m	36m
Fasting blood biochemistry Serum bone markers 2nd void urine bone markers DXA	Fasting blood biochemistry Serum bone markers 2nd void urine bone markers	Fasting blood biochemistry Serum bone markers 2nd void urine bone markers	Fasting blood biochemistry Serum bone markers 2nd void urine bone markers DXA pQCT
Anthropometry 3monthly; blood phenylalanine weekly			

Legend: CGMP-AA, casein glycomacropeptide; AA, amino acid; CGMP100, all protein substitute from casein glycomacropeptide; CGPM50, 50% of protein substitute taken from casein glycomacropeptide and 50% from amino acid protein substitute; DXA, dual x ray absorptiometry; pQCT, peripheral quantitative computerised tomography

2.2.1 Dual Energy X-ray Absorptiometry (DXA) and peripheral quantitative computed tomography (pQCT)

A GE Lunar iDXA and Encore™ software version 13.1g (GE Healthcare, Wisconsin MD USA) was used to measure bone density at enrolment and at the end of 36 months. Trunk thickness and body weight were used as a guide for scanning each child in the most appropriate acquisition mode. Children lay supine on a bed, while the DXA scan was completed. The following parameters were measured: lumbar spine (L2-L4) areal bone mineral density (L2-L4 BMDa) in g/cm^2, lumbar spine (L2-L4) bone mineral content (L2-L4 BMC) in g, total body mineral content (BMC) in g and total body less head BMDa (TBLH) in g/cm^2. Size corrected outcomes measures included lumbar spine bone mineral apparent density (L2-L4 BMAD) in g/cm^3. At 36 months, in addition to the DXA assessment pQCT was also performed.

2.2.2 pQCT

pQCT (Stratec Pfozheim Germany) measures were taken at the 4% and 66% region of the non dominant forearm, evaluating volumetric bone mineral density, together with muscle and bone geometry, size and strength. At the 4% site trabecular and total cross sectional area was measured,

while at the 66% site cortical density, muscle, bone and fat area were measured. pQCT also measured the strength strain index a surrogate marker of bone strength.

2.2.3 Serum blood and urine bone turnover markers

Fasting early morning venous blood samples were collected at enrolment, 6, 12 and 36 months for the following serum bone markers: procollagen type 1 N-terminal propeptide (P1NP), type 1 collagen β cross-linked C-telopeptide (β-CTX) and bone alkaline phosphatase (bone ALP). A urine sample, the second sample of the day was collected at enrolment, 6, 12 and 36 months for urine creatinine adjusted free urine pyridinoline (fPYD/UrC), urine free deoxypyridinoline crosslinks (fDPD/UrC) and urinary calcium:creatinine ratio (Ur Ca/Cr). Urines were collected in containers, which were wrapped in tin foil and put into an envelope to shield them from any light. All samples were taken immediately to the laboratory for processing and urine stored at -80°C. β-CTX and P1NP were analysed using an electro-chemiluminescence immunoassay (ECLIA) on a COBAS e601 analyser (Roche Diagnostics, Mannheim, Germany). Inter-assay coefficient of variation (CV) for β-CTX was <3% across the analytical range between 0.01-6.0 μg/L, with a sensitivity of 0.01 μg/L. Inter-assay CV for P1NP was <3% between 5-1200 μg/L with a sensitivity of 5 μg/L. Serum Bone ALP was determined by MicroVue™ enzyme-linked immunosorbent assay ELISA kit (Quidel Corporation, US), Inter-assay CV for Bone ALP was <5.8% between 0.5-150 U/L, with a detection limit of 0.7 U/L.

Analysis for urinary fPYD and fDPD were performed using liquid chromatography tandem mass spectrometry (LC-MS/MS) method described by Tang et al. In brief, 0.5 mL of urine sample/calibration/quality control materials pretreated with 0.5 mL hydrochloric acid (40% concentrate) was extracted using solid phase extraction (SPE) column packed with cellulose slurry. Pyridinium cross-links were eluted from the SPE columns and analysed by LC–MS/MS coupled with an electrospray ionisation (ESI) source operated in positive mode. Inter-assay CVs were ≤10.3% for PYD between the concentration range of 5-2,000 nmol/L and ≤13.1% for DPD between 2-1,000 nmol/L. The lower limit of quantification was 6 nmol/L for fPYD and 2.5 nmol/L for fDPD.

Urine creatinine was measured to obtain fPYD and fDPD to urine creatinine ratio and urine calcium:creatine ratio. Samples were analysed using Roche kinetic colorimetric assays performed on the COBAS® C501 analyser (Roche, Burgess Hill, UK) according to the manufacturer instructions. The inter-assay CV ranged from 1.3-2.1% across the assay working range for Ur Ca of 0.20-7.5 mmol/L and Ur creatinine of 0.355 mmol/L.

2.2.4. Blood biochemistry markers

Overnight fasting blood samples for serum calcium, magnesium, phosphate, vitamin D and parathyroid hormone were collected at enrolment, 6, 12 and 36 months.

2.2.5 Blood phenylalanine /tyrosine monitoring

Throughout the 36 month study, trained caregivers collected weekly overnight fasting morning blood spots at home for phenylalanine and tyrosine. Blood specimens were sent via the post to the Birmingham Women's and Children's Hospital laboratory. The blood spot filter cards used were Perkin Elmer 226 UK standard NBS; manufactured by Perkin Elmer, Waltham MA, USA. All the cards had a standard thickness and the blood phenylalanine and tyrosine concentrations were calculated on a 3.2 mm punch by tandem mass spectrometry.

2.2.6 Pubertal status

A general medical examination and pubertal status was measured at enrolment using the Tanner picture index. Stage 1 and 2 are classified as pre-pubertal, and stage 3, 4 and 5 as pubertal.

2.2.7Anthropometric measurements

Weight and height were measured once every 3 months by one of two metabolic dietitians. Height was measured using a Harpenden stadiometer (Holtain Ltd, Crymych, UK) and weight on calibrated digital scales (Seca, Medical Measuring Systems and Scales, Birmingham, UK. Model 875); weight was measured to the nearest 0.1kg, and height to the nearest 0.1cm.

2.3 Statistical methods

Continuous data are presented as median and inter quartile ranges and categorical data are presented as frequencies of counts with associated percentages. Longitudinal data are presented graphically using profile plots to show the average change over time with comparisons between treatment groups performed using Analysis of Covariance (ANCOVA) techniques which include baseline measures as adjusting covariates. Models also include covariates for patient gender, age and puberty status. A p-value of 0.05 is used throughout to determine statistical significance. All analyses are performed using R (Version 3).

3. Results

Fifty children (28 boys, 22 girls) with PKU were recruited. Forty-seven children were of European and 3 of Asian origins. Forty-eight completed the study, 29 in the CGMP-AA group and 19 in the AA group. At enrolment the median age (range) in the CGMP100 group was 9.2 years (5-16y) (n=13); in the CGMP50 group, 7.3 years (5-15y) (n=16) and in the AA group, 11.1 years (5-16y) (n=19). All had classical PKU, except two with mild PKU based on dietary phenylalanine tolerance and untreated blood phenylalanine levels at diagnostic confirmation.

3.1.1 Subject drop out

One boy and one girl (both aged 12 years) in the CGMP-AA group were excluded from the study as both failed to comply with the study protocol. One failed to return blood phenylalanine samples and both had poor adherence to the low phenylalanine diet.

3.1.2 Pubertal status

The number of children prepubertal (stage 1 and 2) at enrolment were: CGMP100, 62% (n=8/13); CGMP50, 69% (n=11/16) and AA group, 32% (n=6/19).

The number of children in puberty (stage 3 to 5) were: CGMP100 group, 38% (n=5/13); CGMP50 group, 31% (n=5/16); and AA group, 68% (n=13/19).

3.1.3 Median DXA z scores measurements for CGMP100, CGMP50 and AA groups

Overall there were no significant differences between the groups for any of the measured DXA parameters. Bone density was on the lower side of normal but within normal reference range (Table 1).

Table 1. Median (range) L2-L4 bone mineral density (BMDa), lumbar spine bone mineral apparent density (L2-L4 BMAD) and total body less head BMDa (TBLH) z scores. Other parameters measured included L2-L4 bone mineral content and total bone mineral content for CGMP100, CGMP50 and AA at enrolment and 36 months.

Group	Enrolment z score (range)	36 months z score (range)
	L2-L4 BMDa (g/ cm^2)	
CGMP100	-0.2 (-0.9 to 0.8)	-0.6 (-0.9 to 0.6)
CGMP50	-0.1 (-0.5 to 0.5)	-0.1 (-0.6 to 0.4)
AA	-0.1 (-0.7 to 0.4)	-0.5 (-0.8 to 0.0)
	L2-L4 BMAD (g/cm^3)	
CGMP100	0.2 (-0.9 to 0.6)	0.2 (-0.4 to 0.5)
CGMP50	-0.2 (-0.5 to 0.9)	-0.2 (-0.4 to 0.3)
AA	-0.3 (-0.8 to 0.4)	-0.6 (-1.2 to -0.1)
	TBLH BMDa (g/cm^2)	
CGMP100	-0.6 (-1 to -0.5)	-0.5 (-0.6 to - 0.2)
CGMP50	-0.8 (-1.3 to -0.1)	-0.6 (-0.9 to -0.3)
AA	-0.2 (-0.5 to 0.1)	-0.2 (-0.4 to - 0.1)
	Median values (range) for Total and L2-L4 BMC g	
	Total body BMC g	
CGMP100	832.8 (672.9 to 1543.5)	1258.4 (1082.8 to 1816.9)
CGMP50	604.9 (532.9 to 680.3)	1019.1 (963.4 to 1134.8)
AA	1183.8 (672.9 to 1543.5)	1650.2 (1082.8 to 1816.9)
	L2-L4 BMC g	
CGMP100	18.9 (14.1 to 22.9)	28.1 (24.1 to 38.3)
CGMP50	14.2 (13.0 to 16.6)	22.1 (20.4 to 25.1)
AA	25.6 (15.9 to 34.9)	40.2 (25.0 to 45.4)

Legend: CGMP, casein glycomacropeptide; CGMP100, all the protein substitute as casein glycomacropeptide; CGMP50, a combination of casein glycomacropeptide and amino acid protein substitute; AA, amino acid protein substitute; L2L4 BMD, bone mineral density lumbar vertebrae 2 to 4; BMAD, bone mineral apparent density; TBLH, total body less head; L2L4 BMC, bone mineral content lumbar vertebrae 2 to 4

Table 2. Results from the pQCT scan measuring median z scores (range) for trabecular, cortical and total density at the 4% site and bone, muscle and fat area, strength strain index and bone area/muscle area at 36 months in the CGMP100, CGMP50 and AA groups.

Group	36 months z score (range)
Trabecular density: 4%	
CGMP100	-1.0 (-1.3 to -0.5)
CGMP50	-1.0 (-1.2 to -0.7)
AA	-0.5 (-1.2 to -0.1)
Total density: 4%	
CGMP100	-0.7 (-1.1 to -0.6)
CGMP50	-0.7 (-0.9 to -0.3)
AA	-0.4 (-0.9 to 0.5)
Cortical density: 66%	
CGMP100	0.1 (-0.1 to 0.3)
CGMP50	-0.5 (-1.4 to -0.1)
AA	-0.4 (-1.0 to 0.5)
Bone area: 66%	
CGMP100	1.9 (1.4 to 4.0)
CGMP50	0.9 (0.2 to 1.8)
AA	2.0 (1.5 to 3.7)
Muscle area: 66%	
CGMP100	-1.1 (-1.8 to -0.5)
CGMP50	-1.2 (-1.4 to -0.6)
AA	-1.0 (-1.8 to -0.5)
Fat area: 66%	
CGMP100	0.5 (-0.3 to 0.9)
CGMP50	1.0 (0.4 to 1.8)
AA	1.2 (0.1 to 2.3)
Bone area/muscle area: 66% area	
CGMP100	0.5 (0.2 to 1.1)
CGMP50	-0.4 (-1.2 to 0.5)
AA	0.5 (0.2 to 1.6)
Strength strain index (SSI): 66%	
CGMP100	-0.7 (-1.0 to 1.3)
CGMP50	-0.1 (-0.6 to 0.5)
AA	0.4 (-0.3 to 0.6)

No significant differences were found between the groups for any of the pQCT measurements (Table 2). Overall, all three groups had DXA outcomes within the low-normal range. The only significant change was in increase in cortical density in patients with CGMP100 compared to AA ($p=0.05$).

3.2 Nutritional bone biochemistry markers

Median concentrations for all the biochemistry markers (calcium, phosphate, magnesium, vitamin D and parathyroid hormone) were within normal reference ranges for all the groups over the 36 month study period (Table 3). There was no statistical significant differences within or between the groups.

3.2.1. Measurement for bone formation markers and urine calcium

Urine calcium:creatinine ratio (UrCa/Cr) a measure of renal acid excretion was normal with no indication of excess calcium (Table 4). Similarly serum and urine BTMs showed a physiological decrease with age, and no evidence of a disturbance between formation and resorption.

Table 3 Median (range) biochemical bone markers at enrolment and 36 months for CGMP100, CGMP50 and AA groups.

	Calcium mmol/L (range)		Phosphate mmol/L (range)		Magnesium mmol/L (range)		25 (OH) Vit D nmol/L (range)		PTH ng/L (range)	
	Enrolmen	36m	Enrolm	36m	Enrolme	36m	Enrolment	36m	Enrolme	36m
CGMP100	2.5 (2.3, 2.6)	2.4 (2.3, 2.5)	1.4 (1.0, 1.5)	1.3 (1.0, 1.5)	0.9 (0.7, 1.0)	0.8 (0.8, 0.9)	112 (81, 162)	79 (44, 113)	17 (11, 42)	32 (22, 57)
CGMP50	2.5 (2.3, 2.6)	2.4 (2.3, 2.5)	1.4 (1.1, 1.6)	1.3 (1.1, 1.5)	0.8 (0.8, 1.0)	0.8 (0.8, 0.9)	94.6 (62,135)	95.2 (56,137)	15.5 (6, 37)	31 (19, 46)
AA	2.5 (2.3, 2.6)	2.4 (2.3, 2.5)	1.3 (1.0, 1.5)	1.2 (0.8, 1.7)	0.8 (0.8, 0.9)	0.8 (0.7, 0.9)	93.9 (39,182)	91.8 (60, 161)	21 (6, 44)	31 (19, 46)

Normal reference ranges (reference ranges from Birmingham Children's Hospital Clinical Chemistry Laboratory)

Calcium 2.2-2.7 mmol/L

Phosphate 0.8-1.9 mmol/L

Magnesium 0.7-1.0 mmol/L

25 (OH)D Vitamin D ≥ 50 nmol/L

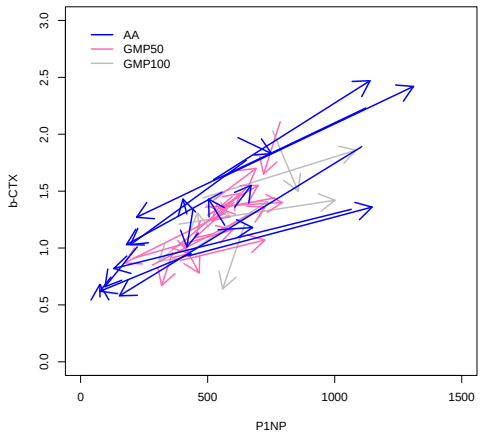
Parathyroid hormone (PTH) 15 -60 10 ng/L

Table 4. Median (range) serum bone and urine turnover markers calculated from enrolment to 36 months for CGMP100, CGMP50 and AA in girls and boys.

	CGMP100 boys	CGMP100 girls	CGMP50 boys	CGMP50 girls	AA boys	AA girls
B-CTX µg/L	1.2 (1.2,1.6)	1.2 (1, 1.5)	1.2 (1.1,1.4)	1.2 (1.2.1.3)	1.4 (1.3,1.4)	1.2 (0.9,1.3)
BAP U/L	86 (76,95)	103 (92,106)	125 (114,131)	108 (76,116)	85 (75,95)	83 (46,97)
P1NP µg/L	503 (488,509)	476 (387,663)	470 (434, 543)	507 (487,649)	522 (418,556)	445 (175,553)
f DPD nmol/L	178 (68,307)	114 (71,338)	207 (91,227)	147 (98,265)	157 (96,247)	107 (93,114)
f DPD/Ur Cr nmol/mmol	22 (9,27)	24 (12,28)	26 (10,30)	23 (13,28)	25 (8,27)	14 (8,26)
f PYD nmol/L	735 (276,1514)	429 (275,700)	825 (310,951)	624 (347,1134)	615 (331,876)	413 (290,436)
f PYD/Ur Cr nmol/mmol	96 (33,118)	90 (40,121)	96 (37,111)	105 (49,110)	94 (27,109)	58 (24,100)
Ur Ca/Cr mmol/L	1 (0.4,1.2)	1.1 (0.8,1.4)	1.3 (0.7,1.5)	0.8 (0.4,1.3)	1.6 (1.3,2.4)	1.9 (1.3,2.5)
Ur Creat mmol/L	12 (1,15)	6 (5,11)	8 (7,9)	8 (8,10)	10 (8,16)	7 (6,8)

Legend. M, males; F, females; CGMP, casein glycomacropeptide; CGMP100, all the protein substitute as casein glycomacropeptide; CGMP50, a combination of casein glycomacropeptide and amino acid protein substitute; AA, amino acid protein substitute; β-CTX, Collagen type1 cross linked C telopeptide; BAP, bone alkaline phosphatase; P1NP, procollagen type 1 N terminal propeptide; fPYD/ urine free pyridinolin; fDPD, urine free deoxypyridinoline; fPYD/Ur, urine creatinine adjusted urine free pyridinoline; fDPD/Ur urine creatinine adjusted urine free deoxypyridinoline; UrCa/Cr, urine calcium:creatinine ratio; UrCr urine creatinine. Standard references for children are not available

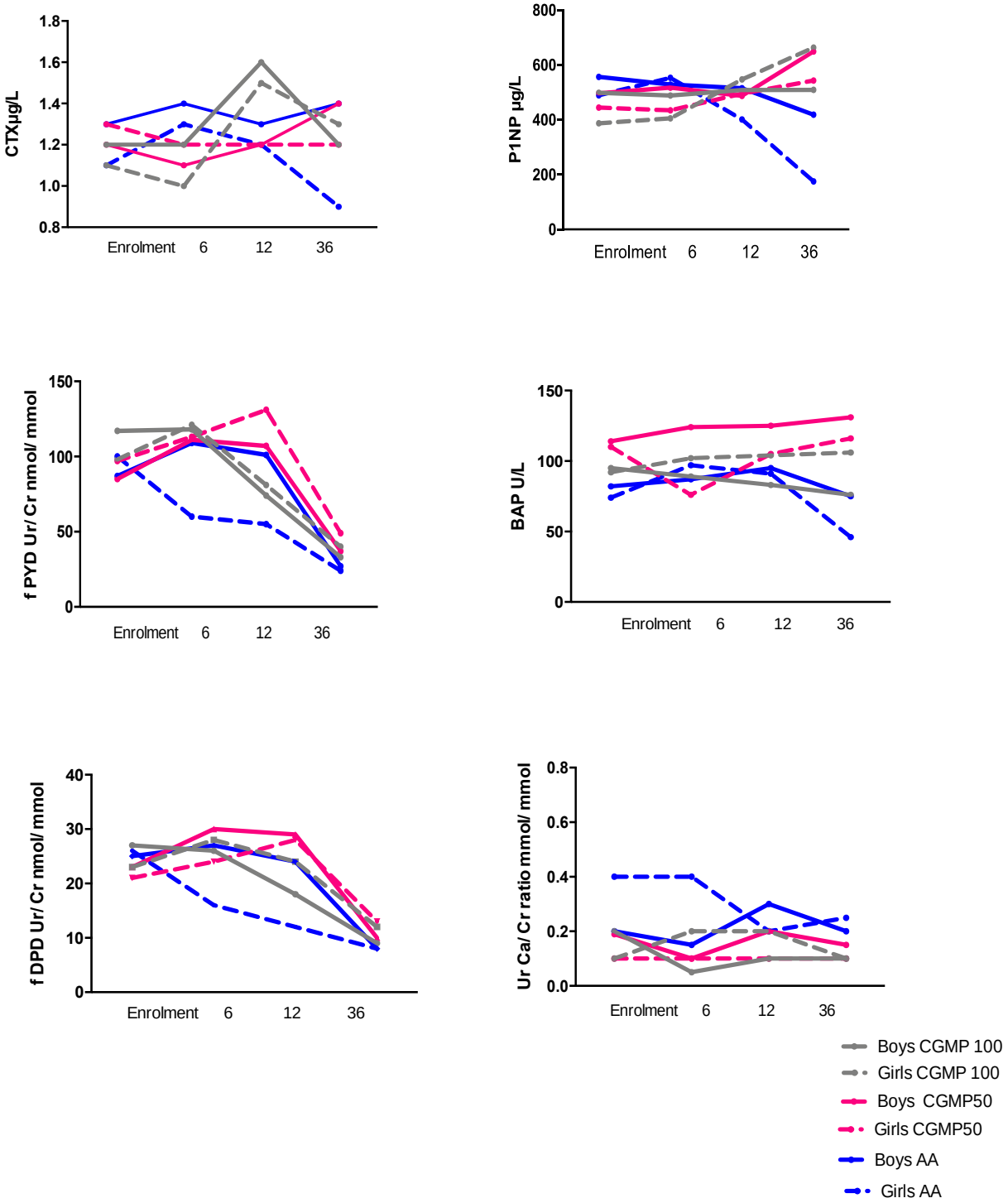
Figure 2. Correlation of β-CTX with PINP for CGMP 100, CGMP 50 and AA at 36 months.



Legend: ↑CGMP 100 (glycomacropeptide only), ↑ CGMP50 (combination of CGMP and AA), ↑ AA only

A strong positive correlation was observed between PN1P and β-CTX at 36 months (r=0.82) (Figure 2). ANCOVA analysis performed on PN1P indicated that the level of PN1P was somewhat dependent on age with older subjects having a lower PN1P level. Further to this there was evidence of an increase in PN1P at 36 months associated with GMP100 when compared to AA (p=0.041) (Figure 3). There was no difference between GMP50 and AA (p=0.80)

Figure 3. Graphs showing serum and urine bone turnover markers at enrolment, 6, 12 and 36 months separated by gender for CGMP100, CGMP50 and AA.



3.3 Anthropometry

We have previously reported height, weight and body mass index in this group of children [24]. At 36 months all groups had a median positive height AA, 0.2 (0 to 0.5), CGMP50 ,0.3 (-0.1 to 0.7) and CGMP100, 0.6 (0.1 to 0.7). Median weight for height z scores and BMI z scores were above the ideal reference mean indicating an overweight group of children.

3.4 Blood phenylalanine concentrations

The median phenylalanine concentrations for the study have been previously reported. Median phenylalanine concentrations were within recommended target reference ranges for children aged ≤11y and ≥ 12 years old [24]

The median daily dose of protein equivalent from protein substitute was 60g/day (range 40-80g), and the median amount of prescribed natural protein was 5.5g protein/day (range 3-30g) or 275mg/day of phenylalanine (range 150-1500mg) in all 3 groups.

4. Discussion

In this 36 month longitudinal study in children with PKU, bone mass, density and geometry was comprehensively examined by DXA and pQCT, in addition to serum BTM and blood biochemistry. No statistical significance was evident between the 3 protein substitute study groups (AA, CGMP50 or CGMP100), suggesting that CGMP-AA had no advantage over AA for bone development.

A strong positive correlation between β-CTX and P1NP was observed in all three study groups, with P1NP being lower in the older age subjects. There was evidence of an increased P1NP in the CGMP100 group independent of age compared to the AA group ($p=0.04$). This synergy between bone formation and resorption shows active bone turnover, and also reflected appropriate bone growth since these markers derive from physiological processes. Our results contrast to those reported by Castro et al [25], which suggested a trend towards increased bone resorption in subjects with PKU. This controlled study, is the first to monitor bone mass and density by two separate imaging technologies (DXA and pQCT), and holistically assesses serum bone, urine and blood biochemistry parameters in PKU. Similar to findings from two systematic reviews [25, 26], the overall bone density for the group was below the population mean but within the normal reference value. Imaging results met the ISCD (International Society for Clinical Densitometry) recommendations (ISCD 2013) [27]. There were no differences among the groups for biochemical or BTM's suggesting no alteration in bone metabolism for any of the three protein substitute groups. Naturallly, BTM concentrations decreased in older adolescents towards lower adult levels, a physiological phenomenon expected in a healthy population [28].

Unlike the findings of Schwahn et al, Mc Murry et al and Fernandez et al [29-31] we found no evidence to suggest that mineralization defects began in childhood and then became more evident in adolescents.

In PKU mouse models, CGMP when compared with AA has been shown to increase bone strength measured by biochemical mechanisms. Solverson [21] gave PKU and wild type mice different dietary regimens: a normal diet, or a low phenylalanine diet supplemented with AA or CGMP protein substitutes. The PKU mice regardless of protein substitute type had lower bone density compared to wild type mice, and those taking AA had inferior bone strength to the CGMP protein substitute group. The authors suggested the peptide structure of CGMP may possibly account for the positive influence on bone radial size improving biochemical performance. Alternatively, the high acid load due to the AA's could decrease bone strength via excreting higher amounts of calcium. However, both these suggestions were conjecture as neither net acid excretion or bone collagen, a marker of bone biomechanical performance, were measured. Results from our study in children with PKU monitored closely over 36 months would suggest neither of these mechanisms are active. BTMs monitoring collagen were physiologically normal and there was no evidence of net acid excretion with a normal calcium creatinine ratio.

Although many studies identified lower BMD in PKU [32-35], not all these studies included a size correction for DXA output and there is little agreement about the lower BMD pathophysiology. Dobrowolski et al [36] studied bone mineralization in PKU mice, showing phenylalanine toxicity inhibits bone mineralization. However, in human studies there is a discord on the link between hyperphenylalaninemia and bone mass, with some studies showing a correlation and others not [32, 34, 37, 38].

Within the three groups (CGMP100, CGMP50 and AA) there were expected physiological changes in the concentrations of BTMs. In adults BTM mainly represent bone remodelling, in children they are released during bone remodelling, modelling and perpendicular growth. Millet et al [39] measured urine DPD and bone ALP in patients with PKU and compared these with a healthy paediatric group; bone remodelling was active in children with PKU aged 7-14 years, and bone ALP, as expected was found to be significantly lower in the oldest group of patients (aged >18 years), although significanlty higher DPD concentrations independent of age were reported. In our study bone resorption and formation markers were consistently lowest in the AA group, particularly noticeable in the AA girls who had reached late puberty with a median age of 17 years (8-18y) at 36 months [28, 40-42]. In contrast the youngest group CGMP50 boys showed an increase in BTMs over the 36 months.

Interpretation of BTM is difficult and their concentrations vary widely affected by a multitude of factors: age, gender, puberty, growth velocity, the rate of mineral accrual, hormonal regulation, nutritional status, circadian and even day-to-day changes [43]. Paediatric reference data is available for some BTM [44-47], although UK specific data is lacking which hampers appropriate interpretation. Specificity for bone tissue and sensitivity and specificity of the measurement assays leads to variation rendering comparisons between study groups difficult [46, 48]. Despite these challenges, in our study in which children were followed for 36 months, BTM followed the expected variations for age with no differences between the groups. These children had an active bone turnover profile, supportive of a normal bone mineral density. The reasons why their bone

mineral density was below the population median was unclear, but this group were not at any increased clinical risk of fractures.

There are limitations to this study; patient numbers in each group were small which reduced the the study power. An extended follow up period of > 3 years may be needed for any differences to emerge between protein substitute sources, as noted P1NP was increased in the CGMP100 group. We also did not have a healthy control group, which would have been beneficial to compare differences with the children with PKU. The ages of the children were significantly different in all 3 groups and CGMP was given at two different concentrations making any absolute differences difficult to recognize, although statistically modelling was used to account for this variable. Age influences bone changes and children entered puberty over the study period. In children no bone marker is specific for any of the three different biological processes of modelling, remodelling and changes in endochondral ossification. However, our findings were consistent: all measurements were taken via DXA or pQCT and showed a below average bone density, with no significant differences between the group taking CGMPAA or AA. Bone markers appear to follow a similar pattern to healthy children. Equally we did not measure exercise activity in this group of children, but a high proportion (60%) did regular activities such as football, dancing and gymnastics.

5. Conclusions

In this detailed and comprehensive study measuring global bone development, using both two and three dimensional imaging in addition to serum BTM and blood biochemistry, a complete assessment of bone mass, density, geometry and bone turnover was assessed. There were no statistical differences in this group of children when taking AA and CGMP-AA protein substitutes and they had satisfactory metabolic control. Bone density was normal and similar to the findings from systematic reviews that suggest it is lower than the population norm, but carries no increased osteoporotic risk. Bone remodelling processes appear active in children with PKU, with both AA and CGMP-AA protein substitutes supporting normal bone growth

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References

1. MacDonald, A., et al., *PKU dietary handbook to accompany PKU guidelines.* Orphanet J Rare Dis, 2020. **15**(1): p. 171.
2. Budek, A.Z., et al., *Dietary protein intake and bone mineral content in adolescents-The Copenhagen Cohort Study.* Osteoporos Int, 2007. **18**(12): p. 1661-7.
3. Ferrari, S.L., et al., *Childhood fractures are associated with decreased bone mass gain during puberty: an early marker of persistent bone fragility?* J Bone Miner Res, 2006. **21**(4): p. 501-7.
4. MacDonald, A., et al., *Adherence issues in inherited metabolic disorders treated by low natural protein diets.* Ann Nutr Metab, 2012. **61**(4): p. 289-95.
5. Walter, J.H., et al., *How practical are recommendations for dietary control in phenylketonuria?* Lancet, 2002. **360**(9326): p. 55-7.
6. Bonjour, J.P., *Protein intake and bone health.* Int J Vitam Nutr Res, 2011. **81**(2-3): p. 134-42.
7. Bonjour, J.P., et al., *Protein intake and bone growth.* Can J Appl Physiol, 2001. **26 Suppl**: p. S153-66.
8. Thorpe, M.P. and E.M. Evans, *Dietary protein and bone health: harmonizing conflicting theories.* Nutr Rev, 2011. **69**(4): p. 215-30.
9. Rizzoli, R., et al., *Benefits and safety of dietary protein for bone health-an expert consensus paper endorsed by the European Society for Clinical and Economical Aspects of Osteopororosis, Osteoarthritis, and Musculoskeletal Diseases and by the International Osteoporosis Foundation.* Osteoporos Int, 2018. **29**(9): p. 1933-1948.
10. Conigrave, A.D., E.M. Brown, and R. Rizzoli, *Dietary protein and bone health: roles of amino acid-sensing receptors in the control of calcium metabolism and bone homeostasis.* Annu Rev Nutr, 2008. **28**: p. 131-55.
11. Rizzoli, R., et al., *Maximizing bone mineral mass gain during growth for the prevention of fractures in the adolescents and the elderly.* Bone, 2010. **46**(2): p. 294-305.
12. Alexy, U., et al., *Long-term protein intake and dietary potential renal acid load are associated with bone modeling and remodeling at the proximal radius in healthy children.* Am J Clin Nutr, 2005. **82**(5): p. 1107-14.
13. Patience, J.F., *A review of the role of acid-base balance in amino acid nutrition.* J Anim Sci, 1990. **68**(2): p. 398-408.
14. Buclin, T., et al., *Diet acids and alkalis influence calcium retention in bone.* Osteoporos Int, 2001. **12**(6): p. 493-9.
15. Kerstetter, J.E., et al., *The impact of dietary protein on calcium absorption and kinetic measures of bone turnover in women.* J Clin Endocrinol Metab, 2005. **90**(1): p. 26-31.
16. New, S.A., *Nutrition Society Medal lecture. The role of the skeleton in acid-base homeostasis.* Proc Nutr Soc, 2002. **61**(2): p. 151-64.
17. Remer, T. and F. Manz, *Potential renal acid load of foods and its influence on urine pH.* J Am Diet Assoc, 1995. **95**(7): p. 791-7.
18. Sebastian, A., et al., *Estimation of the net acid load of the diet of ancestral preagricultural Homo sapiens and their hominid ancestors.* Am J Clin Nutr, 2002. **76**(6): p. 1308-16.
19. Fenton, T.R., et al., *Meta-analysis of the effect of the acid-ash hypothesis of osteoporosis on calcium balance.* J Bone Miner Res, 2009. **24**(11): p. 1835-40.
20. Fenton, T.R., et al., *Phosphate decreases urine calcium and increases calcium balance: a meta-analysis of the osteoporosis acid-ash diet hypothesis.* Nutr J, 2009. **8**: p. 41.
21. Solverson, P., et al., *Low bone strength is a manifestation of phenylketonuria in mice and is attenuated by a glycomacropeptide diet.* PLoS One, 2012. **7**(9): p. e45165.
22. van Wegberg, A.M.J., et al., *The complete European guidelines on phenylketonuria: diagnosis and treatment.* Orphanet J Rare Dis, 2017. **12**(1): p. 162.
23. Daly, A., et al., *Glycomacropeptide in children with phenylketonuria: does its phenylalanine content affect blood phenylalanine control?* J Hum Nutr Diet, 2017. **30**(4): p. 515-523.
24. Daly, A., et al., *The Impact of the Use of Glycomacropeptide on Satiety and Dietary Intake in Phenylketonuria.* Nutrients, 2020. **12**(9).
25. de Castro, M.J., et al., *Bone Status in Patients with Phenylketonuria: A Systematic Review.* Nutrients, 2020. **12**(7).
26. Demirdas, S., et al., *Bone health in phenylketonuria: a systematic review and meta-analysis.* Orphanet J Rare Dis, 2015. **10**: p. 17.

27. Schousboe, J.T., et al., *Executive summary of the 2013 International Society for Clinical Densitometry Position Development Conference on bone densitometry.* J Clin Densitom, 2013. **16**(4): p. 455-66.
28. Mora, S., et al., *Biochemical markers of bone turnover and the volume and the density of bone in children at different stages of sexual development.* J Bone Miner Res, 1999. **14**(10): p. 1664-71.
29. Fernandez Espuelas, C., et al., *[Bone mineral turnover and bone densitometry in patients with a high-risk diet: hyperphenylalaninemia and galactosemia].* An Pediatr (Barc), 2005. **63**(3): p. 224-9.
30. McMurry, M.P., et al., *Bone mineral status in children with phenylketonuria--relationship to nutritional intake and phenylalanine control.* Am J Clin Nutr, 1992. **55**(5): p. 997-1004.
31. Schwahn, B., et al., *Decreased trabecular bone mineral density in patients with phenylketonuria measured by peripheral quantitative computed tomography.* Acta Paediatr, 1998. **87**(1): p. 61-3.
32. Adamczyk, P., et al., *Bone metabolism and the muscle-bone relationship in children, adolescents and young adults with phenylketonuria.* J Bone Miner Metab, 2011. **29**(2): p. 236-44.
33. Al-Qadreh, A., et al., *Bone mineral status in children with phenylketonuria under treatment.* Acta Paediatr, 1998. **87**(11): p. 1162-6.
34. Hillman, L., et al., *Decreased bone mineralization in children with phenylketonuria under treatment.* Eur J Pediatr, 1996. **155 Suppl 1**: p. S148-52.
35. Roato, I., et al., *Bone impairment in phenylketonuria is characterized by circulating osteoclast precursors and activated T cell increase.* PLoS One, 2010. **5**(11): p. e14167.
36. Dobrowolski, S.F., et al., *A bone mineralization defect in the Pah(enu2) model of classical phenylketonuria involves compromised mesenchymal stem cell differentiation.* Mol Genet Metab, 2018. **125**(3): p. 193-199.
37. Mendes, A.B., et al., *Bone development in children and adolescents with PKU.* J Inherit Metab Dis, 2012. **35**(3): p. 425-30.
38. Zeman, J., M. Bayer, and J. Stepan, *Bone mineral density in patients with phenylketonuria.* Acta Paediatr, 1999. **88**(12): p. 1348-51.
39. Millward, D.J., et al., *Protein quality assessment: impact of expanding understanding of protein and amino acid needs for optimal health.* Am J Clin Nutr, 2008. **87**(5): p. 1576S-1581S.
40. Blumsohn, A., et al., *Biochemical markers of bone turnover in girls during puberty.* Clin Endocrinol (Oxf), 1994. **40**(5): p. 663-70.
41. Bollen, A.M. and D.R. Eyre, *Bone resorption rates in children monitored by the urinary assay of collagen type I cross-linked peptides.* Bone, 1994. **15**(1): p. 31-4.
42. Shaw, N.J. and N.J. Bishop, *Mineral accretion in growing bones--a framework for the future?* Arch Dis Child, 1995. **72**(2): p. 177-9.
43. Gennai, I., et al., *Age- and sex-matched reference curves for serum collagen type I C-telopeptides and bone alkaline phosphatase in children and adolescents: An alternative multivariate statistical analysis approach.* Clin Biochem, 2016. **49**(10-11): p. 802-7.
44. Huang, Y., et al., *Establishment of reference intervals for bone markers in children and adolescents.* Clin Biochem, 2011. **44**(10-11): p. 771-8.
45. Rauchenzauner, M., et al., *Sex- and age-specific reference curves for serum markers of bone turnover in healthy children from 2 months to 18 years.* J Clin Endocrinol Metab, 2007. **92**(2): p. 443-9.
46. Szulc, P., E. Seeman, and P.D. Delmas, *Biochemical measurements of bone turnover in children and adolescents.* Osteoporos Int, 2000. **11**(4): p. 281-94.
47. Tise, M., et al., *A biochemical approach for assessing cutoffs at the age thresholds of 14 and 18 years: a pilot study on the applicability of bone specific alkaline phosphatase on an Italian sample.* Int J Legal Med, 2016. **130**(4): p. 1149-1158.
48. Delmas, P.D., *Biochemical markers of bone turnover.* Acta Orthop Scand Suppl, 1995. **266**: p. 176-82.

CHAPTER 6

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THE IMPACT OF THE USE OF GLYCOMACROPEPTIDE ON SATIETY AND DIETARY INTAKE IN PHENYLKETONURIA

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The Impact of the Use of Glycomacropeptide on Satiety and Dietary Intake in Phenylketonuria

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Abstract: Protein is the most satiating macronutrient, increasing secretion of gastrointestinal hormones and diet induced thermogenesis. In phenylketonuria (PKU), natural protein is restricted with approximately 80% of intake supplied by a synthetic protein source, which may alter satiety response. Casein glycomacropeptide (CGMP-AA), a carbohydrate containing peptide and alternative protein substitute to amino acids (AA), may enhance satiety mediated by its bioactive properties. Aim: In a three-year longitudinal; prospective study, the effect of AA and two different amounts of CGMP-AA (CGMP-AA only (CGMP100) and a combination of CGMP-AA and AA (CGMP50) on satiety, weight and body mass index (BMI) were compared. Methods: 48 children with PKU completed the study. Median ages of children were: CGMP100; (n = 13), 9.2 years; CGMP50; (n = 16), 7.3 years; and AA (n = 19), 11.1 years. Semi-quantitative dietary assessments and anthropometry (weight, height and BMI) were measured every three months. Results: The macronutrient contribution to total energy intake from protein, carbohydrate and fat was similar across the groups. Adjusting for age and gender, no differences in energy intake, weight, BMI, incidence of overweight or obesity was apparent between the groups. Conclusion: In this three-year longitudinal study, there was no indication to support a relationship between CGMP and satiety, as evidenced by decreased energy intake, thereby preventing overweight or obesity. Satiety is a complex multi-system process that is not fully understood.

Keywords: phenylketonuria; PKU; glycomacropeptide; satiety

1. Introduction

Phenylketonuria (PKU), due to phenylalanine hydroxylase deficiency, leads to accumulation of phenylalanine and irreversible brain damage if untreated [1,2]. A lifelong phenylalanine/protein restricted diet is essential, and most patients with classical PKU tolerate ≤ 500 mg/day of phenylalanine (equivalent to ≤10 g/day protein). Meats, fish, eggs and cheese are avoided with foods such as potatoes, cereals and peas given in restricted and calculated amounts; special low protein foods together with some fruits and vegetables (containing phenylalanine ≤ 75 mg/100 g) are given without restriction. Therefore, the diet is lacking in high quality protein and protein intake is supplemented with a minimal/phenylalanine-free protein substitute, usually supplying up to 80% of protein requirements.

It is unclear if this synthetic source of protein alters satiety. Satiety, which is a sense of fullness after eating, is important in regulating food intake [3,4]. In general nutrition, there is evidence that the amount and type of dietary protein alters appetite and may influence weight regulation [5,6].

Protein substitutes for PKU are obtained from either casein glycomacropeptide (CGMP) or phenylalanine-free amino acids (AAs). CGMP and AAs are compositionally different. Amino acid-based protein substitutes are composed of free L-amino acids only, whereas CGMP is a glycosylated peptide containing varying amounts of oligosaccharides, mostly sialic acid (N-acetylneuraminic acid), galactosamine and galactose [7]. CGMP has prebiotic, antimicrobial and immunomodulatory effects [8,9] and is prevalent in bovine milk. It constitutes 20–25% of the total protein in whey products [10]. Whey protein has been shown to induce satiety to a greater extent than other protein sources such as casein, soya and egg albumin [11,12]. There is some evidence that CGMP influences hormone responses affecting satiety [13–15]. However, human studies investigating the effect of CGMP on food intake and satiety have resulted in mixed findings [16–19].

Gut absorption of protein is thought to modulate satiety, although the influence of the protein source and individual amino acids on the control of food intake is not completely understood. It involves complex pathways that affect vagus-mediated signals, satiety related hormones and their metabolites (including the peptide ghrelin, cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1), glucose dependent insulinotropic polypeptide (GIP) and peptide tyrosine–tyrosine (PYY)) [11,15,20–23]. Some blood amino acids, particularly leucine, lysine, tryptophan, isoleucine and threonine, are linked to satiety responses [24–27]. CGMP is a small peptide and likely to be quickly absorbed [22,28,29]. A sharp rise in the mean circulating amino-acid concentrations following protein ingestion has been associated with a reduction in appetite [30,31]. Korompokis et al. [32] investigated the absorption kinetics of protein and the impact of amino acids on appetite and satiety related hormones. In a randomized cross over study using liquid preloads with a similar energy density but variable energy from protein, carbohydrate and fat, no postprandial kinetic effect of amino acids on appetite was shown. They concluded protein intake affected the amino acid profile but was not related to appetite regulation.

Equally, it is well established that amino acids bypass degradation by proteases and compared to whole proteins are absorbed faster [33–36]. In our own center, we reviewed plasma amino acid concentrations both fasting and after 2 h following a breakfast meal and 20 g protein equivalent from protein substitute either based on AA or CGMP. Although there were significant differences for individual amino acids, probably related to the amino acid composition of the protein substitutes, postprandial total amino acid concentrations were not different between the protein substitutes [37]. Until the absorption kinetics of CGMP (CGMP with added rate limiting amino acids) has been reported, its influence on satiety remains speculative. Additionally, CGMP and AA supplements modified for PKU usually contain added carbohydrate, which may also influence amino acid kinetics and hormone response [28].

If CGMP did increase satiety in PKU as suggested [38], it may bring important benefits, potentially helping control obesity, commonly reported in PKU [38,39]. In this study, we compared energy intake, weight and body mass index (BMI) over a three-year period in a group of children with PKU taking either CGMP at two different concentrations or amino acid supplements. We hypothesized that, if CGMP influenced satiety, then energy intake should be lower and weight and BMI changes altered between the groups.

Ethical Permission

The South Birmingham Research Ethics committee granted a favorable ethical opinion, referenced 13/WM/0435 and IRAS (integrated research application system) number 129497. Written informed consent was obtained for all subjects from at least one caregiver with parental responsibility and written assent obtained from the subject if appropriate for their age and level of understanding.

2. Methods and Materials

2.1. Subjects

In a three-year long-term prospective study, 50 children (28 boys, 22 girls) with PKU were recruited. Their median recruitment age was 9.2 years (range 5–16 years). Forty-seven children were European and three were of Pakistani origin. Inclusion criteria included: diagnosed by newborn screening, aged 5–16 years, not treated with sapropterin dihydrochloride and adherent to protein substitute. Seventy percent of routine blood phenylalanine concentrations were within phenylalanine target range for six months before study enrolment. Target blood phenylalanine concentrations for children aged 5–11 years was <360 μmol/L and for 12 years and older <600 μmol/L as recommended by the European PKU guidelines [40].

2.2. Protein Substitutes

Two types of protein substitute were studied: one based on phenylalanine-free AA (liquid pouches or powders) and the other on a powdered CGMP-AA supplement (a study product, made by Vitaflo, International Ltd, UK) (see Table 1 for nutritional analysis comparisons). The CGMP contained a residual amount of phenylalanine (36 mg/20 g protein equivalent). It was supplemented with essential and semi-essential amino acids, to provide a balanced amino acid profile to sustain nitrogen requirements and so the term CGMP-AA is used.

Table 1. The nutrient composition of CGMP-AA compared with conventional AA.

Protein Substitute		**CGMP-AA**	**Phe-Free AA ***
Nutrients	**Units**	**Per 20 g PE**	**Per 20 g PE**
Calories	Kcal	120	124
Protein equivalent	g	20	20
Total Carbohydrate	g	6.5	9.4
Sugars	g	2.2	7.8
Total Fat	g	1.5	0.7
Docosahexaenoic acid	mg	84	134
Salt	g	0.53	0.43
Vitamin A	μg RE	283	278
Vitamin D	μg	4.5	10
Vitamin E	mg αTE	6.5	5.2
Vitamin C	mg	38	36
Vitamin K	μg	35	34
Thiamine	mg	0.68	0.70
Riboflavin	mg	0.78	0.77
Niacin	mg	8.4	8.4
Vitamin B6	mg	1.0	0.9
Folic Acid	μg	136	134
Vitamin B12	μg	1.6	1.6
Biotin	μg	63.9	63
Pantothenic acid	mg	2.7	2.6
Choline	mg	204	201
Sodium	mmol	9.0	7.3
Potassium	mmol	6.8	7.9
Chloride	mmol	0.2	3.9
Calcium	mg	407	400

Table 1. *Cont.*

Protein Substitute		CGMP-AA	Phe-Free AA *
Nutrients	**Units**	**Per 20 g PE**	**Per 20 g PE**
Phosphorus	mmol	12	11.4
Magnesium	mg	128	125
Iron	mg	7.3	7.3
Copper	µg	748	730
Zinc	mg	7.3	7.3
Manganese	mg	1.1	1
Iodine	µg	85.7	84
Molybdenum	µg	49	48
Selenium	µg	29.9	29
Chromium	µg	29.9	29
Amino acids			
L-Alanine	g	0.78	0.92
L-Arginine	g	0.95	1.5
L-Aspartic Acid	g	1.12	2.37
L-Cystine	g	0.01	0.61
L-Glutamine [1]	g	2.57	-
Glycine	g	1.2	2.35
L-Histidine	g	0.7	0.92
L-Isoleucine	g	1.35	1.62
L-Leucine	g	3.00	2.54
L-Lysine	g	0.80	1.67
L-Methionine	g	0.28	0.45
L-Phenylalanine	g	0.03	0
L-Proline	g	1.52	1.69
L-Serine	g	0.96	1.04
L-Threonine	g	2.20	1.62
L-Tryptophan	g	0.40	0.5
L-Tyrosine	g	2.24	2.38
L-Valine	g	1.09	1.86

CGMP-AA, casein glycomacropeptide; AA, phenylalanine-free amino acid; PE, protein equivalent; * based on liquid Phe-free amino acids (Vitaflo International Ltd); [1] glutamine content varies according to if the AA protein substitute is a liquid or powder.

2.3. Study Design

The primary aim of this three-year single center, longitudinal study was to compare the efficacy of CGMP-AA compared to AA on bone health in a group of 50 PKU children (this will be reported separately). The secondary aim was to study energy intake, with particular reference to protein intake (from protein substitutes and phenylalanine exchanges), weight and BMI changes between the two groups, exploring the theory that CGMP-AA is actively related to satiety. Following the findings from a pilot study [41], it was clear that not all children in the CGMP-AA group were able to tolerate their entire protein substitute from CGMP-AA, due to its phenylalanine content. Therefore, within this group, there was a further subdivision: CGMP100, those taking all their substitute from CGMP-AA and those taking a combination of CGMP-AA and AA, named CGMP50. A group of children remained on AA only.

2.4. Selection into AA or CGMP-AA Group

The children chose AA or CGMP-AA, depending on their taste preference. They remained on this protein substitute for the three-year duration of the study.

2.5. Dietary Assessment

A three-day semi-quantitative dietary assessment was completed once every three months. The diet diaries were all checked during a face-to-face interview with caregivers by one of two trained metabolic dietitians. Protein containing foods were weighed. A picture book of pre-weighed foods was used to help caregivers estimate the portion size of other foods such as low protein pasta, fruit and vegetables. Twice a year, average food portions were weighed, e.g., low protein pasta, bread and low protein sausages. All children were observed eating at least one meal annually.

2.6. Nutritional Analysis

The dietary assessments were analyzed using Nutritics Nutritional Software (v5.093). The following macronutrients were analyzed: daily energy (Kcal), protein (g), carbohydrate (unrefined and refined) (g) and fat (g). For each subject, the annual median macronutrient value was calculated, and the median value for all subjects in each group was determined, giving the median of the median value for each macronutrient. The results were compared with age and gender specific UK dietary reference values or estimated average requirement (EAR) for energy (UK Scientific Advisory Committee on Nutrition (SCAN)) [42]. The nutrient contribution from CGMP-AA and AA were included in this analysis as well as all special low protein foods.

2.7. Anthropometric Measurements

Weight, height and BMI were measured once every three months by one of two metabolic dietitians. Height was measured with a Harpenden stadiometer (Holtain Ltd, Crymych, UK) and weight on calibrated digital scales (Seca UK model 875); they were measured to the nearest 0.1 cm and 0.1 kg, respectively.

2.8. Blood Phenylalanine Levels

Trained parents/caregivers collected weekly early morning fasted blood spots on filter cards, Perkin Elmer 226 (UK Standard NBS). Blood specimens were sent via first class post to the laboratory at Birmingham Children's Hospital. All the cards had a standard thickness and the blood phenylalanine concentrations were calculated on a 3.2-mm punch by MS/MS tandem mass spectrometry.

2.9. Statistical Analysis

Continuous data are represented as median (IQR) and categorical data were summarized as frequencies of counts and associated percentages. Analyses of study endpoints were performed using Analysis of Covariance (ANCOVA) techniques, which analyzes the data at three years follow-up while adjusting for baseline values. As there was a difference in age between participants between the two groups, all models were adjusted for patient age as well as gender. The impact of CGMP compared to AA was evaluated by comparison of CGMP100 and CGMP50. Descriptive statistics are reported as medians and differences at baseline and follow up were assessed using a paired t test. Analysis was performed using R (Version 3).

3. Results

Of the 50 children recruited, 48 completed the study: CGMP group, $n = 29$; AA group, $n = 19$. The CGMP group was divided into CGMP100, $n = 13$ (45%), and CGMP50, $n = 16$, (55%). The median ages at enrolment were: CGMP100, 9.2 years; CGMP50, 7.3 years; and AA, 11.1 years. There was a significant difference in age between the AA and CGMP50 ($p = 0.005$) and between CGMP50 and CGMP100 ($p = 0.04$).

Prior to starting the CGMP-AA, all patients were prescribed AA as their source of protein substitute. Six subjects took powdered amino acids (XP Maxamum (Nutricia Ltd., Trowbridge, UK), $n = 1$; PKU Anamix first spoon (Nutricia Ltd.), $n = 3$; PKU gel (Vitaflo International Ltd.), $n = 2$) and 44 subjects

took liquid pouches (PKU Lophlex LQ (Nutricia Ltd.), n = 3; PKU Cooler (Vitaflo International Ltd., Liverpool, UK), n = 41). The AA group remained on their usual protein substitute (PKU Lophlex LQ (Nutricia Ltd.), n = 1; PKU Cooler (Vitaflo International Ltd) n = 14) or a powdered preparation (PKU gel (Vitaflo International Ltd.), n = 4) throughout the study. The median (range) phenylalanine concentrations at baseline were not statistically different: CGMP100, 255 µmol/L (170–360); CGMP50, 290 µmol/L (220–430); and AA, 315 µmol/L (215–600). The majority had classical PKU, except two children who were mild based on untreated blood phenylalanine levels at diagnosis and dietary phenylalanine tolerance.

The median daily dose of protein equivalent from protein substitute was 60 g/day (range 40–80 g), and the median amount of prescribed natural protein was 5.5 g protein/day in all groups (range 3–30 g) or 275 mg/day phenylalanine (range 150–1500 mg).

3.1. Subject Withdrawal

One boy and one girl (aged 12 and 11 years, respectively) in the CGMP-AA group were excluded from the study, as both were unable to adhere with the study protocol. One failed to return blood phenylalanine samples and both had poor adherence to their phenylalanine restricted diet.

3.2. Nutritional Intake

Change in Nutrient Intake

In all the groups, the energy intake expressed as a percentage of EAR decreased over the three years. In the AA group: baseline, 106% (77–177), year 3, 95% (80–138); CGMP50, baseline, 105% (90–120), year 3, 100% (88–144); and CGMP100, baseline, 104% (85–126), year 3, 101% (87–118) (Table 2).

ANCOVA analysis adjusting for age, gender, energy intake (Kcal/d) and EAR showed that the difference in three-year EAR between AA and CGMP50 was not statistically significant (p = 0.717) and neither was the difference between AA and CGMP100 (p = 0.673). Further longitudinal analysis showed no significant differences between the groups over the three years for energy intake.

Table 2. Median daily energy intake (range) and %EAR (range) for AA, CGMP50 and CGMP100 from baseline to year 3.

	Median Energy (Kcal/Day)			**Median % EAR**		
Year	**AA (Range) n = 19**	**CGMP50 (Range) n = 16**	**CGMP100 (Range) n = 13**	**AA (Range) n = 19**	**CGMP50 (Range) n = 16**	**CGMP100 (Range) n = 13**
Baseline	1950 (1138–2999)	1701 (1466–2494)	1831 (1612–2591)	106 (77–177)	105 (90–120)	104 (85–126)
1	1957 (1151–3191)	1793 (1560–2681)	1917 (1642–2708)	102 (77–140)	99 (85–132)	96 (73–124)
2	1976 (1517–2669)	1828 (1512–2814)	1965 (1258–2810)	99 (72–130)	105 (90–158)	107 (85–152)
3	2120 (1111–3387)	1966 (1523–2405)	2064 (1672–3144)	95 (80–138)	100 (88–144)	101 (87–118)

AA, amino acid; CGMP, casein glycomacropeptide; EAR, estimated average requirement; CGMP50, patients taking a combination of CGMP-AA and AA; CGMP100, patients taking all their protein substitute from CGMP-AA.

Over the three-year period, the median percentage energy contribution from carbohydrate, protein and fat was not significantly different among the three groups (Table 3).

The median percentage energy contribution from protein (including protein substitute and natural protein from food) was similar for all three groups. Protein provided a median of 15% (75 g) of the total energy intake, and natural protein intake supplied a median intake of 10–16% (8–12 g/day) of the total protein intake. A small but significant difference was noted for the contribution of protein substitute between CGMP50 (88%) and CGMP100 (85%) (p = 0.01) as well as between CGMP50 (88%)

and AA (85%) ($p = 0.007$). Although the protein substitutes still provided >85% of the total protein intake in all groups, there were significant differences in the youngest age group (CGMP50) and in those children unable to tolerate extra phenylalanine from CGMP-AA (Table 3).

Table 4 describes the total protein intake from all foods including fruits and vegetables that are usually allowed without restriction in a UK diet. The actual intake was higher compared to the "prescribed" or allocated phenylalanine exchanges (due to the small amounts of protein present in foods given without restriction). There was a significant increase in the actual intake between baseline and year 3 in AA ($p = 0.002$) and CGMP50 ($p = 0.02$) groups. The median protein intake from food was significantly different between and within the groups. CGMP50 had the lowest natural protein intake, with significant differences between CGMP50 and both AA and CGMP100 (Table 4).

These differences reflect protein tolerance, being lower in the youngest age group and those unable to tolerate the extra phenylalanine from CGMP-AA to meet all their protein substitute requirements.

Table 3. Median three-year percentage (range) energy contribution for protein, carbohydrate and fat from food and protein substitute in AA, CGMP50 and CGMP100.

	Median % Energy from Protein			**Median % Energy from Carbohydrate**			**Median % Energy from Fat**		
	AA ***n* = 19**	**CGMP50** ***n* = 16**	**CGMP100** ***n* = 13**	**AA** ***n* = 19**	**CGMP50** ***n* = 16**	**CGMP100** ***n* = 13**	**AA** ***n* = 19**	**CGMP50** ***n* = 16**	**CGMP100** ***n* = 13**
Baseline (Range)	15 (10–30)	15 (9–26)	15 (11–26)	57 (45–68)	56 (43–70)	57 (45–69)	26 (15–40)	26 (17–37)	28 (17–33)
Year 1–3 (Range)	15 (9–27)	15* (10–22)	16* (12–23)	58 (42–70)	57 (46–67)	58 (48–67)	27 (16–39)	26 (18–37)	27 (18–35)

* $p = 0.02$. AA, amino acid; CGMP, casein glycomacropeptide; CGMP50, patients taking a combination of CGMP-AA and AA; CGMP100, patients taking all their protein substitute from CGMP-AA.

Table 4. Median natural protein intake (g/day) (range) from food sources only and median percentage intake from protein equivalent from substitute from baseline to year 3 in AA, CGMP50 and CGMP100.

	Natural Protein Intake from Food (g/d)			
Year	**AA** ***n* = 19**	**CGMP50** ***n* = 16**	**CGMP100** ***n* = 13**	***p* Value**
Baseline (Range)	11.5 * (5–32)	8 *,§ (4–23)	12 § (5–36)	*,§ $p = 0.001$
1 (Range)	12 * (5–33)	8 *,§ (4–24)	12 § (5–34)	$^{*}p = 0.0001$, § $p = 0.001$
2 (Range)	13 * (5–33)	9 *,§ (3–23)	10 § (5–44)	* $p = 0.014$, § $p = 0.02$
3 (Range)	12 * (6–46)	9 *,§ (3–24)	13 § (6–36)	* <0.0001, § $p = 0.0006$

Table 4. *Cont.*

Median % protein intake from protein substitute equivalent (range)				
Year	**AA**	**CGMP50**	**CGMP100**	***p* Value**
Baseline (Range)	85 (68–92)	87 (72–93)	85 (58–92)	
1 (Range)	86 (72–92)	88 (71–93)	83 (65–91)	
2 (Range)	86 (65–93)	87 (70–91)	86 (59–93)	
3 (Range)	84 (68–90)	88 (75–93)	84 (68–91)	
Year 1–3 (Range)	85 * (65–93)	88 *,§ (70–93)	85 § (59–93)	* $p = 0.01$, § $p = 0.007$

AA, amino acid; CGMP, casein glycomacropeptide; CGMP50, patients taking a combination of CGMP-AA and AA; CGMP100, patients taking all their protein substitute from * significant difference between AA and CGMP50; § significant difference between CGMP50 and CGMP 100.

3.3. Phenylalanine Control

There was a significant increase in blood phenylalanine levels between baseline and year 3 for AA ($p = 0.02$) and CGMP50 ($p = 0.04$), although all groups had median blood phenylalanine control within target (Table 5). This increase is an expected finding due to children reaching teenage years, when dietary adherence is known to deteriorate [43,44].

Table 5. Median (range) blood phenylalanine concentrations from baseline to year 3 in AA, CGMP50 and CGMP100.

	Baseline Median Phe µmol/L			**Year 3 Median Phe µmol/L**		
	AA µmol/L *n* = 19	**CGMP50 µmol/L *n* = 16**	**CGMP100 µmol/L *n* = 13**	**AA µmol/L *n* = 19**	**CGMP50 µmol/L *n* = 16**	**CGMP100 µmol/L *n* = 13**
Phe (Range)	315* (140–600)	255 § (170–360)	290 (200–710)	360 * (210–830)	290 § (220–430)	320 (250–895)

* $p = 0.02$, § $p = 0.04$. AA, amino acid; CGMP, casein glycomacropeptide; CGMP50, patients taking a combination of CGMP-AA and AA; CGMP100, patients taking all their protein substitute from CGMP-AA.

3.4. Anthropometric Data

For weight and BMI measurements, ANCOVA was applied adjusting for age, gender, weight and BMI over the three-year duration (Table 6).

Table 6. Change in weight and BMI Z scores from baseline to year 3 in the AA, CGMP50 and CGMP100 groups applying ANCOVA adjusting for variables in age and gender differences.

BMI Z Score	**Baseline**	**36 m**	**Differences**
AA	−0.15	0.63	0.3
CGMP50	0.17	0.85	0.5
CGMP100	−0.11	0.95	0.6
Wt Z score	**Baseline**	**36 m**	**Differences**
AA	−0.25	0.74	0.2
CGMP50	0.28	0.91	0.2
CGMP100	0.02	0.97	0.4

BMI: body mass index; Wt, weight; AA, amino acid; CGMP, casein glycomacropeptide; CGMP50, patients taking a combination of CGMP-AA and AA; CGMP100, patients taking all their protein substitute from CGMP-AA.

Weight and BMI Z Scores

There was no statistical difference in three-year weight Z scores between AA and CGMP50 ($p = 0.7$), AA and CGMP100 ($p = 0.7$) and CGMP50 and CGMP100 ($p = 0.95$). Similarly, for BMI Z scores there was no statistical difference in the three-year BMI Z scores between AA and CGMP50 ($p = 0.784$), AA and CGMP100 ($p = 0.553$) and CGMP50 and CGMP100 ($p = 407$).

Using the World Health organization (WHO) definition of obesity (BMI equivalent to two standard deviations over the reference median), obesity rates between years 1 and 3 remained unchanged in the AA group (26%, $n = 5/19$), increased in the CGMP50 group from 0% to 19% ($n = 3/16$) and remained at 0% in the CGMP100 group over the three years. Overweight, defined as one standard deviation over the reference median, decreased in the AA group from 37% ($n = 7/19$) to 26%, ($n = 5/19$), remained unchanged in the CGMP50 group at 19% ($n = 3/16$) and increased in the CGMP100 group from 15% ($n = 2/13$) to 46% ($n = 6/13$).

4. Discussion

This three-year longitudinal study in PKU systematically reviewed the macronutrient intake and anthropometry of children taking AA compared to CGMP-AA, with CGMP-AA provided at two different CGMP concentrations. Although satiety was not directly measured through satiety visual analog scales and hormone concentrations, the hypothesis that CGMP would enhance satiety leading to lower energy intake and slower weight gain was not observed. EAR decreased in all three groups over the three years, the most consistent decrease was in the AA group, suggesting the relationship between satiety and CGMP-AA was less convincing. Adjusting for age and gender, no differences in weight or BMI were apparent between the AA and CGMP-AA groups. Although there was no obesity present in the CGMP100 group over the three years of study, the rate of overweight increased from 15% to 45%. Our data suggest that the use of CGMP50 or CGMP100 did not reduce energy intake and thereby appetite.

Protein substitutes based on 60 g of protein equivalent from CGMP-AA provided a typical intake of approximately 45 g/day of CGMP. We cannot evaluate from this study if this quantity may have had a significant impact on hormones such as ghrelin and GLP-1, which are important in controlling satiety. It has been shown that whey protein given to healthy volunteers at 10% of the energy intake suppressed subjective hunger but made no impact on actual energy intake. At a higher protein intake of 25% of energy intake, it increased insulin, and active GLP-1 and incretin hormones were higher, but made no difference to overall satiety and energy intake [5]. In non-PKU human studies, consensus is strong that CGMP has no effect on food intake or satiety [16,45], and weight loss after long-term consumption of CGMP has not been demonstrated [17]. Overall, subjective feelings of appetite indicate that CGMP is not critical for whey-induced satiety or energy intake. In our study, natural protein intake was vegetable based, which remained consistent over the study period for each of the three groups and was not a consideration influencing satiety.

Indirectly, CGMP may influence satiety by its prebiotic properties but a direct relationship on appetite has not been studied [46]. Microbiota play a key role in regulating energy balance and satiety, by the interaction of gut hormones and pathways such as leptin-melanocortin, a critical system controlling appetite and energy balance [47,48]. CGMP can change microbiota. In mice fed CGMP, there was a reduction in Desulfovibrio bacteria, an increase in short chain fatty acids and reduced inflammatory markers [9]. Three products, lactose, commercial CGMP (70% GMP, <2% lactose) and semi-purified CGMP (51% GMP, 4% lactose), were tested on fecal high and low diversity microbiota from healthy and elderly subjects. Both CGMP products resulted in a healthy microbiota, being more pronounced in the lower lactose CGMP preparation [49]. How this translates to satiety remains unanswered.

Only one study has examined the effect of CGMP-AA and satiety in subjects with PKU. MacLeod et al. [50] gave 11 subjects (eight adults and three children) a standard breakfast with either CGMP-AA or phenylalanine-free AA. Plasma insulin, ghrelin and amino acids were measured 180 min after breakfast. Postprandial ghrelin was significantly lowered associated with fullness in the

CGMP group, with total plasma amino acids and insulin concentrations only just reaching significance between the groups. This short four-day study only measured one incretin hormone at one time point. Studies on satiety ideally need to employ a wide variation in time intervals, capturing differences in appetite and plasma amino acid profiles [51]. The carbohydrate intake was higher in the CGMP-AA breakfast, which may sustain insulin concentrations over the short study period. Results from the satiety questionnaire show 60% of the adult group were overweight. It also included only three children. Overall, these limitations render any direct interpretation of the effect of CGMP-AA on satiety challenging.

There were limitations to our study. Firstly, dietary assessments regardless of method are inaccurate, although face-to-face interviews and periodic weighed food intakes were conducted to minimize these difficulties. Satiety visual analog scales post protein substitute consumption were not conducted, although their value is subjective particularly in children and obese subjects. The AA group took different amino acid preparations but 15 of 19 consumed low energy liquid preparations. Age varied between the groups but was statistically accounted for; the influence of growth and exercise on appetite was unmeasured. No biochemical hormone markers were assessed. Both insulin and ghrelin are endocrine mediators of food intake. However, insulin is an anabolic hormone and ghrelin has growth hormone functions, further complicating any clear relationship with satiety in children who are growing and reaching adolescence. Children were not randomized to one of the three protein substitute groups; choice of group was dependent on protein substitute acceptance and blood phenylalanine control when taking CGMP-AA. One further limitation was the lack of a non-PKU control group; although dietary composition would have been different, a comparison of energy intake, weight and BMI would have been useful. Despite these limitations, no obvious impact on satiety was found between our two study groups. There are inherent difficulties in studying appetite given the behavioral and environmental factors that counterbalance the physiological regulators of appetite.

5. Conclusions

In this three-year longitudinal study in children with PKU, CGMP-AA when compared to AA did not appear to influence energy intake, weight gain or BMI and by implication satiety. In PKU, there is little understanding of the optimal dietary composition needed to control appetite preventing overweight and obesity. All macronutrients have unique physiological properties that influence metabolic pathways. The impact of CGMP-AA on satiety, particularly the amounts, timing of ingestion and its effect when combined with other foods related to satiety signals remains to be fully explored.

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References

1. Bickel, H. The first treatment of phenylketonuria. *Eur. J. Pediatr.* **1996**, *155* (Supp. l), 2–3. [CrossRef]
2. Bickel, H.; Gerrard, J.; Hickmans, E.M. Influence of phenylalanine intake on phenylketonuria. *Lancet* **1953**, *265*, 812–813. [CrossRef]
3. Li, T.; Zhao, M.; Raza, A.; Guo, J.; He, T.; Zou, T.; Song, H. The effect of taste and taste perception on satiation/satiety: A review. *Food Funct.* **2020**, *11*, 2838–2847. [CrossRef] [PubMed]
4. Tome, D.; Schwarz, J.; Darcel, N.; Fromentin, G. Protein, amino acids, vagus nerve signaling, and the brain. *Am. J. Clin. Nutr.* **2009**, *90*, 838S–843S. [CrossRef] [PubMed]
5. Veldhorst, M.A.; Nieuwenhuizen, A.G.; Hochstenbach-Waelen, A.; Westerterp, K.R.; Engelen, M.P.K.J.; Brummer, R.-J.M.; Deutz, N.E.P.; Westerterp-Plantenga, M.S. A breakfast with alpha-lactalbumin, gelatin, or gelatin + TRP lowers energy intake at lunch compared with a breakfast with casein, soy, whey, or whey-GMP. *Clin. Nutr.* **2009**, *28*, 147–155. [CrossRef]
6. Sousa, G.T.; Lira, F.S.; Rosa, J.C.; de Oliveira, E.P.; Oyama, L.M.; Santos, R.V.; Pimentel, G.D. Dietary whey protein lessens several risk factors for metabolic diseases: A review. *Lipids Health Dis.* **2012**, *11*, 67. [CrossRef]
7. Walstra, P.; Wouters, J.T.M.; Geurts, T.J. *Dairy Science and Technology*; CRC Press: Boca Raton, FL, USA; Taylor & Francis Group, LLC: Boca Raton, FL, USA, 2006; p. 768.
8. O'Riordan, N.; Kane, M.; Joshi, L.; Hickey, R.M. Structural and functional characteristics of bovine milk protein glycosylation. *Glycobiology* **2014**, *24*, 220–236. [CrossRef]
9. Sawin, E.A.; De Wolfe, T.J.; Aktas, B.; Stroup, B.M.; Murali, S.G.; Steele, J.L.; Ney, D.M. Glycomacropeptide is a prebiotic that reduces Desulfovibrio bacteria, increases cecal short-chain fatty acids, and is anti-inflammatory in mice. *Am. J. Physiol. Gastrointest Liver Physiol.* **2015**, *309*, G590–G601. [CrossRef]
10. Sharma, R.; Rajput, Y.S.; Mann, B. Chemical and functional properties of glycomacropeptide (GMP) and its role in the detection of cheese whey adulteration in milk: A review. *Dairy Sci. Technol.* **2013**, *93*, 21–43.
11. Hall, W.L.; Millward, D.J.; Long, S.J.; Morgan, L.M. Casein and whey exert different effects on plasma amino acid profiles, gastrointestinal hormone secretion and appetite. *Br. J. Nutr.* **2003**, *89*, 239–248. [CrossRef]
12. Veldhorst, M.A.; Nieuwenhuizen, A.G.; Hochstenbach-Waelen, A.; van Vught, A.J.; Westerterp, K.R.; Engelen, M.P.K.J.; Brummer, R.-J.M.; Deutz, N.E.P.; Westerterp-Plantenga, M.S. Dose-dependent satiating effect of whey relative to casein or soy. *Physiol. Behav.* **2009**, *96*, 675–682. [CrossRef]
13. Hoefle, A.S.; Bangert, A.M.; Rist, M.J.; Gedrich, K.; Lee, Y.M.; Skurk, T.; Danier, J.; Schwarzenbolz, U.; Daniel, H. Postprandial metabolic responses to ingestion of bovine glycomacropeptide compared to a whey protein isolate in prediabetic volunteers. *Eur. J. Nutr.* **2019**, *58*, 2067–2077. [CrossRef]
14. Royle, P.J.; McIntosh, G.H.; Clifton, P.M. Whey protein isolate and glycomacropeptide decrease weight gain and alter body composition in male Wistar rats. *Br. J. Nutr.* **2008**, *100*, 88–93. [CrossRef]
15. Cordova-Davalos, L.E.; Jimenez, M.; Salinas, E. Glycomacropeptide Bioactivity and Health: A Review Highlighting Action Mechanisms and Signaling Pathways. *Nutrients* **2019**, *11*, 598. [CrossRef]
16. Gustafson, D.R.; McMahon, D.J.; Morrey, J.; Nan, R. Appetite is not influenced by a unique milk peptide: Caseinomacropeptide (CMP). *Appetite* **2001**, *36*, 157–163. [CrossRef]
17. Keogh, J.B.; Clifton, P. The effect of meal replacements high in glycomacropeptide on weight loss and markers of cardiovascular disease risk. *Am. J. Clin. Nutr.* **2008**, *87*, 1602–1605. [CrossRef]
18. Lam, S.M.; Moughan, P.J.; Awati, A.; Morton, H.R. The influence of whey protein and glycomacropeptide on satiety in adult humans. *Physiol. Behav.* **2009**, *96*, 162–168.
19. Keogh, J.B.; Clifton, P.M. Weight loss using meal replacements high in glycomacropeptide-enriched whey powder. *Aust. J. Dairy Technol.* **2009**, *64*, 32–33.
20. Veldhorst, M.A.; Nieuwenhuizen, A.G.; Hochstenbach-Waelen, A.; Westerterp, K.R.; Engelen, M.P.; Brummer, R.-J.M.; Deutz, N.E.P.; Westerterp-Plantenga, M.S. Comparison of the effects of a high- and normal-casein breakfast on satiety, 'satiety' hormones, plasma amino acids and subsequent energy intake. *Br. J. Nutr.* **2009**, *101*, 295–303. [CrossRef]
21. Bowen, J.; Noakes, M.; Clifton, P.M. Appetite regulatory hormone responses to various dietary proteins differ by body mass index status despite similar reductions in ad libitum energy intake. *J. Clin. Endocrinol. Metab.* **2006**, *91*, 2913–2919. [CrossRef]

22. Batterham, R.L.; Heffron, H.; Kapoor, S.; Chivers, J.E.; Chandarana, K.; Herzog, H.; Le Roux, C.W.; Thomas, E.L.; Bell, J.D.; Withers, D.J. Critical role for peptide YY in protein-mediated satiation and body-weight regulation. *Cell Metab.* **2006**, *4*, 223–233. [CrossRef]
23. Pena, M.J.; Guerreiro, S.G.; Rocha, J.C.; Morais, T.; Pereira, S.S.; Monteiro, M.P.; Borges, N. Acute effect of an amino acid mixture in the rat glycemic profile. *J. Cell. Biochem.* **2019**, *120*, 13056–13065. [CrossRef]
24. Adams, R.L.; Broughton, K.S. Insulinotropic Effects of Whey: Mechanisms of Action, Recent Clinical Trials, and Clinical Applications. *Ann. Nutr. Metab.* **2016**, *69*, 56–63. [CrossRef]
25. Dangin, M.; Boirie, Y.; Garcia-Rodenas, C.; Gachon, P.; Fauquant, J.; Callier, P.; Ballevre, O.; Beaufrere, B. The digestion rate of protein is an independent regulating factor of postprandial protein retention. *Am. J. Physiol. Endocrinol. Metab.* **2001**, *280*, E340–E348. [CrossRef]
26. Poppitt, S.D.; Strik, C.M.; McArdle, B.H.; McGill, A.T.; Hall, R.S. Evidence of enhanced serum amino acid profile but not appetite suppression by dietary glycomacropeptide (GMP): A comparison of dairy whey proteins. *J. Am. Coll. Nutr.* **2013**, *32*, 177–186. [CrossRef]
27. Meyer-Gerspach, A.C.; Hafliger, S.; Meili, J.; Doody, A.; Rehfeld, J.F.; Drewe, J.; Beglinger, C.; Wolnerhanssen, B. Effect of L-tryptophan and L-leucine on gut hormone secretion, appetite feelings and gastric emptying rates in lean and non-diabetic obese participants: A randomized, double-blind, parallel-group trial. *PLoS ONE* **2016**, *11*, e0166758. [CrossRef]
28. Chungchunlam, S.M.; Henare, S.J.; Ganesh, S.; Moughan, P.J. Dietary whey protein influences plasma satiety-related hormones and plasma amino acids in normal-weight adult women. *Eur. J. Clin. Nutr.* **2015**, *69*, 179–186. [CrossRef]
29. Lacroix, M.; Bos, C.; Leonil, J.; Airinei, G.; Luengo, C.; Dare, S.; Benamouzig, R.; Fouillet, H.; Fauquant, J.; Tome, D.; et al. Compared with casein or total milk protein, digestion of milk soluble proteins is too rapid to sustain the anabolic postprandial amino acid requirement. *Am. J. Clin. Nutr.* **2006**, *84*, 1070–1079. [CrossRef]
30. Westerterp-Plantenga, M.S.; Nieuwenhuizen, A.; Tome, D.; Soenen, S.; Westerterp, K.R. Dietary protein, weight loss, and weight maintenance. *Annu. Rev. Nutr.* **2009**, *29*, 21–41. [CrossRef]
31. Mellinkoff, S.M.; Frankland, M.; Boyle, D.; Greipel, M. Relationship between serum amino acid concentration and fluctuations in appetite. *J. Appl. Physiol.* **1956**, *8*, 535–538. [CrossRef]
32. Korompokis, K.; Ostman, E.; Dougkas, A. The impact of liquid preloads varying in macronutrient content on postprandial kinetics of amino acids relative to appetite in healthy adults. *Appetite* **2016**, *107*, 511–520. [CrossRef] [PubMed]
33. Gropper, S.S.; Acosta, P.B. Effect of simultaneous ingestion of L-amino acids and whole protein on plasma amino acid and urea nitrogen concentrations in humans. *JPEN J. Parenter. Enter. Nutr.* **1991**, *15*, 48–53. [CrossRef]
34. Ha, E.; Zemel, M.B. Functional properties of whey, whey components, and essential amino acids: Mechanisms underlying health benefits for active people (review). *J. Nutr. Biochem.* **2003**, *14*, 251–258. [CrossRef]
35. Bos, C.; Metges, C.C.; Gaudichon, C.; Petzke, K.J.; Pueyo, M.E.; Morens, C.; Everwand, J.; Benamouzig, R.; Tome, D. Postprandial kinetics of dietary amino acids are the main determinant of their metabolism after soy or milk protein ingestion in humans. *J. Nutr.* **2003**, *133*, 1308–1315. [CrossRef] [PubMed]
36. Weigel, C.; Rauh, M.; Kiener, C.; Rascher, W.; Knerr, I. Effects of various dietary amino acid preparations for phenylketonuric patients on the metabolic profiles along with postprandial insulin and ghrelin responses. *Ann. Nutr. Metab.* **2007**, *51*, 352–358. [CrossRef]
37. Daly, A.; Evans, S.; Pinto, A.; Jackson, R.; Ashmore, C.; Rocha, J.C.; MacDonald, A. Preliminary Investigation to Review If a Glycomacropeptide Compared to L-Amino Acid Protein Substitute Alters the Pre- and Postprandial Amino Acid Profile in Children with Phenylketonuria. *Nutrients* **2020**, *12*, 2443. [CrossRef]
38. Belanger-Quintana, A.; Martinez-Pardo, M. Physical development in patients with phenylketonuria on dietary treatment: A retrospective study. *Mol. Genet. Metab.* **2011**, *104*, 480–484. [CrossRef]
39. Burrage, L.C.; McConnell, J.; Haesler, R.; O'Riordan, M.A.; Sutton, V.R.; Kerr, D.S.; McCandless, S.E. High prevalence of overweight and obesity in females with phenylketonuria. *Mol. Genet. Metab.* **2012**, *107*, 43–48. [CrossRef]
40. Van Wegberg, A.M.J.; MacDonald, A.; Ahring, K.; Belanger-Quintana, A.; Blau, N.; Bosch, A.M.; Burlina, A.; Campistol, J.; Feillet, F.; Gizewska, M.; et al. The complete European guidelines on phenylketonuria: Diagnosis and treatment. *Orphanet J. Rare Dis.* **2017**, *12*, 162. [CrossRef]

41. Daly, A.; Evans, S.; Chahal, S.; Santra, S.; MacDonald, A. Glycomacropeptide in children with PKU: Does its phenylalalnine content affect blood phenylalanine control? *J. Hum. Nutr. Diet.* **2017**, *30*, 515–523. [CrossRef]
42. Scientific Advisory Committee on Nutrition. *SACN Carbohydrates and Health Report*; Public Health England: London, UK, 2015; p. 384.
43. MacDonald, A. Diet and compliance in phenylketonuria. *Eur. J. Pediatr.* **2000**, *159* (Suppl. S2), S136–S141. [CrossRef]
44. MacDonald, A.; Gokmen-Ozel, H.; van Rijn, M.; Burgard, P. The reality of dietary compliance in the management of phenylketonuria. *J. Inherit. Metab. Dis.* **2010**, *33*, 665–670. [CrossRef]
45. Burton-Freeman, B.M. Glycomacropeptide (GMP) is not critical to whey-induced satiety, but may have a unique role in energy intake regulation through cholecystokinin (CCK). *Physiol. Behav.* **2008**, *93*, 379–387. [CrossRef]
46. Singh, R.K.; Chang, H.W.; Yan, D.; Lee, K.M.; Ucmak, D.; Wong, K.; Abrouk, M.; Farahnik, B.; Nakamura, M.; Zhu, T.H.; et al. Influence of diet on the gut microbiome and implications for human health. *J. Transl Med.* **2017**, *15*, 73. [CrossRef] [PubMed]
47. Crowley, V.E. Overview of human obesity and central mechanisms regulating energy homeostasis. *Ann. Clin. Biochem.* **2008**, *45*, 245–255. [CrossRef]
48. Forte, N.; Fernandez-Rilo, A.C.; Palomba, L.; Di Marzo, V.; Cristino, L. Obesity affects the microbiota-gut-brain axis and the regulation thereof by endocannabinoids and related mediators. *Int. J. Mol. Sci.* **2020**, *21*, 1554. [CrossRef]
49. Ntemiri, A.; Chonchuir, F.N.; O'Callaghan, T.F.; Stanton, C.; Ross, R.P.; O'Toole, P.W. Glycomacropeptide sustains microbiota diversity and promotes specific taxa in an artificial colon model of elderly gut microbiota. *J. Agric. Food Chem.* **2017**, *65*, 1836–1846. [CrossRef]
50. MacLeod, E.L.; Clayton, M.K.; van Calcar, S.C.; Ney, D.M. Breakfast with glycomacropeptide compared with amino acids suppresses plasma ghrelin levels in individuals with phenylketonuria. *Mol. Genet. Metab.* **2010**, *100*, 303–308. [CrossRef]
51. Chungchunlam, S.M.; Moughan, P.J.; Henare, S.J.; Ganesh, S. Effect of time of consumption of preloads on measures of satiety in healthy normal weight women. *Appetite* **2012**, *59*, 281–288. [CrossRef]



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CHAPTER 7

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INVESTIGATION OF PAEDIATRIC PKU BREATH MALODOUR, COMPARING GLYCOMACROPEPTIDE WITH PHENYLALANINE FREE L-AMINO ACID SUPPLEMENTS

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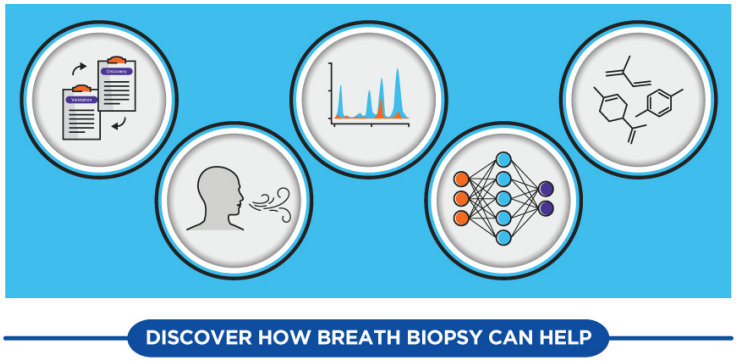
PAPER

Investigation of paediatric PKU breath malodour, comparing glycomacropeptide with phenylalanine free L-amino acid supplements

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Keywords: phenylketonuria (PKU), glycomacropeptide, PKU supplements, breath analysis, breath malodour, GC-IMS, volatile organic compounds (VOCs)

Abstract

In clinical practice, caregivers of children with phenylketonuria (PKU) report that their children have breath malodour. This might be linked to the regular consumption of low phenylalanine (Phe)/Phe-free protein substitutes (PS), which are an essential component of a low-Phe diet. Oral malodour can negatively affect interpersonal communication, lead to bullying, low self-esteem and social isolation. In this longitudinal cross-over study, exhaled volatile organic compounds (VOCs) were measured using gas chromatography-ion mobility spectrometry. 40 children (20 PKU, 20 controls) were recruited. Subjects with PKU took either L-Amino Acid (L-AA) or Casein Glycomacropeptide (CGMP-AA) exclusively for 1 week, in a randomised order. On the seventh day, seven exhaled breath samples were collected over a 10 h period. Subjects then transferred to the other PS for a week and on day seven, provided seven further breath samples. All subjects had a standardised menu using low-Phe food alternatives and all food intake was measured and recorded. In the PKU group, the aim was to collect samples 30 min after consuming PS. In 3 subjects, breath was collected 5 min post-PS consumption. Fasted L-AA and CGMP-AA breath samples contained a similar number of VOC peaks (10-12) as controls. Longitudinal breath testing results demonstrate that there was no significant difference in the number of exhaled VOCs, comparing L-AA or CGMP-AA with controls, or between PS (12-18 VOC peaks). Breath analysed immediately after consumption of PS ($n = 3$) showed an immediate increase in the number of VOC peaks (25-30), but these were no longer detectable at 30 min post-consumption. This suggests PS have a transient effect on exhaled breath. Measurements taken 30 min after consuming L-AA or CGMP-AA were not significantly different to controls. This indicates that timing food and drinks with PS consumption may be a potential solution for carers to reduce or eliminate unpleasant PS-related breath odours.

1. Introduction

Phenylketonuria (PKU) is an inherited metabolic disorder, caused by a deficiency in the enzyme phenylalanine hydroxylase (PAH), which is responsible for converting the amino acid phenylalanine (Phe) into tyrosine. This metabolic disruption causes Phe levels to accumulate in blood, which if untreated, causes neurotoxicity [1] leading to severe and irreversible neurological impairments [2]. PKU has been reported in all ethnic groups, affecting males and females in similar numbers. Incidence rates are around one in every 10 000 births but with a high prevalence in Turkey, Poland and Ireland [3]. In the UK, infants with PKU are detected by new-born screening [4]. While there is no cure for PKU, effective management of the condition is achieved through dietary restriction of Phe (in the form of a strict low-protein diet) and

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supplementation with Phe-free protein substitutes (PS) [5]. PS are essential, supplying adequate nitrogen for growth, suppressing blood Phe concentrations, and most will provide comprehensive vitamin and mineral supplementation meeting age appropriate requirements.

Most children with PKU tolerate $<10\ g\ d^{-1}$ natural protein. This means that high protein foods are avoided with a controlled intake of some vegetables and cereals and children must eat different foods from their friends, compromising normal socialisation. They are usually recommended to take PS 3 times daily with meals. While there have been significant improvements in the taste, appearance and presentation of PS [6], overall PS tastes unpleasant and patients often associate intake with bad breath. This is a concern of parents and caregivers, since oral malodour can result in psychological distress, interfere with social interactions, lower self-esteem and body-image [7]. Breath malodour in PKU has received little clinical or research attention, and it is unknown if this is an issue more common in PKU than a healthy population.

This study focuses on two types of PS; Phe-free L-amino acids (L-AA) and low-Phe casein glycomacropeptide (CGMP-AA). Conventional L-AA consist of single amino acids and have a characteristic odour associated with their pure amino acid composition. Casein glycomacropeptide is a peptide, derived from the whey fraction during the cheese manufacturing process. It is naturally low in phenylalanine and after the addition of some essential amino acids, has been adapted for use as a PS in PKU. CGMP-AA has a different composition from L-AA PS, being made of 60% peptide and 40% L-AA. In PKU, no studies have compared L-AA with CGMP-AA and reported impact on breath malodour. It is suggested that CGMP-AA is associated with less oral malodour.

Since there is limited research, the authors propose three possible causes for PKU-associated breath malodour: (1) residue from the PS in the oral cavity leads to a temporary change in breath composition (i.e. breath smells like the PS); (2) residue and regular replenishment of sulphur-containing amino acids from the PS (such as cystine and methionine) serve as a substrate for bacterial degradation in the oral cavity [8] resulting in the release of volatile sulphur compounds (VSCs) such as hydrogen sulphide, methyl mercaptan, and dimethyl sulphide (DMS), which are typically associated with halitosis [9]; and (3) modifications to the exhaled breath composition of subjects with PKU is caused by changes in metabolism or the gut microbiota due to their low-protein diet [10], resulting in oral malodour. These factors are not necessarily mutually exclusive and could be produced by a combination of these effects.

Currently, three methods for measuring oral malodour are available, including: organoleptic measurement, sulphide monitors and gas chromatography-flame photometric detector (GC-FPD) [11]. The organoleptic method is generally considered the gold standard halitosis test, and involves assessing oral malodour, as perceived by the human nose of a trained investigator [12]. However, this method is expensive (requiring a highly trained professional), subjective (cannot accurately quantify the oral malodour level) and is unsuitable for testing large study groups. Portable commercial instruments such as the OralChroma (Nissha FIS, Osaka, Japan) and Halimeter (Interscan Cor., Simi Valley, CA, USA) provide a relatively inexpensive and easy-to-use alternative to quantify odour levels. The disadvantages of these devices are that they can only measure VSCs with relative concentration measurements and cannot provide chemical identification of other volatile organic compounds (VOCs) in exhaled breath [13]. GC-FPD is a reliable, objective and reproducible method of quantifying breath contents. However, these instruments are bulky, expensive and require trained operators [14]. In recent years, alternative GC-based technologies, such as combining gas chromatography with ion mobility spectrometry (collectively known as GC-IMS), have produced portable analysers for exhaled breath, which can measure VOCs in the low parts-per-billion (ppb) range and are suitable for sampling vulnerable subjects, including children.

Therefore, in a collaborative research effort, undertaken by Birmingham Children's Hospital, the University of Warwick and University Hospitals Coventry and Warwickshire, this study aims to investigate the phenomenon of paediatric PKU breath malodour by analysing exhaled breath using GC-IMS technology.

2. Materials and methods

2.1. Subjects

Forty children (20 PKU, 20 healthy controls) were recruited for this longitudinal cross-over pilot study. This study received ethical approval (IRAS ref: 211894) and was conducted in accordance with the Declaration of Helsinki. Participants were recruited to create a balanced cohort (gender, age, and ethnically matched). The median age was 11 years (range 7–15.5 years), including 18 males and 22 females. The Phe concentrations of subjects with PKU were measured by weekly blood spots 12 months prior to starting the study. Median Phe concentrations for children $\leqslant 12$ years ($n = 17$) was 270 $\mu mol\,l^{-1}$ (range 150–360 $\mu mol\,l^{-1}$), and children >12 years ($n = 3$) was 330 $\mu mol\,l^{-1}$ (range 315–350 $\mu mol\,l^{-1}$). Both these groups show excellent Phe control within the European target recommendations [15]. An overview of the demographic data for the study is shown in table 1.

Eight sets of siblings were involved in this study; 4 sets in both groups and 1 set provided a control and a child with PKU. Recruiting siblings can reduce the

Table 1. PKU breath study demographic data of subjects.

Parameter	Subjects with PKU ($n = 20$)	Controls ($n = 20$)
Age (SD)	10.5 (2.5)	10.6 (2.5)
Gender ratio M:F	9:11	9:11
Siblings (Twins)	3 (1)	4 (0)
Regular medication (No. of subjects)	Ritalin (1) Neurotransmitter medication (1)	N/A

Table 2. Confounding factor groups.

Factor	Groups	PKU	Controls	Total
Age	$\leqslant$10 years	10	10	20
	>10 years	10	10	20
Gender	Male	9	9	18
	Female	11	11	22

possible effects of confounding factors, such as lifestyle and environment [16]. Confounding factors can be accounted for in the design-stage by recruiting a balanced cohort and in the analysis stage by separately investigating factors, such as age and gender. For these purposes, age and gender groups were defined as [$\leqslant$10 versus >10 years] and [male versus female], respectively. The confounding factor groups are shown in table 2.

2.2. Study design

To better understand the effects of paediatric PKU breath malodour, the recruited subjects were asked to complete a questionnaire relating to how they perceive their breath. Subjects with PKU took all their PS requirement from either L-AA or CGMP-AA exclusively for 7 d, in a randomised order. On the seventh day exhaled breath samples were collected over a 10 h period. The subjects arrived fasted and samples were collected first thing, post-morning meal, pre- and post-midday and evening meals, and an afternoon sample taken at a standard time. Subjects with PKU then transferred to the other PS for 7 d and on day seven, provided seven further breath samples as before. When randomised to L-AA, 17 children were on liquid and three on powdered products. The liquid L-AA PS were: PKU Lophlex LQ juicy berry $n = 3$, (Nutricia Ltd), PKU Cooler red $n = 12$, PKU Cooler Air red $n = 2$, (Vitaflo, Liverpool, UK). The flavours of the L-AA powder PS were: PKU express raspberry $n = 1$, PKU gel raspberry $n = 1$, (Vitaflo, Liverpool, UK), PKU First Spoon unflavoured $n = 1$ (Nutricia Ltd). They were all reconstituted with water. CGMP-AA was also a powdered PS, available in vanilla $n = 2$ and berry $n = 18$ flavours (Vitaflo Ltd, Liverpool, UK) and was reconstituted with water.

For the first breath sample (before morning meal) all children attended the research unit without brushing their teeth. Breath samples were collected in a staggered timetable to maintain standard interval times between samples for all subjects. A maximum of four subjects were sampled each day, including control, L-AA and CGMP-AA subjects on most days. All subjects consumed a standardised menu on the days of breath sampling using low-Phe alternatives, e.g. low-protein pasta for subjects with PKU and regular pasta for controls. All food intake was measured and recorded. The cross-over study design and staggered timetable are shown in figure 1 and appendix respectively.

In the PKU group, the aim was to collect samples 30 min after consuming the PS to allow subjects to digest and metabolise their meals and PS. This enabled measurements in potential changes in exhaled breath composition related to the metabolism of PS (i.e. pharmacokinetic changes), instead of residue from the PS.

2.3. Gas chromatography-ion mobility spectrometry (GC-IMS)

This study utilised a commercially-available, GC-IMS instrument (G.A.S. BreathSpec, Dortmund, Germany). This technology uses GC as a pre-separator, followed by a drift-tube IMS detector. The BreathSpec GC-IMS was equipped with a MXT-200 midpolarity column (Thames Restek, Saunderton, UK) and fitted with a circulator gas flow unit (G.A.S. CGFU, Dortmund, Germany). This unit recirculated and filtered the air and allowed the unit to operate without the need of an external gas supply. When the breath sample was introduced to the GC, chemical interactions with the column cause individual compounds to elute at different times—known as the retention time. This stage was followed by the IMS detector, where the pre-separated analytes were ionised and injected into a drift-tube. The ions drifted against a buffer gas, under influence of a uniform electric field (400 V cm^{-1}). This enabled ions to achieve different velocities, inversely proportional to their size, mass and charge [17]. They were then collected on a Faraday plate, to provide a time-dependent signal corresponding with ion mobility. The buffer gas flow rate was 150 ml min^{-1} and carrier flow rate through the instrument was 15 ml min^{-1}, while the sample was being introduced. The carrier flow rate was then ramped to 50 ml min^{-1} over the 11 min measurement time. The temperatures of the GC column and IMS were set to 45 °C. The device can measure substances down to the low ppb range, depending on the proton affinity of the compound. The instrument was suitable for a clinical setting and was placed on a work surface (dimensions: 45 × 50 × 30 cm; mass: 20 kg).

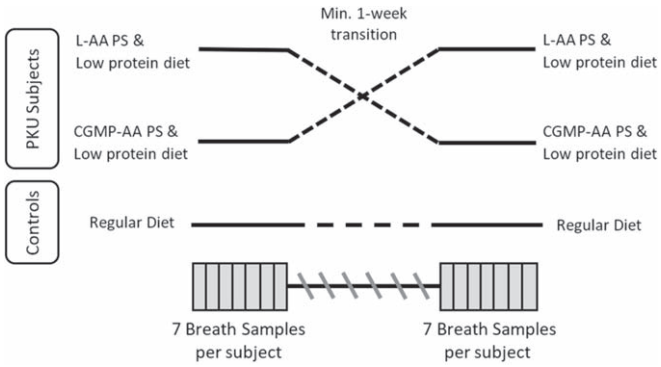


Figure 1. Longitudinal cross-over study design: subjects with PKU consumed L-AA or CGMP-AA exclusively, by random order for 7 d, and on day seven, provided seven breath samples. Subjects with PKU then transferred to the other PS for 7 d and provided seven further breath samples on day seven.

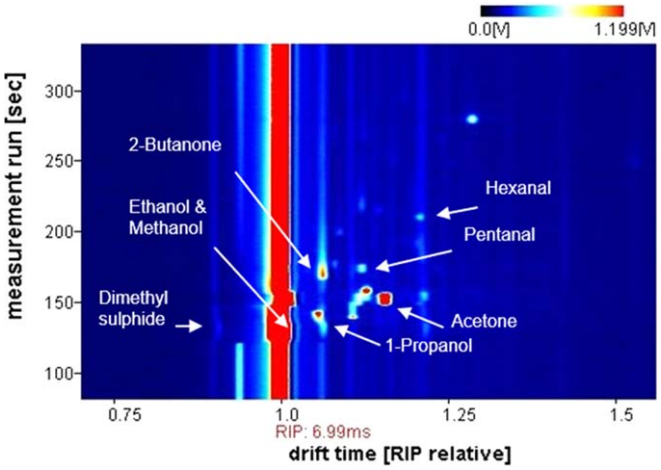


Figure 2. Typical GC-IMS BreathSpec output chromatogram (control subject).

2.4. Breath sampling

The BreathSpec instrument collects end-tidal breath using an integrated sampling system. Subjects were provided with a disposable polypropylene mouthpiece (G.A.S., Dortmund, Germany), which attached to a holder (connected to the front-panel of the instrument). A new mouthpiece was provided for each sample. The mouthpiece is a tapered and open-ended tube, with a one-way valve. As the subject exhales through the tube, air is displaced, which separates dead-space from end-tidal breath. This portion of exhalate is pulled into the 10 ml sample loop, prior to being introduced to the GC column. The transfer tubing between the mouthpiece holder and instrument/sample loop was heated, with a temperature set to 45 °C. The sampling procedure only required around 4 s of exhaled breath. Subjects were not required to exhale until their lungs were as empty as possible; instead, sampling only required a single normal breath. This procedure improved reproducibility and was appropriate for paediatric subjects.

2.5. Data analysis

A typical output response (chromatogram) from the GC-IMS is shown in figure 2; specifically, a fasted sample from a control subject. The chromatogram is represented as a topographic map, whereby each data point was characterised by the retention time in the chromatographic column (in seconds), the drift time (in milliseconds) and the intensity of the ion current signal (in volts), as indicated by colour. The reactant ion peak (RIP) refers to the constant peak in the spectrum that results from the carrier gas being always present in the measurement process. This peak can be used to align samples for data analysis. As a result, the drift time is represented as a RIP relative value. Laboratory Analytical Viewer software (v2.2.1, G.A.S., Germany) was used to analyse chromatograms.

For data analysis, a custom program was written in MATLAB (R2018b, MathWorks, Natick, MA, USA) to detect, count and extract VOC information. This data analysis approach attempts to represent breath samples as VOC 'fingerprints' by quantifying the number and types of endogenous VOCs (compounds that originate from within the body). This method has been

previously used to demonstrate that patients with Alzheimer's disease have different exhaled breath VOC fingerprints than healthy controls [18]. The first step involves a pre-processing stage. The aim of which was to remove the background (that contains no useful information). A typical GC-IMS dataset contains 11 million data points. The individual regions of high intensity, which correspond to individual VOC peaks, are thus made up of tens of single data points. Since we were only interested in these areas, we could remove the background by setting a static threshold. The remaining clusters of non-zero data points can then be considered as individual VOC peaks and totalled. In addition, their locations and maximum intensities can be extracted. Maximum peak intensities were selected, as we are able to correspond this with the abundance of the compound and it is independent of the applied background threshold. Using the locations of the VOC peaks, GC-IMS Library Search software (v1.0.1, G.A. S., Dortmund, Germany) was used to identify the compounds, based on gas chromatographic retention times and ion mobility drift times. These were linked to a NIST database, which included 400 000 annotated retention indices and an estimated 83 000 compounds entries [19]. Some common breath VOCs have been labelled in figure 2.

2.6. Quality assurance and control

For quality assurance, the position and quality of the RIP on the GC-IMS was regularly checked for signs of contamination. Samples were collected in the same setting, by the same operator, throughout the study. This ensured consistent sampling procedures since the collection process was manually triggered by the operator, while the subject exhaled through the mouthpiece. Additional quality control procedures were implemented. This involved collecting four room air samples at suitable intervals between breath samples (8:00, 11:00, 15:15 and 18:45) to monitor changes in ambient air and identify exogenous VOCs (compounds that do not originate from within the body).

To match the GC-IMS Library Search software with the equipped column, the GC-IMS instrument was normalised using a standard ketone mix (2-butanone, 2-pentanone, 2-hexanone, 2-heptanone, 2-octanone). The locations of common breath VOCs, such as acetone, 2-butanone, pentanal, hexanal and 1-propanol were also verified using chemical standards. The locations of other compounds, such as DMS and limonene were identified using the GCxIMS Library Search software. In addition to normalising the column for compound identification, it is possible to develop quantification curves for some VOCs. This was achieved using a dilution series of the target compound to generate a calibration function, which allows the voltage signal from the GC-IMS output to be converted into ppb concentrations.

3. Results

3.1. Questionnaire responses

The questionnaire responses are shown in figures 3–8. The results indicate that 50% of subjects with PKU dislike or strongly dislike the taste of L-AA, compared to 35% with CGMP-AA (figure 3). Subjects with PKU worry more about their breath smelling when consuming L-AA, over CGMP-AA (figures 4 and 8). Controls stated that they worried little or not at all about their breath and that others do not comment on their breath (figures 4 and 5). This suggests that the problem is associated with PS consumption. While most subjects (controls and subjects with PKU) noticed their breath smelling when fasting in the morning, subjects with PKU also observed their breath odour in the afternoon and after consuming PS (figure 6). A limited number of subjects from both PS groups worried their breath smell might affect friendships (figure 7). The questionnaire results indicate that subjects with PKU experience breath malodour in greater numbers than controls and that these subjects notice their breath smelling more when consuming L-AA, compared to CGMP-AA.

Descriptive statistics were applied to the questionnaire results, using one-way ANOVA analysis and Wilcoxon matched t-test. A significant difference between the L-AA compared to the CGMP-AA and control groups was evident when children were asked 'Q2: Do you worry about your breath smelling?' ($p = 0.02$), 'Q3: Do others notice/comment your breath smells?' ($p = 0.03$), 'Q5: Does your breath affect your friendships?' ($p = 0.04$), 'Q6 + 7: Do you notice your breath smells more with L-AA than CGMP-AA, or CGMP-AA than L-AA?' ($p < 0.0001$).

3.2. Headspace gas and exhaled breath outputs

To demonstrate that the BreathSpec GC-IMS could measure VOCs from the PS, analysis was conducted of the headspace gas generated from heating up a small portion (5 ml) to 40 °C (DB-2D Dri-Block, Techne, Stone, UK) in a 20 ml glass vial, for 10 min To illustrate the change in the GC-IMS output, an empty vial was also analysed.

For comparison, the exhaled breath of one subject with PKU was analysed, 5 min after consuming the PS. To demonstrate the change in the GC-IMS output, the exhaled breath of the same subject was analysed in a fasted state, 30 min after consuming the PS, and 105 min after consuming the PS. The results for L-AA and CGMP-AA are shown in figures 9 and 10, respectively.

The VOC analysis program detected >75 VOC peaks in the headspace gas of supplement L-AA, compared to 25 in exhaled breath from a subject with PKU, 5 min post-consumption of PS. Supplement CGMP-AA had >140 VOC peaks in headspace gas and 30 VOC peaks 5 min post-consumption of PS. By comparison, there were nine and 11 VOC peaks in the fasted breath samples of the same subject,

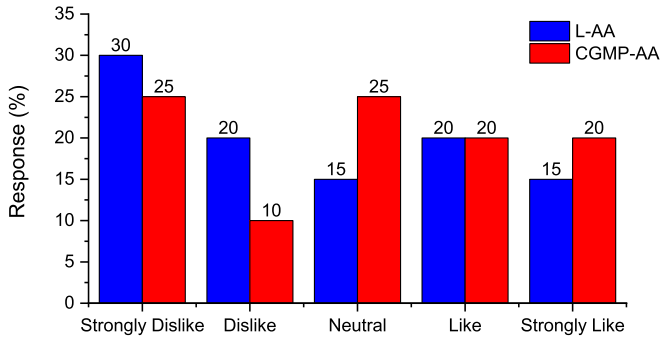


Figure 3. Questionnaire Responses to Question 1 (Q1): Do you like/dislike the taste of your L-AA or CGMP-AA supplements?

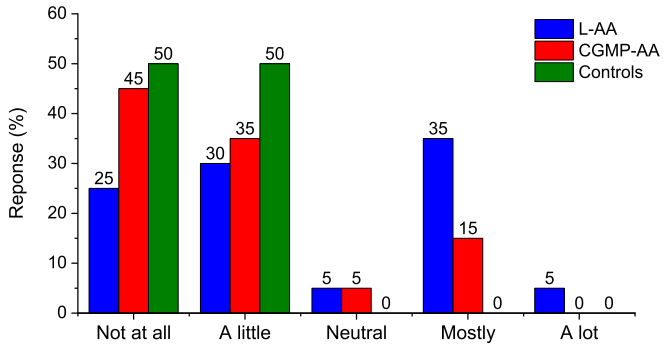


Figure 4. Questionnaire Responses to Question 2 (Q2): Do you worry about your breath smelling?

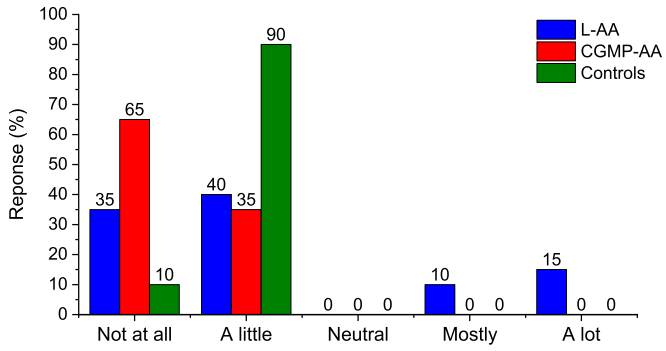


Figure 5. Questionnaire Responses to Question 3 (Q3): Do others notice/comment your breath smells?

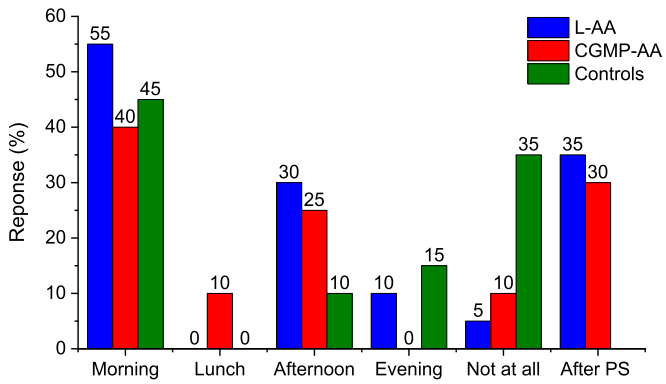


Figure 6. Questionnaire Responses to Question 4 (Q4): When does your breath smell more?

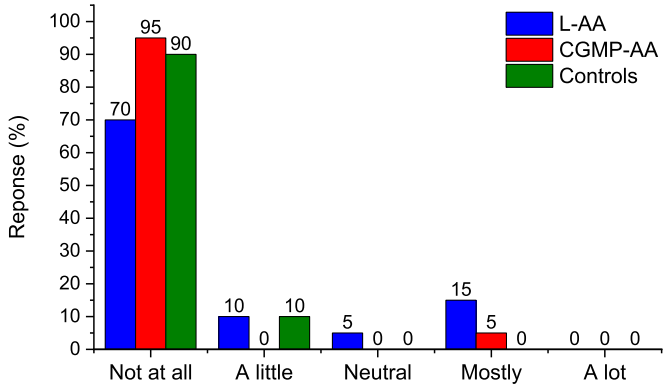


Figure 7. Questionnaire Responses to Question 5 (Q5): Does your breath affect your friendships?

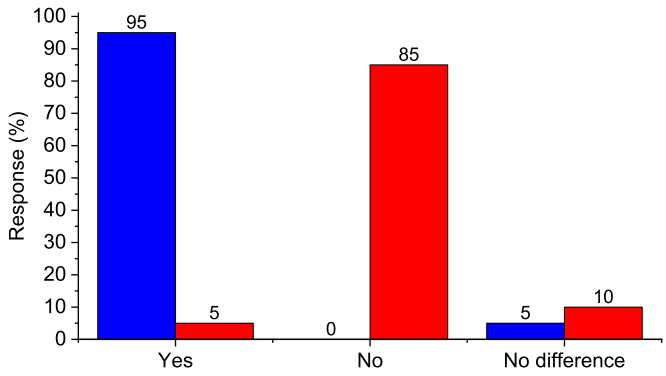


Figure 8. Questionnaire Responses to Questions 6 and 7 (Q6 + 7): Do you notice your breath smells more with L-AA than CGMP-AA (blue) or CGMP-AA than L-AA (red)?

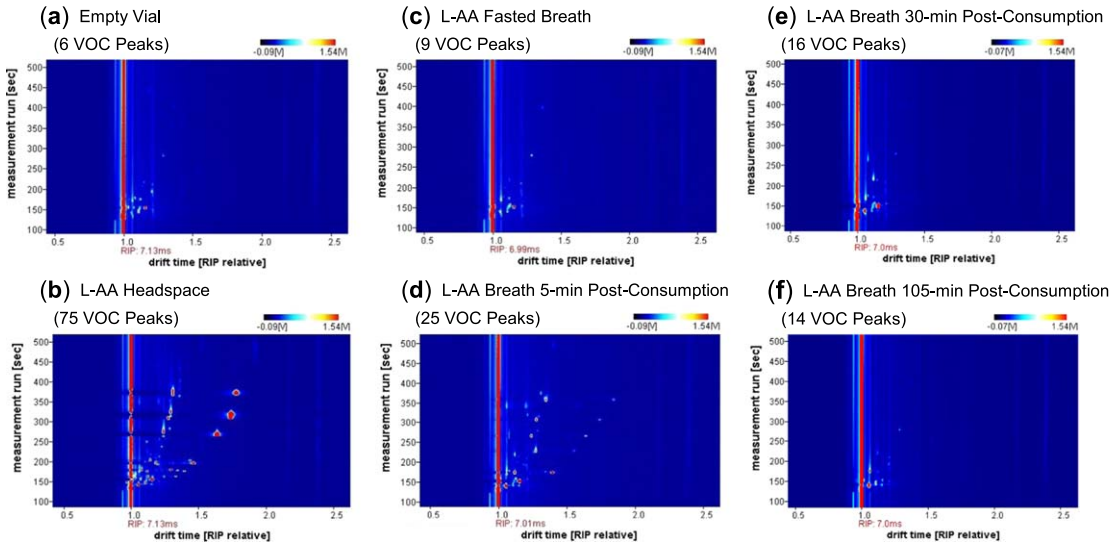


Figure 9. L-AA: (a) Empty vial; (b) L-AA headspace gas; (c) subject with PKU fasted exhaled breath; (d) subject with PKU exhaled breath 5 min post-consumption of L-AA; (e) subject with PKU exhaled breath 30 min post-consumption of L-AA; (f) subject with PKU exhaled breath 105 min post-consumption of L-AA.

respectively. The number of VOC peaks in samples collected 30 and 105 min post-consumption of either PS (11-16 VOC peaks) are significantly lower than in samples collected 5 min post-consumption. The extracted VOC peak locations from the PS established a strong alignment between VOCs found in the headspace gas and exhaled breath, shortly after consumption.

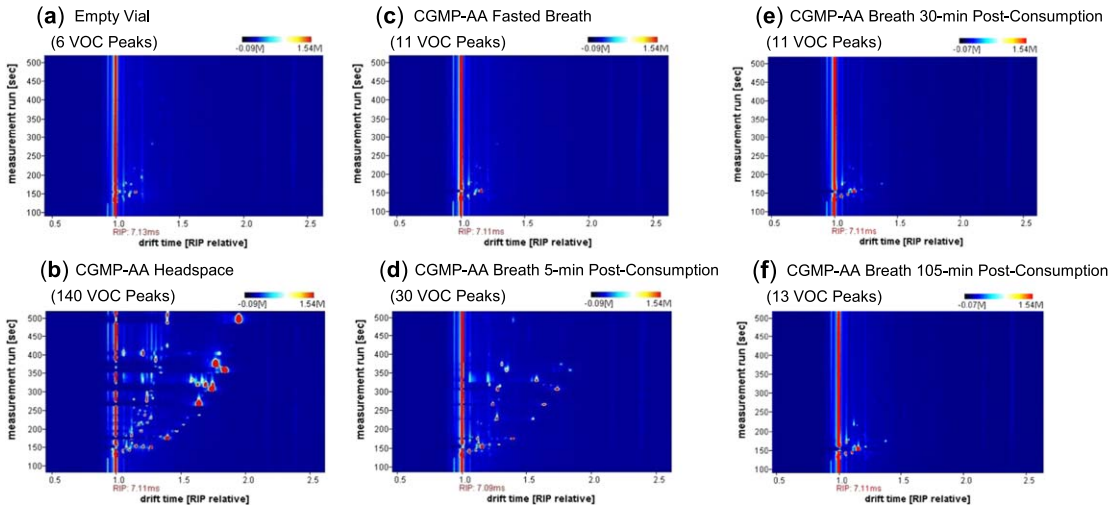


Figure 10. CGMP-AA: (a) empty vial; (b) CGMP-AA headspace gas; (c) subject with PKU fasted exhaled breath; (d) subject with PKU exhaled breath post-consumption of CGMP-AA; (e) subject with PKU exhaled breath 30 min post-consumption of CGMP-AA; (f) subject with PKU exhaled breath 105 min post-consumption of CGMP-AA.

3.3. Longitudinal breath samples

Figure 11 shows the longitudinal trends of the number of VOC peaks in the exhaled breath samples of controls, and subjects with PKU consuming L-AA and CGMP-AA, across all seven sampling intervals. The plot represents mean values, with standard error whiskers.

Closer examination of the fasted sampling interval demonstrated that the number of VOC peaks for all groups start at similar levels. A box plot of the number of VOC peaks for the fasted samples is given in figure 12. This demonstrated that the average number of VOC peaks in controls was slightly lower than L-AA and CGMP-AA, but not significantly different. CGMP-AA was associated with the greatest variance.

In addition to monitoring the number of VOCs peaks, it was possible to track the intensity of specific VOCs, such as acetone, DMS and 2-butanone (figures 13–15, respectively). DMS was of particular interest, since it is a compound typically associated with halitosis.

The intensity trends shown in figures 13 and 14 indicate that acetone and DMS exhibit similar trends across all groups. The intensity trends of 2-butanone (figure 15) show some differences between subjects with PKU and controls. The post-morning meal (post-fasted) sample is associated with a sharp increase in 2-butanone for subjects with PKU (both L-AA and CGMP-AA). To better characterise this behaviour, the quantification procedure described in section 2.6 was completed for 2-butanone to convert voltage signals from the GC-IMS output into ppb concentrations. The quantification model indicates that the concentration of 2-butanone in the post-fasted samples for subjects with PKU was around 3.9 ppb, compared to 2.0 ppb for controls. Subsequent samples are comparable in intensity (around 3.0 ppb) for all groups and follow similar increasing trends for the rest of the day.

3.4. Confounding factors

The analysis in figure 11 was repeated after categorising the subjects based on confounding factor groups, instead of intervention groups, e.g. younger versus older subjects, instead of controls versus L-AA versus CGMP-AA. The longitudinal trends of the number of VOC peaks in exhaled breath samples, based on age and gender, are shown in figures 16 and 17, respectively.

4. Discussion

The results of this study provide an important insight into how subjects with PKU perceive their breath after consuming PS. The questionnaire results suggest that control subjects worried little or not at all about their breath, but subjects with PKU were concerned about breath odour and associated this with PS. However, the two types of PS were not perceived in the same way, since 95% of subjects with PKU stated that they notice their breath more with L-AA than CGMP-AA. Unlike CGMP-AA, L-AA was also associated with others noticing breath malodours. Study results from the questionnaire suggests that the effects of L-AA and CGMP-AA on exhaled breath are likely to be different, immediately post-consumption. The effects of PS consumption were analysed by locating and extracting VOC information from the headspace gas and breath samples for both PS.

Analysis of headspace gas from L-AA and CGMP-AA was associated with over 75 and 140 VOC peaks, respectively; while only 25-30 VOC peaks were

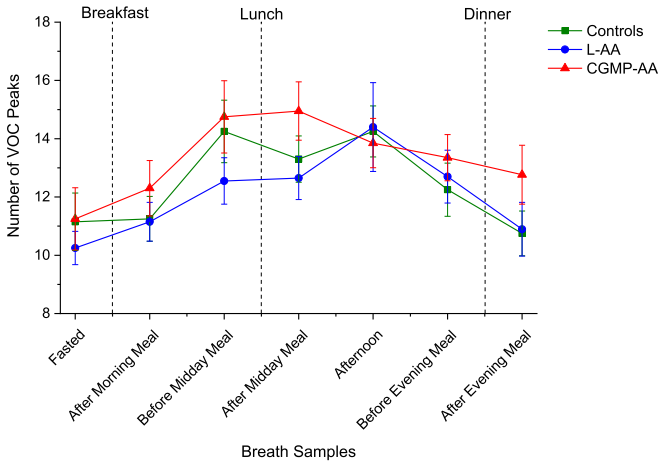


Figure 11. Number of VOC peaks by intervention groups.

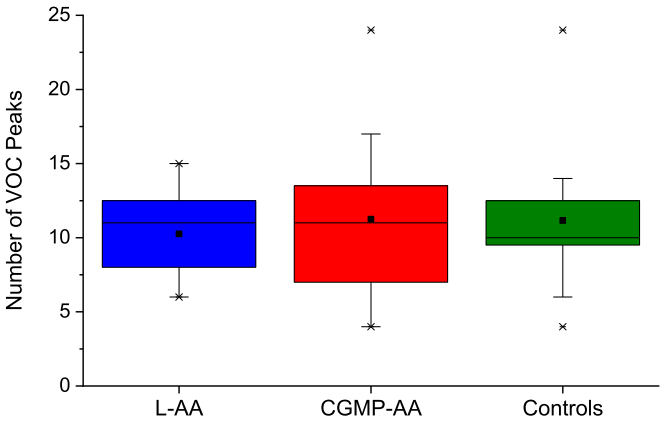


Figure 12. Box plot for number of VOC peaks from fasted sampling interval.

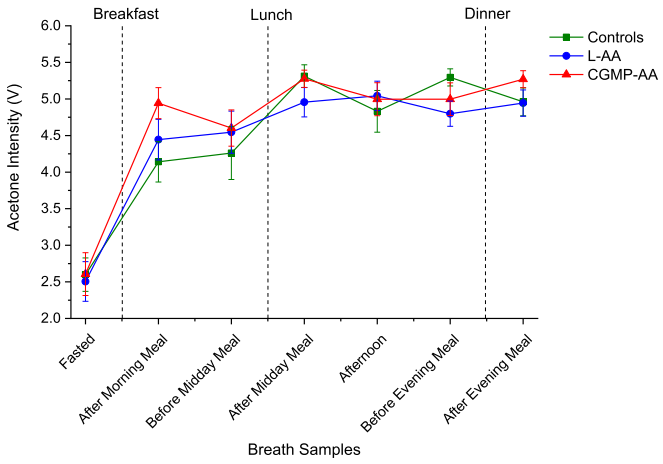


Figure 13. Acetone intensity changes by intervention groups.

detected in the exhaled breath from subjects with PKU, 5 min after PS consumption. VOCs in PS thus appear in reduced numbers and lower concentrations in exhaled breath, shortly post-consumption. CGMP-AA is likely associated with greater numbers of VOC peaks, due to additional flavourings and sweeteners,

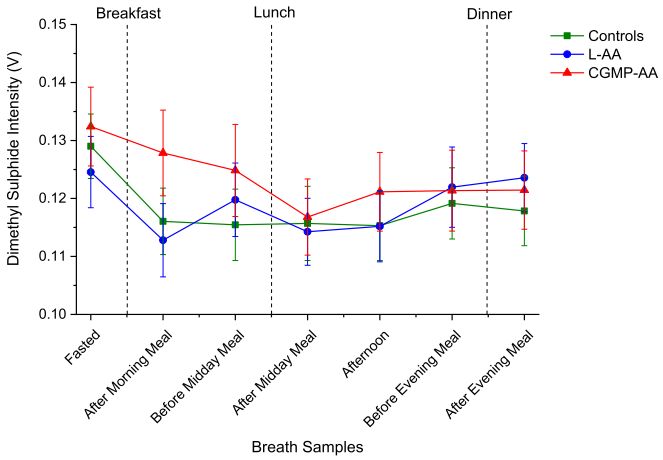


Figure 14. Dimethyl sulphide intensity changes by intervention groups.

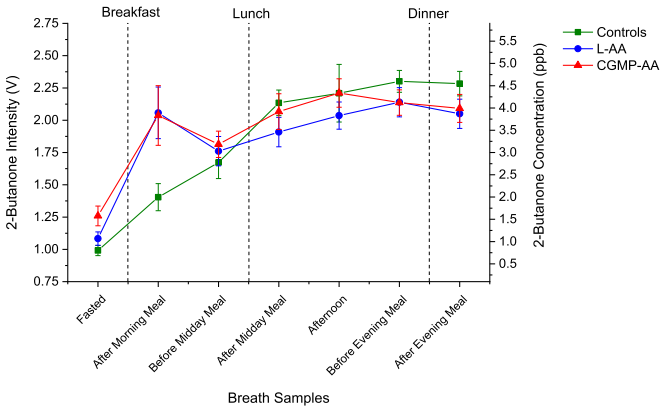


Figure 15. 2-butanone intensity/concentration changes by intervention groups.

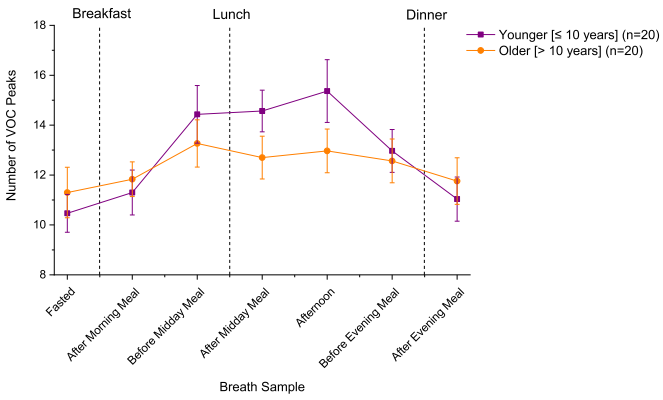


Figure 16. Number of VOC peaks: younger versus older (controls and subjects with PKU).

compared with L-AA. This is reflected in the questionnaire results (figure 3) regarding PS taste, as 50% of subjects with PKU stated they dislike or strongly dislike the taste of L-AA, compared to 35% with CGMP-AA. Subjectively, CGMP-AA is described to have a sweet smell (like ice cream) and taste less like traditional L-AA. Experiments measuring breath odour post-PS administration demonstrate that there is an immediate and objective change to the breath content of subjects with PKU, 5 min after consuming

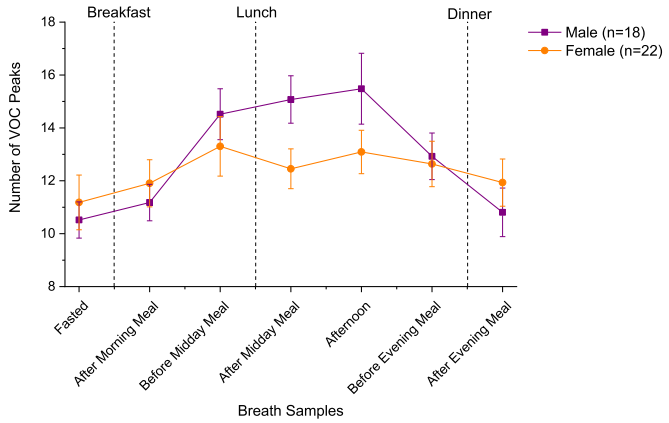


Figure 17. Number of VOC peaks: male versus female (controls and subjects with PKU).

supplements. The strong alignment of VOC peaks in both the headspace gas and breath suggest that for a short duration, the subject's breath is perceived similarly to the PS. However, the immediacy of this intense taste in the mouth is likely to lead to subjective differences, since perceived taste and measurable difference in exhaled breath are not the same.

Longitudinal breath testing results show that the mean number of VOC peaks in exhaled breath increase after the fasted breath sample, in all three groups. Likewise, the initial number of VOC peaks in fasted breath are similar. This indicates that there is no fundamental difference in breath composition between controls and subjects on a low-protein diet, supplemented with PS—detectable using our analytical platform. Common breath compounds, such as acetone, 2-propanol, hexanal, 2-butanone and 1-propanol were observed in fasted samples, including the control group.

The longitudinal trends of control and L-AA groups are characterised by two increases in the number of measured VOC peaks; specifically, during the 'before midday meal' and 'afternoon' samples. In the CGMP-AA group, the number of VOC peaks were observed to increase until the 'after midday meal' sample and decrease thereafter. The increase in exhaled VOCs following meals, and throughout the day generally, is partially associated with the intake of food and drinks. For example, limonene was observed in postprandial samples from both control and subjects with PKU, especially those who drank orange cordial. It is well known that some dietary products can leave a residual taste and cause transient breath odours (e.g. onions, garlic and spicy foods) [9]. The increase in the number of exhaled VOCs throughout the day is also linked to day-time metabolic processes and the microbiome in the gastrointestinal tract, which generates additional VOCs during the metabolism of nutrients [20, 21]. Subjects with PKU are not observed to have significantly more, or different types of VOCs in exhaled breath, when sampled 30 min after consumption of either PS.

The headspace gas analysis enabled identification of the locations of VOC peaks relating to the PS. The locations of these VOCs were tracked across the longitudinal samples in figure 11. However, none of the VOC peaks directly related to those in the headspace gas were detected in any breath samples from subjects with PKU, for either PS. These findings suggest that the effects of the PS disappear in less than 30 min after consumption. This strongly indicates that breath malodour from the supplements is reduced, or entirely removed, by the intervention of food and drinks. Comparing samples from subjects with PKU to those from controls, we did not observe any additional VOC peaks, which could be linked to by-products of pharmacokinetic effects and metabolic breakdown of the supplement.

Analysis of possible confounding factors indicate that differences in the number and trends of VOCs throughout the day are more likely related to factors of age and gender, rather than PKU-associated effects, since the differences in the results from confounding factor groups are greater than in the intervention groups. This provides further evidence to suggest that the composition of breath between subject with PKU and controls is not fundamentally different. The trends in figures 16 and 17 are quite similar, which could be related to more females being recruited (22F: 18M) and the mean age of female subjects being marginally higher than males; 11.0 years (standard deviation: 2.6) and 9.8 years (standard deviation 2.2), respectively.

Intensity monitoring of specific compounds, such as acetone and DMS, demonstrate similar trends across all groups. There is, however, an interesting difference in the concentration of 2-butanone between subjects with PKU and controls in the post-morning meal sample. While controls are associated with a gradual increase throughout the day, both L-AA and CGMP-AA are associated with a sharp rise after their

morning meal (around 3.9 ppb for subjects with PKU, for both PS, compared to 2.0 ppb for controls). These levels drop subsequently and maintain similar trends to controls for the rest of the day. This could be a metabolic response to the PS and requires further investigation. Similar peaks are not observed following the midday and evening meal PS, which suggests that this only occurs post-fasting. The quantification results for 2-butanone can be used for comparison with other studies or serve as a reference for future studies. Regarding DMS, the intensity trend is shown to decrease throughout the day, after the fasted sample. It is possible that this compound is associated with malodorous breath upon awakening, known as 'morning breath'. These unpleasant odours are VSCs, which are produced by bacteria during sleep, as a result of decreased salivation (i.e. dry mouth) [22, 23]. This effect usually lasts a few hours and can be reduced or relieved through oral hygiene (brushing of teeth) and/or consumption of breakfast. Findings from this study suggests that DMS concentrations in exhaled breath are similar in subjects with or without PKU, for both PS.

There are some limitations associated with this study. For example, the scope of this study focuses only on the PS and not on blood Phe levels. In future studies, blood Phe levels could be monitored in conjunction with breath measurements, to determine any links between exhaled breath composition and Phe levels. It has also been suggested that Phe could be converted to phenyl ketones, which may contribute to malodour [24]. This is also worth investigating.

A further limitation of this study is that targeted monitoring of sulphur-based compounds using dedicated VSC-monitors may provide insight beyond the generally screening approach taken here using GC-IMS technology. Another drawback is that only a small number of subjects ($n = 3$) provided breath samples, 5 min after consuming the PS. While we were able to demonstrate that it was possible to measure the immediate effect of consuming L-AA and CGMP-AA PS, longitudinal breath testing using GC-IMS could not establish any significant and measurable changes between controls and subjects with PKU, for either PS.

These findings strongly suggest that the reported effects of breath malodour are transient in nature and can be reduced, or completely removed, by the intervention of food and drinks. Further work should include an investigation of intervention methods to determine which are most effective at reducing or completely removing temporary breath malodour, following the consumption of L-AA or CGMP-AA. For example, a simple intervention study comparing the composition of exhaled breath of subjects with PKU who rinse their mouth with water post-consumption of PS, to those who do not.

5. Conclusions

In this longitudinal cross-over pilot study, exhaled VOCs were measured using GC-IMS technology when subjects took both L-AA and CGMP-AA protein substitutes. Headspace gas from L-AA and CGMP-AA were associated with over 75 and 140 VOC peaks, respectively; while only 25-30 VOC peaks were detected in the exhaled breath from subjects with PKU, 5 min after consuming the PS. The strong alignment of VOC peaks in both headspace gas and breath suggest that consuming PS has an immediate impact on breath composition. Fasted L-AA and CGMP-AA breath samples contained a similar number of VOC peaks (10-12) as controls. Monitoring of specific VOCs indicate that acetone and DMS demonstrate similar trends across groups. Post-fasted samples from subjects with PKU, for both PS, were associated with a sharp increase in 2-butanone concentration, which was not observed in the control group. This should be further investigated, as it could be a metabolic response relating to the post-fasted consumption of PS. Intensity tracking of DMS suggests a subsiding trend of 'morning breath' across all groups. Longitudinal breath testing showed that there were no significant differences in the number and types of exhaled VOCs compared to controls or between PS (12-18 VOC peaks). PS are thus shown to have a transient effect on exhaled breath. This indicates that any PS-related unpleasant breath odours are eliminated by food or drinks.

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Appendix. Staggered breath sampling time

Time	Subject 1	Subject 2	Subject 3	Subject 4
08:00	Room Air Sample 1			
08:15	Arrive	Arrive	Arrive	Arrive
08:30	Fasted Sample	Break	Break	Break
08:45	Morning Meal	Fasted Sample		
09:00		Morning Meal	Fasted Sample	
09:15			Morning Meal	Fasted Sample
09:30	After Morning Meal			Morning Meal
09:45	Break	After Morning Meal		
10:00		Break	After Morning Meal	
10:15			Break	After Morning Meal
10:30 – 10:45				Break
11:00	Room Air Sample 2			
11:15 – 11:30	Break	Break	Break	Break
11:45	Before Midday Meal			
12:00	Midday Meal	Before Midday Meal		
12:15		Midday Meal	Before Midday Meal	
12:30			Midday Meal	Before Midday Meal
12:45	After Midday Meal			Midday Meal
13:00	Break	After Midday Meal		
13:15		Break	After Midday Meal	
13:30			Break	After Midday Meal
13:45				Break
14:00	Afternoon Sample			
14:15	Break	Afternoon Sample		
14:30		Break	Afternoon Sample	
14:45			Break	Afternoon Sample
15:00				Break
15:15	Room Air Sample 3			
15:30 – 16:15	Break	Break	Break	Break
16:30	Before Evening Meal			
16:45	Evening Meal	Before Evening Meal		
17:00		Evening Meal	Before Evening Meal	
17.15			Evening Meal	Before Evening Meal
17.30	After Evening Meal			Evening Meal
17.45	Finished	After Evening Meal		
18.00		Finished	After Evening Meal	
18.15			Finished	After Evening Meal
18.30				Finished
18.45	Room Air Sample 4			

Author contributions

AM, JH, JAC and AD conceptualised and designed the study. Patients were recruited by AD and AM. The questionnaire was designed and conducted by AD and AM. Dietary control was overseen by AD, AP, SE, CA and AM. Breath samples were collected by AT. Data analysis was conducted by AT, JH and JAC. Original draft preparation, review and editing of the manuscript was completed by AT, JAC, JH, AD and AM.

Conflicts of interest

The authors declare no conflict of interest.

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References

[1] Palermo L, Geberhiwot T, MacDonald A, Limback E, Hall S K and Romani C 2017 Cognitive outcomes in early-treated adults with phenylketonuria (PKU): a comprehensive picture across domains *Neuropsychology* **31** 255–67
[2] Blau N, Bélanger-Quintana A, Demirkol M, Feillet F, Giovannini M, MacDonald A, Trefz F K and Spronsen F van 2010 Management of phenylketonuria in Europe: survey results from 19 countries *Mol. Genet. Metab.* **99** 109–15
[3] Matalon R and Michals K 1991 Phenylketonuria: screening, treatment and maternal PKU *Clin. Biochem.* **24** 337–42
[4] Korenev S, Lemonde H, Cleary M and Chakrapani A 2019 Newborn screening for inborn errors of metabolism *Paediatrics and Child Health* **29** 105–10
[5] Al Hafid N and Christodoulou J 2015 Phenylketonuria: a review of current and future treatments *Transl. Pediatr.* **4** 304–17
[6] MacDonald A, Lilburn M M, Davies P, Evans S, Daly A, Hall S K, Hendriksz C, Chakrapani A and Lee P 2006 'Ready to drink' protein substitute is easier is for people with phenylketonuria *J. Inherit. Metab. Dis.* **29** 526–31
[7] Sanz M, Roldán S and Herrera D 2001 Fundamentals of breath malodour *J. Contemp. Dent. Pract.* **2** 1–17
[8] Tangerman A and Winkel E G 2013 Volatile sulfur compounds as the cause of bad breath: a review *Phosphorus, Sulfur Silicon Relat. Elem.* **188** 396–402
[9] Aylikci B and Çolak H 2013 Halitosis: from diagnosis to management *J. Nat. Sci. Biol. Med.* **4** 14–23
[10] Ma N, Tian Y, Wu Y and Ma X 2017 Contributions of the interaction between dietary protein and gut microbiota to intestinal health *Curr. Protein Pept. Sci.* **18** 795–808
[11] Cortelli J R, Barbosa M D S and Westphal M A 2008 Halitosis: a review of associated factors and therapeutic approach *Braz. Oral Res.* **22** 44–54
[12] Kameyama A, Ishii K, Tomita S, Tatsuta C, Sugiyama T, Ishizuka Y, Takahashi T and Tsunoda M 2015 Correlations between perceived oral malodor levels and self-reported oral complaints *Int. J. Dent.* **2015** 1–6
[13] Alasqah M, Khan S, Elqomsan M, Gufran K, Kola Z and Bin H M 2016 Assessment of halitosis using the organoleptic method and volatile sulfur compounds monitoring *J. Dent. Res. Rev.* **3** 94–8
[14] Rosenberg M, Septon I, Eli I, Bar-Ness R, Gelernter I, Brenner S and Gabbay J 1991 Halitosis measurement by an industrial sulphide monitor *J. Periodontol.* **62** 487–9
[15] van Wegberg A M J *et al* 2017 The complete European guidelines on phenylketonuria: diagnosis and treatment *Orphanet J. Rare Dis.* **12** 162
[16] Blanchet L, Smolinska A, Baranska A, Tigchelaar E, Swertz M, Zhernakova A, Dallinga J W, Wijmenga C and van Schooten F J 2017 Factors that influence the volatile organic compound content in human breath *J. Breath Res.* **11** 16013
[17] Ruzsanyi V, Mochalski P, Schmid A, Wiesenhofer H, Klieber M, Hinterhuber H and Amann A 2012 Ion mobility spectrometry for detection of skin volatiles *J. Chromatogr.* B **911** 84–92
[18] Mazzatenta A, Pokorski M, Sartucci F, Domenici L and Di Giulio C 2015 Volatile organic compounds (VOCs) fingerprint of Alzheimer's disease *Respir. Physiol. Neurobiol.* **209** 81–4
[19] G.A.S. 2016 GCxIMS Library Search *G.A.S. Gesellschaft für Anal. Sensorsysteme* (Accessed: 2 March 2019)
[20] Smolinska A, Baranska A, Dallinga J W, Mensink R P, Baumgartner S, van de Heijning B J M and van Schooten F J 2019 Comparing patterns of volatile organic compounds exhaled in breath after consumption of two infant formulae with a different lipid structure: a randomized trial *Sci. Rep.* **9** 554
[21] Bogusław B, Martyna K, Tomasz L and Anton A 2007 Human exhaled air analytics: biomarkers of diseases *Biomed. Chromatogr.* **21** 553–66
[22] Suarez F L, Furne J K, Springfield J and Levitt M D 2000 Morning breath odor: influence of treatments on sulfur gases *J. Dent. Res.* **79** 1773–7
[23] McDowell J D and Kassebaum D K 1993 Diagnosing and treating halitosis *J. Am. Dent. Assoc.* **124** 55–64
[24] Schulz S and Dickschat J S 2007 Bacterial volatiles: the smell of small organisms *Nat. Prod. Rep.* **24** 814–42

CHAPTER 8

DISCUSSION

8.1 OVERALL FINDNGS

Casein glycomacropeptide (CGMP), a bioactive low phenylalanine protein, is given as an alternative to phenylalanine free amino acids (AA) in PKU. This thesis examined the efficacy of CGMP compared with AAs. The focus was particularly on exploring its efficacy and potential health benefits in PKU.

It was evident over the 3 year study duration not all children with PKU could completely replace all their AA protein substitute with CGMP. Providing additional CGMP increased blood phenylalanine concentrations, particularly in patients with a low phenylalanine tolerance. In a randomised crossover study, examining 24-hour phenylalanine variability, blood phenylalanine concentrations were more stable with CGMP compared with AA. This only became evident after phenylalanine intake was lowered in the diet so that the overall daily phenylalanine intake was not increased. Comparing body composition in three groups (group 1, AAs only; group 2, CGMP and AA; and group 3, CGMP only), there was a trend that children taking CGMP only were taller, had improved lean body mass and decreased fat mass. However, this was not statistically significant. Bone mineral density for the three groups was clinically normal although the median z score was below the population mean. The bone turnover markers, type 1 collagen cross-linked C telopeptide (β-CTX) (resorption marker) and procollagen type 1 terminal propeptide (P1NP) (formation marker), were significantly correlated showing active bone breakdown and formation. At 36 months after adjusting for age, there was evidence of an increase in P1NP in the CGMP100 group compared to the AA group. Exhaled organic volatile compounds measured via gas chromatography ion mobility spectrometry showed CGMP and AA protein substitutes had a transient effect on exhaled breath. No differences were observed when compared to a group

of healthy control subjects. However, children in the CGMP group perceived their breath to have less odour compared with AAs.

8.2 CHAPTER 3A. GLYCOMACROPEPTIDE IN CHIDREN WITH PHENYLKETONURIA: DOES ITS PHENYLALANINE CONTENT AFFECT BLOOD PHENYLALANINE CONTROL?

Prior to 2012, only two short-term human studies and one case report [1-3] had described the use of CGMP compared to AAs as an alternative protein substitute in PKU. These trials were of short duration (8 days) with small patient numbers (mainly adults) who had variable phenylalanine tolerance and metabolic control. No dietary phenylalanine adjustments were made to compensate for the phenylalanine content of CGMP.

In 2009, van Calcar et al [2] compared AA with CGMP in subjects with PKU, evaluating plasma concentrations of pre and postprandial AAs, insulin, glucose and urea. CGMP was given as a food and AAs as a liquid. CGMP was supplemented with histidine, leucine, methionine, tryptophan and tyrosine. All the amino acids except tyrosine provided 130% of the USA dietary reference values for amino acids (expressed per gram of protein); tyrosine provided 150% (IOM 2002) [4]. Each subject took a different type of AA supplement with variable AA composition (not referenced in their original paper); except for tyrosine which was higher in AAs compared with CGMP. They suggested that CGMP was associated with more stable blood phenylalanine concentrations and an improvement in blood urea nitrogen suggested better protein utilization. Although CGMP was supplemented with indispensable amino acids, several differences in the plasma amino acid profile between AA and CGMP were described. In the CGMP group, fasting concentrations of tyrosine and arginine were significantly lower and threonine higher. The nutritional composition of the

CGMP studied, contained 40% less tyrosine compared to the AA protein substitutes. CGMP contained 71 mg tyrosine / g of protein compared to 120 mg/ g of protein in the AAs. The authors concluded that the lower blood urea nitrogen, provided evidence of a slower rate of AA absorption from CGMP. However, total AA's, insulin and urea were only measured at one time point, missing the peak or nadir concentrations. Similarly, the carbohydrate content of the protein substitutes was not reported, and CGMP was given as a food and AAs as a liquid, which may have altered the insulin response [5].

Although the initial results of the CGMP studies conducted primarily in adults were encouraging, there was concern that the phenylalanine content of CGMP may adversely affect blood phenylalanine control in children. Chapter 3A reported a 6-month pilot study comparing the use of low phenylalanine CGMP (30 mg phenylalanine/ 20g protein equivalent) with phenylalanine-free AAs. This was conducted in well controlled PKU children aged 5 to 16 years. The aim was to compare the effect of the two different sources of protein substitute on blood phenylalanine, tyrosine and phenylalanine to tyrosine ratios. These markers were monitored weekly together with monthly weight, height and dietary assessments. Comparing the essential amino acid composition with the WHO/FAO/UNU 2007 requirements, expressed as mg/ g of protein, essential amino acids were consistently higher in the AA protein substitutes (mean 138%, range 60-200), compared to CGMP (mean 67%, range 3-300).

This 6-month study was the first long-term controlled study using CGMP in children. After 6 months, the CGMP group had a statistically significant increase in blood phenylalanine concentrations and phenylalanine to tyrosine ratio with a decrease in tyrosine

concentrations compared to the AA group. Every child using CGMP had an increase in blood phenylalanine concentrations. In 70% of the children in the CGMP group the protein equivalent from CGMP was reduced. This was necessary to keep phenylalanine concentrations within the European therapeutic target ranges [6]. Energy intake in both groups met estimated average requirements [7].

Three combined factors probably explained these findings: 1) patients were unable to metabolise the extra phenylalanine supplied by CGMP, 2) the CGMP contained lower amounts of large neutral amino acids (LNAA) compared to AAs, and 3) the added AAs (40%) in the CGMP were less efficiently absorbed compared to whole protein [8, 9].

Blood AA concentrations are influenced by the amount, and ratio of amino acids in protein substitutes. Tyrosine, considered an essential amino acid in PKU, was 55% lower in CGMP than in AA's. In healthy subjects, 67-90% of phenylalanine in protein is converted to tyrosine [10]. The tyrosine added to protein substitutes should adequately meet this conversion and allow for the inefficient absorption and retention of amino acids. The amount of tyrosine, 55mg/g protein in CGMP failed to meet the physiological demands in PKU. It is hypothesised that an increase in the phenylalanine to tyrosine ratio leads to dopamine deficiency because of the competition of amino acids at the blood brain barrier [11-13]. The CGMP formulation used in this pilot study appeared to provide an imbalance of essential LNAA and was functionally less effective.

Overall, the patient numbers in this study were low due to the rarity of PKU. It was not possible to conduct a randomised controlled trial as only some of children found the

CGMP protein substitute acceptable in the prescribed amounts. Also, mutation analysis was not available to confirm the severity of the PKU.

8.3 CHAPTER 3B. GLYCOMACROPEPTIDE: LONG TERM USE AND IMPACT ON BLOOD PHENYLALANINE, GROWTH AND NUTRITIONAL STATUS IN CHILDREN WITH PKU

In the second part of this work, a revised CGMP formulation (CGMP2) was developed. It had an amino acid pattern similar to conventional AA supplements, with increased amounts of indispensable LNAA: tyrosine, histidine, tryptophan and leucine. The exception was threonine which is naturally higher in CGMP. There were lower quantities of methionine and valine so the total amino acids per g of protein remained consistent with CGMP1. To test the efficacy of the CGMP2, a follow on 12-month prospective, parallel study was conducted.

The aim of this study was to re-evaluate blood phenylalanine, tyrosine and phenylalanine to tyrosine ratios between AA and CGMP2 in children with PKU. Twenty-nine children were in the CGMP2 group (median age 8.4 years, range 5-16y) and 19 in the AA group (median age 11.1 years, range 5-16y). This study measured weekly blood phenylalanine and tyrosine by blood spots, 3 monthly anthropometric measurements (height and weight), and nutritional biochemistry at enrolment and 26 weeks.

After 12 months of using CGMP2, there were no significant differences between the groups for: blood phenylalanine, tyrosine or phenylalanine to tyrosine ratios. [14]. Blood tyrosine significantly improved, and phenylalanine concentrations were lower. Only forty-eight per cent of the children were able to take CGMP2 as their single source of protein

substitute without adverse effect on blood phenylalanine concentrations. Blood phenylalanine control deteriorated in 52% when CGMP2 provided > 33% to 66% of intake. A combination of CGMP2 and AA was needed to maintain phenylalanine concentrations within therapeutic target ranges. Nutritional blood biochemistry (zinc, selenium, vitamin D, calcium, phosphate, magnesium, ferritin, B12 and haemoglobin) for both groups were within reference ranges, with selenium being significantly higher in the CGMP2 group. Weight and BMI z scores significantly increased in the CGMP2 group, but no differences were observed between the groups.

The small changes in amino acid composition that were made to CGMP2, led to improved tyrosine and stabilised phenylalanine concentrations. It also increased the proportion of CGMP2 that could be given in the protein substitute regimens, a mean of 75% of daily total protein equivalent intake. The only difference between the amino acid composition of CGMP2 and the CGMP study product used by van Calcar et al [2] was a higher arginine content in the latter. Van Calcar et al [2] had described low blood arginine concentrations and so added additional amounts to their CGMP formulation. Arginine is a conditional essential amino acid synthesised from citrulline which is derived from glutamine. It has a diverse role in the production of enzymes, growth hormone, insulin and nitric oxide. Nitric oxide has an important function contributing to antioxidant status [15, 16]. The AA product in our study contained 50% more arginine than CGMP2. To prevent a breach of manufacturing patent, additional arginine was not added to CGMP2.

The additional amino acids added to CGMP2 were all LNAA. These have received considerable attention over recent decades as potential treatment options in PKU [17-21].

They improve brain biochemistry [22], lowering blood and brain phenylalanine concentrations in PKU mice. This effect may be due to the competitive inhibition of phenylalanine uptake at the gut-blood barrier [18, 23, 24]. It could also be an effect of improved protein synthesis associated with the additional amino acids from LNAA [20]. Lower brain phenylalanine concentrations support findings of a competitive effect of phenylalanine across the blood brain barrier [19]. Blood and brain tyrosine concentrations were lower in mice, a surprise finding given the additional tyrosine supplied by the LNAA's. However, norepinephrine synthesised from tyrosine was significantly increased. This suggested tyrosine was being utilized to restore this depleted neurotransmitter in the PKU mice. There was also an improvement in brain tryptophan, methionine and histidine. Only brain threonine concentration was significantly reduced in PKU mice, but this was not added to the LNAA formula. Threonine is naturally higher in CGMP. Sanjurjo et al [25] has shown that adding threonine supplementation (50mg/ kg/ day extra to the amounts provided by regular protein substitute intake) significantly reduced blood phenylalanine concentrations.

Human studies giving LNAA supplements in PKU have reported inconsistent results. Some have reported reduced brain and blood phenylalanine with improved tyrosine concentrations, but with variable effects on psychometric testing [18, 24, 26-29]. Typically, AA supplements contain 470 to 617 mg LNAA/ g protein, and CGMP2 contained 563 mg/ g protein. LNAA have an important role to play in restoring altered brain biochemistry but the composition of LNAA needed for optimal brain biochemistry is not fully determined.

Our research in contrast to other studies has clearly shown a detrimental effect of the residual phenylalanine present in CGMP on blood phenylalanine concentrations in children

[30-34]. In a study by Ney et al [31] in older subjects (aged 15-49 years) with PKU, there was an increase (+62 μmol/L) in blood phenylalanine with CGMP compared to a reduction (-85 μmol/L) with AA supplements. This was reported as non-significant. Zaki et al [34] used CGMP in children but a direct comparison with our study was not possible, as only an equal combination of AA and CGMP were provided, rather than CGMP only. The CGMP was given in the form of food (cheese), which was spread on low protein bread not as a liquid supplement. Pinto et al [35] reviewed 11 subjects (median age 27 years), with variable PKU phenotypes taking 57% of their protein substitute as CGMP. After a mean time of 13 months taking CGMP they reported no adverse changes in blood phenylalanine and a significant increase in tyrosine. A further extension of this study, after 29 months using CGMP, showed that biochemical markers remained unchanged except for HbA1c which, was no longer significantly different. Blood tyrosine concentrations continued to be statistically significant in the CGMP group, despite AA containing higher amounts of tyrosine compared to CGMP [32]. The authors speculated this might be due to improved adherence and/ or bioavailability either because of improved solubility in CGMP compared to AA or via gut microbiota. Ney et al [36] has shown microbiome derived compounds synthesised from tyrosine e.g., phenol sulphate and from tryptophan e.g., quinolinic acid, are higher in AA supplements, suggesting less degradation. Further work is needed to explore this finding.

In a systematic review, Pena et al [37] concluded there was no difference in blood phenylalanine concentrations when giving CGMP compared with AA. However only two studies (Ahring, et al [30] and Ney et al [31]) qualified for comparable investigation. Pena et al [37] acknowledged there were several limitations, such as small sample sizes, short study duration, different ages and phenotypes used in the studies. The recommendation was

to improve the study design to show if blood phenylalanine increased with CGMP. It was also highlighted that in the adult studies, the blood phenylalanine concentrations were generally above target therapeutic range. Patient adherence was difficult to assess, and long-term phenylalanine concentrations were not studied making it difficult to evaluate intervention outcomes. In addition, some subjects with PKU were using adjunctive therapy such as sapropterin, which naturally decreased phenylalanine concentrations. Thus, any impact of the phenylalanine content of CGMP would have been concealed.

In our study, blood selenium concentrations were significantly higher in the CGMP2 group. Kelleher et al [38] demonstrated that CGMP improved the absorption of zinc in monkeys. It was suggested that the negative charge in CGMP had a positive effect on mineral and trace element absorption, assisting in chelation and transport of minerals into the intestinal epithelium.

A limitation of this study in Chapter 3B, was the age difference between the AA and CGMP2 groups. Although these children were monitored very closely, there was an element of uncertainty, particularly regarding control and dietary adherence [39, 40]. An extension of the nutritional biochemistry to 12 months would have confirmed if an improved selenium status could have been sustained over time in the CGMP2 group.

8.4 CHAPTER 4A. THE EFFECT OF GLYCOMACROPEPTIDE VERSUS AMINO ACIDS ON PHENYLALANINE AND TYROSINE VARIABILITY OVER 24 HOURS IN CHILDREN WITH PKU: A RANDOMISED CONTROLLED TRIAL

The comparison between CGMP and AA supplements was explored in a randomised controlled cross over study in children with PKU. The hypothesis was that CGMP (as an

‘intact’ protein) would improve blood phenylalanine stability and decrease phenylalanine fluctuations compared with AA supplements. The potential advantages of stable blood phenylalanine are improved protein utilization, and a steady release of phenylalanine at the blood brain barrier balancing brain neurotransmitter synthesis. Literature suggests that the cumulative effect of day-to-day phenylalanine fluctuations may impact on cognition [41, 42].

In this study, children were randomised into three groups: regimen R1, CGMP2 only with no dietary adjustment to phenylalanine, regimen R2, CGMP2 only with dietary adjustments to phenylalanine and regimen R3, AA only with no dietary adjustment to phenylalanine. Each child took the protein substitutes and dietary regimens allocated in regimen 1, 2 and 3 for two weeks respectively. On day 13 and 14 of each assessment period, four hourly blood spots for phenylalanine and tyrosine concentrations were collected over 48 hours. Children followed the same standardised food menu during the two days of testing.

The results showed the median phenylalanine blood concentrations were significantly different between the three regimens. Blood phenylalanine was highest in regimen 1 (CGMP2) and lowest in regimen 3 (AA), both with no adjustment for phenylalanine intake. The most stable blood phenylalanine concentrations were observed in regimen 2 (CGMP2) after dietary phenylalanine adjustment. Blood phenylalanine concentrations lowered over 24-hours in regimen 2 and 3 but increased over 24 hours in regimen 1. Phenylalanine concentrations within the therapeutic target range occurred in 44% of all results in regimen 1, 52% in regimen 2 and 39% in regimen 3. In regimen 1, most blood phenylalanine concentrations were above the upper target range of 360 μmol/L in

contrast to regimen 3 in which the majority were below the lower target range <120 μmol/L. Therefore, the additional phenylalanine source in CGMP2 appeared to adversely affect blood phenylalanine concentrations.

In PKU, the 24-hour diurnal variation in phenylalanine and tyrosine concentrations are more variable than in non-PKU subjects in which phenylalanine does not vary by more than 50%. In non-PKU subjects phenylalanine concentrations are maintained within a narrow range 30-70 μmol/L [43, 44]. When comparing regimens 2 and 3, the same daily amount of phenylalanine was consumed. Only the protein substitute varied. There was improved blood phenylalanine stability with CGMP2 after lowering the phenylalanine intake from food, which compensated for the amount of phenylalanine supplied from CGMP2. The observed improvement of phenylalanine stability in regimen 2 may be accounted for by the composition of the macropeptide base. In regimen 2, CGMP2 provided a proportion of phenylalanine intake, which is part of the glycomacropeptide structure and therefore, was possibly utilized differently from free amino acids.

In contrast, in regimen 1 there was a steady increase in blood phenylalanine concentrations over 24 hours unlike regimen 2 and 3. It is likely that the additional phenylalanine supplied in regimen 1, exceeded requirements and so was not metabolised. However, the 3-D structure of CGMP is unknown but it is considered to be a random coil or an intrinsically disordered peptide, which changes in shape depending on the acidic environment and presence of other molecules. How phenylalanine is imbedded into this structure and released is unknown [45, 46] and further studies are needed to explain these differences.

MacDonald et al [47] has shown administration of an AA supplement in 4 hourly doses over 24 hours stabilised blood phenylalanine concentrations with minimal variability. When the protein substitute was taken in the day as 3 or 4 divided doses there was greater variability. Although, protein substitutes were not administered 4 hourly in this CGMP2 study, in regimen 2 a greater percentage of phenylalanine concentrations were within the target therapeutic range, providing evidence of improved stability.

Tyrosine concentrations were highest for all three regimens at 12-midday, after the post morning protein substitute ingestion suggesting tissue replenishment after an overnight fast. Regimen 1 showed a progressive decline of tyrosine over 24 hours, unlike regimen 2 and 3, showing a rise and fall over the observation period. In all regimens, the lowest concentration was at 8.00 am after overnight fasting. At no point were levels below physiological reference ranges. It is unknown why tyrosine concentrations declined steadily over 24 hours in regimen 1, but not in 2 and 3 but this may correlate with the phenylalanine concentrations rising steadily in regimen 1.

Although this was a randomised controlled study, measurement of essential amino acids over 24 hours may have helped explain the findings. Amino acids are gluconeogenic precursors and a decrease in their plasma concentrations may occur after an overnight fast [48]. In addition, insulin and glucose measurements would have provided additional information about amino acid utilisation. Other factors influencing phenylalanine variability such as energy intake, exercise and the timing of protein substitute intake, were not standardised between subjects and only a small number of children were studied. Ideally a

control group of age and gender matched healthy children would have provided information about normal amino acid patterns over 24 hours. However, this study methodology was intensive and burdensome for children with PKU and their parents.

This study provided further evidence that in a well-controlled group of children with PKU, that additional phenylalanine provided by CGMP2 had a significant negative impact on blood phenylalanine control. There is some evidence that the macropeptide structure of CGMP2 may help stabilise blood phenylalanine concentrations. Developing a CGMP product without phenylalanine is an important development for the future.

8.5 CHAPTER 4B. PRELIMINARY INVESTIGATION TO REVIEW IF A GLYCOMACROPEPTIDE COMPARED TO L-AMINO ACID PROTEIN SUBSTITUE ALTERS THE PRE AND POSTPRANDIAL AMINO ACID PROFILE IN CHILDREN WITH PHENYLKETONURIA

Van Calcar et al [2] suggested that CGMP improved protein retention and phenylalanine utilisation compared with AA supplements. This was not a kinetic study and only measured amino acids, urea and insulin at 180 minutes post CGMP consumption. In our study, we aimed to evaluate the pre- and postprandial blood amino acid profiles. Subjects were given 20g protein equivalent of CGMP1 (from chapter 3A) or CGMP2 (from chapter 3B) or AA together with a low protein breakfast. Blood samples for amino acids were taken after an overnight fast and 120 minutes postprandial after protein substitute and breakfast consumption.

No significant postprandial changes for total, large neutral, essential or branch chain amino acids were observed between the three groups. This suggested a similar rate of absorption and delivery of amino acids into the circulation for all three supplements.

However, individual blood amino acids for the three protein substitutes were different, particularly between CGMP1 and CGMP2. At 120 minutes, tyrosine, histidine and leucine, added to the CGMP2, were significantly higher compared to CGMP1, and leucine, threonine and tyrosine concentrations were higher in CGMP2 than AA. This study demonstrated that the amino acid composition of protein substitutes modified the blood amino acid concentrations and ultimately tissue utilization.

Individual amino acid changes were most prominent for tyrosine and histidine. Fasting concentrations of tyrosine were lowest in CGMP1 compared to CGMP2 and AA. Postprandial concentrations of tyrosine with CGMP1 were approximately 50% of CGMP2 and AA. This reflected the lower amount of tyrosine in CGMP1. Histidine remained unchanged after 120 minutes in CGMP1 but increased significantly with CGMP2 and AA supplements. This lack of pre and postprandial change in histidine suggested insufficient supplementation in CGMP1, potentially with rapid tissue replenishment once ingested. Nitrogen balance is only maintained when all amino acids are present in adequate quantities. There is a suggestion the CGMP1 amino acid profile was potentially unable to meet this function. Interestingly, the postprandial arginine concentration, which was not added to CGMP2 (unlike the CGMP used in van Calcar's study) [2], was similar in CGMP1 and AA. Although higher in CGMP2, this was not significant. These findings are important, as protein synthesis and support of metabolic pathways is dependent on a balanced supply and ratio of amino acids [49]. Optimal nitrogen balance is also dependent upon sufficient energy intake, but this was adequate in our group.

AA supplements deliver amino acids rapidly into the circulation, bypassing protein digestion [50]. The rate, duration and quantity of amino acids delivered will determine muscle and whole-body protein synthesis in addition to sustaining metabolic pathways [51]. Protein substitutes have an anabolic benefit promoting muscle synthesis, although anabolism is only established when protein breakdown and synthesis are measured, and assessment of muscle protein breakdown is only possible via invasive techniques [50-52]. Therefore, essential AA are used to measure protein synthesis by isotope markers [53, 54]. The protein synthetic response to an intact protein is sustained over a longer time than free amino acids, [51] suggesting a potential benefit for CGMP. However, this will only occur if it is utilised in a similar manner to intact/ natural protein. Interestingly the anabolic impact of free essential AA is greater than a comparable amount in whey protein. The difference being due to free essential AAs being absorbed more rapidly [53]. However, maintaining a balance of essential AAs that is in proportion to the demand of each essential AA is important. Tyrosine is necessary for the synthesis of monoaminergic transmitters and leucine muscle protein synthesis. A balanced formulation of amino acids combined with a high quality intact/ natural protein would in theory provide prolonged availability of all the necessary precursors for protein synthesis and metabolic pathways.

Kinetic studies are necessary to fully compare the fate of amino acids from AA and CGMP with measurements at least over 240 minutes. We did not measure minimum or maximum concentrations of each amino acid. We also did not control for the consumption of energy, carbohydrate, and fat intake provided at breakfast. The breakfast meal provided a mean phenylalanine intake of 30%. Urea, glucose, insulin, leucine/ non-oxidative leucine disposal were not measured, which would have given an indicator of protein synthesis. To

enhance this study, net protein balance could have been measured, which assesses whole body protein synthesis minus protein breakdown. The fractional synthetic rate (FSR) [53], which looks at the rate a labelled amino acid precursor is incorporated into protein would be a useful indicator, together with net protein balance to measure the effectiveness of protein substitute supplementation.

8.5.1 A 36-month review of blood phenylalanine concentrations using CGMP2 compared to AA

Historical data overwhelmingly supports the evidence for maintaining blood phenylalanine concentrations above the normal reference range. It is recommended to maintain phenylalanine concentration between a therapeutic target range of 120-360 umol/L [55-57] in children aged <12 years and 120-600 μmol/L aged >12 years. Despite early intervention with a low phenylalanine diet and protein substitutes, neuropsychological deficits, behavioural and social issues still occur [58-60]. Evidence is lacking about the optimal blood phenylalanine concentrations in PKU, [12, 61] but agreement is unanimous that any intervention leading to higher blood phenylalanine concentrations are detrimental.

Reviewing blood phenylalanine concentrations over 36 months using AA or the reformulated CGMP2 either as CGMP100 (CGMP only) or CGMP50 (a combination of CGMP and AA) there was an increase in blood phenylalanine concentrations in all groups over the 36 months. A significant increase was noted for AA and CGMP50, but not CGMP100. Despite the increase, median blood phenylalanine concentrations were within target therapeutic range demonstrating good metabolic control. This supports our findings from chapter 3B that modification of CGMP2 was able to maintain blood phenylalanine concentrations within target therapeutic range over the longer time period. However, not all

children could metabolise the additional phenylalanine from CGMP2 and could only replace part of their protein substitute from this source. An increase in blood phenylalanine concentrations with age could also be associated with a decline in adherence to a stringent, arduous and relentless low phenylalanine diet, which was particularly noticeable in the oldest (AA) age group studied [39, 40]

8.6 CHAPTER 5A. GROWTH AND BODY COMPOSITION IN PKU CHILDREN A 3-YEAR PROSPECTIVE STUDY COMPARING THE INFLUENCE OF L-AMNIO ACID ON GLYCOMACROPEPTIDE PROTEIN SUBSTITUTES

Growth, body composition, bone mass, density and geometry in children with PKU remains a contentious issue. Some have reported normal body composition, bone density and growth while others suggest sub optimal outcomes [62-66]. Protein substitutes provide nitrogen and essential micronutrients needed to support these processes. In this longitudinal, controlled study, over 36 months, height was measured 3 monthly and body composition parameters (fat and lean body mass and % body fat) were measured by dual X ray absorptiometry (DXA) at enrolment and 36 months. The children were divided into 3 groups: AA, CGMP100 (CGMP only) and CGMP50 (CGMP and AA).

There was no statistical significance between height, lean body or fat mass between any of the groups. No correlation was found with age, pubertal status or blood phenylalanine concentrations. However, in the CGMP100 group statistical modelling indicated a trend towards improved growth in height, a reduction in total body percentage fat and improved lean body mass. It is difficult to explain this trend, the inference, when CGMP is given at a higher dose there is a physiological impact improving growth and body composition. CGMP a bioactive glycosylated peptide with naturally higher amounts of threonine and isoleucine

may improve muscle synthesis. Branched chain amino acids (BCAA) are important nutrient signals yet interventional studies on BCAA present a growing paradox. They may have a beneficial effect on health regulating release of hormones and stimulating insulin and muscle growth. Mammalian target of rapamycin complex (mTORC1) and protein kinase may be activated stimulating muscle growth [67]. BCAA also have a negative association with obesity, insulin resistance and diabetes type 2, although it is thought insulin resistance increases BCAA and not vice versa. Similarly, essential AAs are the stimuli for an increase in muscle protein synthesis [51]. This is a graded response with a higher amount of extracellular compared to intercellular essential AA, favouring protein synthesis. These mechanisms have not been verified in PKU with children taking either CGMP or AA supplements. If CGMP is more slowly absorbed, and oxidation of amino acids reduced compared to AA supplements, higher amounts of essential AA and BCAA may be available which may further increase protein accretion.

Accumulating evidence from long term cohort studies indicate that body composition in childhood tracks into adult life. Early feeding choices (breast versus formula milk) may further influence this finding, as macronutrient content and bioactive factors differ between breast and formula milk [68]. Breast feeding is advocated in PKU but only in combination with a phenylalanine free infant protein substitute. In a longitudinal study assessing growth and body composition in healthy boys from birth to 15 years of age, height was determined by foetal and infant growth, while fat/ lean body mass ratios were more strongly influenced by later growth [69]. In PKU, it is unknown if the protein substitute source or dose introduced in the first few days of life alters this pattern of growth and body composition in children with PKU. In an age and gender matched controlled longitudinal

study, Evans [70] examined growth over 2 years in 20 infants with PKU and described a normal growth pattern similar to non-PKU children.

When measuring growth and body composition in children with PKU several other confounding factors need consideration. These include frequency of administration and adherence with protein substitute. Also, carbohydrate consumption in children with PKU is higher compared to Scientific Advisory Committee of Nutrition (SACN) [7] recommendations as evidenced in several studies [5, 70, 71]. This altered dietary pattern (a higher carbohydrate and lower fat diet) may also change body composition, increasing carbohydrate intolerance and insulin resistance. Evans [70] noted this higher carbohydrate intake was evident in infants with PKU from the age of 1 year compared to a control group. These findings enforce the need for long-term studies in PKU examining the effect of dietary intake on body composition and associated biochemical parameters.

The numbers of children in the CGMP50 and CGMP100 groups were small and higher numbers in all groups would have improved the power of the study and possibly would have shown a statistical difference in body composition. Reference data comparing body composition with healthy children was unavailable and a healthy control group was not studied. The ratio of lean and fat mass influences health outcomes, a greater fat mass leading to overweight and obesity. The risk of cardiometabolic syndrome, diabetes and hyperlipidaemia are increased. These parameters were not measured in our group but should be routinely reviewed.

8.7 CHAPTER 5B. A THREE-YEAR LONGITUDINAL STUDY COMPARING BONE MASS, DENSITY AND GEOMETRY MEASURED BY DXA, pQCT AND BONE TURNOVER MARKERS IN CHILDREN WITH PKU TAKING EITHER L-AMINO ACID OR GLYCOMACROPEPTIDE PROTEIN SUBSTITUTES

Artificial protein substitutes supply 50-80% of total protein requirements in addition to essential vitamins, minerals and micronutrients. In PKU, there is concern that dependency on an artificial protein supplement may impact on bone mass, density and geometry. Of three published systematic reviews examining bone density in PKU [72-74], two (Demirdas et al [73] and Castro et al [72]) indicate that bone density is lower than the population norm but was within the normal reference range and of no clinical concern. These results contrasted with older, individual studies that suggested bone mass and density was compromised in PKU [75-77].

In this study described in Chapter 5B, bone density in children with PKU was comprehensively measured over 36 months. The aims were to compare the efficacy of AA and CGMP on bone mass, density and geometry in children, by measuring a series of parameters systematically over 36 months. No human studies in PKU have investigated the effects of two different protein substitutes on bone mass, density and geometry. Bone mineral density was assessed at baseline and 36 months, via dual X ray absorptiometry (DXA). Also, at 36 months, bone mineral mass, density and geometry was assessed by peripheral quantitative computer tomography (pQCT). DXA only measures a composite of trabecular and cortical bone, whilst pQCT measures these components separately and is not affected by body size and so results in a true volumetric bone density [78]. Blood bone biochemistry and bone turnover markers (BTM) were measured at baseline, 6, 12 and 36

months. These included formation markers [bone alkaline phosphatase (BAP), procollagen type 1 N terminal propeptide (P1NP)] and resorption markers [β cross linked telopeptide (β-CTX), urine pyridinoline (PYD), and urine deoxypyridinoline (DPD)]. Blood biochemistry included vitamin D, calcium, magnesium and phosphate. Measurements were taken in three study groups of children with PKU on different protein substitutes: 1) children taking AA only, 2) CGMP only (CGMP100) and 3) CGMP50 (a combination of CGMP and AA).

The results of this study show bone density, mass and geometry were within the normal reference range, but below the population norm, similar to the finding of the systematic review by Demidras et al and Castro et al [72, 73]. Bone resorption and formation markers (β-CTX v P1NP) were positively correlated, showing a synergy between formation and resorption. At 36 months after adjusting for age there was evidence of an increase in P1NP in the CGMP100 group. Clinically, these children had a normal bone density. Formation and resorption bone markers were active and in equilibrium. Bone biochemistry was normal and therefore this group were not at risk of increased bone fractures. Bone turnover markers showed an expected decrease with age, and this is confirmed by other studies [79]. Reference values were higher than adult ranges showing active bone formation, skeletal growth and bone remodelling.

In human subjects, CGMP when compared to AA did not appear to influence bone density. These results conflict with a mouse study [80], which showed a positive effect of CGMP on bone health. It is difficult to compare animal and human studies. The strengths of our study were systematic follow up of subjects over 36 months in a well-controlled group of children with PKU. In addition to regular review of BTM and blood nutritional

biochemistry, bone density, mass and geometry were measurement by both DXA and pQCT. There was a synergy between bone formation and resorption markers which indicates a correlation with a high bone turnover and active bone growth, which in children includes remodelling, modelling and epiphyseal growth (the main site of bone growth in children) [81, 82].

Bone mineral density reflects only bone mineral status but not the dynamics of bone turnover. BTM reflect changes in bone metabolism more rapidly that bone mineral density. They are higher in children due to skeletal growth velocity and rapid bone turnover in mid puberty when linear growth is maximised [81, 83].

Despite a bone density within the normal reference range, it is unclear why all three groups had a median bone density lower than the population norm. This may be due to a number of molecular and biochemical factors. Protein substitutes are not as efficiently utilized compared to whole protein [84, 85]. Therefore, the availability of amino acids for sustaining lean body mass, maintaining bone structure and increasing insulin like growth factor (IGF1) (an important mediator of osteoblastic activity) [86] may be suboptimal. Some argue that the high acid load of amino acids in protein substitutes is detrimental to bone density, it lowers the pH of urine and increases calcium excretion decreasing bone density. This theory has largely been disproven, and the measurement of urine creatinine/calcium ratio in our study was normal. Other studies have reported no adverse effects of a higher protein intake [87-89].

There is some evidence supporting the role of individual amino acids in optimising bone mineral density namely arginine, lysine, alanine, proline, leucine and glutamine. In vitro studies have demonstrated that amino acids stimulate insulin secretion, which promotes osteoblast growth and differentiation [90, 91]. Arginine stimulates growth hormone secretion promoting IGF1 [92]. Arginine, lysine and glycine are associated with improved collagen formation or synthesis [93]. Leucine directly affects messenger ribonucleic acid (mRNA) translation and is thought to be the most efficient at increasing muscle protein synthesis essential in maintaining bone strength and density [94]. If protein substitutes fail to deliver these amino acids efficiently bone density may be compromised.

In the UK, there are no paediatric age and gender matched reference curves for bone markers available to appropriately assess and compare bone turnover. A further limitation was the failure to include a healthy control group matched for age and gender. Also, within the three groups with PKU, we were unable to study children at the same pubertal stage. Ideally this study should have included a larger cohort of children with PKU to improve the statistical power. To prove a stronger relationship between bone formation and breakdown markers in children taking CGMP only, a longer term study is necessary over at least 5 years.

8.8 CHAPTER 6. THE IMPACT OF THE USE OF GLYCOMACROPEPTIDE ON SATIETY AND DIETARY INTAKE IN PHENYLKETONURIA

Protein is the most satiating nutrient and gut absorption of protein is thought to modulate satiety. The protein leverage hypothesis suggests that a change in the ratio of protein to fat and carbohydrate in the diet may influence energy intake. In a 4 day randomised controlled study, in healthy lean adults the intake of energy from protein carbohydrate and fat was measured. When a lower amount of protein was provided, energy intake from

carbohydrate and fat increased. A diet providing 10% of energy as protein led to an increase in the consumption of carbohydrate and fat, but the same response did not occur when 15% of the energy came from protein [95]. Normal satiety mechanisms activated by food may be absent in a low phenylalanine diet, which is high in carbohydrate and lower in fat. Eighty percent (80%) of the protein usually comes from an amino acid source [96]. CGMP influences cholecystokinin (CCK) a hormone responsible for satiety [97-100]. Animal studies have shown a reduction in fat mass when given a CGMP diet compared with whey protein isolate [101], although this has not been demonstrated in human studies.

If CGMP altered satiety, it would benefit weight control and help prevent obesity, an increasingly reported problem in PKU [102, 103]. Chapter 6 investigated if CGMP compared to AA influenced satiety by altering energy intake and weight gain. Over the three year period dietary data was collected once every 3 months, with macronutrient intake (energy, protein, fat and carbohydrate) assessed together with weight and BMI (body mass index) in children taking AA, CGMP only (CGMP100) and CGMP50 (a combination of CGMP and AA).

Analysis over 36 months showed that all groups had the same total energy intake from protein, carbohydrate and fat. Likewise, there were no statistically significant differences in energy intake, weight or BMI between groups. The incidence of overweight and obesity was prevalent in all the groups with a noticeable increase over the study period. The assertion that CGMP enhanced satiety was not observed in this study.

There is evidence to support CGMP stimulating CCK, but this is a short-term incretin hormone. Human studies investigating CGMP on food intake have given inconsistent and inconclusive findings [98, 100, 101]. There is agreement that high protein meals cause the greatest reduction in hunger [97, 104, 105]. The mechanism how protein controls satiety remains unclear. It is multifaceted but an important influence is the gut-endocrine system, which plays a major role in regulating eating behaviour. Incretin hormones ghrelin, CCK (cholecystokinin), glucagon like peptide (GLP 1) and peptide YY3-36 (PYY3-36) [104, 106, 107], all have varying influences on satiety. The release of gut hormones depends on the amount and composition of foods consumed. PYY appears to be a potent incretin hormone, and given intravenously acutely decreases food intake [104, 107, 108]. The satiating effects of protein depends on the gut endocrine partnership. This is greater than just the effect of CCK making the link between CGMP and satiety less likely. Suppression of food intake is observed only when protein is administered orally [109], in PKU natural protein intake is limited and mostly in the form of amino acids which, may alter these endocrine responses.

Despite the association of CGMP and CCK, the results from the study in Chapter 6, confirm the findings from other studies and do not identify any relationship between CGMP and satiety. This longitudinal study over 36 months, contrasted with the majority of satiety studies, which are mainly carried out in adults, over one or a few days only, and conducted in very controlled environments. Satiety related incretin hormones such as ghrelin, insulin, PYY or CCK were not measured; similarly, data using a visual analog appetite scale was not collected. However, dietary assessments and anthropometry were consistently measured, but showed no changes between the groups. Satiety is a complex study area, influenced by physiological and psychological factors. Measuring satiety is challenging in PKU. In

children food is often provided rather than chosen and in PKU natural protein is restricted. It is important to establish if satiety mechanisms are altered by the restricted phenylalanine intake and abnormal food patterns in PKU.

8.9 CHAPTER 7. INVESTIGATION OF PAEDIATRIC PKU BREATH MALODOUR COMPARING GLYCOMACROPEPTIDE WITH PHENYLALANINE FREE L-AMINO ACID SUPPLEMENTS

Breath malodour in PKU has received minimal clinical or research attention. Children, teenagers, adults and care givers complain of malodour post protein substitute ingestion. It is unknown if this problem is specific to PKU compared to a healthy population. Despite considerable improvement in protein substitute presentation and taste, they continue to have an underlying unpleasant taste, and this is subjectively associated with oral malodour. In a randomised controlled crossover study, we measured exhaled volatile organic compounds (VOCs) using gas chromatography-ion mobility spectrometry (GC-IMS). Twenty children with PKU were randomised to take both AA and CGMP protein substitutes for one week prior to testing, and 20 healthy children were used as a comparative group. Samples were collected over 10 hours, early morning fasting and pre and 30 minutes postprandial, with a random sample mid-afternoon. All subjects had a standardised menu adapted for children with PKU.

Despite subjective evidence to suggest differences in the breath signatures of PKU compared to a control group of children, fasting VOCs were similar in all children regardless of protein substitute intake. Longitudinal results measured over 10 hours showed no significant difference in the number of exhaled VOCs comparing AA, CGMP and controls. Similarly, no differences were found between protein substitutes. Interestingly, a questionnaire asking the children how they perceive their breath after taking AA or CGMP

showed that 95% associated less breath malodour with the CGMP. In three children who had their breath tested immediately after taking their protein substitute there was an immediate increase in the VOCs, but these were not detected after 30 minutes. There was one observable difference between the CGMP and AA groups compared to the control group. The concentration of 2-butanone rose sharply post-fasted morning sample with the AA and CGMP groups. This was not observed in the control group, although all groups had a similar concentration at midday. Butanone is a methyl ethyl ketone and the increase was identical with AA and CGMP. This would suggest that the increase is 1) due to the protein substitute although this is less likely as CGMP and AA were structurally different, or 2) it could be explained as a metabolic difference in PKU possibly related to phenylketones following a period of overnight fasting. Unfortunately, this observation is not easily explained.

VOCs are gases classified depending on their organic structure such as nitrogen containing dimethylamine ammonia and sulphur containing ethyl mercaptane dimthylsulphide. These can be identified in breath possibly producing a unique breath signature. These endogenous volatiles are generated by biochemical reactions. The incomplete metabolism of methionine in the transamination pathway produces a sulphur compound. Acetone is produced from decaboxylation of acetoacetate derived from lipolysis [110-112]. It is possible that the observed rise in 2-butanone characteristic in the subjects with PKU could be derived from such a reaction.

We determined that protein substitutes had a transient effect on exhaled breath. Measurements taken 30 minutes after a meal were not significantly different to non-PKU subjects. To prevent breath odour, food or water should be taken immediately post protein

substitute intake. The results were in contrast to a study in children with cystic fibrosis that observed distinct breath pattern differences compared to healthy children [113].

We did not measure concurrent blood phenylalanine concentrations in this study and therefore do not know the impact of blood phenylalanine on breath and the possibility of excess phenylalanine producing phenylketones. The use of a volatile sulphur monitor may have given more insight into specific exhaled sulphur compounds. The CG-IMS is a general screening tool and did not assess all volatile compounds. Not all the children took the same brand of amino acid supplement and the flavours were different. Manufacturers were not able to clearly identify all the artificial components of each protein substitute making identification of all the compounds difficult. In future studies it would be beneficial to monitor the effect of taking a protein substitute and measuring breath VOC before and after drinking water only. The compound 2-butanone should be more closely investigated as this increased in subjects with PKU regardless of the type of protein substitute and therefore likely to be a specific metabolic process in PKU.

Table 1.8. Key findings, clinical implications and future studies that should be conducted

Key findings	Clinical implications	Future research
• The residual phenylalanine in CGMP can lead to a deterioration in blood phenylalanine concentrations particularly in PKU subjects with limited enzyme activity	• Deterioration of blood phenylalanine decreases IQ scores and leads to neurological deterioration	• The development of a CGMP phenylalanine free protein substitute suitable for all patients with PKU • The development of phenylalanine free CGMP infant formula. It would then be possible to examine if CGMP improves learning (due to its sialic content) and alters stool quality and microbiota in infants with PKU
• Suggested improvement in blood phenylalanine stability when using CGMP (with dietary phenylalanine adjustment)	Potential clinical benefits: • may mimic whole protein delivering amino acids slowly into the systemic circulation • may decrease entry of phenylalanine at the blood brain barrier (BBB) • improves amino acid utilization	• Aim to demonstrate if CGMP improves amino acid absorption and potentially improves protein synthesis. This could be done by kinetic studies that compare CGMP-AA, AA, casein and whey protein. Studies should be conducted over a minimum time of 240 minutes. Measurements should include amino acids, blood glucose, insulin, blood urea nitrogen, and urine amino acids • An indicator amino acid study would allow the amino acid profile of protein substitutes to be studied over time and help direct the ideal amino acid profile

• Positive trend showing improved body composition and growth in those using CGMP exclusively	• CGMP may improve height, body composition, lean muscle mass, and decrease fat mass with long term use	• Extended longitudinal study on growth and body composition in children taking CGMP and AA with routine monitoring of body composition to study impact of different protein substitutes
• All groups had a positive correlation with bone resorption and formation (β-CTX compared to P1NP) markers indicating a balance bone metabolism	• In the CGMP100 group P1NP was larger compared to the AA group after adjusting for age	• The question remains why BMD although clinically normal was below the reference norm and warrants further study. This finding leaves unanswered questions: 1) is there an underlying physiological reason when BMD does not achieve a median normality? 2) if CGMP was used for longer would there be a difference as potentially shown in body composition? 3) studies on net protein balance would provide an indication on the effectiveness of protein substitute supplementation and protein synthesis
• Satiety is not influenced by CGMP	• There was no evidence to suggest a link between CGMP and satiety	• Although satiety was unaffected, there is evidence to suggest CGMP favourably alters gut microbiota, which is linked to satiety • Future research on gut microbiota would be beneficial in this group of children in which a mixed fibre intake is restricted
• Despite subjective evidence breath malodour did not occur post protein	• There was suggestion that any malodour immediately post protein supplement	• Systematic investigation of breath malodour immediately post protein

substitute consumption when volatile organic compounds were measured 30 minutes post consumption.	consumption dissipated after drinking water or food	substitute examining the impact of mouth water 'wash-outs.' • Further investigation to review volatile organic compounds not captured in the first study such as sulphur volatile compounds

CLINIC INDICATIONS AND CONCLUSIONS

This compilation of studies over 36 months in a group of well controlled children with PKU compares the novel bioactive CGMP to conventional AA protein supplements, which are central to dietary management. In PKU, this is the only prospective, controlled, longitudinal research completed over this duration. The studies provide comprehensive, compelling, and systematic data that deliver a strong foundation outlining the impact of CGMP in children. The key findings and their clinical implications together with a summary of future studies which should be conducted on CGMP in PKU are given in Table 8.1. The residual phenylalanine in CGMP is a limitation to its use in children with PKU especially in those with minimal phenylalanine hydroxylase activity. This has been shown to lead to a deterioration in blood phenylalanine control. However, there was a trend (although not statistically significant) that children taking all their protein substitute as CGMP had improved height and lean body mass with a reduced fat mass. At 36 months independent of age, the bone formation marker, PINP, was increased in those taking CGMP only compared to the AA group. This suggests that the bioactive properties of CGMP may infer some clinical advantages. However, until the residual phenylalanine is fully eliminated from CGMP, its associated potential clinical benefits remain inaccessible.

8.11 References

1. Ney, D.M., et al., *Nutritional management of PKU with glycomacropeptide from cheese whey.* J Inherit Metab Dis, 2009. **32**(1): p. 32-9.
2. van Calcar, S.C., et al., *Improved nutritional management of phenylketonuria by using a diet containing glycomacropeptide compared with amino acids.* Am J Clin Nutr, 2009. **89**(4): p. 1068-77.
3. MacLeod, E.L., et al., *Breakfast with glycomacropeptide compared with amino acids suppresses plasma ghrelin levels in individuals with phenylketonuria.* Mol Genet Metab, 2010. **100**(4): p. 303-8.
4. *Institute of Medicine. Food and Nutrition Board Dietary Reference Intakes for Energy, Carbohydrates, Fiber, Fat, Protein and Amino Acids.* 2002: Washington DC: National Academy Press.
5. Couce, M.L., et al., *Carbohydrate status in patients with phenylketonuria.* Orphanet J Rare Dis, 2018. **13**(1): p. 103.
6. van Wegberg, A.M.J., et al., *The complete European guidelines on phenylketonuria: diagnosis and treatment.* Orphanet J Rare Dis, 2017. **12**(1): p. 162.
7. Scientfic Advisory Committee on Nutrition, *SACN Dietary Reference Values for Energy* P.H.E. Nutrition Science Team, Editor. 2016, PHE publication gateway number:2016202: London
8. Gropper, S.S., et al., *Nutrient intakes of adolescents with phenylketonuria and infants and children with maple syrup urine disease on semisynthetic diets.* J Am Coll Nutr, 1993. **12**(2): p. 108-14.
9. Monch, E., et al., *Utilisation of amino acid mixtures in adolescents with phenylketonuria.* Eur J Pediatr, 1996. **155 Suppl 1**: p. S115-20.
10. Lenke, R.R., et al., *Tyrosine supplementation during pregnancy in a woman with classical phenylketonuria. A case report.* J Reprod Med, 1983. **28**(6): p. 411-4.
11. Diamond, A., et al., *Prefrontal cortex cognitive deficits in children treated early and continuously for PKU.* Monogr Soc Res Child Dev, 1997. **62**(4): p. i-v, 1-208.
12. Jahja, R., et al., *Neurocognitive evidence for revision of treatment targets and guidelines for phenylketonuria.* J Pediatr, 2014. **164**(4): p. 895-899 e2.
13. Sharman, R., et al., *Depressive symptoms in adolescents with early and continuously treated phenylketonuria: associations with phenylalanine and tyrosine levels.* Gene, 2012. **504**(2): p. 288-91.
14. Daly, A., et al., *Glycomacropeptide in children with phenylketonuria: does its phenylalanine content affect blood phenylalanine control?* J Hum Nutr Diet, 2017. **30**(4): p. 515-523.
15. Morris, S.M., Jr., *Arginine Metabolism Revisited.* J Nutr, 2016. **146**(12): p. 2579S-2586S.
16. Tharakan, J.F., et al., *Adaptation to a long term (4 weeks) arginine- and precursor (glutamate, proline and aspartate)-free diet.* Clin Nutr, 2008. **27**(4): p. 513-22.
17. Koch, R., et al., *Large neutral amino acid therapy and phenylketonuria: a promising approach to treatment.* Mol Genet Metab, 2003. **79**(2): p. 110-3.
18. Matalon, R., et al., *Large neutral amino acids in the treatment of phenylketonuria (PKU).* J Inherit Metab Dis, 2006. **29**(6): p. 732-8.
19. Pietz, J., et al., *Large neutral amino acids block phenylalanine transport into brain tissue in patients with phenylketonuria.* J Clin Invest, 1999. **103**(8): p. 1169-78.

20. van Spronsen, F.J., et al., *Large neutral amino acids in the treatment of PKU: from theory to practice.* J Inherit Metab Dis, 2010. **33**(6): p. 671-6.
21. Pietz, J., et al., *Effect of high-dose tyrosine supplementation on brain function in adults with phenylketonuria.* J Pediatr, 1995. **127**(6): p. 936-43.
22. van Vliet, D., et al., *Large Neutral Amino Acid Supplementation Exerts Its Effect through Three Synergistic Mechanisms: Proof of Principle in Phenylketonuria Mice.* PLoS One, 2015. **10**(12): p. e0143833.
23. Hidalgo, I.J. and R.T. Borchardt, *Transport of a large neutral amino acid (phenylalanine) in a human intestinal epithelial cell line: Caco-2.* Biochim Biophys Acta, 1990. **1028**(1): p. 25-30.
24. Matalon, R., et al., *Double blind placebo control trial of large neutral amino acids in treatment of PKU: effect on blood phenylalanine.* J Inherit Metab Dis, 2007. **30**(2): p. 153-8.
25. Sanjurjo, P., et al., *Dietary threonine reduces plasma phenylalanine levels in patients with hyperphenylalaninemia.* J Pediatr Gastroenterol Nutr, 2003. **36**(1): p. 23-6.
26. Burlina, A.P., et al., *Large Neutral Amino Acid Therapy Increases Tyrosine Levels in Adult Patients with Phenylketonuria: A Long-Term Study.* Nutrients, 2019. **11**(10).
27. Scala, I., et al., *Large Neutral Amino Acids (LNAAs) Supplementation Improves Neuropsychological Performances in Adult Patients with Phenylketonuria.* Nutrients, 2020. **12**(4).
28. Schindeler, S., et al., *The effects of large neutral amino acid supplements in PKU: an MRS and neuropsychological study.* Mol Genet Metab, 2007. **91**(1): p. 48-54.
29. Yano, S., K. Moseley, and C. Azen, *Large neutral amino acid supplementation increases melatonin synthesis in phenylketonuria: a new biomarker.* J Pediatr, 2013. **162**(5): p. 999-1003.
30. Ahring, K.K., et al., *Comparison of Glycomacropeptide with Phenylalanine Free-Synthetic Amino Acids in Test Meals to PKU Patients: No Significant Differences in Biomarkers, Including Plasma Phe Levels.* J Nutr Metab, 2018. **2018**: p. 6352919.
31. Ney, D.M., et al., *Glycomacropeptide for nutritional management of phenylketonuria: a randomized, controlled, crossover trial.* Am J Clin Nutr, 2016. **104**(2): p. 334-45.
32. Pena, M.J., et al., *Continuous use of glycomacropeptide in the nutritional management of patients with phenylketonuria: a clinical perspective.* Orphanet J Rare Dis, 2021. **16**(1): p. 84.
33. Pinto, A., et al., *Dietary practices in isovaleric acidemia: A European survey.* Mol Genet Metab Rep, 2017. **12**: p. 16-22.
34. Zaki, O.K., et al., *The Use of Glycomacropeptide in Dietary Management of Phenylketonuria.* J Nutr Metab, 2016. **2016**: p. 2453027.
35. Pinto, A., et al., *Nutritional status in patients with phenylketonuria using glycomacropeptide as their major protein source.* Eur J Clin Nutr, 2017.
36. Ney, D.M., et al., *Metabolomic changes demonstrate reduced bioavailability of tyrosine and altered metabolism of tryptophan via the kynurenine pathway with ingestion of medical foods in phenylketonuria.* Mol Genet Metab, 2017. **121**(2): p. 96-103.
37. Pena, M.J., et al., *The Use of Glycomacropeptide in Patients with Phenylketonuria: A Systematic Review and Meta-Analysis.* Nutrients, 2018. **10**(11).

38. Kelleher, S.L., et al., *Glycomacropeptide and alpha-lactalbumin supplementation of infant formula affects growth and nutritional status in infant rhesus monkeys.* Am J Clin Nutr, 2003. **77**(5): p. 1261-8.
39. MacDonald, A., et al., *Adherence issues in inherited metabolic disorders treated by low natural protein diets.* Ann Nutr Metab, 2012. **61**(4): p. 289-95.
40. Walter, J.H., et al., *How practical are recommendations for dietary control in phenylketonuria?* Lancet, 2002. **360**(9326): p. 55-7.
41. Vilaseca, M.A., et al., *Quality of dietary control in phenylketonuric patients and its relationship with general intelligence.* Nutr Hosp, 2010. **25**(1): p. 60-6.
42. Waisbren, S.E., et al., *Phenylalanine blood levels and clinical outcomes in phenylketonuria: a systematic literature review and meta-analysis.* Mol Genet Metab, 2007. **92**(1-2): p. 63-70.
43. Milsom, J.P., M.Y. Morgan, and S. Sherlock, *Factors affecting plasma amino acid concentrations in control subjects.* Metabolism, 1979. **28**(4): p. 313-9.
44. Scriver, C.R., et al., *Normal plasma free amino acid values in adults: the influence of some common physiological variables.* Metabolism, 1985. **34**(9): p. 868-73.
45. O'Riordan, N., et al., *Structural and functional characteristics of bovine milk protein glycosylation.* Glycobiology, 2014. **24**(3): p. 220-36.
46. Sharma, N., et al., *Distinction between glycomacropeptide and beta-lactoglobulin with 'stains all' dye on tricine SDS-PAGE gels.* Food Chem, 2021. **340**: p. 127923.
47. MacDonald, A., et al., *Administration of protein substitute and quality of control in phenylketonuria: a randomized study.* J Inherit Metab Dis, 2003. **26**(4): p. 319-26.
48. Chourpiliadis, C. and S.S. Mohiuddin, *Biochemistry, Gluconeogenesis*, in *StatPearls*. 2021: Treasure Island (FL).
49. Millward, D.J., et al., *Protein quality assessment: impact of expanding understanding of protein and amino acid needs for optimal health.* Am J Clin Nutr, 2008. **87**(5): p. 1576S-1581S.
50. Boirie, Y., et al., *Slow and fast dietary proteins differently modulate postprandial protein accretion.* Proc Natl Acad Sci U S A, 1997. **94**(26): p. 14930-5.
51. Church, D.D., et al., *Essential Amino Acids and Protein Synthesis: Insights into Maximizing the Muscle and Whole-Body Response to Feeding.* Nutrients, 2020. **12**(12).
52. Bos, C., et al., *Postprandial kinetics of dietary amino acids are the main determinant of their metabolism after soy or milk protein ingestion in humans.* J Nutr, 2003. **133**(5): p. 1308-15.
53. Park, S., et al., *Anabolic response to essential amino acid plus whey protein composition is greater than whey protein alone in young healthy adults.* J Int Soc Sports Nutr, 2020. **17**(1): p. 9.
54. Volpi, E., et al., *Essential amino acids are primarily responsible for the amino acid stimulation of muscle protein anabolism in healthy elderly adults.* Am J Clin Nutr, 2003. **78**(2): p. 250-8.
55. Bickel, H., *The effects of a phenylalanine-free and phenylalanine-poor diet in phenylpyruvic oligophrenis.* Exp Med Surg, 1954. **12**(1): p. 114-7.
56. Woolf, L.I., R. Griffiths, and A. Moncrieff, *Treatment of phenylketonuria with a diet low in phenylalanine.* Br Med J, 1955. **1**(4905): p. 57-64.
57. Woolf, L.I., *Nutrition in relation to phenylketonuria.* Proc Nutr Soc, 1962. **21**: p. 21-9.

58. Blau, N., et al., *Management of phenylketonuria in Europe: survey results from 19 countries.* Mol Genet Metab, 2010. **99**(2): p. 109-15.
59. de Groot, M.J., et al., *Phenylketonuria: reduced tyrosine brain influx relates to reduced cerebral protein synthesis.* Orphanet J Rare Dis, 2013. **8**: p. 133.
60. Enns, G.M., et al., *Suboptimal outcomes in patients with PKU treated early with diet alone: revisiting the evidence.* Mol Genet Metab, 2010. **101**(2-3): p. 99-109.
61. Schmidt, H., et al., *Intelligence and professional career in young adults treated early for phenylketonuria.* Eur J Pediatr, 1996. **155 Suppl 1**: p. S97-100.
62. Acosta, P.B., et al., *Nutrient intakes and physical growth of children with phenylketonuria undergoing nutrition therapy.* J Am Diet Assoc, 2003. **103**(9): p. 1167-73.
63. Dokoupil, K., et al., *Optimising growth in phenylketonuria: current state of the clinical evidence base.* Clin Nutr, 2012. **31**(1): p. 16-21.
64. Hoeksma, M., et al., *The intake of total protein, natural protein and protein substitute and growth of height and head circumference in Dutch infants with phenylketonuria.* J Inherit Metab Dis, 2005. **28**(6): p. 845-54.
65. Ilgaz, F., et al., *Long-Term Growth in Phenylketonuria: A Systematic Review and Meta-Analysis.* Nutrients, 2019. **11**(9).
66. Weglage, J., et al., *Growth in patients with phenylketonuria.* Eur J Pediatr, 1994. **153**(7): p. 537-8.
67. Lynch, C.J. and S.H. Adams, *Branched-chain amino acids in metabolic signalling and insulin resistance.* Nat Rev Endocrinol, 2014. **10**(12): p. 723-36.
68. Gale, C., et al., *Effect of breastfeeding compared with formula feeding on infant body composition: a systematic review and meta-analysis.* Am J Clin Nutr, 2012. **95**(3): p. 656-69.
69. Victora, C.G., et al., *Methodological aspects of the 1993 Pelotas (Brazil) Birth Cohort Study.* Rev Saude Publica, 2006. **40**(1): p. 39-46.
70. Evans, S., et al., *Growth, Protein and Energy Intake in Children with PKU Taking a Weaning Protein Substitute in the First Two Years of Life: A Case-Control Study.* Nutrients, 2019. **11**(3).
71. Moretti, F., et al., *Dietary glycemic index, glycemic load and metabolic profile in children with phenylketonuria.* Nutr Metab Cardiovasc Dis, 2017. **27**(2): p. 176-182.
72. de Castro, M.J., et al., *Bone Status in Patients with Phenylketonuria: A Systematic Review.* Nutrients, 2020. **12**(7).
73. Demirdas, S., et al., *Bone health in phenylketonuria: a systematic review and meta-analysis.* Orphanet J Rare Dis, 2015. **10**: p. 17.
74. Hansen, S., et al., *Bone geometry, volumetric bone mineral density, microarchitecture and estimated bone strength in Caucasian females with systemic lupus erythematosus. A cross-sectional study using HR-pQCT.* Calcif Tissue Int, 2014. **95**(6): p. 530-9.
75. Allen, J.R., et al., *Decreased bone mineral density in children with phenylketonuria.* Am J Clin Nutr, 1994. **59**(2): p. 419-22.
76. Hillman, L., et al., *Decreased bone mineralization in children with phenylketonuria under treatment.* Eur J Pediatr, 1996. **155 Suppl 1**: p. S148-52.
77. Koura, H.M., et al., *A long-term study of bone mineral density in patients with phenylketonuria under diet therapy.* Arch Med Sci, 2011. **7**(3): p. 493-500.
78. Shaw, N. and N. Crabtree, *Bone density in children: what are we measuring?* Arch Dis Child, 2019. **104**(11): p. 1108-1111.

79. Szulc, P., E. Seeman, and P.D. Delmas, *Biochemical measurements of bone turnover in children and adolescents.* Osteoporos Int, 2000. **11**(4): p. 281-94.
80. Sawin, E.A., et al., *Differential Effects of Dietary Fat Content and Protein Source on Bone Phenotype and Fatty Acid Oxidation in Female C57Bl/6 Mice.* PLoS One, 2016. **11**(10): p. e0163234.
81. Gennai, I., et al., *Age- and sex-matched reference curves for serum collagen type I C-telopeptides and bone alkaline phosphatase in children and adolescents: An alternative multivariate statistical analysis approach.* Clin Biochem, 2016. **49**(10-11): p. 802-7.
82. Schoenau, E., et al., *The development of bone strength at the proximal radius during childhood and adolescence.* J Clin Endocrinol Metab, 2001. **86**(2): p. 613-8.
83. Cavalier, E., et al., *The role of biochemical of bone turnover markers in osteoporosis and metabolic bone disease: a consensus paper of the Belgian Bone Club.* Osteoporos Int, 2016. **27**(7): p. 2181-2195.
84. Gropper, S.S., D.M. Gropper, and P.B. Acosta, *Plasma amino acid response to ingestion of L-amino acids and whole protein.* J Pediatr Gastroenterol Nutr, 1993. **16**(2): p. 143-50.
85. Metges, C.C., et al., *Kinetics of L-[1-(13)C]leucine when ingested with free amino acids, unlabeled or intrinsically labeled casein.* Am J Physiol Endocrinol Metab, 2000. **278**(6): p. E1000-9.
86. Jennings, A., et al., *Amino Acid Intakes Are Associated With Bone Mineral Density and Prevalence of Low Bone Mass in Women: Evidence From Discordant Monozygotic Twins.* J Bone Miner Res, 2016. **31**(2): p. 326-35.
87. Conigrave, A.D. and D.T. Ward, *Calcium-sensing receptor (CaSR): pharmacological properties and signaling pathways.* Best Pract Res Clin Endocrinol Metab, 2013. **27**(3): p. 315-31.
88. Fenton, T.R., et al., *Meta-analysis of the effect of the acid-ash hypothesis of osteoporosis on calcium balance.* J Bone Miner Res, 2009. **24**(11): p. 1835-40.
89. Fenton, T.R., et al., *Phosphate decreases urine calcium and increases calcium balance: a meta-analysis of the osteoporosis acid-ash diet hypothesis.* Nutr J, 2009. **8**: p. 41.
90. Liu, Z., et al., *Dose- and Glucose-Dependent Effects of Amino Acids on Insulin Secretion from Isolated Mouse Islets and Clonal INS-1E Beta-Cells.* Rev Diabet Stud, 2008. **5**(4): p. 232-44.
91. Yang, J., et al., *Insulin stimulates osteoblast proliferation and differentiation through ERK and PI3K in MG-63 cells.* Cell Biochem Funct, 2010. **28**(4): p. 334-41.
92. Chevalley, T., et al., *Arginine increases insulin-like growth factor-I production and collagen synthesis in osteoblast-like cells.* Bone, 1998. **23**(2): p. 103-9.
93. Fini, M., et al., *Effect of L-lysine and L-arginine on primary osteoblast cultures from normal and osteopenic rats.* Biomed Pharmacother, 2001. **55**(4): p. 213-20.
94. Fujita, S. and E. Volpi, *Amino acids and muscle loss with aging.* J Nutr, 2006. **136**(1 Suppl): p. 277S-80S.
95. Gosby, A.K., et al., *Testing protein leverage in lean humans: a randomised controlled experimental study.* PLoS One, 2011. **6**(10): p. e25929.
96. MacDonald, A., et al., *PKU dietary handbook to accompany PKU guidelines.* Orphanet J Rare Dis, 2020. **15**(1): p. 171.

97. Bowen, J., M. Noakes, and P.M. Clifton, *Appetite regulatory hormone responses to various dietary proteins differ by body mass index status despite similar reductions in ad libitum energy intake.* J Clin Endocrinol Metab, 2006. **91**(8): p. 2913-9.
98. Gustafson, D.R., et al., *Appetite is not influenced by a unique milk peptide: caseinomacropeptide (CMP).* Appetite, 2001. **36**(2): p. 157-63.
99. Keogh, J.B. and P. Clifton, *The effect of meal replacements high in glycomacropeptide on weight loss and markers of cardiovascular disease risk.* Am J Clin Nutr, 2008. **87**(6): p. 1602-5.
100. Veldhorst, M.A., et al., *A breakfast with alpha-lactalbumin, gelatin, or gelatin + TRP lowers energy intake at lunch compared with a breakfast with casein, soy, whey, or whey-GMP.* Clin Nutr, 2009. **28**(2): p. 147-55.
101. Royle, P.J., G.H. McIntosh, and P.M. Clifton, *Whey protein isolate and glycomacropeptide decrease weight gain and alter body composition in male Wistar rats.* Br J Nutr, 2008. **100**(1): p. 88-93.
102. Belanger-Quintana, A. and M. Martinez-Pardo, *Physical development in patients with phenylketonuria on dietary treatment: a retrospective study.* Mol Genet Metab, 2011. **104**(4): p. 480-4.
103. Burrage, L.C., et al., *High prevalence of overweight and obesity in females with phenylketonuria.* Mol Genet Metab, 2012. **107**(1-2): p. 43-8.
104. Batterham, R.L., et al., *Critical role for peptide YY in protein-mediated satiation and body-weight regulation.* Cell Metab, 2006. **4**(3): p. 223-33.
105. Poppitt, S.D., et al., *Evidence of enhanced serum amino acid profile but not appetite suppression by dietary glycomacropeptide (GMP): a comparison of dairy whey proteins.* J Am Coll Nutr, 2013. **32**(3): p. 177-86.
106. Badman, M.K. and J.S. Flier, *The gut and energy balance: visceral allies in the obesity wars.* Science, 2005. **307**(5717): p. 1909-14.
107. Degen, L., et al., *Effect of peptide YY3-36 on food intake in humans.* Gastroenterology, 2005. **129**(5): p. 1430-6.
108. Scott, V., et al., *Intravenous peptide YY3-36 and Y2 receptor antagonism in the rat: effects on feeding behaviour.* J Neuroendocrinol, 2005. **17**(7): p. 452-7.
109. Walls, E.K., A.E. Willing, and H.S. Koopmans, *Intravenous nutrient-induced satiety depends on feeding-related gut signals.* Am J Physiol, 1991. **261**(2 Pt 2): p. R313-22.
110. Jia, Y., et al., *Butyrate stimulates adipose lipolysis and mitochondrial oxidative phosphorylation through histone hyperacetylation-associated beta3 -adrenergic receptor activation in high-fat diet-induced obese mice.* Exp Physiol, 2017. **102**(2): p. 273-281.
111. Jia, Z., et al., *Critical Review of Volatile Organic Compound Analysis in Breath and In Vitro Cell Culture for Detection of Lung Cancer.* Metabolites, 2019. **9**(3).
112. Pereira, J., et al., *Breath analysis as a potential and non-invasive frontier in disease diagnosis: an overview.* Metabolites, 2015. **5**(1): p. 3-55.
113. Weber R, Haas N, Baghdasaryan A, Bruderer T, Inci D, Micic S, et al. Volatile organic compound breath signatures of children with cystic fibrosis by real-time SESI-HRMS. ERJ Open Res. 2020;6(1).

APPENDICES

Appendix 1. Dietary assessment diary

Dietary Assessment Diary

Study Patient
Number

…….

Subject initials:

…….

Date

……..

Instructions

- Please **weigh all food and drink eaten** rather than what is offered on the days
- Remember to include all snacks including sweets, biscuits and cakes.
- State the brand names of manufactured foods e.g., Heinz tomato sauce, Walkers plain crisps.
- State method of cooking e.g., grilled, fried, boiled etc.
- Please weigh the butter or margarine used over the period of recording the food intake.
- Record all food in the diet diary at the time of eating and not all together at the end of the day.
- Please save any ingredient labels/nutritional analysis from the sides of manufactured foods and return these with the diet diary.
- Please record any type of vegetable oils or fats that you have used
- Please record if you have used normal foods or low protein equivalent.
- Please record if you have used any low fat foods.

General Questions

Please tick appropriate answers

1. What type of milk replacement is given?

Sno Pro	
Loprofin PKU Drink	
Prozero	
Calogen	
Dalia	
Duocal	
Plant milk e.g., rice milk	
Coffee creamer	
Full cream milk/ semi skimmed/ skimmed	

2. How much milk replacement does your child drink (on cereals, drinks etc?)

_____

3. What type of low protein bread is eaten?

Home-made	
Manufacturers LP sliced bread	
LP bread rolls	
Other – please specify	

4. What type of fat/oil is put on bread?

Margarine	
Butter	
Low fat spread	
Oil (please state type)	
Other	

5. How is this spread?

Thinly	
Medium	
Thickly	

6. Do you put butter/margarine on vegetables?

Yes	
No	

7. Please specify the amount of butter/margarine used over the period of recording food intake.

_____

8. Do you put oil/salad dressings on salads?

Yes	
No	

EXAMPLE MENU

Date:-_____

Time	Weight of Food or Drink	Type of Food
8.00 am	**20g**	**Weetabix**
	10g	**Sugar**
	100 ml	**Prozero milk**
	30g	**Fate low protein white toast**
	8g	**Butter (Lurpak)**
	15g	**Jam**
	PKU Sphere	**Protein substitute**
	200 ml	**Water**
	200 ml	**Coca Cola**
10.00 am	**1 (10g)**	**Mevalia Frollini**
	55g	**Tinned pasta (spaghetti) Tesco**
12.30 pm	**1 slice**	**Fate low protein white toast**
	16g	**Butter (Lurpak)**
	30g	**Vanilla ice cream (normal) Walls**
	30 ml (before dilution)	**Ribena (sugar containing)**
	20g	**Starbursts sweets**
3.00 pm	**25g**	**Crisps Walkers Plain**
	30 ml (before dilution)	**Tesco's High Juice orange squash**
	2 sachets	**PKU gel raspberry**
5.00 pm	**90g**	**Oven chips (Tesco French Fries)**
	90g	**Grilled vegetable fingers (Birds Eye)**
	20g	**Baked beans (Heinz)**
	1 x 200 ml carton	**Apple Ribena (not low sugar)**
8.00 pm	**PKU cooler 20**	**Protein substitute**
	200 ml	**Water**

MENU PLAN DAY 1

Date:-_____

Time	Weight of Food or Drink	Type of Food or Drink
Breakfast		
Mid-Morning		
Midday		
Mid-afternoon		
Evening Meal		
Bedtime		

MENU PLAN DAY 2

Date:-_____

Time	Weight of Food or Drink	Type of Food or Drink
Breakfast		
Mid-Morning		
Midday		
Mid-afternoon		
Evening Meal		
Bedtime		

MENU PLAN DAY 3

Date:-_____

Time	Weight of Food or Drink	Type of Food or Drink
Breakfast		
Mid-Morning		
Midday		
Mid-afternoon		
Evening Meal		
Bedtime		

Appendix 2. Low protein food frequency questionnaire

LOW PROTEIN

FOOD FREQUENCY

QUESTIONNAIRE

DATE

……

SUBJECT NUMBER

……

INITIALS

……

FOOD FREQUENCY QUESTIONNAIRE

- This questionnaire asks you questions about what you/your child eats.
- We would like you to tell us about all foods/drinks regularly eaten by you/your child.
- The questionnaire lists 92 commonly eaten foods, food groups or drinks.
- For each food/food group there is a portion size suggested e.g., teaspoon, tablespoon, bowl, glass, slice.
- A portion size booklet is also provided. This will help you complete the food diary.

PLEASE MAKE SURE YOU PUT A NUMBER IN EVERY BOX ON EVERY LINE IN THE QUESTIONNAIRE.

- Write the number of times each food is taken each day in the 'in a DAY' box.
- Then write the number of days a week this amount is eaten in the 'per WEEK' box.
- If a food is eaten less than once a week then put a '0' in both boxes for this food.
- For food items made up of different ingredients e.g., jam sandwich, divide into individual ingredients e.g., 2 slices white bread, 1 teaspoon margarine, 2 teaspoons jam.
- If you/your child's portion size is double the suggested size, count this as 2 portions or if their portion size is smaller count it as ½ a portion.
- If there are food/drinks you/your child has regularly that are not listed, add these in the last section of each food group under 'other'.
-

FOR EXAMPLE:
If you/your child has:

- **½ a small pot of yoghurt once a day,**
- **a jam sandwich (2 slices of bread with butter and jam) 5 days a week,**
- **fruit once every 2 weeks**

your answers should look like this:

Food	Portion size	Number of portions in a DAY	Number of times per WEEK
Regular Flavoured or plain yoghurt	1 small pot (60g)	½	7
Regular Bread – sliced, toast	1 slice (30-40g)	2	5
Butter/margarine/oils	1 teaspoon (5g)	2	5
Sweet spreads e.g., Jam/marmalade/honey/icing	2 teaspoons (10g)	1	5

MILK AND MILK REPLACEMENTS			
Food	**Portion size**	**Number of portions in a DAY**	**Number of times per WEEK**
Regular Milk – full fat, semi-skimmed, skimmed, condensed, soya	30ml		
Other plant milks – coconut, oat, rice, almond, hemp	200-250 ml		
Low protein milk replacement e.g., Prozero, Sno Pro, Loprofin, Dalia	200-250 ml		
Milk flavouring e.g., chocolate, strawberry	1 tablespoon (15ml)		
Other: please list			

PLEASE MAKE SURE THERE IS <u>A NUMBER IN EVERY BOX ON EVERY LINE</u> BEFORE CONTINUING

DAIRY PRODUCTS AND ALTERNATIVES			
Food	**Portion size**	**Number of portions in a DAY**	**Number of times per WEEK**
Regular Cheese e.g., cheddar, Edam, Emmental	15g (1 small cube)		
Regular soft cheese, cream cheese, cheese spread	15-20g (1 tablespoon)		
Low protein cheese e.g., Violife	1 slice (20g)		
Soya cheese	15g (1 small square)		
Regular flavoured or plain yoghurt, drinking yoghurt, soya yoghurt, fromage frais, dairy desserts, custard, mousse, instant dessert	Small pot (40-60g)		
Low protein yoghurt, protein-free custard, mousse, dessert mix	1 small serve (60g)		
Cream (all types)	30ml		
Other: please list			

PLEASE MAKE SURE THERE IS <u>A NUMBER IN EVERY BOX ON EVERY LINE</u> BEFORE CONTINUING

BREADS AND CEREALS			
Food	**Portion size**	**Number of portions in a DAY**	**Number of times per WEEK**
Regular Bread – sliced, toast, small wrap	1 slice (30g-40g)		
Regular bread roll, English muffin, crumpet, pitta, naan, ciabatta, large wrap	1 small (50-70g)		
Low protein bread- sliced, toast, small wrap	1 slice (30g-40g)		
Low protein bread roll, English muffin, crumpet, pitta, naan, ciabatta, large wrap	1 small (50-70g)		
Gluten-free bread- sliced, toast, small wrap	1 slice (30g-40g)		
Gluten-free bread roll, English muffin, crumpet, pitta, naan, ciabatta, large wrap	1 small (50-70g)		
Regular Breakfast cereals – **wheat & oat based** e.g., muesli branflakes, multigrain, shredded wheat, Weetabix, gluten-free	10g		
Regular or gluten-free Breakfast cereal – **corn or rice based** e.g., cornflakes, rice krispies, Cheerios, sugar puffs, Frosties,	15-20g		
Low protein breakfast cereal	1 small bowl (30g)		
Other: please list			

PLEASE MAKE SURE THERE IS <u>A NUMBER IN EVERY BOX ON EVERY LINE</u> BEFORE CONTINUING

SPREADS			
Food	**Portion size**	**Number of portions in a DAY**	**Number of times per WEEK**
Sweet spreads e.g., Jam/marmalade/honey/icing	2 teaspoons (10g)		
Regular chocolate/nut spread	1 tablespoon (20g)		
Low protein spreads e.g., choc spread, low protein cheese spread	1 tablespoon (20g)		
Regular Savoury spreads/sandwich fillers e.g., Peanut butter/hummus/cheese spread	1 tablespoon (20-30g)		
Butter/margarine/oils	1 teaspoon (5g)		
Low fat spread/butter/margarine	1 teaspoon (5g)		
Other: please list			

PLEASE MAKE SURE THERE IS <u>A NUMBER IN EVERY BOX ON EVERY LINE</u> BEFORE CONTINUING

PASTA, RICE AND POTATO			
Food	**Portion size**	**Number of portions in a DAY**	**Number of times per WEEK**
Regular & gluten-free pasta, noodles, rice, couscous (cooked)	Rice cooked (45g), pasta/ noodles/couscous cooked (15g)		
Low protein pasta, noodles, rice, couscous (cooked)	Cooked 80-100g		
Tinned pasta	2 tablespoons (50g –70g)		
Potato (boiled, mashed, jacket, baked)	80g		
Potato cooked in fat (roast, chips, fried)	Roast (55g), chips (45g)		
Processed potatoes – waffles, croquettes, wedges, Alphabites,	1 piece (30g- 40g)		
Other: please list			

PLEASE MAKE SURE THERE IS <u>A NUMBER IN EVERY BOX ON EVERY LINE</u> BEFORE CONTINUING

VEGETABLES (fresh, raw, cooked, frozen and canned)			
Food	**Portion size**	**Number of portions in a DAY**	**Number of times per WEEK**
Broad beans, peas, spinach, Spring greens, sweetcorn, mixed vegetables	Broad beans (20g), peas (25g), spinach (25g), Spring greens (35g), sweetcorn (35g), mixed vegetables (30g)		
Asparagus, beansprouts, broccoli, Brussel sprouts, cauliflower, okra, yam, sugar snap peas, mange tout	60g		
All other vegetables e.g., carrots, sweet potato, tomatoes, mushroom, cucumber, peppers, salad	60g		
Legumes/pulse e.g., baked beans, Lentils, chickpeas, dried peas/ beans	20g cooked		
Other: please list			

PLEASE MAKE SURE THERE IS <u>A NUMBER IN EVERY BOX ON EVERY LINE</u> BEFORE CONTINUING

FRUIT			
Food	**Portion size**	**Number of portions in a DAY**	**Number of times per WEEK**
All fresh, canned or frozen fruit	1 piece (80-100g)		
Dried fruit e.g., apricot, dates, cranberries, banana chips, mango, raisins, sultanas, fruit bars	2 tablespoons (30-40g)		
Other: please list			

PLEASE MAKE SURE THERE IS <u>A NUMBER IN EVERY BOX ON EVERY LINE</u> BEFORE CONTINUING

MEAT AND ALTERNATIVES			
Food	**Portion size**	**Number of portions in a DAY**	**Number of times per WEEK**
Meat e.g., beef, lamb, pork, bacon, veal, chicken, turkey	1 small piece (15g)		
Fish e.g., tuna, salmon, cod, haddock, kippers, mackerel, sardines prawns, crab, scallops, mussels	1 small piece (15g)		
Processed meats/ fish e.g., **sausage**, **chicken nuggets, fish fingers**, ham, sandwich slices, burgers, battered/crumbed fish, scampi	1 small piece (15g)		
Tofu/Quorn	1 small piece (15g)		
Eggs – boiled, poached, fried, scrambled, omelette	1 (50-60g)		
Low protein sausage/burger	1 (80g)		
Other: please list			

PLEASE MAKE SURE THERE IS <u>A NUMBER IN EVERY BOX ON EVERY LINE</u> BEFORE CONTINUING

SAUCES			
Food	**Portion size**	**Number of portions in a DAY**	**Number of times per WEEK**
Regular table top sauces, **ketchup**, dressings, chutney, pickles, mint sauce, vinaigrette	1 tablespoon (20-30g)		
Gravy	3 tablespoons (50ml)		
Mayonnaise, salad cream, oil based dressings	1 tablespoon (20-30g)		
Other milk based sauces e.g., cheese, white, parsley	2 tablespoons (50ml)		
Low protein sauce e.g., LP cheese sauce	2 tablespoons (50ml)		
Cook-in/meal based sauces e.g., curry, sweet & sour	5 tablespoons (100ml)		
Other: please list			

PLEASE MAKE SURE THERE IS <u>A NUMBER IN EVERY BOX ON EVERY LINE</u> BEFORE CONTINUING

SWEET SNACKS/PUDDINGS			
Food	**Portion size**	**Number of portions in a DAY**	**Number of times per WEEK**
Regular biscuit/cookie	1-2 (15-20g)		
Regular cake, muffin, doughnut, tart, scone, pastry	1 (50-70g)		
Regular cereal bar	1 (20-40g)		
Low protein biscuit/cookie	1-2 (15-20g)		
Low protein cake, muffin, doughnut, tart, scone, pastry	1 (50-70g)		
Low protein cereal bar	1 (20-40g)		
Regular Puddings – cheesecake, crumble, pie, tart, sponge, trifle, rice	Small serve (80-100g)		
Low protein Puddings – cheesecake, crumble, pie, tart, sponge, trifle, rice	Small serve (80-100g)		
Regular ice cream	30g		
Low protein ice cream/sorbet	30g		
Regular chocolate/chocolate bars	3-5 squares (15-25g)		
Low protein chocolate/chocolate bars	1 bar (25g)		
Gummy sweets or marshmallows (contain gelatine)	4-8 sweets (30g)		
Vegetarian gummy sweets	4-8 sweets (30g)		
All other sweets e.g., boiled, chewy, mints, candy	4-6 sweets (30g)		
Regular Jelly with gelatine	100g		

Low protein jelly	100g		
Ice lolly	1 lolly (45-80ml)		
Sugar added to drinks or cereals	1 teaspoon (5g)		
Other: please list			

PLEASE MAKE SURE THERE IS A NUMBER IN EVERY BOX ON EVERY LINE BEFORE CONTINUING

SAVOURY SNACKS			
Food	**Portion size**	**Number of portions in a DAY**	**Number of times per WEEK**
Potato crisps, corn snacks, rice snacks, popcorn	1 small packet (15-25g)		
Nuts and seeds	1 tablespoon (10-15g)		
Low protein crisps	1 packet (25g)		
Regular crackers, crispbreads, breadsticks, rice cakes	2 (10-15g)		
Other: please list			

PLEASE MAKE SURE THERE IS A NUMBER IN EVERY BOX ON EVERY LINE BEFORE CONTINUING

TAKEAWAYS/READY MEALS/SOUPS			
Food	**Portion size**	**Number of portions in a DAY**	**Number of times per WEEK**
Regular Pizza	1 slice (40-50g)		
Low protein pizza	1 slice (40-50g)		
Vegetarian burger (with bread roll)	1 (50g) burger (60g) 1 bread roll		
Quiche	1 slice (40-50g)		
Meat based dish e.g., lasagne, curry, chow mein, stir fry	1 small bowl (200g)		
Vegetable based dish – lasagne, curry, stir fry	1 small bowl (200g)		
Meat based pie, sausage roll, pastie, samosa, pakora, spring roll	1 small (50 -80g)		
Vegetable based pie, sausage roll, pastie, samosa, pakora, spring roll	1 small (50-80g)		
Low protein dish e.g., pasta & sauce, snack pot, pot noodles	1 small bowl/1 pot (200g)		
Soup	1 small bowl (200 ml)		
Other: please list			

PLEASE MAKE SURE THERE IS A NUMBER IN EVERY BOX ON EVERY LINE BEFORE CONTINUING

DRINKS			
Food	**Portion size**	**Number of portions in a DAY**	**Number of times per WEEK**
Water (tap, mineral)	1 glass (200 ml)		
Natural fruit juice (no added sugar)	1 glass (200 ml)		
Vegetable juice	1 glass (200 ml)		
Regular sweet drinks, squash, fizzy drinks, coke	1 glass (200 ml)		
Sugar-free drinks, squash, fizzy drinks, coke	1 glass (200 ml)		
Tea/coffee	1 small cup (200 ml)		
Powdered hot chocolate	15g (1 tablespoon)		
Non-dairy creamer e.g., coffee whitener	15g (1 tablespoon)		
Other: please list			

This validated food frequency questionnaire for PKU has been developed by Sharon Evans, a dietetic PhD student.

Appendix 3. Dietary assessment diary for breath study

Dietary Assessment Diary

BREATH STUDY

Study Patient
Number

…….

Subject initials:

……

Date

……

Instructions

- Please weigh all food and drink eaten rather than what is offered on the day
- Remember to include all snacks including sweets, biscuits and cakes.
- State the brand names of manufactured foods e.g., Heinz tomato sauce, Walkers plain crisps.
- State method of cooking e.g., grilled, fried, boiled etc.
- Please weigh the butter or margarine used over the period of recording the food intake.
- Record all food in the diet diary at the time of eating and not all together at the end of the day.
- Please save any ingredient labels/nutritional analysis from the sides of manufactured foods and return these with the diet diary.
- Please record any type of vegetable oils or fats that you have used
- Please record if you have used normal foods or low protein equivalent.

EXAMPLE MENU

Date:-_____

Time	Weight of Food or Drink	Type of Food
8.00 am	**20g**	**Weetabix**
	10g	**Sugar**
	200 ml	**Prozeo milk**
	30g	**Fate low protein white toast**
	8g	**Butter (Lurpak)**
	15g	**Jam**
	1 sachet	**PKU sphere**
	200 ml	**Water**
10.00 am	**200 ml**	**Coca Cola**
	1 (10g)	**Mevalia fruit bar**
12.30 pm	**198g**	**Low protein pasta (Mevalia)**
	1 slice 55g	**Mavalia baguette**
	16g	**Garlic butter Tesco**
	30g	**Vanilla ice cream (normal) Walls**
	30 ml (before dilution)	**Ribena (sugar containing)**
	1 sachet	**PKU sphere**
3.00 pm	**20g**	**Skittles**
	25g	**Crisps Walkers (plain)**
	30 ml (before dilution)	**Tesco's High Juice orange squash**
5.00 pm	**90g**	**Oven chips French Fries (Tesco)**
	90g	**Hooba sausage**
	35g	**Lettuce**
	28g	**Cucumber**
	48g	**Tomato**
	27g	**Mayonnaise (Heinz)**
	1 x 200 ml carton	**Apple Ribena (not low sugar)**
	1 sachet	**PKU sphere**

MENU PLAN

Date:-_____

Time	Type of Food or Drink	Weight of Food or Drink
Breakfast Drinks		
Mid-Morning Drinks		
Lunch Drinks		
Mid afternoon Drinks		
Tea time Drinks		

Notes

Appendix 4. Breath study questionnaire

Birmingham Children's Hospital NHS
NHS Foundation Trust

CHAIRMAN
Joanna Davis

CHIEF EXECUTIVE OFFICER
Sarah-Jane Marsh

BREATH STUDY QUESTIONNAIRE

Please answer these questions (Please tick ✓ each of the boxes below)

QUESTION 1

I like the taste of my coolers/ gel/ twist?

Not at all ☐ A little ☐ Not bothered ☐ Most of the time ☐ Very much ☐

Comments:

QUESTION 2

I worry my breath smells?

Not at all ☐ A little ☐ Not bothered ☐ Most of the time ☐ Very much ☐

Comments:

QUESTION 3

Other people comment on my breath?

Not at all ☐ A little ☐ Not bothered ☐ Most of the time ☐ Very much ☐

Comments:

QUESTION 4

I notice my breath smells more?

Mornings ☐ Lunchtime ☐ After my cooler ☐ Afternoon ☐ Evening ☐ Not at all ☐

Comments:

QUESTION 5

I worry that this might affect my friendships?

Not at all	A little	Not bothered	Most of the time	Very much
☐	☐	☐	☐	☐

Comments:

QUESTION 6

The worry I have about my breath stops me taking my protein substitute (cooler/ gel/ lophlex/ twist)?

Not at all	A little	Not bothered	Most of the time	Very much
☐	☐	☐	☐	☐

Comments:

QUESTION 7

I notice my breath smells more with my cooler/ gel/ lophlex than twist?

Yes	No	No difference	Don't know
☐	☐	☐	☐

QUESTION 8

I notice my breath smells more with my twist than cooler/ gel/ lophlex?

Yes	No	No difference	Don't know
☐	☐	☐	☐



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