Nutritional Issues and Impact of Treatment in Patients with Phenylketonuria

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Author's Declaration

I declare that the work contained within this thesis is original and is the work of the author Hani A. Alfheeaid. I have been solely responsible for the organisation and day to day running of the studies as well as clinical measurements, laboratory analyses, and data processing, unless otherwise stated.

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I certify that the work reported in this thesis has been performed by Hani A. Alfheeaid and that during the period of study he has fulfilled the conditions of the ordinances and regulations governing the Degree of Doctor of Philosophy, University of Glasgow

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Abbreviation List

ADP	Air-displacement plethysmography
ANR	Above normal range
AUC	Area under the curve
BBB	Blood-brain barrier
BH4	Tetrahydrobiopterin
BIA	Bioelectrical impedance analysis
BMI	Body mass index
BNR	Below normal range
BWt	Body weight
CCK	Cholecystokinin
CHO	Carbohydrates
CHOO	Carbohydrate oxidation
CO_2	Carbon dioxide
CV	Coefficient of variation
D_2O	Deuterium oxide
DEXA	Dual-energy X-ray absorptiometry
ECD	European Commission Directive of foods for special medical purposes
EE	Energy expenditure
EI	Energy intake
ESPKU	The European Society for Phenylketonuria and Allied Disorders Treated as Phenylketonuria
FFM	Fat-free mass
FM	Fat mass
FO	Fat oxidation
FTIR	Fourier Transform Infrared spectrophotometer
GGC	Greater Glasgow and Clyde
GLP-1	Glucagon-like peptide 1
GMP	Glycomacropeptide

GRI	Glasgow Royal Infirmary
HPA	Hyperphenylalaninemia
ICP-MS	Inductively coupled plasma mass spectrometry
kg	Kilograms
kJ	Kilo Joules
LNAA	Large neutral amino acids
MRC	PKU Working Group in the Medical Research Centre
NDNS	National Diet and Nutrition Survey (NDNS 2008-2009)
NHS	National Health Service
NLB	New Lister Building
NR	Normal ranges
NSPKU	The National Society for Phenylketonuria
O_2	Oxygen
PA	Physical activity
PAH	Phenylalanine hydroxylase
PFC	Prospective food consumption
PHE	Phenylalanine
PKU	Phenylketonuria
PYY	Peptide YY
RBC	Red blood cells
RER	Respiratory exchange ratio
RHSC	Royal Hospital for Sick Children
RMR	Resting metabolic rate
RNI	Recommended Nutrient Intake
SLPF	PKU special low protein foods
TAG	Triglyceride
TBW	Total body water
TDEE	Total daily energy expenditure
TEF	Thermic effect of feeding
TOBEC	Total body electrical conductivity
TRYP	Tryptophan
TYR	Tyrosine

VAS	Visual analogue scale
$\dot{V}CO_2$	Rate of carbon dioxide production
$\dot{V}O_2$	Rate of oxygen consumption

Abstract

In the early days of PKU, micronutrient deficiencies, undernutrition and growth failure were common features of patients with the condition. This was mainly due to the limited availability of engineered special low protein foods (SLPF) and PKU protein substitutes to use. Nowadays, SLPF foods and micronutrient-enriched PKU protein substitutes have become widely available and are free on prescription in most countries. These SLPF are high in carbohydrates and often in fat content, have a higher glycaemic index and provide more energy per weight compared with the protein-containing equivalent normal foods.

Advancement in the PKU management, including dietary practices, led to nutritional problems that had never been reported before, but have become more frequent in the recent years. Overweight and obesity, rather than undernutrition, have become increasingly reported in patients with PKU, with some studies suggesting higher prevalence in females than males. Data on body composition in patients with PKU are inconsistent with some studies showing that patients with PKU have higher FM and lower FFM compared to healthy controls. This suggests that for a given body weight and height, patients with PKU might be fatter and look bigger. However, there is very little research looking at the determinants of nutrient status, body composition and obesity in patients with PKU.

Hence, among the aims of this thesis was to investigate the impact of a PKU SLPF-based meal on appetite ratings, gut appetite hormones, thermic effect of feeding (TEF) and fat oxidation (Chapter 2). Twenty-three healthy adults (mean ± SD age: 24.3 ± 5.1 years; BMI: 22.4 ± 2.5 kg/m^2) participated in a randomised crossover study. Each participant conducted two (PKU and Control) experimental trials which involved consumption of a PKU SLPF-based meal and protein substitute drink or an

isocaloric and weight matched ordinary meal and protein-enriched milk drink. Appetite, metabolic rate, fat oxidation measurements and blood collections were conducted for the duration of 300 minutes. On completion of the measurements, an *ad libitum* buffet dinner was served. Responses of appetite ratings, plasma concentrations of GLP-1 and PYY (P>0.05, trial effect, two-way ANOVA) and energy intake during ad libitum buffet dinner (P>0.05, paired t-test) were not significantly different between the two trials. The TEF (PKU, 10.2 ±1.5%; Control, 13.2 ± 1.0%) and the total amount of fat oxidised (PKU, 18.90 ± 1.10 g; Control, 22.10 ± 1.10 g) were significantly (P<0.05, paired t-tests) lower in the PKU than in the Control trial. The differences in TEF and fat oxidation were significant (P<0.05, paired t-tests) for the post-meal period. Therefore, from this first study we concluded that consumption of a meal composed of SLPF has no detrimental impact on appetite and appetite hormones but produces a lower TEF and postprandial fat oxidation than an ordinary meal. We hypothesised that these metabolic alterations may contribute to the increased prevalence of obesity reported in patients with PKU on contemporary dietary management.

In the second experimental chapter, we tested the hypothesis generated from the study above and measured TEF, fasting and postprandial fat and CHO oxidation in 13 patients with PKU and 10 healthy controls of similar age and BMI. Participants in the PKU group were provided an SLPF-based meal while those in the Control group were provided an isocaloric normal meal. It was found that TEF, and postprandial fat and CHO oxidation were not signifcantly different between the PKU and the Control groups. In addition, this study compared body composition characteristics (measured by Deuterium Oxide dilution technique) between PKU patients and healthy controls and revealed that differences in body composition are not significant between the two groups despite a tendency of PKU patients having

higher percentage of body fatness (*P*=0.08). However, data generated from this study should be interpreted with caution and requires confirmation from studies with larger sample size.

Micronutrient imbalance has been noted in patients with PKU despite their high provision through the PKU protein substitutes. Recent studies showed high blood levels of vitamin B12 and folate, but simultaneously deficient plasma levels of selenium and zinc in PKU patients prescribed with micronutrient-enriched PKU protein substitutes. Factors associated with micronutrient imbalance have rarely been studied in the literature. Therefore, the last chapter of this thesis aimed to evaluate the micronutrient status of children with PKU and explore factors associated with micronutrient imbalances and deficiencies. This was analysis of a large clinical dataset with serial measurements obtained from PKU children (≤16 years) attending the metabolic medicine clinic at the Glasgow Royal Hospital for Sick Children between 1990 and 2013. The study included 81 patients who provided a total of 512 blood samples for their routine annual micronutrient screening. Data on blood micronutrient measurements was available for vitamins A, B12, D, E, serum folate, and erythrocyte folate and the trace elements copper, selenium, zinc and serum ferritin as a biomarker of iron stores. Status of vitamin B12, E, and serum and erythrocyte folate measurements were above the normal range (NR) in 27%, 54%, 46% and 35% of the blood samples, respectively. However, 44% of selenium and 14% of zinc measurements were below the NR. Moreover, when we compared results with those from the UK National Dietary and Nutritional Survey, selenium and zinc deficiencies were specific to PKU condition and not a reflection of the epidemiology in the general UK population. In our PKU sample, poor metabolic control, PKU severity, and low adherence to PKU protein substitutes predicted low selenium status; while deficient zinc status was solely predicted by low adherence

to PKU protein substitutes. Yet, these predictors, collectively, explained a small (5.8 – 8.8 %) variation in the status of selenium and zinc in this group of patients. Selenium and zinc deficiencies are common in PKU patients despite high levels of other nutrients including vitamin B12, E and folate. The findings of this study suggest that selenium and zinc deficiencies reported in patients with PKU may be attributed to other factors which we were unable to measure in this retrospective study, such as low bioavailability of these nutrients from the artificial PKU protein substitutes.

Chapter One: General Introduction

1.1 Introduction

This chapter aims to provide relevant scientific background to the studies conducted in this thesis and to establish the theoretical basis and justification for these studies. This chapter begins with an overview about phenylketonuria (PKU) and its dietary management. The chapter also discusses available evidence on the potential impact of PKU disease and/or PKU special foods on appetite regulation, habitual energy intake (EI), fat oxidation, and components of energy expenditure such as resting metabolic rate (RMR), thermic effect of feeding (TEF) and habitual physical activity. These will be discussed in manner to establish their potential contribution to the increasing prevalence of overweight and obesity in PKU. In addition, micronutrient deficiencies and factors associated with micronutrient imbalance reported in patients with PKU will be discussed.

1.2 Phenylketonuria

Phenylketonuria (frequently called PKU; OMIM 261600) is an inborn error of phenylalanine (PHE) metabolism, characterised by mutations of the phenylalanine hydroxylase (PAH) (EC 1.14.16.1) gene, an essential liver-specific enzyme that metabolises dietary L-Phenylalanine to L-Tyrosine [1]. Deficiency or dysfunction of this enzyme leads to accumulation of PHE resulting in hyperphenylalaninemia (HPA) and abnormalities in metabolism of many compounds derived from other amino acids [2]. The degree of PHA deficiency varies among cases from complete absence of activity to residual activity ranging from 2% to 70% [3].

Phenylketonuria was first described in 1934 by AsbjØn FØlling, a Norwegian physician and biochemist, who discovered phenylketones in urine samples from two siblings presented with neurological impairments [4]. FØlling suggested that presences of phenylketones are the cause of their neurological symptoms [1]. The

mother of siblings Liv and Dag Egeland from Oslo, went to FØlling to examine the cause of their mental retardation and noted that children had abnormal odour in their urine. During the examination of the children, a routine urine check detected phenylpyruvic acid.

FØlling went further to test urine from other 430 children showing mental retardation and discovered phenylpyruvate in urine from eight of them. He then speculated that the finding of the ketone acids in the urine was the result of the body's inability to metabolise the amino acid; phenylalanine [5]. All eight children whose phenylpyruvate was found in their urine samples had similar symptoms notably: eczema, fair skin, a spastic gait and severe intellectual impairment [1]. In his publication, FØlling described this condition as "imbecillitas phenylpyrouvica" [5].

Few years later, Jervis and Penrose identified similarly effected individuals in the USA and the UK, respectively. In 1937, Jervis discovered that the deficiency of the enzyme PAH was the cause of phenylketones presence in the urine samples of affected individuals [1]. Review on the family history of the effected children suggested that the condition is an inherited recessive autosomal disease. The term phenylketonuria has been used since then for this condition despite the fact that hyperphenylalaninaemia is more accurate for identifying the range of conditions treated today [6].

In 1950, Dr Horst Bickel proposed a low-PHE diet that was effective at lowering blood PHE concentrations [7]. The low-PHE diet improved common PKU symptoms that was caused by PHE intoxication resulting in better neurological and mental outcomes [8]. First use of a synthetic diet using a PKU free-PHE protein substitute in combination with low-PHE foods was introduced in 1954 in a study that was conducted on five children with PKU by Armstrong and Taylor. The study showed

that the synthetic low-PHE diet improved development, behaviour, mental ability, and progression of seizures of the studied PKU sample [9].

1.3 Phenylalanine metabolism

Phenylalanine cannot be synthesised in the human body, thus it is an essential amino acid that should be included in the diet. All dietary proteins contain 4 to 6 % phenylalanine of their total amino acid content [10]. In the body, PHE is mainly sourced from dietary intake of protein or endogenous metabolism of protein and amino acid stores [1]. On the other hand, PHE as all other amino acids is used for protein syntheisis, oxidation to tyrosine (TYR) or conversion to other metabolites [1]. Thus, free PHE distribution across body and concentration in circulation depends on dietary protein intake and rate at which PHE is utilised.

After ingestion of dietary proteins, their digestion starts with the activation of pepsinogen to pepsin by hydrochloric acid in the stomach. At this stage, proteins are hydrolysed to peptides and amino acids. The pancreas then secretes proteases including trypsin, chymotrypsin and carboxypeptidase in the duodenum and small intestine to further digest the proteins by breaking specific sites of the peptide chain. The amino acid PHE is released with TYR and tryptophan (TRYP) (all belong to a group which is called aromatic amino acids) after the enzyme chymotrypsin cleaves the internal carboxyl bond at the peptide chains. Amino acids are then absorbed via intestinal epithelial cells and released into the bloodstream.

Phenylalanine concentrations are controlled constantly by dynamic input and runout flux [1]. Alteration in the PHE concentrations results from any disturbance to this dynamic influx system. The circulating phenylalanine is hydroxylated to tyrosine in the liver by the phenylalanine hydroxylase (PAH) enzyme in the presence of oxygen (O_2) and iron (Fe^{+2}), and oxidation of cofactor tetrahydrobiopterin (BH4) to a 4a-

hydroxy-BH4 [2]. The 4a-hydroxy-BH4 is then recycled to BH4 through quinonoid (q) dihydrobiopterin by the enzymes carbinolamie-4a- dehydratase (PCD) and the NADH-dependent dihydropteridine reductase (DHPR) (Figure 1.1) [11]. The cofactor BH4 is produced from guanosine triphosphate (GTP) in the presence of three enzymes GTP cyclohydrolase I (GTPCH), 6-pyruvoyL-tetra-hydropterin synthase (PTPS), and sepiapterin reductase (SR) [11]. Deficiency of BH4 can result from mutations in gene coding of enzymes used for its production or recycling (PCD, DHPR, GTPCH, PTPS, and SR) [2, 11].

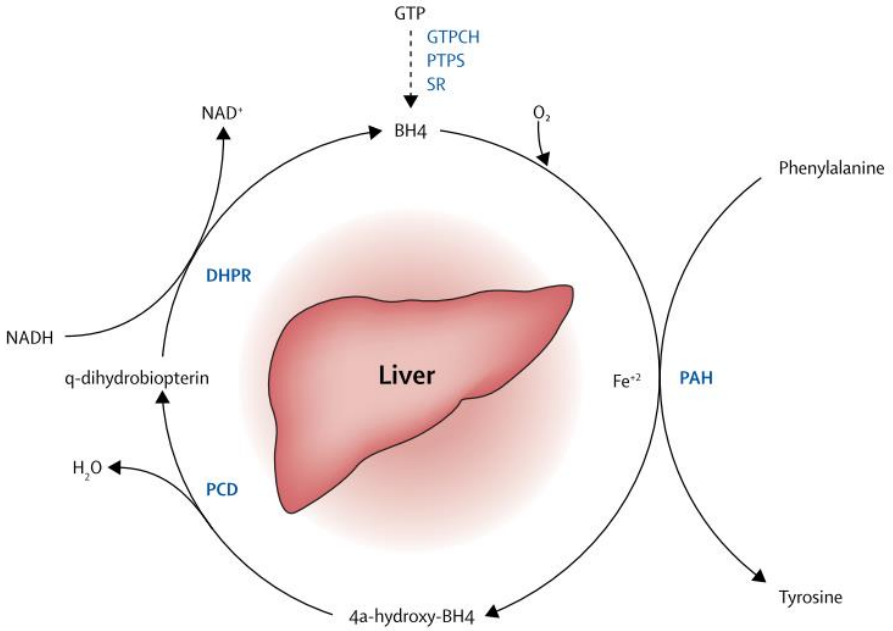


Figure 1.1: The phenylalanine hydroxylation metabolic pathway

Reproduced from Phenylketonuria [11]

Phenylalanine may be further decreased and decarboxylated when hydroxylation to tyrosine is disrupted and thus converted to phenylpyruvic acid (a ketone excreted in high amounts in the urine, from which the term phenylketonuria or PKU is used for this condition) [2].

Hydroxylation of TYR and TRYP by action of BH4 enables synthesis of melanin, dopamine, catecholamine and serotonin (Figure 1.2) [2]. Thus, diminished TYR and TRYP concentrations lead to the reduced production of these biologically important molecules which are linked to most common PKU symptoms such as skin and iris pigmentation [2], neurotransmitters imbalance, behavioural and neurological impairments [12]. In addition, absence of regular tyrosine and tryptophan metabolism impairs the synthesis of nitric oxide synthase and alkylglycerol monooxygenase enzymes [13]. The alkylglycerol monooxygenase enzyme has been suggested to play a physiological role in ether lipid metabolism [2]. Lack of nitric oxide synthase reduces nitric acid synthesis which relates to vascular function and therefore impacts blood pressure regulation [14].

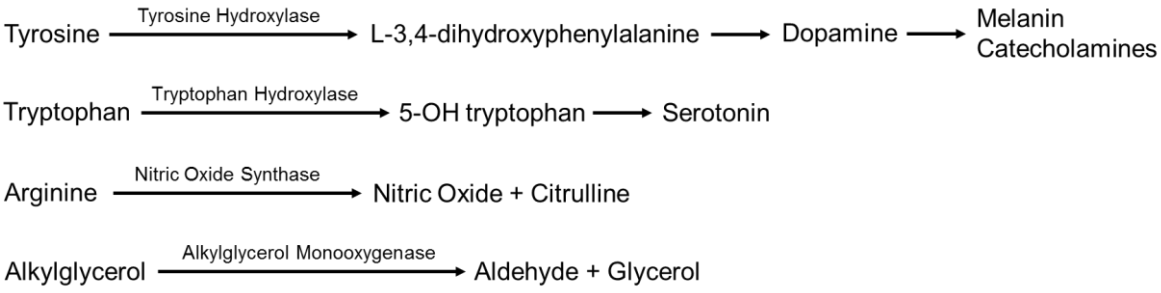


Figure 1.2: Involvement of the cofactor tetrahydrobiopterin (BH4) in metabolic pathways for Tyrosine and Tryptophan metabolism, and synthesis of alkylglycerol monooxygenase (AGMO) and nitric oxide synthase enzymes

Reproduced from Hyperphenylalaninaemia, In Inborn Metabolic Diseases, Diagnosis and Treatment [2]

1.4 Genetics of PKU

Phenylalanine hydroxylase (PAH) deficiency is an autosomal recessive disorder caused by inherited mutation in the PAH gene. The PAH gene is based in the region of band q22-124 of the long arm in the chromosome number 12 [15]. More than 950 different PAH mutations have been identified. The majority of the affected patients

are compound heterozygotes (i.e. biallelic mutation) in which each patient conveys two different mutations [16].

1.5 Epidemiology of PKU

Phenylketonuria is the most common inborn error of amino acid metabolism [17]. The prevalence of PKU varies geographically and among different ethnic groups [11, 18, 19]. In Europe, the estimated incidence is one per 10,000 livebirths [20]. The incidence is higher in other areas such as in Turkey and Northern Ireland at rate of 1 per 4, 000 births due to high consanguinity within their populations [21-23], while the prevalence drops to its lowest in Finland among other European countries with one case per 10,000 births [24]. Within the UK, the incidence of PKU is one per 8,000 in Scotland to one per 10,000 in England and Wales [18, 20]. In the US, the incidence is one case per 15,000 births [25] and the rate varies among Latin America from one case per 50,000 to one case per 25,000 births with a higher incidence rate in Southern Latin America compared to the rest of the region [11, 26]. In Asia, the incidence rate differs from one per 70,000 in Japan [27], one per 20,000 in Thailand [28] and one per 15,000 to one per 100,500 in China [29, 30]. In Saudi Arabia, the largest conutry in the Arabic Gulf region, the incedence rate ranges between 3 to 7 cases per 100,000 live births [31, 32]. Africa may have the lowest incidence rate of PKU worldwide [11].

1.6 Diagnosis of PKU

In early years, PKU was diagnosed clinically after symptoms are noticed resulting in major health and developmental impairments. First efforts of population-based screening for inborn errors of metabolism started in the 1950s with PKU after improved outcomes resulting from dietary treatment that was proposed by Dr Horst Bickel [33]. In 1957, Willard Centerwall developed diaper test which was used to

evaluate high PHE in urine samples of individuals with PKU by applying a ferric chloride solution to a wet diaper [34]. However, this method was mostly used in older infants by attending paediatric clinics, consequently delaying diagnosis and treatment of PKU [5, 35].

In 1960, Dr Robert Guthrie established first sensitive method to detect PKU in early days of life using dry blood spots collected from heel prick in a filter paper (so-called Guthrie cards) [35, 36]. The assay is based on that presence of high PHE concentrations in dry blood spots encourages bacterial growth. This microbiological inhibition assay was sensitive enough to detect PHE concentrations greater than 180–240 μmol/L (3–4 mg/dL) [36]. This method was practical and applicable at population level for early detection (1 to 5 days of life) of newborns with PKU.

The success of PKU detection led to expanding the single diagnosis to national based screening programmes that cover many other inborn disorders. This had a significant impact on preventing morbidity and mortality rates within countries utilising such programmes [37]. Tandem Mass Spectrometry has been used since 1990 for application of national newborn screening programmes. Tandem Mass Spectrometry uses electrical and magnetic fields to separate and measure the mass of the charged particles to quantify both amino acids and carnitine esters [38]. It is fast and reliable approach for detection of PKU by quantification of PHE and TYR concentrations [39]. Besides PKU, MS/MS allows to detect other 29 disorders of metabolic inborn errors in a single dried blood spot card including disorders of amino acids, organic acids, fatty acid oxidation, urea cycle, carnitine cycle and ketone body synthesis [40-44].

In most developed countries, PKU is detected via newborn national screening programmes. At birth, blood PHE is normal but elevates quickly within first days of

life. Cases are considered PKU when high blood PHE and/or PHE to TYR ratio were present in dry blood spots collected for the diagnosis of inborn errors. PKU detection protocols and PHE cut-offs vary between countries (Table 1.1) [43, 45-49]. These variations may be attributed to the differences in age at which diagnosis is performed, and techniques used for the determination of PHE concentrations (Guthrie microbiological inhibition assay, Tandem Mass Spectrometry or High-performance liquid chromatography) [2].

Table 1.1: Examples of PHE cut-offs and age of screening used in national screening programmes in different countries for diagnosis of individuals with PKU

Country of screening programme	PHE screening cut-off (µmol/L)	Age of screening (days)
UK [47]	240	5 – 8
Germany [46]	150	3 – 7
Portugal [48]	150	3 – 6
Australia [49]	150	2 – 3
USA (North Carolina) [43]	250	1 – 2
USA (New England) [45]	139	1 – 3

In the UK, PKU is detected via newborn national screening programme in which dry blood spots are collected in quadruplicate cards during the first 5 – 8 days of life. When PHE concentration is above 200 µmol/L in the first measurement, both PHE and TYR are requested for repeated measurement in the other dry blood spots [47]. Phenylketonuria is suspected when the PHE is higher and the TYR is lower than 240 µmol/L. Flowchart for routine newborn screening protocol of PKU in the UK is shown in Figure 1.3.

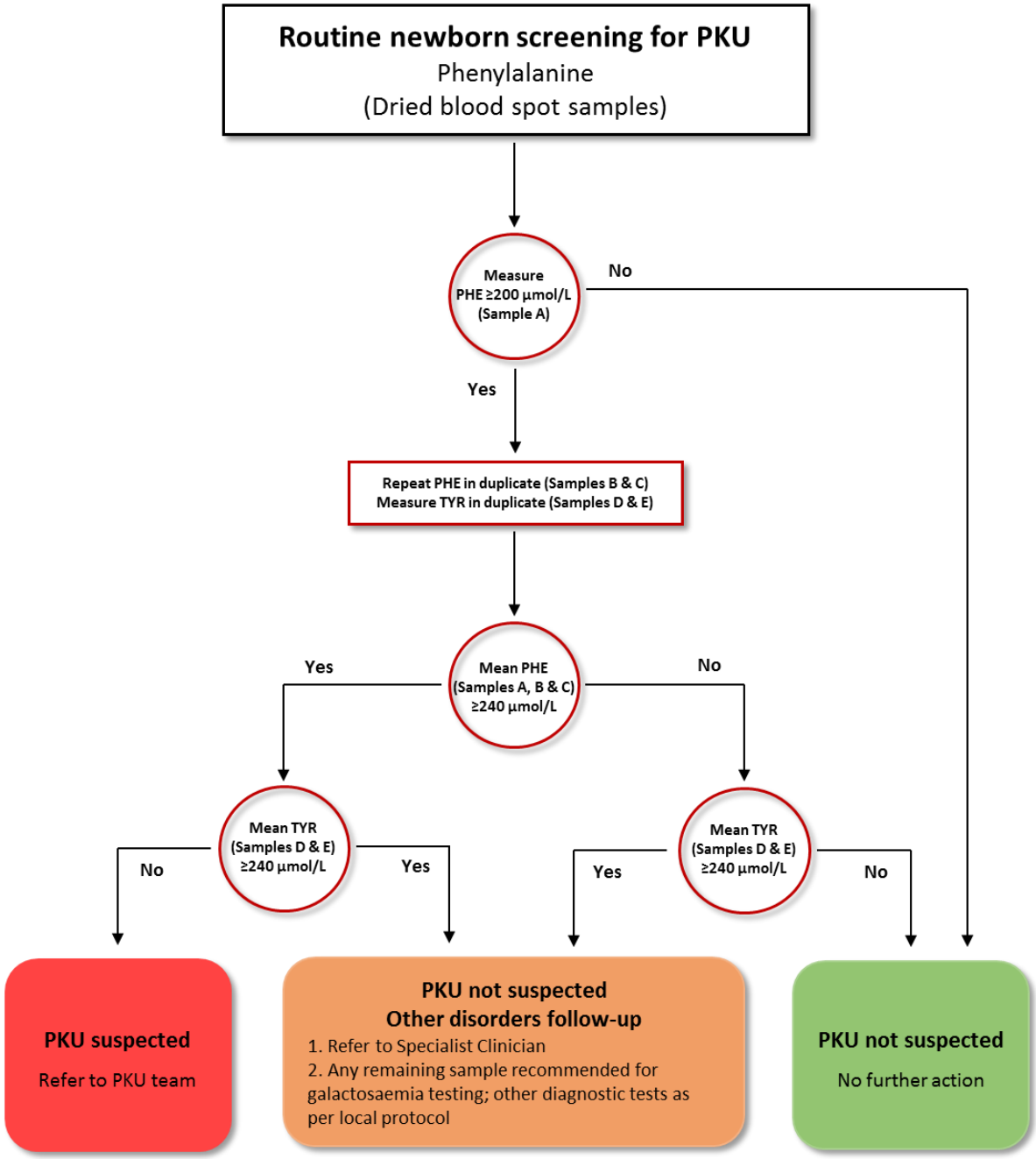


Figure 1.3: PKU Newborn Screening Protocol in the UK

Adapted from A laboratory Guide to Newborn Blood Spot Screening for Inherited Metabolic Diseases, NHS Newborn Blood Spot Screening Programme [50]

PHE, phenylalanine; TYR, tyrosine

Deoxyribonucleic acid (DNA) [51] testing is performed to confirm PKU cases and identify the type of mutation in the PAH gene [47]. Determination of PHE/TYR ratio may be useful to avoid false-negative results of PHE and TYR concentrations in blood spots obtained at the newborn screening [15, 52]. PHE/TYR ratio of more than 3 is used as a cut-off value for confirmation of cases with PKU at diagnosis [6]. The PHE/TYR ratio may also be used as a marker for disease severity [53]. However, a differential diagnosis is necessary to exclude BH4 deficiencies from other types of HPA and is performed by measurement of pterins in blood or urine, and dihydropteridine reductase activity in dried blood spot samples [15, 54]. The outcome from these tests is crucial for more effective clinical and dietary management [39, 53].

Phenylketonuria is classified into four severity categories according to initial PHE concentrations obtained at newborn screening. The severity classes include classical PKU (i.e. most severe type), moderate PKU, mild PKU and mild non-PKU hyperphenylalaninaemia (Table 1.2) [47]. Recently, the European Society for Phenylketonuria and Allied Disorders Treated as Phenylketonuria (ESPKU) proposed a new classification approach based on intervention or treatment type required to maintain blood PHE within recommended range [15]. The new ESPKU approach classifies patients as either (a) not requiring treatment, (b) or requiring diet or (c) BH4, (d) or both.

Table 1.2: Classification of PKU severity types

PKU severity type	PHE concentration (µmol /L)
Classical PKU	> 1200
Moderate PKU	900 – 1200
Mild PKU	600 – 900
Mild hyperphenylalaninaemia or Non-PKU hyperphenylalaninaemia	< 600

Reproduced from Phenylketonuria (PKU), Review of Initial Clinical Referral Guidelines and Standards, Newborn Blood Spot Screening in the UK [47]

1.7 Clinical symptoms and pathology

Chronically elevated PHE concentrations lead to dynamic and irreversible physical, behavioural and psychological impairments. These are frequently reported in untreated, late-treated or poorly controlled patients and have significant impact on patient's quality of life [55]. The PKU metabolic control (blood PHE concentrations) determines the severity of the reported impairments with untreated patients of classical form of PKU showing most severe symptoms. Untreated patients typically have low intelligence quotient (IQ) scores (often below 50) [56], but other common symptoms of persistent HPA include eczema, seizures, odor (in urine and body), hypopigmentation (in skin, hair and iris), intellectual disability [57], depression, anxiety, agoraphobia, hyperactivity and self-injury [58]. Other studies have also shown an association between PKU and autism [59] and attention deficit-hyperactivity disorder (ADHD) [60]

Early detection and treatment of PKU usually results in favourable outcome, but at individual level, even in well controlled, some patients may present poor executive functioning [61-63], low self-esteem, and emotional and behavioural disorders [64].

Since the discovery of PKU, mechanisms by which the brain function is disturbed via PHE neurotoxicity have yet to be described [65, 66]. Elevated PHE concentrations and lack of Large Neutral Amino Acids (LNAA) in the brain are the cause of neurotoxicity [11, 66] despite the fact that the outcome of cognitive function is correlated with metabolic control [67, 68]. Although the development of brain mass and the creation of synaptic connections occur mainly during the first year of life, concerns from complex effect of poor metabolic control continue until the end of infancy [68-70].

Damage to the central nervous system is the result of the neurotoxic effect of uncontrolled PKU and associated with its typical symptoms seen in untreated or poorly controlled patients. Untreated PKU patients present with microcephaly in which the brain size is 80% of the healthy individuals [71]. Morphological changes in the brain affecting both white and grey matter has also been reported in this group of patients. These are caused by altered myelin structure anomalies that lead to reduced myelin volume, abnormal neuronal development and protein synthesis [58, 71, 72]. The multidirectional impact of elevated PHE concentrations and reduced LNAA concentrations on cognitive, neurophysiological and neuropsychological functions are illustrated in Figure 1.4 [15, 73].

In HPA, the ability for LNAA uptake into the brain is decreased due to the high PHE concentrations passing the blood-brain barrier (BBB). An LNAA carrier, the L-amino acid transporter 1 (LAT-1), works as transporter in the brain for PHE and other amino acids such as TYR and TRYP [74-77]. In case of high PHE concentrations, the delivery of other LNAA by the LAT-1 transporter is decreased since the delivery rate is affected by the blood concentrations of all other transported amino acids [78]. Thus, in PKU condition, protein synthesis in the brain is reduced [12]. In addition, synthesis of catecholamines, dopamine and serotonin in the brain is negatively

affected by the high PHE concentrations due to alterations in TYR and TRYP metabolism which lead to behavioural impairments and depressive symptoms commonly reported in PKU (Figure 1.4) [58, 79]. These are also known contributors to energy and nutrient balance regulation [80-83].

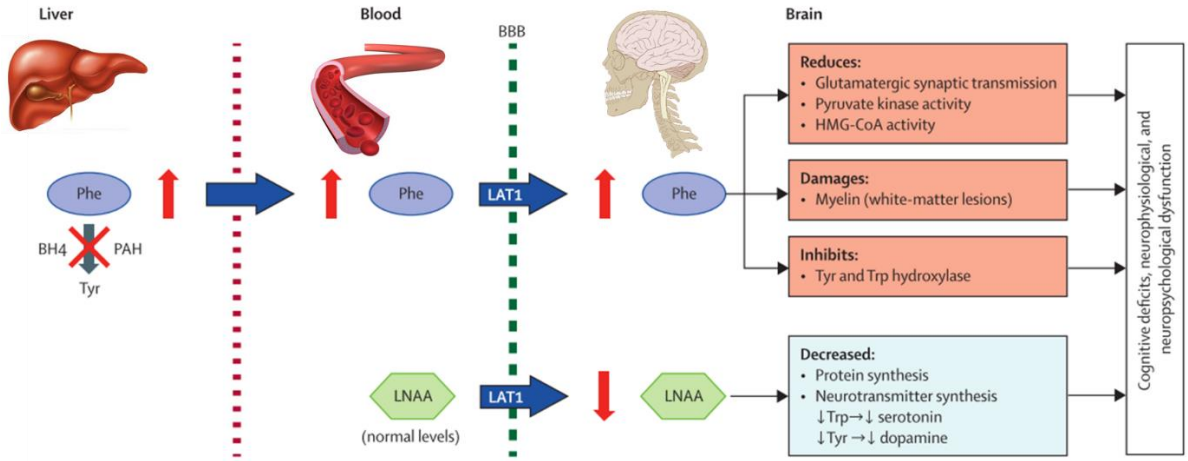


Figure 1.4: Mechanisms of PKU pathology

Reproduced from Challenges and Pitfalls in the Management of Phenylketonuria; and Key European guidelines for the diagnosis and management of patients with phenylketonuria [15, 73]

Phe, phenylalanine; PAH, phenylalanine hydroxylase; BBB, blood–brain barrier; LNAA, large neutral amino acids; LAT1, L-type amino acid carrier 1; BH4, tetrahydrobiopterin; HMG-CoA, 3-hydroxy-3-methylglutaryL-coenzyme A; Tyr, tyrosine; Trp, tryptophan

Disturbance in dopamine synthesis affects the activity of dopaminergic neurons in the prefrontal cortex of the brain [75]. Lack of dopamine, secondary to tyrosine deficiency, is associated with neurotransmitter imbalance which may be responsible for emotional problems, neurocognitive and executive dysfunction reported in patients with PKU [67, 69, 84].

Cholesterol synthesis is necessary for optimal neuronal signal transmission and myelin production in the brain. Active 3-hydroxy-3-methylglutaryl coenzyme A

(HMG-CoA) reductase is needed for the formation of cholesterol particularly in myelinating process of PHE-sensitive oligodendrocyte cells located in the brain. Abnormal concentrations of PHE and related metabolites inhibit the activity of HMG-CoA and thus alter cholesterol synthesis which eventually results in neurocognitive dysfunction [84-86].

1.8 Dietary management of patients with PKU

Dietary management with a low-PHE diet has been successful in treating manifestations of PKU when started at early age (Figure 1.5) [5, 87, 88]. The predominant goal of dietary treatment is to provide sufficient intake of energy and protein including PHE to prevent the breakdown of body tissues and maintain normal body functioning and development [10, 89, 90]. This, however, can increase PHE concentrations in the blood [91]. Adequate intake of amino acids including PHE and energy [91, 92], as well as optimal growth are important to control PHE concentrations [93, 94]. High PHE concentrations may also result from catabolic state during the disease course [94]. Thus, providing adequate protein and energy ‘’overall optimal nutrition’’ for patients with PKU while maintaining their PHE concentrations within treatment targets can be challenging.

Figure 1.5: Two siblings with Phenylketonuria, untreated and treated cases. The 11 years boy, left, is severely retarded; while the 2.5 years girl, right, (normal) was early diagnosed and treated with a low PHE diet

Reproduced from The Discovery of Phenylketonuria: The Story of a Young Couple, Two Retarded Children, and a Scientist [5]

Dietary treatment should be started as soon as PKU or HPA is confirmed. It was common belief that PKU treatment with a low-PHE diet can be discontiued at age of 6 years whithout effect on later development [95-97]. Nonetheless, following studies indicated that best clinical outcome is achieved when patients are treated with diet for life [15, 98, 99]. The PKU dietary management involves consuming a restricted amount of natural protein [100] depending on the PKU severity ‘’PHE tolerance’’ and patient’s adherence to dietary treatment [101, 102]. It also includes the use of PKU special low protein foods (SLPF), and eating foods that naturally contain very low amounts of protein such as most fruits and vegetables (PKU free foods) [100]. To cover protein and other nutritional requirments, the diet is therefore supplemented with PHE-free PKU protein substitutes (in form of L-amino acids) which are enriched with micronutrients [102].

1.8.1 PKU special low protein foods

Phenylketonuria special low protein foods (SLPF) are central in dietary management of patients with PKU [103]. These foods are manufactured to provide more diverse selection of food and cover energy requirements of patients under dietary restriction of natural protein-containing foods in the low-PHE diet. The SLPF are mainly made from CHO and fat sources [102, 103]. These foods have higher glycaemic index [104] and provide more energy when compared to their equivalent protein-containing ‘’normal’’ foods [103, 105]. However, SLPF are often of poor nutritional value [106-108] and their contribution to micronutrient supply in the low-PHE diet has not yet been evaluated [102]. Thus, the SLPF impact on long-term nutritional status should be investigated.

In recent years, the SLPF have significantly improved in terms of their availability, accessibility, variety and palatability [100] and contributed to improvement in patients’ adherence to dietary treatment [108]. Currently available SLPF include breakfast cereals, bread, flour, cake mixes, chocolate bars, jelly, cookies, ice cream, milk replacers, egg replacers, pasta, rice and savoury foods [100, 103]. Consumption of the SLPF depends on the disease severity in which 50% of non-protein energy requirements is supplied by the SLPF in most sever type of PKU [103]. Patients with mild PKU are less likely to be dependent on SLPF as higher amount of natural protein is allowed in their diet compared to patients with severe PKU [99]. However, the SLPF may sometimes contain very small amounts of PHE (<25 mg /100 g of food weight) [109].

The cost cover of SLPF varies between countries. In the US, the majority of patients with PKU pay for their SLPF, but in some other states these are covered by health insurance [110]. Other countries such as Denmark, Belgium, Norway and Turkey,

an annual or monthly allowance is paid to the patients to cover costs of SLPF [111]. In Spain and Poland, patients receive no government funding, while in Germany and Netherlands only patients on welfare programmes or low income receive financial assistance. Patients with PKU in the UK and Italy are fully reimbursed by which SLPF are prescribed through the general practitioners (GPs) and dispensed to the patients by their local pharmacists [111]. However, in England, free SLPF prescription is limited to patients under 16 years old.

The National Society for Phenylketonuria (NSPKU) has published recommendations on maximum monthly number of SLPF unites as a guideline for GPs to ensure adequate supply and necessary variety of SLPF needed for patients on low-PHE diet (Table 1.3) [112].

Table 1.3: Recommended monthly maximum number of SLPF units* according to age groups*

Age group	Monthly maximum SLPF units
4 months to 3 years	15
4 – 6 years	25
7 – 10 years	30
≥11 years + Pre-pregnancy/Pregnancy	50

*Adapted from The Prescription of Low Protein Foods in PKU, National Society for Phenylketonuria [112]
SLPF, special low protein food
Examples of 1 SLPF unit: 4 sachets of sup; 1 packet of biscuits; 300 g of pasta

1.8.2 PKU protein substitutes

Phenylketonuria protein substitutes are the main source of protein equivalents for patients on low-PHE diet (52 - 80% of total protein intake, varies depending on disease severity ‘’PHE tolerance’’ and dietary adherence) and aim to prevent protein deficiency [101, 113] and maintain good metabolic control [92, 114]. The PKU

protein substitutes contain all essential amino acids including TYR which becomes an indispensable amino acid in PKU condition [99]. These protein substitutes are also considered as source for carbohydrate and sometimes fat in the low-PHE diet [115]. They may contribute to approximately 30% of total EI in patients with PKU [102].

Early research [116] investigating micronutrient status of patients with PKU has led to supplementation of protein substitutes with vitamins and minerals [117]. Before this, vitamins and minerals were prescribed or given separately as syrup for infants and/or tablets for older children or sometimes as mineral mixtures to be added to the free-PHE protein feeds during preparation (Figure 1.6) [118-120]. Full addition of vitamin and minerals to protein substitutes started at the beginning of the 90's [117]. Poor adherence to dietary treatment and absence of disease-specific vitamin and mineral supplements led to the addition of micronutrients to the currently most available PKU protein substitutes using age-specific profiles to meet requirements of infants, children and adults including pregnant women [102, 117]. However, the micronutrient content of these substitutes exceeds the UK's Recommended Nutrient Intake (RNI) for healthy population [121] and limits set by European Commission Directive (ECD) of foods for special medical purposes [102]. PKU protein substitutes are the main source for micronutrients in the low-PHE diet [102, 117]. Thus, the micronutrient supply in the low-PHE diet depends on the prescribed amount of PKU protein substitutes and patients' adherence to their intake [102, 117].

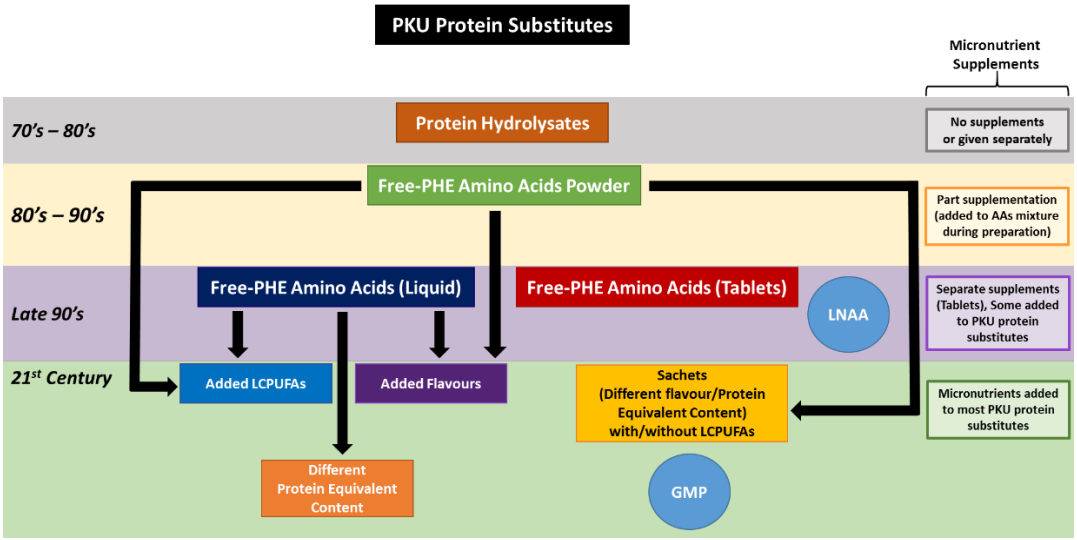


Figure 1.6: Chronological development of PKU free-PHE protein substitutes

Adapted from Protein Substitutes for Phenylketonuria in Europe: Access and Nutritional Composition [115]

PHE, Phenylalanine; LNAA, Large neutral amino acids; GMP, Glycomacropeptide; LCPUFA, Long-chain polyunsaturated fatty acids

Historically, poor adherence to PKU protein substitutes has been an issue in PKU dietary management for many years due to their poor palatability [122, 123]. However, recent versions of the protein substitutes have been immensely improved in terms of their forms, composition, taste, and palatability [87, 124]. They became available in various forms including powder, tablets, bars and ready-to-drink liquids with wide selections of flavours (Figure 1.6) [87, 115]. The nutritional profile of the current PKU protein substitutes has been further improved in terms of type of amino acids or protein used, for example use of LNAA and glycomacropeptide (GMP), and inclusion of other functional nutrients such as long-chain polyunsaturated fatty acids (LCPUFA) and dietary fibre [87, 115].

In absence of defined protein requirements for patients with PKU, total protein intake should meet the age-specific safe values of protein intake (FAO/WHO/UNU 2007) [15]. However, published guidelines on PKU management are in favour of a higher protein intake than general population [10, 125]. Hence, an additon of 40% of protein

requirements should be added to the low-PHE diet in people with PKU. This can be added in the form of natural protein, within their protein exhange allowance or as L-amino acids from their PKU protiein susbtitutes [15]. This includes additional 20% for what is called digestible indispensable amino acid score and 20% to promote the optimising impact of L-amino acids on blood PHE control. Furthermore, some studies indicate that higher free-PHE amino acid intake is associated with better PHE tolerance from ingestion of natural protein [92, 114, 126]. Inadequate L-amino acid intake limits their contribution to body protein synthesis and thus results in prevalence of catabolic protein metabolism. During protein catabolism, PHE is not used for protein synthesis but is excessively released in the blood [15]. Thus, adequate intake of L-amino acids reduces the blood PHE concentrations [127].

Consumption of amino-acid based protein substitutes has been reported to be associated with abdominal pain, constipation, and diarrhoea in children with PKU [128]. Some adult patients experienced proteinuria and had a reduced glomerular filtration rate when consuming the amino-acid based PKU protein substitutes in high doses over long period of time [129]. There is convincing evidence that even and frequent distribution of PKU protein substitute intake during the day results in positive effect on metabolic control [114, 130, 131]. On the other hand, intake of amino acid-based protein substitutes stimulates insulin secretion [132] and may result in hypoglycaemia if they are ingested in a single dose [133]. Thus, dietary metabolic booklets advise patients with PKU to divide their daily protein substitute intake to three equal parts and to combine the intake with their daily prescribed natural protein [10]. This was seen to reduce fluctuations in blood PHE variability [91].

1.8.3 Natural protein prescription (PHE allocation) and PKU-free foods

Dietary PHE is required to ensure adequate protein synthesis from all essential amino acids, which are provided by the PKU protein substitutes [101, 131, 134]. To meet PHE requirements, the diet should include naturally low-protein foods (PKU-free foods i.e. contain <50 mg PHE /100 g) such as most fruits and vegetables, and foods with moderate protein content such as potatoes [135]. Therefore, patients with PKU are prescribed specific amounts of daily PHE by their metabolic dieticians based on disease severity ‘’PHE tolerance’’ and dietary adherence [101, 136]. The majority of patients with severe PKU type can be prescribed no more than 5 grams of natural protein per day (i.e. 250 mg of PHE/day) [102, 114]. However, it is often easy for patients on such restrictive diet to excess their daily prescribed natural protein (i.e. tolerable PHE/day) by overconsuming foods of moderate protein content and/or PKU-free foods [91, 106, 135] which can significantly increase their blood PHE concentrations. Thus, these foods must be weighed or measured to keep PHE concentrations within treatment targets [99].

In early 1980s, a system based on protein exchanges was introduced in the UK to simplify calculation of PHE content of foods that have not been analysed. This system assumes that 1 gram of protein contains 50 mg of PHE. The protein exchange system is easily understood and is more flexible compared to other systems applied in the US, Australia and some European countries which only allow foods of pre-calculated PHE content (as 15 mg PHE/ 1 g protein) or use at least a two-tier system of PHE exchanges for the diet plan [123, 135]. A study found that some children with PKU may increase their mean protein intake by 50 to 100% from PKU-free foods using the protein exchange system [91]. However, PHE concentrations remained within treatment targets in this group of patients. The

protein exchange system is effective and allows individuals with PKU a generous food selection in their diet plan while achieving good metabolic control [123].

Recently, the NSPKU [137] has adapted a traffic-light labelling system (Figure 1.7) for popular foods to help patients and their carers to vary their diet and decide what to avoid, to eat freely or to count within their allowance of natural protein exchanges (tolerable PHE). The NSPKU published lists of foods that are categorised under three colours: red, amber and green. The red colour represents foods that must be avoided due to their high natural protein content (thus PHE) such as meat, egg, fish, nuts and seeds. The amber colour represents foods that contain some protein (1 g protein or 50 mg PHE) and can be consumed in measurable amounts within daily allowance of protein exchanges (tolerable PHE). Examples of foods in the amber list include potatoes, broccoli, beans, banana, dates, figs, corn, rice and oatmeal. The green colour represents foods that contain small amounts of PHE and can be eaten in normal quantities with indication to avoid excessive consumption of any food in the list. Foods in the green list include mostly fruits such as apples, apricots, bilberries, blackberries, blueberries, cherries and clementines; and vegetables such as aubergine, cabbage, all green beans, butternut-squash, lettuce, mushrooms, cucumber, leek, garlic, onion and carrots. In general, foods containing <50 mg PHE /100 g can be permitted without measurement (referred as PKU-free foods) [109]. These foods include sugars, sweets, honey, jam, oils, vinegars, and species. However, patients with PKU should avoid using the artificial sweeter aspartame due to its high PHE content [15].

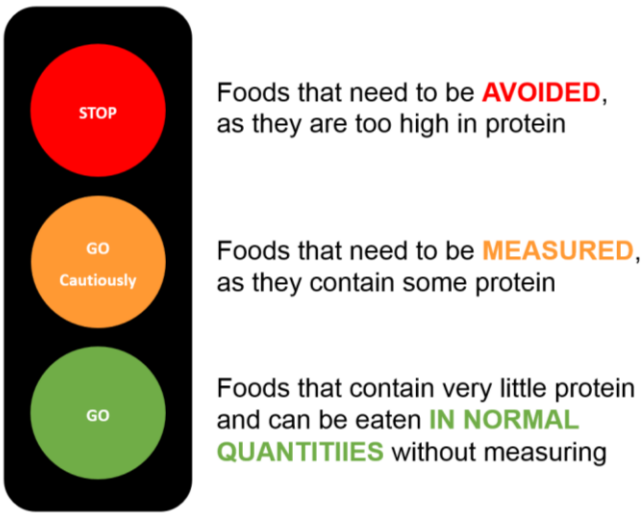


Figure 1.7: A representation for traffic-light system introduced by the NSPKU in 2017 to control PHE intake for dietary management of PKU

1.8.4 Alternative therapies for PKU

Despite the success of dietary treatment for management of PKU, alternative therapies to replace or as a part of the low-PHE diet is the current research focus and most desirable by the patients [135]. A number of treatment alternatives have already been utilised to improve adherence to dietary treatment such as the use of LNAA, Glycomacropeptide (GMP), and BH4 therapy, while other advanced clinical therapies including phenylalanine ammonia lyase (PAL) enzyme replacement, gene therapy and liver transplantation are still under experimental trials [6].

Large Neutral Amino Acids

The use of Large Neutral Amino Acids (LNAA) within the PKU protein substitutes may be the best example of effective utilisation of new alternative or supportive treatments in PKU dietary management [115]. The common LNAAs used for PKU supplementation include tyrosine, tryptophan, threonine, isoleucine, leucine, valine, methionine, lysine, arginine, and histidine [138, 139]. Due to competition between PHE and LNAA to pass the BBB, supplementation with the LNAA may help to improve their presence and reduce PHE concentrations in the brain [11]. Similar mechanism may also happen in the gut in which lower PHE is absorbed when high amounts of LNAA is consumed [6]. In a double blind placebo controlled study, short-term supplementation of LNAA was effective in lowering blood PHE by 39% from baseline [138]. Another study showed that executive functioning was significantly improved in 16 patients with classical PKU when supplemented with LNAA alone compared to or when taken with regular PKU protein substitute [140]. In addition, the LNAA products have been shown to improve adherence to dietary treatment in adult patients [141, 142] although they are unlikely to be used as sole treatment [6]. However, the availability of LNAA products is still limited [115] and their optimal composition has not yet been identified [108].

Glycomacropeptide

Glycomacropeptide (GMP) is a whole protein that is extracted from cheese whey, and contains all essential amino acids except PHE, TYR and TRYP [143]. There is an extensive ongoing research on the use of GMP in management of PKU to improve adherence to dietary treatment and reduce the need of L-amino acid based PKU protein substitutes [144]. Studies conducted on patients with PKU reported that GMP products have better palatability compared to usual PKU protein substitutes, and improved PHE utilisation and protein retention [145]. The GMP food products have also lower acidity load compared to PKU protein substitutes which was found to enhance bioavailability of some micronutrients such as calcium and magnesium [146].

Sapropterin

Some patients, particularly those with mild PKU type, can be responsive to treatment with pharmacologically prepared doses of BH4 i.e. sapropterin dihydrochloride (Kuvan) [147]. The BH4 works by activating the residual PAH activity which leads to reduction in blood PHE concentration in treated patients. Several studies showed that patients on BH4 treatment significantly increase their natural protein intake (PHE tolerance) up to 30 protein exchanges (i.e. 1500 mg of PHE/day) [148] and have reduced blood PHE concentrations [149-152]. Long-term studies have also shown improved quality of life [153, 154] in patients receiving BH4 doses with positive impact on executive functioning in responsive PKU patients exhibiting symptoms of attention deficit-hyperactivity disorder (ADHD) [60]. In addition, BH4-treated PKU patients presented better micronutrient status; including zinc, selenium and copper concentrations; compared to those not receiving the treatment, although their intake of micronutrient-enriched PKU protein substitutes is reduced or stopped due to high PHE tolerance [102, 155, 156]. Pharmacological

BH4 is safe with patients showing good adherence to doses of the treatment [147, 157]. The BH4 is an expensive treatment option [158] and have been approved in the USA and most of the European countries [6, 73] with ongoing campaign groups to start the treatment in the UK.

1.9 PKU monitoring

Successful management of patients with PKU depends on sufficient clinical and nutritional monitoring. The dietary regimen is adjusted to keep PHE and TYR concentrations within the treatment targets. PKU metabolic control is monitored through blood concentrations of PHE [125]. However, the frequency of blood spots collected for PHE monitoring varies between clinics and countries [159]. In the UK, the PKU Working Group in the Medical Research Centre (MRC) recommends to measure PHE weekly during the first six months of life, fortnightly between age of 6 months until 4 years and monthly for 5 years old patients or above [125]. Recommendations from the US [160] and recently published ESPKU guidelines [15] for management of PKU state that PHE should be monitored weekly in patients under 1 year old, and fortnightly or monthly in those above 12 years old. However, more frequent PHE monitoring is associated with better metabolic control [135, 161].

Pre-adulthood PHE treatment targets in the US, Germany and the ESPKU guidelines are stricter when compared to those in the UK and France (Table 1.4). In the US [160], the PHE treatment target is 120 – 360 µmol/L through the lifecycle while the range relaxes with increasing age, particularly after age of 10 years, in the UK [125], France [136], Germany [162] and the ESPKU guidelines [15] (Table 1.4). However, the UK has endorsed the recently published ESPKU guidelines and metabolic clinics through the country will be applying them.

Table 1.4: Blood PHE treatment targets and daily tolerance of PHE across age groups and lifecycles in different countries for management of patients with PKU

<table>
<tr><th rowspan="2">Age group (years)</th><th rowspan="2">PHE tolerance (mg/d)</th><th colspan="5">Target blood PHE (µmol/L)</th></tr>
<tr><th>UK</th><th>Germany</th><th>France</th><th>USA</th><th>ESPKU</th></tr>
<tr><td>0 – 1</td><td>130 - 330</td><td rowspan="2">120 – 360</td><td rowspan="3">40 - 240</td><td rowspan="5">120 – 600</td><td rowspan="6">120 – 360</td><td rowspan="3">120 – 360</td></tr>
<tr><td>2 – 3</td><td>200 - 320</td></tr>
<tr><td>4 – 5</td><td rowspan="5">200 - 1100</td></tr>
<tr><td>6 – 10</td><td>120 – 480</td><td rowspan="2">120 – 480</td></tr>
<tr><td>11 – 12</td><td rowspan="3">120 – 700</td><td rowspan="2">40 - 900</td><td rowspan="3">120 - 600</td></tr>
<tr><td>13 – 15</td></tr>
<tr><td>Adolescent/ adult</td><td>40 - 1200</td><td>120 – 1200</td></tr>
<tr><td>Pregnancy T1</td><td>265 – 700</td><td colspan="5" rowspan="4">120 – 360</td></tr>
<tr><td>Pregnancy T2</td><td>400 – 1650</td></tr>
<tr><td>Pregnancy T3</td><td>700 – 2275</td></tr>
<tr><td>Lactation</td><td>700 – 2275</td></tr>
</table>

T1, first trimester of pregnancy; T2, second trimester of pregnancy; T3, third trimester of pregnancy
Adapted from Hyperphenylalaninaemia, In Inborn Metabolic Diseases, Diagnosis and Treatment [2] and Recommendations for the Nutrition Management of Phenylalanine Hydroxylase Deficiency [99]

Adherence to dietary treatment and clinical follow up is vital in PKU management to achieve best long-term outcomes. The term adherence is preferred over compliance, and is used to describe the extent to which the patient's behaviour coincides with or matches the agreed recommendations from the prescriber [163], in terms of following the treatment diet, not exceeding prescribed natural protein intake, taking PKU protein substitute, or attending clinical follow ups [164]. However, the dietary restriction creates social pressure and financial burden, the PKU-type foods are unpalatable, and meal preparation is time-consuming and requires high

skills [6]. In addition, patients with PKU have to provide frequent blood spots for PHE and TYR monitoring, and regularly attend metabolic clinics [15, 125, 160]. Thus, adherence to PKU treatment is challenging.

Several indicators can be used to measure adherence to dietary treatment including self-reporting dietary records, qualitative surveys, and interviews with clinical staff [164]. Yet, these methods suffer from over/under reporting by the patients or estimation by the interviewing staff member [165, 166]. Prescriptions of PKU protein substitutes by GPs could be another potential measure to determine patient's adherence to dietary treatment, but this does not necessary mean that these patients will also adhere to their prescribed protein exchanges [167, 168]. However, blood PHE concentrations (metabolic control) may be the best overall measure for adherence to PKU dietary treatment although there is no agreed number of measurements that should be within treatment targets or the frequency and timing of measurements [164]. The quality of adherence can be expressed as percentage of PHE (%PHE) measurements above treatment targets or annual mean/median PHE concentrations. Most recent guidelines by ESPKU for management of PKU recommend using the %PHE measurements above treatment targets during long term periods to determine the extent and type of intervention needed to improve an individual's metabolic control [15]. However, some patients intentionally tend to improve their adherence to dietary treatment during time before blood sampling or delay sampling if they know that their PHE would be high [164]. Thus, blood PHE can sometimes be misrepresentative unless samples are collected in frequent manner.

Phenylketonuria treatment is based on a very restrictive diet and supplemented with PKU protein substitutes that are enriched with micronutrients. Yet, suboptimal growth [169], overweight/obesity [105] and micronutrient deficiencies [117] have

been reported in patients with PKU. Therefore, an annual nutritional review is required for any patient who is on a prescribed low-PHE diet or is self-restricting high protein foods [15]. Recent guidelines suggest that annual nutritional review should include assessment of anthropometric parameters, plasma amino acids, plasma homocysteine or methylmalonic acid, haemoglobin, mean corpuscular volume, and ferritin [15, 160]. Other micronutrients such as vitamins, selenium, zinc, copper and calcium should be measured only when clinically requested after a comprehensive dietary assessment indicating insufficient intake or patient over-relying on nutritionally incomplete SLPF-based foods [15, 160]. However, MRC guidelines in the UK recommend to assess micronutrient status on yearly basis [125].

Risk for neurocognitive and psychological impairments is increased in patients with PKU [170], thus regular examination for mental health is necessary. The mental health examination should include assessment of developmental and intellectual ability, behavioural/emotional and executive functioning [15, 160]. The timeframe in which neurocognitive and psychological assessments are performed differs between guidelines. The US guidelines [160] recommend that assessment of neurocognitive and psychological functions should be performed every 2 – 3 years or when clinically indicated following cognitive and socio-emotional screening test to identify patients who are in need for more frequent follow ups. On the other hand, requirement for psychological and social intervention in the ESPKU guidelines is based on long-term status of metabolic control. The ESPKU guidelines [15] recommend that psychology consultation or social worker intervention in patients younger than 12 years should be considered when more than 50% of the blood PHE concentrations are above treatment targets over a period of 6 months. Hospitalisation, re-education and more frequent clinical and PHE monitoring are

recommended to help patients re-stabilising their blood PHE. Moreover, social services and child safeguarding measures should be conducted when about 100% of the measured PHE concentrations are above treatment targets over a period of 6 months.

1.10 Issues in nutritional management of patients with PKU from the past to the present

Research in the four decades up until the early 1990s showed that patients with PKU had impaired growth [171-173] and malnutrition [174]. These were attributed to undernutrition including PHE deficiency caused by over-restricting the dietary treatment in early days of the PKU management [173, 175]. Micronutrient deficiencies, including vitamin A [176], vitamin B12 [177], vitamin D [178], folate [179], iron [180], calcium [120], selenium [181], zinc and copper [116] deficiencies, were also present.

Despite the increasing focus of research on non-dietary treatments for PKU, the low-PHE diet remains the mainstay of PKU management [135]. Starting from 1990 up until now, there was an enormous progress in development of the low-PHE diet and managing practices of PKU [135, 182]. Phenylketonuria SLPF became available in wider range [100, 103] with more selections from the PKU-free foods being allowed for patients on a low-PHE diet [137]. The palatability, forms and availability of PKU protein substitutes, and their macro and micronutrient composition have also significantly changed [115, 117, 122]. The patients' accessibility to these PKU medical foods, including SLPF and protein substitutes, has been easier than before with flexible governmental charge cover and hassle-free prescription and delivery systems in some countries [111, 168]. In addition, evidence-based guidelines for PKU management [15, 160] and strategies to improve patients' adherence to dietary treatment have been progressively implemented into clinical practice [164, 183].

Advancement in the PKU management, including dietary practices, led to nutritional problems that had never been reported before but become more frequent in the recent years. Overweight and obesity have become increasingly reported in patients with PKU, with some studies suggesting higher prevalence in female than male patients [107, 134, 184-187]. The reported prevalence of obesity and overweight in PKU varies between countries and ranges between 15.2- 55% in adults and 0-45% in children [185, 186, 188]. The estimated prevalence of overweight and obesity in British adults with PKU is 55% [186]. However, the existing evidence suggests that overall trend for overweight and obesity in patients with PKU is similar to that in the general population [105, 134, 185, 186, 188]. Micronutrient imbalance is another issue that has been noticed in this group of patients. High levels of vitamin B12 and folate have been reported in patients with PKU on prescription with micronutrients-enriched PKU protein substitutes but deficiencies in other nutrients, particularly selenium and zinc, still persist [121, 155, 189]. Yet, factors contributing to the aetiology of obesity and micronutrient imbalance in PKU have not been properly studied. Summary of the issues related to nutritional management of patients with PKU are presented in the following figure (Figure 1.8).

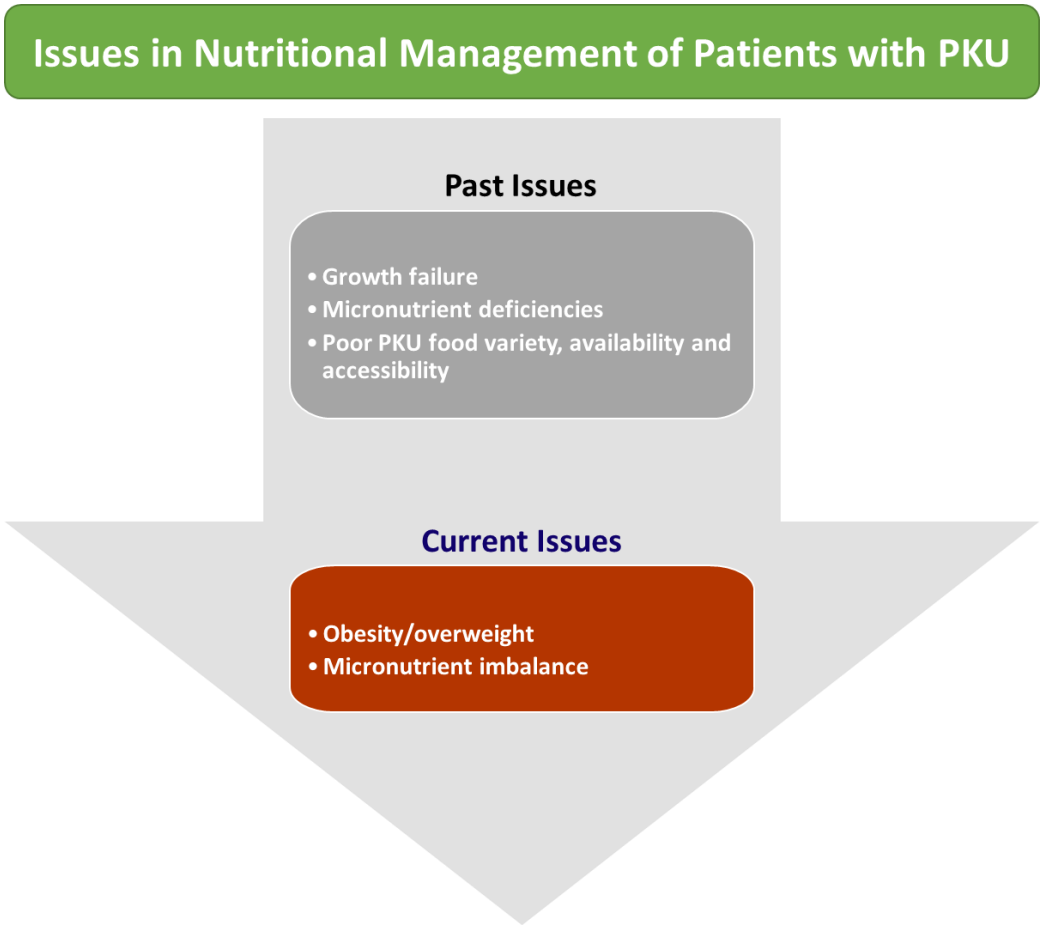


Figure 1.8: Issues in nutritional management of patients with PKU

1.10.1 Obesity and overweight in PKU

The earliest studies demonstrating tendency to being overweight in patients with PKU were published during 1970's and 1980's [190, 191], showing a higher prevalence than in normal children. Subsequent research suggest that issue of overweight and obesity is a growing problem in PKU [107, 134, 156, 184-187]. A study by Burrage and colleagues revealed that the proportion of overweight and obese PKU females were 55% and 33%, respectively [184]. Two previous studies [134, 192] reported that the tendency of increased body weight in PKU females was more pronounced during the post-puberty stage. A retrospective longitudinal study on 97 children with PKU and mild HPA (Overweight 25%) showed that high

birthweight may be used as a predictor for overweight or obesity in later life [193]. Despite the increasing trend of overweight and obesity in PKU, its prevalence is similar to that in healthy population [105, 134, 185, 186, 188].

The most convenient approach to measure overweight and obesity is using body mass index ‘’BMI’’ (weight kg/ height m^2) [194]. Obesity is essentially an excess accumulation of body fat, however BMI does not differentiate between fat and lean tissues [195]. Therefore, people with higher proportion of muscle mass are at increased risk of BMI misclassification. Accurate assessment of body composition is important in patients with chronic illnesses since it has been associated with poor clinical outcome [196]. Presence of body fatness in PKU patients were measured in several studies in which different body composition techniques were used for these measurements. Data obtained using Dual-energy X-ray absorptiometry (DEXA), bioelectrical impedance analysis (BIA) and total body electrical conductivity (TOBEC) showed no significant differences in measured body composition between patients with PKU and healthy controls [107, 197-200]. Similarly, measures of body fatness by skinfold thickness [201] or waist circumference [185] did not differ between PKU patients and healthy individuals. However, in some studies patients with PKU tended to have higher fat mass (FM) and lower fat-free mass (FFM) compared to healthy people [107, 192, 202]. A study showed that patietns with PKU had significanlty higher percentage of body fat than healthy controls when air-displacement plethysmography (ADP) technique was used [192]. This suggests that for a given weight and height people with PKU might be fatter. Yet, BIA and skinfold thickness were the most frequently used techniques to measure body composition of the patients with PKU in the privious studies. These techniques are based on predictive equations developed from healthy population, which are subject to inaccuracy and imprecision bias, particularly when these are applied to people with

chronic illness [195]. It has been reported that body protein and fluids are altered during disease course, thus induce changes in body composition of the affected individuals [194, 196, 203]. Hence, more studies applying gold standard or reference techniques to measure body fatness and lean mass are needed to compare body fatness in patients with PKU and age and gender matched healthy controls.

Despite the intensive and continuous nutritional care for patients with PKU, when compared to general population, they still have a higher probability of becoming overweight or obese [184]. Obesity is associated with several comorbidities such as cardiovascular disease [204], insulin resistance [205] and type-II diabetes [206]. In general, obesity is related to repeated and cumulative effects of positive energy balance due to either increased energy intake (EI) or reduced energy expenditure or both [207, 208]. Although the most modifiable components of energy balance are EI and energy expenditure (EE) of physical activity [209], other contributors to energy expenditure such basal metabolic rate (BMR) and thermic effect of feeding (TEF) are also important to be considered in relation to obesity aetiology [210]. In addition, there is some evidence suggesting that diminished ability to oxidise fat may also contribute to obesity development [210]. The contribution of these factors to the increasing prevalence of obesity in patients with PKU has not been studied yet.

Thus, the following section will discuss the evidence on the potential impact of PKU disease and/or SLPF-based meals on appetite regulation, energy intake, fat oxidation, and components of energy expenditure such as RMR, TEF and habitual physical activity to establish their contribution to the increasing prevalence of overweight and obesity in PKU.

1.10.2 Potential impact of PKU disease and PKU SLPF-based foods on appetite regulation and energy intake

Regulation of energy intake (EI), a part of energy balance, is complex and involves interactions between hormonal, neuroendocrine, central nervous system (CNS), body organ systems, environmental and external factors [211]. Peripheral signals regulate food intake by integration of complex mechanisms in the CNS (Figure 1.9) [211-213]. These signals include sensory properties of foods, mechanical and chemical receptors in the gastrointestinal tract, gut hormones, and circulating metabolites [214, 215].

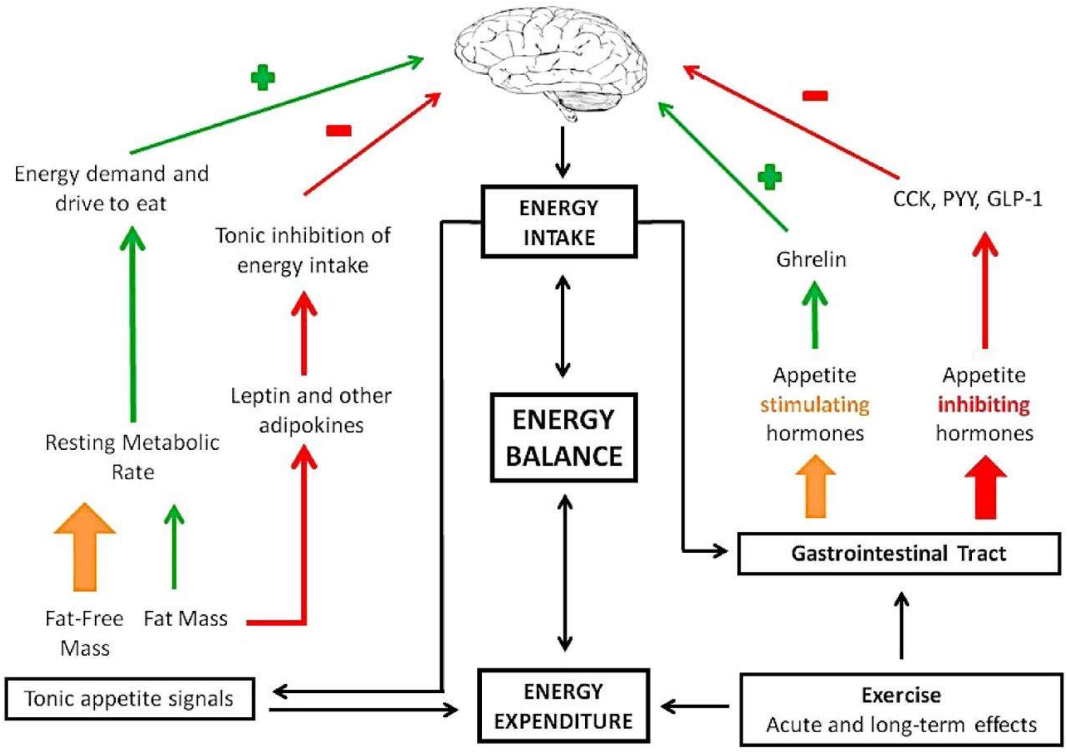


Figure 1.9: Formulation of the major influences on appetite control using an energy balance framework

Reproduced from The biology of appetite control: Do resting metabolic rate and fat-free mass drive energy intake? [216]

Information on subsequent meal size, total EI and probably composition of food are translated through integration of peripheral signals in the hypothalamus, brainstem, and cortex [211]. These contribute for determining EI, amount and duration of food

ingestion. Insulin and leptin are involved in the long-term regulation of food intake by exerting their action in the hypothalamus [211]. However, the role of these hormones in appetite and EI regulation is still under investigation [217].

Gastrointestinal (GI) appetite hormones contribute to short-term food intake independently and by integration with insulin and leptin [211]. The gut hormones involved in short-term appetite regulation has either anorexigenic or orexigenic effect on regulation of food intake. The anorexigenic gut appetite hormones include glucagon-like peptide 1 (GLP-1), peptide YY (PYY) and cholecystokinin (CCK) which are released in response to meal ingestion and involved in postprandial satiety [218]. On the other hand, ghrelin, secreted in stomach, is an orexigenic peptide hormone which is secreted in stomach during fasting or pre-meal conditions, and is suppressed postprandially [219].

GI peptide hormones activate vagal afferent nerves that innervate brain regions involved in the immediate need for food intake [220]. The release of these gut appetite hormones is not only proportional to the size of the ingested meal, but may also be related to meal macronutrient composition [220]. GLP-1 is produced in intestinal ‘’L’’ cells in the distal jejunum and ileum [220]. GLP-1 enhances satiety by reducing rate of gastric emptying and thus slows nutrient absorption which also leads to reduction in postprandial glycaemia [221, 222]. Carbohydrate and fat intakes have been shown to stimulate the GLP-1 release [221, 223]. GLP-1 is secreted in phases in which the earliest phase occurs within 10-15 mins following food intake, while the second peak occurs within 30-60 minutes after food intake [221]. GLP-1 is quickly deactivated after release in which its half-life in plasma is less than 2 minutes [220].

PYY is co-secreted with GLP-1 by L cells in the lower intestine [220]. PYY plays role in gastrointestinal tract motility and inhibits gastric acid secretion, gall bladder emptying and pancreatic enzyme secretion [224-226]. It may reduce gastric emptying and intestinal transit by acting as part of ‘’ileal brake’’ to increase absorption of nutrients, fluid and electrolytes in the ileum after meal ingestion [227]. PYY secretion is stimulated more by fat intake than isocaloric protein and carbohydrate rich-meals [220, 226-228]. Protein rich-meals induce the highest elevation in PYY concentrations compared to other macronutrients [229, 230].

Cholecystokinin (CCK) is produced by I cells in the duodenal and jejunal, and it is secreted in response to luminal nutrient intake [220]. The main function of CCK on the gastrointestinal tract is to facilitate nutrient absorption, enhance pancreatic enzyme secretion, stimulate gall bladder contraction, and slow gastric emptying. The rate of nutrient delivery from the stomach to the small intestine is regulated by CCK [225, 231, 232]. CCK concentrations increase within 30 minutes after the start of meal ingestion. The concentrations fall gradually and require up to 5 hours to reach baseline levels. Proteins and fats (rather than amounts of calorically equivalent of carbohydrates) have been shown to stimulate the CCK release [231].

Evidence suggests that under normal body weight conditions, body fatness is negatively correlated to EI, while fat-free mass (FFM) is positively correlated to energy demand and drive to eat [217, 233]. Enhancing effect of FFM on EI can at least be partly explained by its impact on RMR which is known as independent factor contributing to EI [216, 217]. By all means, RMR not only depends on FFM, but also on other body organs to determine body energy demand [233, 234]. It has been noted that inhibitory impact of fat mass (FM) on EI can be diminished under conditions of obesity due to leptin resistance [216, 220].

Phenylketonuria as a condition and metabolic control may alter appetite regulation in affected patients. It was suggested that increased serum amino acid concentrations is associated with reduction in appetite [235]. This was attributed to the enhancing effect of blood amino acid concentrations on satiety centres in the brain, thus suppressing the feeling of hunger [236, 237]. Therefore, chronically elevated PHE concentrations may potentially impact appetite regulation in patients with PKU.

Catecholamine requires TYR to produce the neurotransmitters dopamine, adrenaline and noradrenaline by sympathetic nervous system in the brain [238]. The decreased catecholamine levels found in patients with PKU, particularly those with poor metabolic control, causes major brain dysfunction and effects the levels of adrenaline and noradrenaline neurotransmitters [239, 240] which could be expected to reduce leptin secretion [241]. However, a study by Schulpis and colleagues showed higher leptin plasma concentrations in PKU patients with poor metabolic control compared to those with good metabolic control or healthy subjects [238]. This suggests that patients with PKU may have leptin resistance which was reported to be present in obese individuals [242, 243]. High PHE concentrations could stimulate leptin secretion by active uptake and release of this amino acid on the adipose tissues [238, 244]. These results suggest that elevated leptin concentrations found in patients with PKU may be due to loss of sensitivity to leptin. Thus, poor sensitivity to leptin might be one of the underlying reasons for the development of obesity in patients with PKU.

Secretion of ghrelin may also be affected by defects in catecholamine metabolism and neurotransmitters production in PKU. A study by Schulpis et al., found lower fasting plasma ghrelin concentrations in PKU patients with poor metabolic control in comparison to those with good metabolic control or healthy subjects [240]. The

study also reported negative correlation between blood PHE and ghrelin concentrations in patients with PKU. However, it remains unclear how low catecholamine production relates to low ghrelin concentrations.

Findings from above-mentioned studies suggest that disturbance in appetite regulation may exist in patients with PKU. In addition, it has been reported that PKU SLPF have more energy content compared to normal type foods [103, 105]. This can potentially contribute to increase overall EI in patients with PKU since a positive energy balance of 3 to 4% (~ 350 kJ/day i.e. equal to one slice of bread) could result in weight gain of 1 kg in a single year [245]. Therefore, patients with PKU can be expected to have high EI.

Limited studies, however, evaluated EI of patients with PKU and found no significant difference from healthy controls [104, 238, 240] or estimated average energy requirements of healthy population [104, 105, 133]. The findings are hardly available regarding difference in EI between patients of different disease severity [185, 193] or dietary adherence [238, 240, 246]. However, it should be mentioned that dietary intake studies are not reliable due to under reporting bias particularly in overweight individuals [247, 248] or those on restricted diet [201, 249].

Due to protein restriction, low-PHE diet is high in CHO and sometimes fat [250]. In addition, reliance on SLPF and poor adherence to PKU protein substitutes would result in diminished protein intake and thus higher contribution of CHO and fat to total EI [104]. It is known that meals with enhanced protein content stimulate satiety more than isocaloric intake of CHO or fats-rich meals [251]. It is also known that the response of gut peptide hormones, such as GLP-1, CCK, PYY and ghrelin, varies according to macronutrient composition of the ingested meals. Consumption of high protein meals resulted in significantly lower ghrelin [252, 253], and higher CCK

[253], GLP-1 [252-254] and PYY [253, 254] concentrations when compared to consumption of isocaloric meals with lower protein content but higher proportions of CHO or fat. In addition, lower hunger, and higher satiety and fullness sensation scores were reported following consumption of high protein than isocaloric high CHO [252, 255] or fat meals [256]. Appetite responses also vary according to protein quantity given, with studies showing that appetite and satiety measures, and GLP-1 and PYY gut peptide hormones being protein dose-dependent [254].

Source of macronutrients in the meal may not have impact on appetite response or spontaneous EI. Protein composition, when compared between isocaloric soy, whey, or gluten based-preloads, had no effect on measured appetite hormones or at *ad libitum* EI [253]. Similarly, source of protein, animal or plant-based, of similar quantity did not affect appetite or spontaneous EI [257, 258]. However, contribution of other macronutrients to total EI can affect the extent to which appetite responses to the ingested meal. Despite the protein content being high in both test meals (30% of EI), hunger was significantly higher following consumption of meal containing CHO compared to that without CHO [259]. Fullness, on the other hand, was lower following consumption of meals with high protein containing no CHO compared with a meal of lower protein content and high CHO (60% of EI).

Ingestion of amino acids from PKU protein substitutes and amount of natural protein may aid satiety and suppress appetite. High PHE concentrations have been reported to associate with increased body mass index (BMI) [133, 186]. Overconsuming natural protein and/or not taking the prescribed PKU protein substitutes can cause poor metabolic control (high PHE concentrations) [164]. A study by Burrage et al., compared prevalence of increased body weight in 45 patients with PKU who were adherent or non-adherent to PKU protein substitutes [184]. The study reported that obesity and overweight, with higher BMI z-scores,

were more prevalent among patients who were non-adherent to PKU protein substitutes. Another study conducted by Rocha and colleagues demonstrated that intake of protein equivalent as amino acids, but not intact protein, was significantly lower in PKU patients with central obesity than those without central obesity [185]. These findings can be attributed to the potential satiating effect of PKU protein substitutes on overall daily food and EI [184].

The above evidence suggests that diminished protein intake, which is common in PKU, in favour of the other macronutrients, may impact gut appetite hormones and reduce satiety, thus increasing EI. The amount of protein used in the experimental studies, however, was much higher than optimal and recommended protein intake [260]. For example, in several studies meals provided 30% or even 70% of EI as protein [253, 254, 256] while the recommend protein intake is 10-15% of total EI [260, 261]. In addition, some of the studies used preload liquid-based meals [253] and sometimes meal volume differed between trials [254]. So far there are no studies examined how appetite hormones, subjective appetite and EI are influenced by foods which either lack protein or have protein content which is significantly lower than recommended intake.

It is possible for PKU type foods lacking whole protein to induce lower postprandial thermic effect compared to foods high in protein which has been suggested to be an independent appetite regulator [236, 256, 262]. The lack of protein intake also induces lower demand for oxygen consumption which is suggested to promote postprandial hunger [236, 263]. In addition, diminished protein intake can be expected to suppress hepatic gluconeogenesis and consequently reduce glucose concentrations [236]. The less satiating effect of the diminished protein compared to high protein meals may also be the result of this impaired glucose homeostasis [236]. Independent of glucose homeostasis, insulin may produce appetite

suppressing effect. Protein has an insulinotropic effect, thus increased hunger may result from diminished protein intake [264, 265].

Dietary fibre is another nutrient that induces postprandial satiety in human diet [133]. It has been reported that intake of fibre is low in patients with PKU [133, 238] and that SLPF have, lower fibre [115, 266], higher CHO and glycaemic index compared to normal type foods [104, 105]. This type of dietary pattern is known to increase hunger and reduce satiety, thus contributes to aetiology of obesity in healthy individuals [267].

Based on the above evidence, it can be hypothesised that patients with PKU not adhering, or partly adhering to the intake of their PKU protein substitutes and/or over-relying on SLPF may have impaired appetite regulation and thus have higher EI. However, no previous study has investigated the impact of PKU protein substitutes or meals composed of SLPF on postprandial appetite sensations or gut appetite hormones and EI.

1.10.3 Potential impact of PKU disease and body composition on resting metabolic rate

Resting metabolic rate (RMR) is the largest component (60 – 75%) of total daily energy expenditure (TDEE) [268] and refers to the minimum amount of metabolised energy that the body requires when lying in physiological and mental rest [269]. Resting metabolic rate may vary between individuals according to body weight, body composition, age, gender, growth rate and genetics [270, 271]. In addition, illness, nutritional status and body temperature can influence RMR [271, 272].

Resting metabolic rate is mainly determined by body FFM. The total sum of two distinct moieties, active and low active organs or tissues determine the difference in value for RMR of the FFM [234]. Muscle and none-muscle organ mass, such as

brain and visceral organs, considerably differ regarding their mass and rates of energy expenditure [234]. The metabolic rate of brain, kidney and liver as per kg organ weight is 10 – 20 times of the whole body metabolic rate. Therefore, approximately 70 – 80 % of RMR comes from organs that comprise only 5% of total body mass [234]. The lowest metabolically active tissue in the body is fat (adipose tissue) [273]. However, RMR of adipose tissue may be increased by 10% or more in obese individuals due to bigger body FM and FFM.

Despite its significance in nutritional management, very little information is available on resting energy metabolism of patients with PKU with RMR being measured only in two studies [201, 274]. Allen and colleagues evaluated RMR in 30 patients with PKU and found no significant difference from healthy controls with tendency of PKU females having lower RMR than control females (4703 vs 5167 kJ/d) [201]. However, the control group was not properly matched as patients in PKU group were younger, and had significantly lower FFM. Another study conducted by Quirk et al, measured RMR in 36 female adolescents with PKU but did not compare to a control group [274].

Phenylketonuria causes damage to the brain and defects in metabolic pathways of many substantial components including those related to energy metabolism and balance [15, 82]. Research on the effect of PKU on several metabolites showed reduced levels of catecholamines, serotonin [275] and leptin [238] in patients with PKU, particularly those with poor metabolic control. Catecholamines are known to directly affect oxygen uptake by different body tissues, thus contribute to regulation of resting metabolic rate and fat oxidation [80, 276]. The neurotransmitter serotonin plays role in harmonising activity of many autonomic pathways and neuropeptide systems in the brain, consequently regulates metabolism of energy stores and thermogenesis [81]. Leptin may also interact with serotonin to achieve energy

balance by inhibiting brainstem-derived serotonin (BDS) synthesis (increase energy expenditure) or inactivation of the leptin receptor in serotonergic neurons (decrease energy expenditure) [82]. However, leptin itself may contribute to regulation of energy balance during periods of reduced EI by activating the sympathetic nervous system to prevent decline in metabolic rate [277].

Alteration to any compartment of the body composition can affect RMR. It has been reported that patients with PKU tend to have higher FM and lower body protein or FFM when compared to healthy controls [107, 192, 201, 202]. The RMR is determined mostly by body weight and body composition [270, 271], with FFM being a much greater predictor of RMR than FM. Although obese individuals have higher FM but may also have higher FFM, they tend to have higher RMR compared to non-obese individuals [271]. However, the RMR becomes similar between obese and non-obese individuals when adjusted to differences in FFM [270, 278]. Despite the adjustment for body composition, variation in RMR values persists [210, 279-281]. Therefore, relatively low metabolic rate may contribute to weight gain. Since the RMR is the largest component of TDEE, reduced RMR, due to altered body composition, growth, and levels of energy balance-related metabolites may potentially contribute to the increased prevalence of overweight and obesity in patients with PKU.

1.10.4 Potential impact of PKU special foods on thermic effect of feeding and fat oxidation

Thermic effect of feeding (TEF), also known as diet-induced thermogenesis (DIT), is a component of energy expenditure (EE) and comprises 10 – 15% of TDEE [282]. TEF is the increase in body temperature that results from activation of intraoral sensory nervous system, including the senses of taste and smell, and converting food into components for use or storage via the activity and consumption of energy

by enzymes and transporters [269, 283]. Thus, it is calculated as amount or percentage of increase in EE above RMR or proportional to EI after meal or food ingestion [284-286]. Elevation of EE after food or meal consumption can last up to 4 to 8 hours [286]. Protein is the most thermogenic nutrient with longest-lasting effect, followed by CHO and fat [287, 288]. Diminished protein intake may result in reduced TEF due to absence of high energy demand for absorption, peptide-bond synthesis, gluconeogenesis and urea production which are involved in protein metabolism [261, 289]. Although TEF may be a small contributor to aetiology of obesity, but on long term duration it may have impact on body weight changes. For instance, a diminished daily EE by 50 kcal may induce body weight gain of approximately 1 kg in a period of a single year [220].

Diminished fat oxidation has also been appreciated as a factor contributing to development of excess fat accumulation [210, 290]. Some studies attributed this to reduced activity of enzymes related to fat oxidation in the mitochondrial skeletal muscle of the obese individuals [283, 291]. On the other hand, it is not clear whether fat oxidation of obese individuals in postprandial state is altered or not. However, in normal weight individuals, fat oxidation is inhabited after food consumption, while this inhibitory effect in obese individuals may be diminished due to insulin resistance [292, 293]. The mechanisms by which fat oxidation is inhibited during the postprandial state is due to increase in glucose oxidation which reduces transportation of fatty acids into mitochondria [293-295] with numerous studies showing an inverse relationship between glucose and fat oxidation [296]. Shifting between fuel sources, to either energy production or fat storage, is mainly regulated by the mitochondria. Increased glucose concentrations are associated with insulin secretion which enhances glycolysis and glucose entry into cells [293]. This also suppresses lipolysis and fatty acid oxidation, and promotes fat storage.

It has been reported that high protein meals stimulate TEF and postprandial fat oxidation more than isocaloric CHO or fat-rich meals [251, 252, 259, 288, 297]. However, TEF and postprandial fat oxidation are influenced by macronutrient composition of ingested meal or food [286]. Replacement of CHO in favour of fat diminished effect of higher proportion of protein (20% of EI difference) on TEF [259]. However, there was no significant difference in TEF between isocaloric high protein diets containing or not containing CHO. Fat oxidation, on the other hand, was significantly lower when CHO was present in high protein diet compared to an isocaloric diet with similar proportion of protein but without CHO [259]. At constant protein intake, full fat meal with lower CHO content induced higher fat oxidation in comparison to an isocaloric reduced fat meal with higher CHO content, but had no impact on TEF [298].

Source of macronutrients in the meal may have impact on the long-term thermogenesis. Although there was no significant difference in TEF between animal and vegetable based diets that was estimated for 5-hours following diet intake, EE for 24 hours was significantly higher after consumption of the animal protein based diet [257]. However, there is no study reporting effect of macronutrient source on fat oxidation. Limited data is available on the effect of protein composition on responses of TEF and postprandial fat oxidation. A study by Acheson and colleagues examined, in 32 healthy adults, the effect of three different isocaloric protein-based preloads, whey, casein or soy (protein, 50% of EI) on TEF and postprandial fat oxidation [251]. The TEF was significantly higher after ingestion of whey-based preload compared to the other isocaloric preloads composed of casein or soy proteins. However, difference in protein composition between the three tested preloads did not affect rate of fat oxidation [251]. These studies imply that TEF and

postprandial fat oxidation may response differently to PKU medical foods that are composed of PHE-free L-amino acids.

The discussed evidence above considered studies in which test meals were high in protein and did not investigate the effect of diminished rather than increased protein intake on TEF and postprandial fat oxidation. It can be hypothesised that SLPF-based meals that are protein-diminished and high in CHO to have detrimental impact on TEF and postprandial fat oxidation. In addition, it can be expected that TEF and postprandial fat oxidation may response differently to the synthetic PHE-free L-amino based PKU protein substitutes which are poorly adhered by patients with PKU. Therefore, research is needed to investigate effect of PKU type foods and protein substitutes on TEF and fat oxidation to understand their contribution to obesity aetiology in patients with PKU.

1.10.5 Potential impact of PKU disease on habitual physical activity

Physical activity (PA) is the most variable component in energy balance and accounts for 20% and up to 40% of total daily EE of inactive and active persons, respectively [299]. Physical inactivity has been associated with increased risk of overweight and obesity in different populations [209, 300-303]. Evidence also suggests that sedentary lifestyle can independently promote development of obesity in general population [304].

Poor functional and motor skills in early life, and risk of depressive and anxiety disorders are associated with reduced habitual PA and exercise in normal children [305-308]. Previous studies in PKU have shown a negative correlation between elevated PHE plasma concentrations (uncontrolled PKU) with depression, anxiety, and poor mood [309, 310]. Patients with PKU have also been shown to have poor executive skills [311] and quality of life and less social interaction. Thus, they might

be less likely to pursue an active lifestyle, engage in exercise and have social interaction [64, 187, 311, 312].

There is, however, only one study which tried to describe patterns of PA in patients with PKU [107] and compared to Centers for Disease Control and Prevention (CDC) recommendations for PA using the self-reported International Physical Activity Questionnaire (known as IPAQ). The study [107] found that PA as metabolic equivalents (METs) in minutes per week was 429 and 1306 for adults and children with PKU. These figures meet the recommended MET minutes per week suggested by CDC for healthy children and adults. Despite this, the intensity type of PA in 85% and 54% of adult and child patients, respectively, was light [107]. The study measured PA using a subjective approach and did not compare to a control group. Therefore, there is a need to objectively measure PA in patients with PKU and compare to healthy matched controls to understand the contribution of PA energy expenditure and pattern to the aetiology of obesity in this group of patients. The contribution of PA component to the aetiology of obesity in patients with PKU is out of scope of this PhD thesis.

1.10.6 Micronutrient imbalance in PKU

Micronutrient deficiencies in patients with PKU, including vitamin A [176], vitamin B12 [177], vitamin D [178], folate [179], iron [180], calcium [120], selenium [181], zinc and copper [116] deficiencies have been reported years ago. However, advances in PKU dietary management during the last two-decades have shifted the paradigm for some but not all micronutrients. Hence, recent data suggests that the status of some micronutrients in blood is deficient whereas for other ones this exceeds the normal ranges [121, 155, 156, 189, 313]. Recent studies showed high blood levels of vitamin B12 and folate, but simultaneously deficient plasma levels of

selenium and zinc, implying micronutrient imbalance in patients with PKU prescribed micronutrient-enriched PKU protein substitutes [121, 155, 189].

The PKU protein substitutes are the main source for micronutrients in the low-PHE diet [102, 117] with small contribution from PKU free foods [314, 315]. Although their contribution to micronutrient supply has not yet been evaluated, the SLPF are known to have poor nutritional value as they are made mainly from processed starches and are not fortified with micronutrients [102]. Thus, the micronutrient supply in the low-PHE diet depends almost exclusively on their prescribed amount of PKU protein substitutes and patients' adherence to their intake [102, 117].

Predictors associated with micronutrient imbalance in patients with PKU have rarely been investigated [155, 169, 315-319]. The PKU phenotype (PKU severity) and PHE tolerance were associated with micronutrient status in patients with PKU with some studies showing significant association to increased levels of folate or reduced levels of selenium [155, 156]. Previous studies also suggested that micronutrient status in PKU patients is associated with PHE concentrations, a biomarker commonly used to reflect metabolic control [320]. Poor metabolic control reflects low adherence to dietary treatment [321] and often intake of PKU protein substitutes and subsequently low micronutrient intake [121]. However, it is interesting that studies reporting low levels of selenium and zinc tend to show high or normal levels of other micronutrients such as folic acid, and vitamin B12 and E [121, 155, 156, 189, 313]. Recent studies have also reported an association between PHE control and micronutrient status with studies showing that poor PHE control was associated with selenium and zinc deficiencies while some other studies showed opposite findings in patients below 4 years old [121, 155]. If poor adherence to dietary treatment was assumed to be the cause of reported deficiencies, it would be expected that other micronutrients would also be deficient or low. Indeed, other

studies reported that high adherence to low-PHE diet could be a risk factor for developing micronutrient deficiency such as selenium and vitamin B12 [155, 322, 323].

Most studies [155, 156, 314, 316, 317, 324, 325], however, used mean or median blood concentrations of PHE to explore associations between metabolic control (or dietary adherence) with micronutrient status. This approach may be biased by the number or frequency of PHE measurements considered in these studies. Percentage of raised PHE (%PHE) measurements above treatment targets may be a more reliable measure of metabolic control because it considers difference in PHE treatment targets between various age groups and accounts for fluctuation in PHE concentrations over the studied period. The use of % raised PHE measurements rather than mean or median PHE concentrations is also less likely to be affected by frequency or number of PHE measurements. Thus, it may provide better indication on how changes in metabolic control associate with micronutrient status. Indeed, %PHE over long-term period is recommended in the most recent PKU management guidelines to determine the extent and type of intervention needed to control PHE concentrations in patients with PKU [15].

Very little data is available on interactions between micronutrients in patients with PKU. Relationships between dietary intake of micronutrient and plasma levels of micronutrients have rarely been investigated. Two studies have shown that the ratios of dietary calcium, phosphorus, iron, and copper to zinc intake were negatively correlated to serum levels of zinc [326, 327]. Some studies in PKU reported no association between dietary intake of micronutrients such as selenium, zinc and copper, and blood concentrations of these micronutrients [315]. Lack of association may be explained by difficulties in dietary intake assessment since dietary intake is not always reliable due to over or under reporting bias, particularly in individuals

following a restricted diet [201, 249]. Alternatively, correlations between micronutrient blood levels can be used to explore their interactions to each other. To our knowledge, only one cross-sectional study [328] has utilised this approach and analysed micronutrient interactions in a single blood sample obtained from 40 patients. Of these patients, 20 were with PKU and were taking micronutrient-enriched PKU protein substitutes, while other 20 patients were with HPA and were not prescribed with micronutrient-enriched PKU protein substitutes. The study reported positive correlations between blood concentrations of selenium with potassium and cobalt in patients taking PKU protein substitutes and no correlations were observed between other trace elements considered in this study [328]. This suggests that micronutrient interactions may be induced by the high supply of chemically formed micronutrients from the PKU protein substitutes. Previous studies [155, 169, 315-319] which have explored factors associated with micronutrient deficiency, including micronutrient interactions, dietary adherence, PHE tolerance and metabolic control, in patients with PKU were mainly based on case-control or cross-sectional studies. Thus, these studies did not investigate how changes in these factors may affect micronutrient status in patients with PKU. In addition, previous studies looked individually at the potential factors associated with micronutrient status, but none have explored how these factors together may explain micronutrient deficiencies in this group of patients.

It is known that nutritional needs and dietary adherence change with age [121, 329], thus the association between micronutrients status and age should also be investigated, preferably in a prospective manner. Optimal micronutrient profile for PKU protein substitutes has not yet been defined [102]. Exploring factors associated with micronutrient status may assist identifying micronutrient issues within subgroups and determine micronutrient requirements in certain groups of patients

with PKU. Therefore, research is needed to explore how factors such as age, gender, metabolic control, PKU severity, body weight status, and adherence to clinical and nutritional follow-ups relate to micronutrient status in this group of patients. More research is also needed to identify potential interactions between micronutrients, and how various factors together explain micronutrient deficiencies in patients with PKU. In addition, longitudinal follow-up studies would allow to understand how changes in those potential factors may predict micronutrient deficiencies reported in patients with PKU.

1.11 Summary, hypothesis and aims

Accumulating evidence suggests that prevalence of overweight and obesity is increasing among patients with PKU. However, factors underlying obesity aetiology in PKU are still unclear. The existing knowledge suggests that foods or meals lacking whole protein, such as SLPF-based meals, may have lower TEF and postprandial fat oxidation. In addition, they may induce lower satiety leading to higher energy and food intake. However, these known contributors to aetiology of obesity were tested using meals composed of normal type foods and contained high proportions of protein well above recommendations. Therefore, there is a need to examine the mechanisms by which the SLPF-based meals, lacking whole protein and high in CHO, may contribute to the prevalence of overweight and obesity in patients with PKU.

Furthermore, studies showed that patients with PKU tend to have higher fat mass (FM) and lower lean mass compared to healthy people, which suggests that for a given weight and height people with PKU will be fatter. However, these studies used poor and inacurate techniques to measure body composition in this group of patietns. Previous studies have also indicated that PKU disease may have impact

on metabolites related to energy balance and nutrient substrate metabolism. Yet, very limited evidence is available on energy metabolism of patients with PKU. Thus, there is a need to study energy and substrate metabolism in PKU while applying gold standard or reference techniques to measure body composition, and whether these contribute to obesity prevalence in the effected patients.

Recent studies have shown that micronutrient deficiencies are prevalent among patients with PKU, but simultaneously high blood concentrations of other micronutrients have been observed. Factors associated with this micronutrient imbalance in PKU were not properly studied.

Hypothesis:

- Lack of whole protein in SLPF-based meals reduces TEF, postprandial fat oxidation and detrimentally impacts appetite and gut appetite hormones.

- Patients with PKU will have higher fat mass compared to their age/BMI and gender matched healthy controls.

- Diminished TEF and fasting and postprandial fat oxidation contribute to the presence of higher fat mass and unfavourable body composition in patients with PKU.

- Phenylketonuria disease severity, metabolic control, dietary adherence, age, and body weight status are associated with micronutrient status in patients with PKU.

Therefore, the main objectives of the subsequent research chapters of this thesis are:

- To investigate the impact of PKU protein substitutes and meal based on SLPF on subjective appetite scores, gut appetite hormones, thermic effect of feeding (TEF), postprandial fat oxidation and spontaneous energy and macronutrient intake in a group of healthy individuals (Chapter 2).

- To investigate compartments of body composition in patients with PKU and sociodemographic and age-gender/BMI-matched healthy controls (Chapter 3).

- To investigate components of energy expenditure such as RMR and TEF, along with energy substrate oxidation and their contribution to body fatness in patients with PKU and sociodemographic and age-gender/BMI-matched healthy controls (Chapter 3).

- To evaluate the micronutrient status of children with PKU and explore factors which associate with micronutrient imbalances and deficiencies using a large retrospective dataset of patients with serial measurements (Chapter 4).

Chapter Two: Impact of Phenylketonuria SLPF Based-Meal on Appetite, Appetite Hormones, and Thermic Effect of Feeding

Some of the results from this chapter have been presented in the following meetings:

Invited oral presentations:

Alfheeaid, H., Gerasimidis, K., Nastase, A. M., Elhauge, M., Cochrane, B. & Malkova, D. 2016, Impact of PKU-type Diet on Appetite, Appetite Hormones and Thermogenesis, *The 30th Annual Conference of The European Society for Phenylketonuria and Allied Disorders Treated as Phenylketonuria (ESPKU 2016)*, Dublin, Ireland

Alfheeaid, H., Gerasimidis, K., Nastase, A. M., Elhauge, M., Cochrane, B. & Malkova, D. 2017, Impact of Phenylketonuria Type-Meal on Appetite, Postprandial Fat Oxidation and Thermogenesis, *Scottish Inherited Metabolic Disease Group (SIMDIG) Symposium*, Stirling, UK

Published paper:

Alfheeaid, H., Gerasimidis, K., Nastase, A. M., Elhauge, M., Cochrane, B. & Malkova, D. 2017. Impact of Phenylketonuria Type Meal on Appetite, Thermic Effect of Feeding and Postprandial Fat Oxidation. *Clinical Nutrition* (2017) doi: 10.1016/j.clnu.2017.03.005.

2.1 Background

Phenylketonuria (PKU) is an autosomal recessive disorder of phenylalanine (PHE) metabolism resulting from the dysfunction of the enzyme phenylalanine hydroxylase and leading to accumulation of PHE to neurotoxic levels [16]. The PHE levels are controlled by a low-PHE diet [88, 330] which does not permit intake of natural protein foods rich in PHE, such as meat, cheese, poultry, eggs and milk, and restricts plant or vegetable proteins such as potatoes and cereals to small amounts depending on disease severity and patient's compliance to the dietary treatment [135]. Thus, the diet of patients with PKU comprises predominantly of foods low in natural protein and includes prescribed PKU special low protein foods (SLPF), which are high in carbohydrate (CHO) and fat [133], and PHE-free protein substitutes [103, 315, 331].

Despite the very restrictive nature of the low-PHE diet, prevalence of overweight and obesity in patients with PKU is similar to general population with more female PKU patients developing obesity than males [184, 186, 187]. From an energy balance point of view, increased body fatness in patients with PKU should mainly relate either to enhanced energy intake (EI) and/or reduced energy expenditure (EE). With respect to EI, it has been reported that SLPF, such as bread, pasta, flour and breakfast cereals, provide more energy than their protein-containing equivalent amount of food [103, 105]. Reduction in EE can also be expected in patients with PKU since the reported social anxiety and isolation of these patients [312, 332, 333] may lead to the avoidance of sporting activities. However, the aetiology of obesity in individuals with PKU may be also associated with effects of the PKU dietary regimen on a wider range of factors contributing to energy balance regulation.

Previous research in healthy people suggested that, in comparison to meals providing the recommended protein amounts, high protein meals enhance satiety

and postprandial responses of anorexigenic hormones such as PYY, GLP-1 and CCK [252-254]. It is also known that protein has the highest and most prolonged thermic effect of all macronutrients, followed respectively by carbohydrate and fat [261, 334]. In addition, enhanced protein content of the meal was reported to upregulate fat oxidation [252, 261, 297]. Thus, meals based on SLPF can be expected to have a detrimental impact on appetite and satiety, leading to an enhanced EI, and simultaneously attenuate TEF and fat oxidation, factors known to contribute independently to increased obesity risk [210].

This is a mechanistic study aimed at investigation of the impact of meal based on SPLF on subjective appetite scores, gut appetite hormones, thermic effect of feeding and postprandial fat oxidation in healthy individuals.

2.2 Methods

2.2.1 Participants

Twenty-three healthy adults (12 males, 11 females) aged 24.3 ± 5.0 years (Range: 18.0 – 44.0) and with BMI of 22.4 ± 2.5 kg/m^2 (Range: 19.0 – 27.6) were recruited to participate into this study. Participants were recruited by means of an advertisement via university's email-broadcasting and word of mouth in the campus of the University of Glasgow, and other public areas. All participants were healthy, non-smokers, not on any medication or any nutritional supplement, had their weight stable and not have been aiming to reduce their weight or been on a special diet for at least the previous four months. Female participants were required to have a regular menstrual cycle. Pregnant or lactating women were excluded from participation. Before enrolling in the study, the participants underwent a screening visit which included height and weight measurements and filling out a detailed health screen questionnaire (Appendix A.3) regarding the participant's health to exclude

chronic illness, eating disorders and gastrointestinal operations which could interfere with the results of the study. The health screening visit took place at the Metabolic Research Unit in Human Nutrition Department, New Lister Building, Glasgow Royal Infirmary. Before the screening visit, all potential participants were provided with volunteer information sheet (Appendix A.2) describing all the details of the purpose of the study, inclusion criteria for the study, screening procedures of the study, what the participant would to do prior to the study, experimental tests involved in the study, possible benefits for taking part in the study and possible disadvantages and risks of taking part in the study. All participants were required to give written informed consent (Appendix A.4). The study was approved by the Research Ethical Committee, College of Medical Veterinary and Life Sciences, University of Glasgow (Reference Number: 200130139) and was performed in accordance with the Helsinki Declaration. Ethics documents for the study are shown in (Appendix A.1). The study was registered at ClinicalTrials.Gov database with identifying number NCT02440932.

2.2.2 Study design

The study applied a randomised, crossover design with two sequenced experimental trials, separated by one week. A randomisation scheme was generated using the website (randomization.com) to allocate participants to the experimental trials. Trials were marked as Control and PKU by the researcher. Each participant started first trial according to allocated trial randomisation. On the morning of each experiment trial, participants reported to the metabolic research unit between 8:00 and 9:00 after an overnight fast. Height, body mass, body fat, and resting metabolic rate (RMR) were measured. A cannula was introduced into an antecubital vein and after an interval of 10 min, a baseline blood sample was obtained. Subsequently, an appetite questionnaire was completed. Within 5 min,

participants were then asked to consume 174 ml of either a PKU protein substitute drink (PKU Trial) or whole protein skimmed milk enriched with a mixture of protein powder (Control Trial). The drinks were isocaloric, volume-matched and had a similar macronutrient composition (Figure 2.3) and (Table 2.1). Ninety minutes after drink consumption, the lunch meal was served. Foods introduced in the lunch meal were based on trial-type and comprised cheese sandwich, crackers and chocolate cookies with a glass of tap water. The meals were isocaloric and weight-matched but differed in macronutrient composition (Figure 2.4) and (Table 2.1). Participants were asked to consume the entire meal within 20 min. Appetite questionnaires were obtained and blood samples were collected at 30, 60 and 90 minutes post-drink, and at 120, 150, 180, 210, 240 and 300 minutes post meal, whilst metabolic rate was measured every 30 seconds for the duration of 20 minutes after each blood sample. After the completion of the last measurement, the participants were presented with an *ad libitum* buffet test meal to assess subsequent food and energy intakes. Water was available throughout the trial, but intake was replicated in the second trial and consumption time was matched. During the two days before the first trial, participants weighed and recorded all foods and drinks consumed and were asked to replicate this intake during the two days preceding the second trial. In addition, for two days prior the experimental trials participants refrained from exercise and alcohol intake (Figure 2.1). A schematic diagram of experimental trial is presented in Figure 2.2.

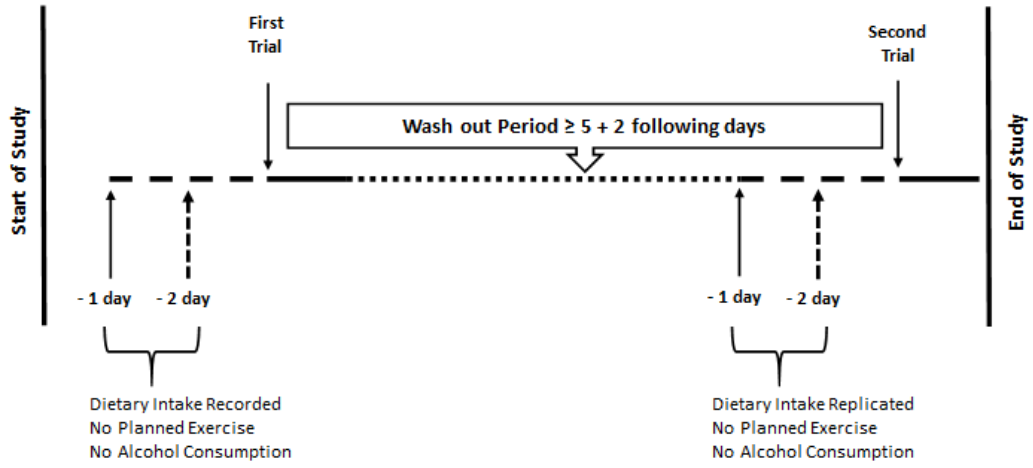


Figure 2.1: Preparation for Experimental Trials

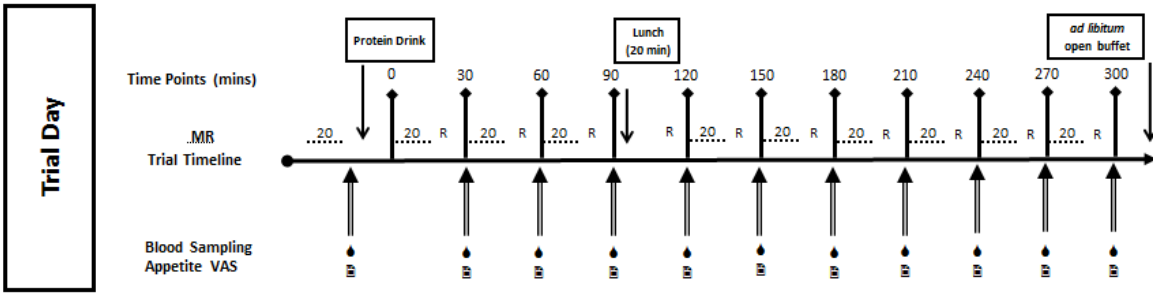


Figure 2.2: Schematic Diagram of Experimental Trial

20 metabolic rate measurements (MR); R, in between measurements resting time (10 min); ♦ , blood sampling; 🗎, appetite rating questionnaire (VAS)

2.2.3 Anthropometric and body composition measurements

All measurements were taken upon arrival in the fasted state while the participants wearing light cloths and without shoes at the metabolic research unit. All participants were measured in privacy.

2.2.3.1 Height

Height was measured by a portable stadiometer (Seca, Leicester, Cranela, UK), using a stretch stature method. Participants were measured while standing barefooted, with their back against a fixed backboard and their arms hanging laterally by their body. The head was positioned with the line of eyesight perpendicular to the backboard. Participants were instructed to relax, and a moveable headboard was lowered to the top of the head with light pressure added to compress the hair. Gentle upward pressure was applied to the lower jaw and height was measured to the nearest 0.5 cm. The same height scale was used by the researcher throughout the experimental study.

2.2.3.2 Body mass and composition

Body mass was measured using a Tanita foot to foot balance scale (TANITA-TBF-310, UK). All participants were weighed while wearing minimal clothing, typically lightweight shorts and a t-shirt, whilst standing with bare feet in contact with the electrodes of the balance and their arms hanging laterally by their body. All footwear, substantial jewellery and accessories, e.g. watches, and items within pockets were removed prior to any measurement. Tanita scales determine the opposition to the flow of electric current through the body tissues i.e. bioelectrical impedance, which is then used to estimate the total body water. Total body water is then used to calculate fat-free body mass, and through difference in body weight, body fat is estimated. The body mass index (BMI) was derived as weight (kg) divided by height (m) squared (kg/m^2).

2.2.4 Protein drinks

PKU protein substitute (PKU Cooler20, white) drink was provided by Vitaflo®, UK. Protein content of the PKU Cooler drink was based on free amino acids (L-amino

acids). This drink was introduced to the participants during their PKU trials. In Control trials, participants received a whole-protein skimmed milk drink enriched with whey protein isolate (ISO:PRO 97 Whey, MYPROTEIN®). Both drinks were isocaloric and isovolumetric, and matched for macronutrient composition (Figure 2.3) and (Table 2.1). PKU protein substitute drink provided 7.8 gm of sugar and 1.6 g of complex of CHO, while in Control drink all 8.4 g of CHO were sugars. Participants were asked to consume the drinks within 5 minutes before the first post-prandial metabolic rate measurement.

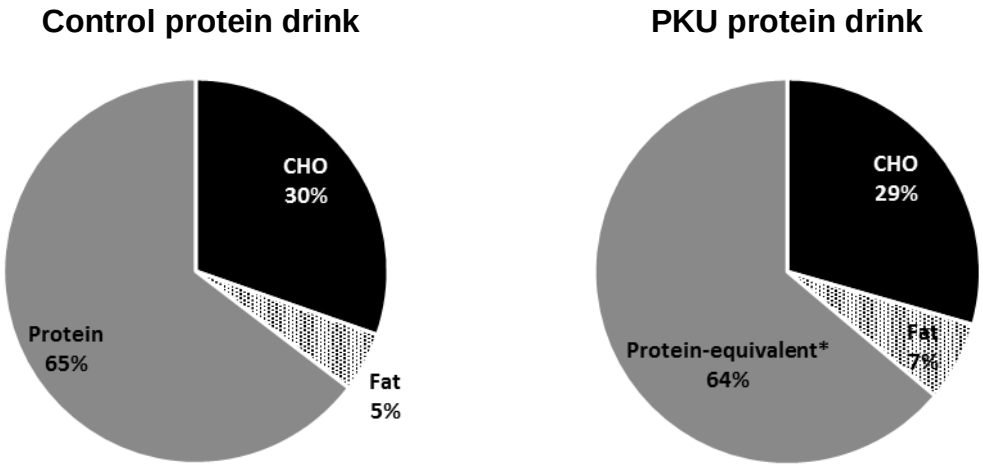


Figure 2.3: Macronutrient composition of Control and PKU protein drinks

* PHE-free L-amino acids based protein equivalent
Energy content: Control drink 0.51 MJ, PKU drink 0.52 MJ

2.2.5 Lunch meals

Trial lunch meals were selected from common local foods and that for PKU meal were obtained from most popular PKU medical food providers Vitaflo® and Juvela®. The PKU foods were suggested by senior metabolic dieticians based on typical lunch-meals consumed by patients with PKU. Lunch meal in the PKU trial (Figure 2.4) was based on PKU special low protein foods and comprised of cheese sandwich (2 slices of bread Juvela®, 60 g; and 2.5 cheese slices Violife®, 50 g);

mini crackers Vitaflo®, 20 g; chocolate cookies Juvela®, 10 g; (all protein-free) and a glass of tap water. The lunch meal in the Control trial (Figure 2.4) comprised of cheese sandwich of (2 slices of white bread Warburtons, 57 g; and 2 Lighter Mature cheese slices Tesco, 50 g); crackers Ritz®, 20 g; Everyday Value chocolate chip cookies Tesco, 10 g; and a glass of tap water.

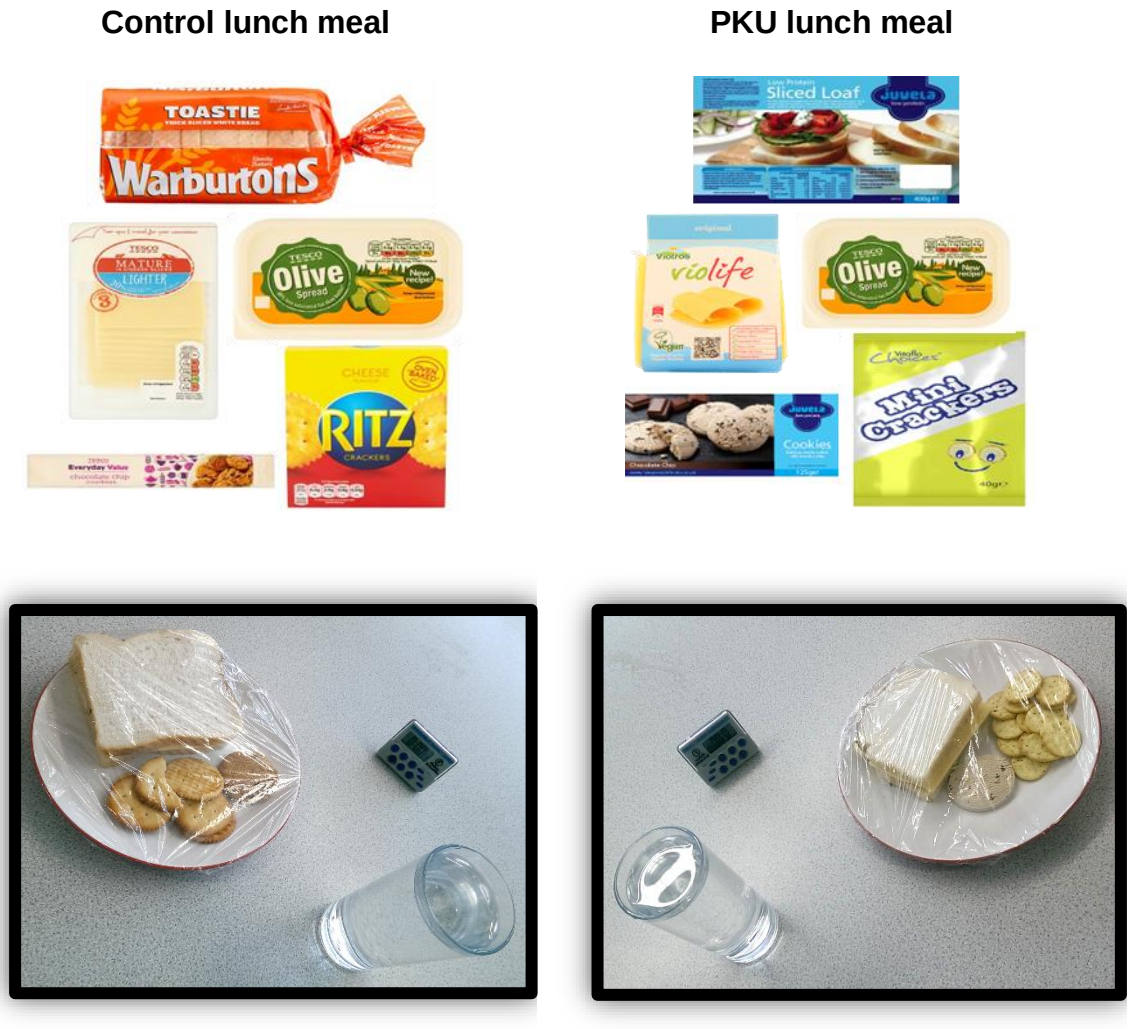


Figure 2.4: Normal and PKU foods provided in lunch meals of the Control and PKU trials

Energy content: Control lunch meal 2.0 MJ, PKU lunch meal 2.0 MJ

The lunches were isocaloric, and matched for weight content, but differed in macronutrient composition (Figure 2.5) and (Table 2.1). In Control trial, lunch meal

provided 22.6 grams of protein (18% of energy intake), which is close to average protein content of a typical lunch [260], while the meal based on PKU special low protein foods (SLPF), protein content was negligible and consisted only of 0.31 grams (0.2% of energy intake). Fibre content was 2.0 g and 2.5 g for Control and PKU lunch meals, respectively. All participants were asked to consume their entire lunch meal within 20 minutes.

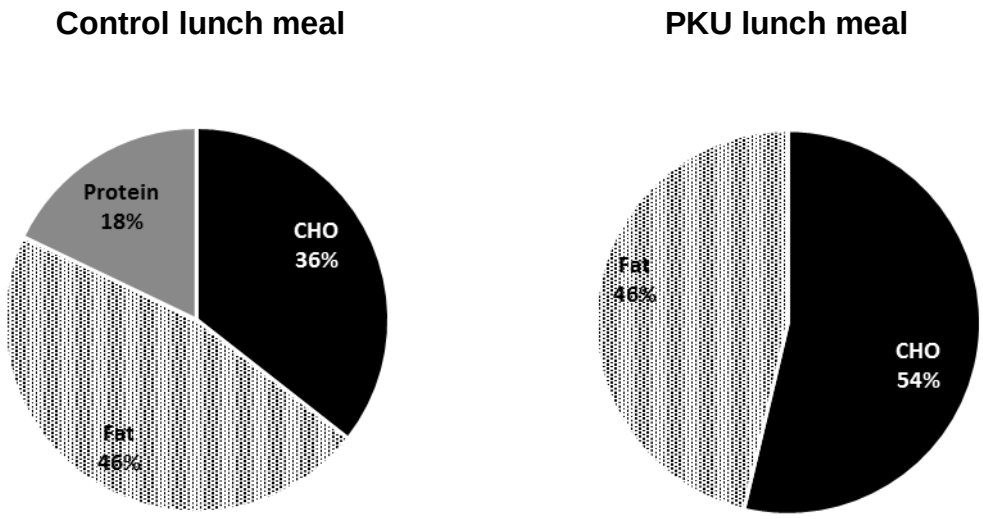


Figure 2.5: Macronutrient energy distribution of Control and PKU lunch meals

Energy content: Control lunch meal 2.0 MJ, PKU lunch meal 2.0 MJ

Table 2.1: Energy, fat, carbohydrate and protein provided by drinks and meals in the Control and the PKU trials.

	Drink		Meal	
	Control	**PKU**	**Control**	**PKU**
Energy (MJ)	0.51	0.52	2.0	2.0
Fat (g)	0.7	0.9	25.7	24.4
CHO (g)	9.4	9.2	44.5	63.7
Protein (g)	20	20[a]	22.6	0.31

a, grams of protein equivalent

2.2.6 *Ad libitum* open buffet meals

Participants were presented with an *ad libitum* buffet test meal which is an established and reproducible method to assess under laboratory conditions the spontaneous EI and macronutrient preferences [335]. Foods used for at the *ad libitum* buffet dinner consisted of a variety of standardised foods and included starter (green salad with tomatoes and onions), main dish, two side dishes and sweet providing a total energy of approximately three times what participants were expected to consume (~5 MJ/1200 kcal). Before starting their first trials, participants were given food menus from which they can choose variety of available meals to consume during *ad libitum* buffet dinner. The main dishes included selections of Beef Lasagne; Lamb Moussaka; Rich Beef Bolognese Pasta and Focaccia Crostoni; Fajita Chicken with Pitta Bread; Tikka Chicken with Pilau Rice; Roasted Butternut Squash Lasagne (vegetarian); or Vegetable Paneer Korma with Pilau Rice (vegetarian). The side dishes included selections of grapes, apple, orange, and chips with flavours of cheddar cheese, vinegar or light salt. Sweets included selections of strawberry tart or cheese cake with flavours of chocolate, strawberry or raspberry. Water was provided during the buffet meal. They were served (Figure 2.6) in the same setting, serving the same type of food in the same coloured dishes scheduling meals at the same time, and at the same table to avoid any bias in eating behaviour. Food menu for ad libitum buffet meal provided to study participants is shown in Appendix A.6. The participants were given ultimate time to consume their meal and advised to eat according to their appetite until satisfied and comfortably full. In both Control and PKU trials, *ad libitum* meals were identical as the same foods of same weight were introduced to insure an equal provision of energy and macronutrients.

Figure 2.6: Two examples of *ad libitum* buffet meals

The food was cut into smaller pieces to eliminate portion related cues. All offered and remaining food was weighed by the researcher using an electronic kitchen scale (Salter Housewares Ltd., Tonbridge, UK) to estimate amount of food consumed. The researchers were not present when the participants ate their meals to avoid any potential effect of the researcher on eating behaviour [336-338]. Activities such as watching TV [338], using smart gadgets (e.g. listening to music) [339], or reading were not permitted during ad libitum buffet meal as these may influence food consumption [340]. The participants were blinded to the actual purpose of buffet meals i.e. measurement of food and energy intake. If the participants knew the actual purpose for the buffet meal i.e. monitoring food intake, they may be conscious of eating and potential bias then could not be avoided [340].

2.2.7 Appetite ratings

Previously validated and reliable visual analogue scales (VAS) with a line of 100 mm were used to assess appetite sensations during trials [341]. Using paper and pen VAS format, the participants were asked to place a vertical mark on the relative horizontal line corresponding to their feelings of hunger, satiety, fullness,

prospective food consumption and the desire to eat at each time point [341]. The lines were anchored by most-positive (e.g. I am not hungry) and most-negative (e.g. never been hungrier) feeling words from the right to the left respectively (Figure 2.7) and Appendix A.5. Distance from the left end to the participant's mark was measured to quantify rate of each feeling. Upon completion of each time appetite rating, appetite scales were removed from the front of the participants so they could not refer to their previous ratings.

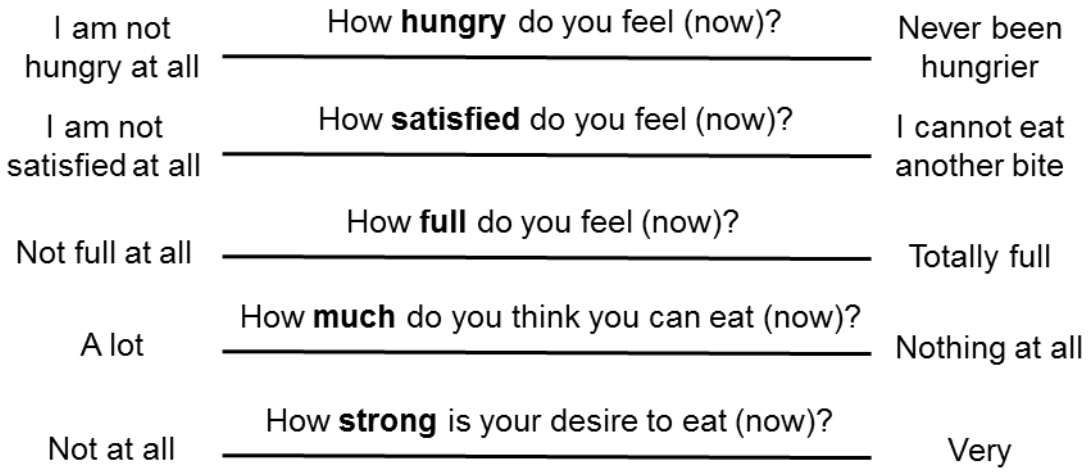


Figure 2.7: Appetite visual analogue scales (VAS)

Reproduced from Reproducibility, power and validity of visual analogue scales in assessment of appetite sensations in single test meal studies [341].

2.2.8 Measurement of macronutrient and energy intake

Introduced foods during ad libitum buffet were covertly weighed pre- and post-consumption using a standard kitchen scale (accuracy ± 1 g). Food and macronutrient information were obtained from product information labels provided by the manufacturing company. The information was entered into the dietary software Windiets 2010 (The Robert Gordon University, Aberdeen, Scotland, UK) to calculate macronutrient and energy intakes.

2.2.9 Measurement of metabolic rate

2.2.9.1 Instrument (Metabolic cart)

The resting metabolic rate (RMR) and thermic effect of feeding were measured by indirect calorimetry, with a computerised open-circuit ventilated hood system (*Oxycon Pro*®, Jaeger GmbH, Hoechberg, Germany, running JLab Software version 5.20.1.1) (Figure 2.8). The *Oxycon Pro*® metabolic cart consists of a clear plastic canopy that is connected to the mixing chamber by a flexible plastic tube. It has a bi-directional rotary digital volume sensor consisting of an electronic amplifier, Triple V flow transducer with a twin sample tube and all connect to an automatic ventilator in the back of the mixing chamber. The *Oxycon Pro*® metabolic cart uses online stream paramagnetic (accuracy <0.2 %) and infrared analysers (accuracy <0.05 %) for determination of fractional oxygen (O_2) and carbon dioxide (CO_2) concentrations, respectively, in the expired air samples. The metabolic cart is also provided with multisensory ambient condition module by which temperature, humidity and pressure in the testing environment can be determined.

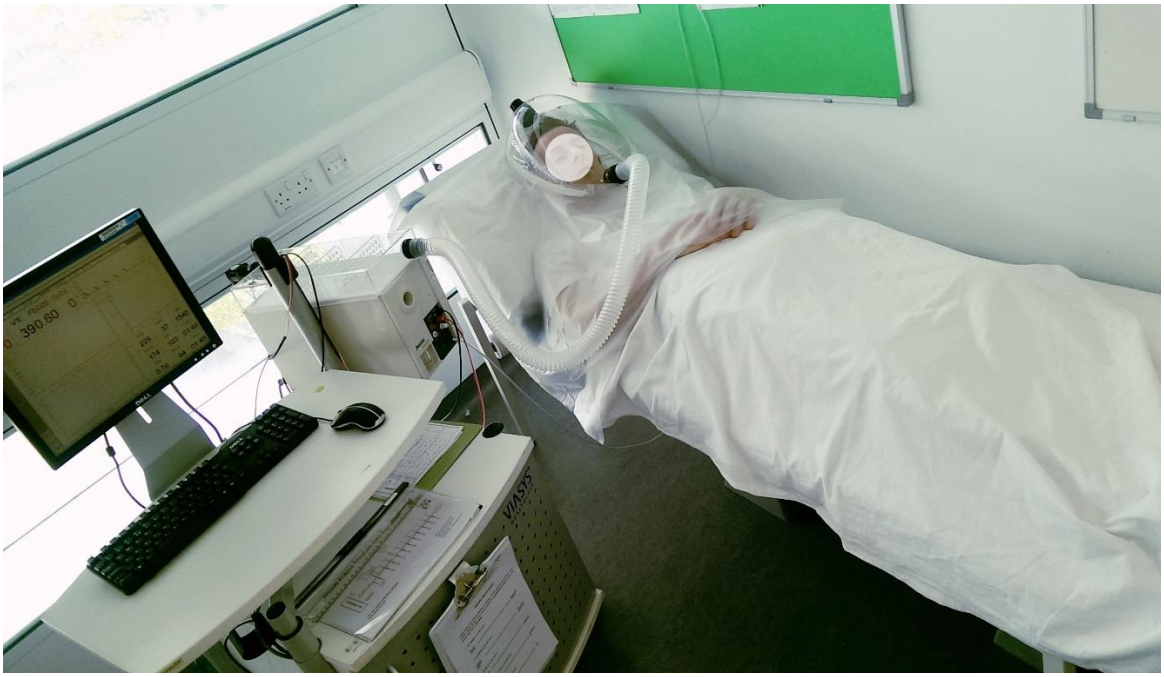


Figure 2.8: Indirect calorimetry ventilated system (*Oxycon Pro*, Jaeger GmbH, Hoechberg, Germany)

On trial day, the *Oxycon Pro*® system was switched on 2 hours prior to use, and volume and gas calibrations were performed before each measurement. For volume calibration, an automatic program is initiated and the flow values are measured at 2-point calibration (0.2 litre/second and 2 litre/second). The calibration values were accepted if the percentage difference between the recent and previous volume calibration was less than 1 % [342]. The gas analyser and delay time calibrations are involved in another automatic process window. Provided gas cylinder containing precisely known (±0.02% absolute) concentrations of O_2 (16%) and CO_2 (5%) is used for the standard two-point gas calibration procedure recommended by the manufacturer. The automatic procedure was repeated until the difference between current and previous values, delay time and offset were within 1 % [342]. In addition, ambient conditions including temperature, pressure and humidity are automatically measured on daily bases through integrated ambient module located in the compact

housing of the metabolic cart. Step by step description of metabolic rate measurement protocol by the *Oxycon Pro* system is shown in Appendix A.9.

2.2.9.2 Principles of indirect calorimetry

Indirect calorimetry technique is based on quantification of oxygen (O_2) and carbon dioxide (CO_2) concentrations in the collected expired gas samples with known flow rate at standard temperature and pressure dry (STPD). Basically, indirect calorimetry equipment measures the ventilation rate of the expired air ($\dot{V}_e$) (litres per minute) and, the fractions of oxygen (F_eO_2) and carbon dioxide (F_eCO_2) concentrations in the expired air. Fractions of oxygen (F_iO_2) and carbon dioxide (F_iCO_2) concentrations in the inspired air are assumed to be constant at 0.2093 and 0.0003, respectively [343]. Utilising Haldane transformation assumption and equations, the measured values of F_eO_2 and F_eCO_2 fractions in the expired air are corrected for STPD and used to calculate rates of whole body oxygen consumption ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) in units of litres per minute (Equations 1 and 2) [343]. Developed indirect calorimetry equations can then be used to calculate resting energy expenditure (in Kcal or kJ) and rates of substrate oxidation (g/min) [344].

Rate of oxygen consumption ($\dot{V}O_2$) [Equation 1]:

$= \dot{V}O_2\ (L \cdot min^{-1}) = \dot{V}_e \times (F_iO_2 - F_eO_2)$

$\therefore \dot{V}O_2\ (L \cdot min^{-1}) = \dot{V}_e \times (0.20930$''Constant value of $F_iO_2 - F_eO_2)$

Rate of carbon dioxide production ($\dot{V}CO_2$) [Equation 2]:

$= \dot{V}CO_2\ (L \cdot min^{-1}) = \dot{V}_e \times (F_eCO_2 - F_iCO_2$'')

$\therefore \dot{V}CO_2\ (L \cdot min^{-1}) = \dot{V}_e \times (F_eCO_2 - 0.0003$''Constant value of F_iCO_2'')

Where

$\dot{V}_e$= ventilation rate of the expired air

F_eO_2 = fractions of oxygen in the expired air

F_eCO_2 = fractions of carbon dioxide in the expired air

F_iO_2 = fractions of oxygen in the inspired air

F_iCO_2 = fractions of carbon dioxide in the inspired air

2.2.9.3 Measurements and calculations

The resting metabolic rate (RMR) of the participant was measured in the morning between 8:00 to 9:00 hrs in the metabolic research unit, after overnight fasting and in abstinence from physical activity for at least two days prior to each trial. The Metabolic Research Unit, New Lister Building at Glasgow Royal Infirmary, in which all tests were conducted, was quiet, semi-dark and where background noise was kept to a minimum level. The atmospheric temperature was automatically controlled in the whole research unit and kept at neutral thermic degrees (20-25°C). Before starting metabolic measurements, the participants were asked to lie relaxed for at least 15 minutes in supine position with arms placed straight on the side and straight legs. [345]. A plastic ventilated canopy was then placed over the participants' head and expired gas was drown by the automatic ventilator through the plastic tube to the mixing chamber and Triple V connector including all sensors. The rate of O_2 consumption ($\dot{V}O_2$) and rate of CO_2 production ($\dot{V}CO_2$) were recorded every 30 seconds for the duration of 20 minutes in which the first 5 minutes were disregarded and steady state is achieved [345] [defined as 5-minute period coefficient of variation (CV) for the $\dot{V}O_2$ and $\dot{V}CO_2$ values being ≤10%]. Measurements were recorded for 10 minutes prior to placing the canopy on the participant and these measurements were called pre-drifts. Similarly, 10 minutes of measurements were taken when the participant was outside the canopy during the lunch time (Midday)

and again after the trial was completed which act as post-drifts. Then the mean values of drift measurements were calculated and used for the correction of the participant's measurements. During trials, participants were closely monitored so that talking, excess movement and sleeping would be avoided. The participants had a resting time between measurements in 10 minutes intervals in which they were allowed to perform their sanitary activities.

The corrected mean values of $\dot{V}O_2$ and $\dot{V}CO_2$ were used to calculate rates of fat and CHO oxidation, respiratory exchange ratio and rate of energy expenditure using indirect calorimetry equations developed by Frayn and Macdonald [344].

Rate of fat oxidation ($g \cdot min^{-1}$)= [($\dot{V}O_2$ - $\dot{V}CO_2$) / 57] x 100

Rate of carbohydrate oxidation ($g \cdot min^{-1}$)= [1.40 x ($\dot{V}CO_2$ - $\dot{V}O_2$) / 30] x 100

Rate of energy expenditure ($kJ \cdot min^{-1}$)= [(Rate of fat oxidation x 39.4) + (Rate of carbohydrate oxidation x 15.6)] x100

Respiratory exchange ratio (RER)= $\dot{V}CO_2$ / $\dot{V}O_2$

Where:

$\dot{V}O_2$= rate of oxygen consumption ($L \cdot min^{-1}$)

$\dot{V}CO_2$= rate of carbon dioxide production ($L \cdot min^{-1}$)

Thermic effect of feeding (TEF) in the Control and the PKU trials was calculated as percentage (%) increase in energy expenditure above RMR. The TEF was also calculated as relative increase in EE expressed as percentage of energy intake provided [284] by drink for the post-drink period (0-90 min), by meal for the post-

meal period (90-300 min) and by both drink and meal for the entire period of the experiment trial (0-300 min), Equations 6, 7 and 8.

Relative increase in EE as percentage (%) of EI (post-drink 0-90 min) [Equation 6]:
= (TEF during post-drink period in kJ ×100) / EI from drink in kJ

Relative increase in EE as percentage (%) of EI (post-meal 90-300 min) [Equation 7]:
= (TEF during post-meal period in kJ ×100) / EI from meal in kJ

Relative increase in EE as percentage (%) of EI (entire trial 0-300 min) [Equation 8]:
= (TEF during entire trial in kJ ×100) / EI from drink and meal in kJ

2.2.9.4 Validation of indirect calorimetry system

Accuracy of indirect calorimetry system (*Oxycon Pro*®) was validated using alcohol (99.9% absolute Ethanol) burning test [346, 347]. The validation tests were conducted regularly through the study period and according to the laboratory standard operating procedure. Each test lasted for 15 – 20 minutes and steady state of measurement values (defined as ≤ 10% CV) [345] was verified during all tests. The principal of the alcohol burning validation test is that at STPD, combustion of 1 mole of ethanol, 3 moles of O_2 is consumed and 2 moles of CO_2 is produced (Equation 9) which results in respiratory quotient (RQ) of 0.667. The accuracy is then validated by comparing the theoretical value against the measured value of RQ. Step by step description of Alcohol burning test protocol for validation of the *Oxycon Pro*® system is shown in Appendix A.10.

Chemical principal of alcohol burning test [Equation 9]:

$C_2 H_5OH + 3 O_2 = 2 CO_2 + 3 H_2O$

Molecule weight of Ethanol (99.9% absolute) is 46.08 g. Therefore, theoretically consumed O_2 and produced CO_2 can be calculated as follow:

Theoretical O_2 consumption: 46.08 g Ethanol= 3 moles of O_2=67.23 L of O_2 ∴1 g Ethanol 67.23/46.08=1.459 L of O_2

Theoretical CO_2 production: 46.08 g Ethanol= 2 moles of CO_2=44.82 L of CO_2 ∴1 g Ethanol 44.82/46.08= 0.973 L of CO_2

Theoretical RQ value is 0.973/1.459= 0.667

2.2.10 Blood sampling and plasma preparations

During day of the experimental trial, venous blood samples were collected from the participants. Participants reported to the metabolic research unit in the morning between 08:00 to 09:00 after an overnight fast. After recording the resting-metabolic rate (RMR) measurement, the participant rested in a supine position and was informed about the intravenous cannulation. Then an indwelling 20GA cannula (BD Venfoln™, Helsingborg, Sweden) was inserted into the antecubital vein of the forearm. Fasting (baseline) venous blood sample was collected into a pre-chilled 4 ml ethylenediamine tetra-acetic acid (EDTA) Vacuette® tubes (Greiner Bio-One, Kremsmünster, Austria). After each sample collection, the cannula was flushed using 5 mls of 0.9% Saline solution (Braun Mini-Plasco®, Melsungen, Germany) to maintain patency. About 8 mls of venous blood was collected at 0, 30, 60, 120, 150, 180, 210, 240 and 300 minutes during the entire period of each experimental trial.

Blood samples for the analysis of insulin, glucose, and triglyceride concentrations were centrifuged at 4°C, 3000 rpm for 15 minutes in a refrigerated centrifuge. After centrifugation, the plasma supernatant was aspirated using a pipette and then 200 µL of plasma aliquoted into four labelled 2 ml Eppendorf tubes (Alpha laboratories Ltd, UK) and frozen at - 80°C for the analysis. For the determination of gut peptides, a 1.8 ml of blood from EDTA tube was aliquoted into a centrifuge tube (Corning®, NY, USA) containing aprotinin (Sigma-Aldrich, UK) to give concentration of 400 kallikrein inactivator units per ml blood (kIU/ml) [348]. Then the sample was centrifuged at 4°C, 3000 rpm for 15 minutes in a refrigerated centrifuge. Subsequently, 200 µL of plasma was aliquoted into 5 labelled Eppendorf tubes for measurement of GLP-1 and PYY concentrations. Immediately all the aliquoted samples were frozen at - 80°C until assayed.

2.2.11 Blood analyses

2.2.11.1 Active glucagon-like peptide 1

Plasma active glucagon-like peptide 1 (GLP-1: 7-36 amide and 7-37) measurements were performed in the Biochemistry laboratory, Level 2, West Medical Building, at the Institute of Cardiovascular and Medical Sciences, University of Glasgow. Active GLP-1 concentrations were measured by using a commercially available chemifluorescenct enzyme-linked immunosorbent assay (ELISA) kit (Cat. # EGLP-35K, Merck EMD Millipore, Millipore, Billerica, MO, USA) and analyses were performed according to the manufacturer's instructions. GLP-1 ELISA kit is based on sandwich technique and used for non-radioactive quantification of GLP-1 active forms (GLP-1 ''7-36 amide'' and GLP-1 ''7-37'') in plasma or serum with a sensitivity of 2 pg /mL using a 100 µL sample size. The assay involves capturing of active GLP-1 from the sample by a monoclonal antibody, immobilised in the wells of a microtiter plate that binds specifically to the N-terminal region of the active GLP-1 molecule.

Unbound antibodies and materials are then washed out. An anti GLP-1-alkaline phosphatase detection conjugate is then added to bind the immobilised GLP-1 molecules and followed by a second wash to remove free enzyme. Immobilised GLP-1 monoclonal antibody-enzyme conjugates are then quantified by monitoring GLP-1-alkaline phosphatase activities in the presence of the substrate methyl umbelliferyl phosphate (MUP). Finally, the acid was added to stop the reaction and to get a fluorescent product umbelliferon which was read fluorescently by the relative increase in fluorescence units (emitted fluorescent light) with an excitation/emission wavelength of 355 nm/460 nm. The increase in fluorescence is directly proportional to the amount of captured active GLP-1 (GLP-1 ‘’7-36’’ amide’’ and GLP-1 ‘’7-37’’) in the unknown sample, thus the concentration of active GL-1 can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of active GLP-1.

Before analysis, all samples and reagents were brought to room temperature. The micro-plate wells were planned to accommodate the calibrators, controls and samples in duplicate. At first, the coated plates (active GLP-1 monoclonal antibodies) are activated by multiple washes using provided wash buffer [Phosphate-buffered Saline (PBS)], 300 µL each time. Following this, assay buffer (100 µL) containing proprietary protease inhibitor, Borate Saline, Sodium Azide, Bovine Serum Albumin (BSA) and PBS, is added to the microtiter wells. Calibrators (100 µL), controls (100 µL) and plasma samples (100 µL) are then poured into appropriate assay wells. The pates were then incubated for 24 hours at 4°C to allow capturing of active GLP-1 molecules in the samples by monoclonal antibodies immobilised on the microtiter wells. After incubation, plates were washed 5 times using buffer solution (300 µL) and dried after each wash to eliminate any unbound materials. Then, a 200 µL of GLP-1-alkaline phosphatase detection conjugate was

added to each well and plates were incubated for 2 hours at room temperature (18-25°C). The plates were then washed 3 times to remove unbound enzyme conjugates. A 200 µL of freshly prepared substrate was added to all wells and incubated for 20 minutes in the dark at room temperature (18-25°C). The enzymatic activity was stopped after incubation in each well by adding 50 µL of stop solution. Subsequently using fluorescence plate reader (SpectraMax® M2e, Molecular Devices Corporation, California, USA) the produced fluorescent units (emitted fluorescent light) of every well was read with an excitation/emission wavelength of 355 nm/460 nm. By comparing the light absorption (fluorescent units produced) of the samples to the standard curve for every plate, active GLP-1 concentration in the samples was obtained. All the samples from one subject were analysed in a single run to minimise inter-assay variation. The coefficients of variation for the assay was <5%.

2.2.11.2 Human total Peptide YY

Plasma total peptide YY (PYY; 1~36 and 3~36 molecules) analysis was performed in the Main laboratory, at the Human Nutrition, Level 3, New Lister Building, Glasgow Royal Infirmary, University of Glasgow. PYY concentrations were measured by using a commercially available Enzyme-linked immunosorbent assay (ELISA) kit (Cat. # EZHPYYT66K, Merck EMD Millipore, Millipore, Billerica, MO, USA) and analyses were performed according to the manufacturer's instructions. For analysis, inter-assay and intra-assay coefficient of variance were 8% and 3%. The total PYY ELISA kit is based on sandwich technique and used for non-radioactive quantification of human PYY molecules (both 1~36 and 3~36) in plasma or serum with a sensitivity of 6.5 pg/mL using a 20 µL sample size. The assay involves binding the human PYY molecules in the sample by rabbit anti-human PYY IgG. The resulting complex is then immobilised to the wells of the microtiter plate

coated with a pre-titered amount of anti-rabbit IgG antibodies. Simultaneously, a second biotinylated antibody is bound to the PYY molecules. Unbound antibodies and materials are then washed out. A conjugation of horseradish peroxidase enzyme is added to the immobilised biotinylated antibodies followed by a second wash to remove free enzyme. Immobilised antibody-enzyme conjugates are then quantified by monitoring horseradish peroxidase activities in the presence of the substrate 3,3',5,5'-tetra-methylbenzidine (TMB). Finally, the acid was added to stop the reaction and to get a calorimetric endpoint which was read spectrophotometrically by the increased absorbency at 450 nm and corrected from the absorbency at 590nm. The increase in absorbency is directly proportional to the amount of captured human PYY (both 1~36 and 3~36) in the unknown sample, thus the concentration of total PYY can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of human PYY.

Before analysis, all samples and reagents were brought to room temperature. The micro-plate wells were planned to accommodate the calibrators, controls and samples in duplicate. At first, the coated plates (anti-rabbit IgG antibodies) are activated by multiple washes using provided wash buffer (Tris Buffered Saline, 300 µL each time). Following this, serum matrix (20 µL) containing dipeptidyl-peptidase IV (DPP IV) inhibitor and assay buffer (20 µL) [containing Borate Saline, EDTA, Sodium Azide, and Bovine Serum Albumin (BSA)] are added to the microtiter wells. Calibrators (20 µL) controls (20 µL) and plasma samples (20 µL) are then poured into appropriate assay wells. A blocking solution (20 µL) was added to prevent false positive signals, and the plates are then incubated for 30 minutes at room temperature (18-25°C) on a plate shaker (at 400- 500 rpm). Next, a 50 µL of antibody mixture (rabbit capture antibody with human PYY detection antibody) was

added to all wells for the capture of PYY modules in the samples. Plates were again incubated for one hour and a half at room temperature (18-25°C) on a plate shaker (at 400- 500 rpm). Afterwards, plates were washed 3 times using buffer solution (300 µL) and dried after each wash to eliminate any unbound enzyme labelled antibodies. Following this, a 100 µL of freshly prepared enzyme conjugate (Streptavidin-Horseradish Peroxidase) was added to each well and plates were then incubated for one hour at room temperature (18-25°C) on a plate shaker (at 400-500 rpm). During incubation, human PYY module present in samples reacts with the peroxidase-conjugated anti-body mixture and these antibodies attach to the plate wells. This is followed by a final wash (300 µL x 6 times) to remove unbound antibodies. Subsequently, a 100 µL of substrate TMB was added to each well to detect bound conjugates in the wells. The plates were again incubated for 20 minutes at the room temperature to permit the reaction between bound conjugates and substrate TMB. The reaction was stopped after incubation in each well by adding 100 µL of stop solution containing 0.5 M hydrochloric acid. A yellowish tint colour is developed form the conjugate-substrate complex concentration. Subsequently using a plate reader (Multiskan® Spectrum, M1500, Thermo Scientific, Vantaa, Finland) the optical density of every well was read at two wavelengths, once at 450 nm and then corrected from the absorbency at 590 nm. By comparing the optical density of the samples to the standard curve for every plate, total human PYY concentration in the samples was obtained. All the samples from one subject were analysed in a single run to minimise inter-assay variation. The coefficients of variation for the assay were <5%.

2.2.11.3 Insulin

Plasma insulin concentration measurements were performed in the Main laboratory, at the Human Nutrition, Level 3, New Lister Building, Glasgow Royal Infirmary,

University of Glasgow. Quantitative insulin analysis was performed by using a commercially available Enzyme-linked immunosorbent assay (ELISA) kit (Cat. # 10-1113-01) with pro-insulin (Mercodia AB, Uppsala, Sweden) and with less than 0.01% cross-reactivity. Mercodia insulin ELISA is based on direct sandwich technique which utilises the principle of solid phase two-site enzyme immunoassay. Monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. Insulin present in the samples reacts with the per-oxidase conjugated anti-insulin antibodies, and these anti-insulin anti- bodies then bound to the micro-titration well. Unbound enzymes labelled anti-bodies are then removed by a simple washing step. The bound conjugates are detected by reaction with substrate 3,3',5,5'-tetramethylbenzidine (TMB). Finally, the acid was added to stop the reaction and to get a calorimetric endpoint which was read spectrophotometrically.

Before analysis, all samples and reagents were brought to room temperature. The micro-plate wells were planned to accommodate the calibrators, controls and samples in duplicate. Plasma samples (25 µL) controls (25 µL) and calibrators (25 µL) were poured into appropriate assay wells. Then a 100 µL of freshly prepared enzyme conjugate was added to each well. Following this, plates were incubated for one hour at room temperature (18-25°C) on a plate shaker (at 700- 900 rpm). During incubation, insulin present in samples reacts with peroxidase-conjugated anti-insulin antibodies and these anti-insulin antibodies attach to the plate wells. Afterwards, plates were washed 6 times using buffer solution (350 µL) and dried after each wash to eliminate any unbound enzyme labelled antibody. Subsequently, a 200 µL of substrate 3,3',5,5'-tetramethylbenzidine (TMB) was added to each well to detect bound conjugates in the wells. The plate was again incubated for 15 minutes at the room temperature to permit the reaction between bound conjugates

and substrate TMB. The reaction was stopped after incubation in each well by adding 50 µL of stop solution containing 0.5 M sulphuric acid. The plate was then placed on the shaker for approximately 5 seconds to ensure proper mixing. A yellowish tint colour is developed form the conjugate-substrate complex concentration. Subsequently using a plate reader (Multiskan® Spectrum, M1500, Thermo Scientific, Vantaa, Finland) the optical density of every well was read. By comparing the optical density of the samples to the standard curve for every plate, insulin concentration in the samples was obtained. All the samples from one subject were analysed in a single run to minimise inter-assay variation. Human low and high quality controls sera (Mercodia Diabetes Antigen Control, Mercodia AB, Uppsala, Sweden) were used to determine the precision and accuracy of the assays. The coefficients of variation for assay were <4%.

2.2.11.4 Development of micro-volume plasma glucose analysis

A new protocol which uses micro-volumes and allows analysis of large number of samples in a single run was developed to measure plasma glucose concentrations. This protocol was developed using the enzymatic (Hexokinase) calorimetric method from commercially available glucose analysis kit. The glucose determination principle is based on the calorimetric reaction presented below. Glucose analysis kit consists of standard, buffer and enzyme reagent (Cat. # GL1611) (Randox Laboratories Ltd., Crumlin, UK). The required working micro-volumes of the reagents with the samples were proportionally calculated from suggested values in the kit manual to be measured in 300 µL microtiter-plate wells. The new volumes of reagent (150 µL) with samples (5 µL plasma) were then accommodated into 96 microtiter plates of 300 µL maximum capacity wells (Costar®, ME, USA). The analysis was performed using the spectrophotometer (Multiskan® Spectrum, M1500, Thermo Scientific, Vantaa, Finland). The precision and accuracy of the

assays were monitored with human levels 1 and 2 multi-sera quality controls (Randox Laboratories Ltd., Crumlin, UK). The analysis of all the samples from each subject was performed on a single run and in duplicate with the coefficient of variation of less than 3%. The protocol was practical, cost-effective (saved 85% of total reagents volume) and allowed measurement of large number of samples in shorter time. The development of the protocol and measurements were conducted in the Main Laboratory, at the Human Nutrition, Level 3, New Lister Building, Glasgow Royal Infirmary, University of Glasgow. The protocle for micro-volume measurmet of plasma glucose cocnentrations is shown in Appendix A.7.

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{Hexokinase}} \text{glucose-6-phosphate} + \text{ADP}$$

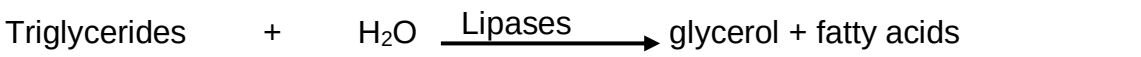
$$\text{Glucose-6-phosphate} + \text{NAD} \xrightarrow{\text{G-6-PDH}} \text{gluconate-6-P} + \text{NADH} + \text{H}^{+}$$

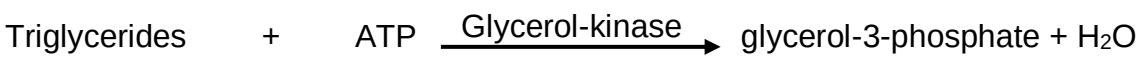
Where G-6-PDH= glucose-6-phosphate dehydrogenase

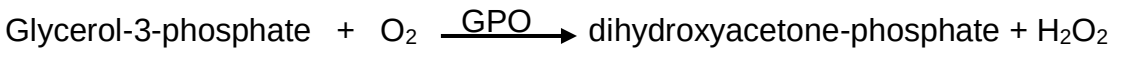
2.2.11.5 Development of micro-volume plasma triglyceride analysis

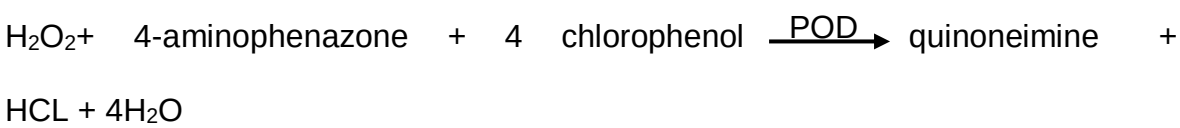
A new protocol which uses micro-volumes and allows analysis of large number of samples in a single run was developed to measure plasma triglyceride (TAG) concentrations. This protocol was developed using the enzymatic (GPO-PAP) calorimetric method from commercially available TAG analysis kit. The TAG determination principle is based on the calorimetric reaction presented below: TAG analysis kit consists of standard, buffer and enzyme reagent (Cat. # TR210) (Randox Laboratories Ltd., Crumlin, UK). The required working micro-volumes of the reagents with the samples were proportionally calculated from suggested values

in the kit manual to be measured in 300 µL microtiter-plate wells. The new volumes of reagent (150 µL) with samples (5 µL plasma) were then accommodated into 96 microtiter plates of 300 µL maximum capacity wells (Costar®, ME, USA). The analysis was performed using the spectrophotometer (Multiskan® Spectrum, M1500, Thermo Scientific, Vantaa, Finland). The precision and accuracy of the assays were monitored with human multi-sera levels 1 and 2 quality controls (Randox Laboratories Ltd., Crumlin, UK). The analysis of all the samples from each subject was performed on a single run and in duplicate with the coefficient of variation of less than 3%. The protocol was practical, cost-effective (saved 85% of total reagents volume) and allowed measurement of large number of samples in shorter time. The development of the protocol and measurements were conducted in the Main Laboratory, at the Human Nutrition, Level 3, New Lister Building, Glasgow Royal Infirmary, University of Glasgow. The protocle for micro-volume measurmet of plasma TAG cocnentrations is shown in Appendix A.8.









Where

GPO= glycerol-3-phosphate oxidase

POD= peroxidase

2.2.12 Statistical analyses

Data were assessed for normality of distribution using Anderson-Darling test and revealed that data was normally distributed except GPL-1 values which have been log-transformed and then was analysed using parametric tests. Data for the responses during the experimental trials were analysed using two-way repeated measures ANOVA, followed by post-hoc Tukey test. Time averaged values for the post-drink (0-90 minutes), post-meal (90-300 minutes) and the entire period of the experiment trial (0-300 minutes), calculated as the time averaged areas under the variable versus time curve (AUC), and the EI of the buffet meal were compared by paired *t*-test. Statistical analyses were performed using Statistica (version 10.0; StatSoft, Inc., Tulsa, OK) and Minitab (version 17.3.1; Minitab, Inc., State College, PA).

2.3 Results

2.3.1 Participants

Of 30 eligible participants, 4 individuals declined to take part in the study because of time commitments, and thus 26 participants underwent randomization. Two participants were excluded, as prior to the second experimental trial they did not replicate their food intake while one participant dropped out due to having difficulty to ingest the PKU protein substitute drink (Figure 2.9). Thus, the study was completed by 23 participants, of which 11 were women with a mean (±SD) age of 22.9 ± 1.0 years, BMI of 21.3 ± 1.8 kg/m^2 and body fat mass of 13.0 ± 5.4 kg, and 12 men with age of 25.7 ± 6.7 years, BMI of 23.3 ± 2.7 kg/m^2 and body fat mass of 11.9 ± 5.5 kg.

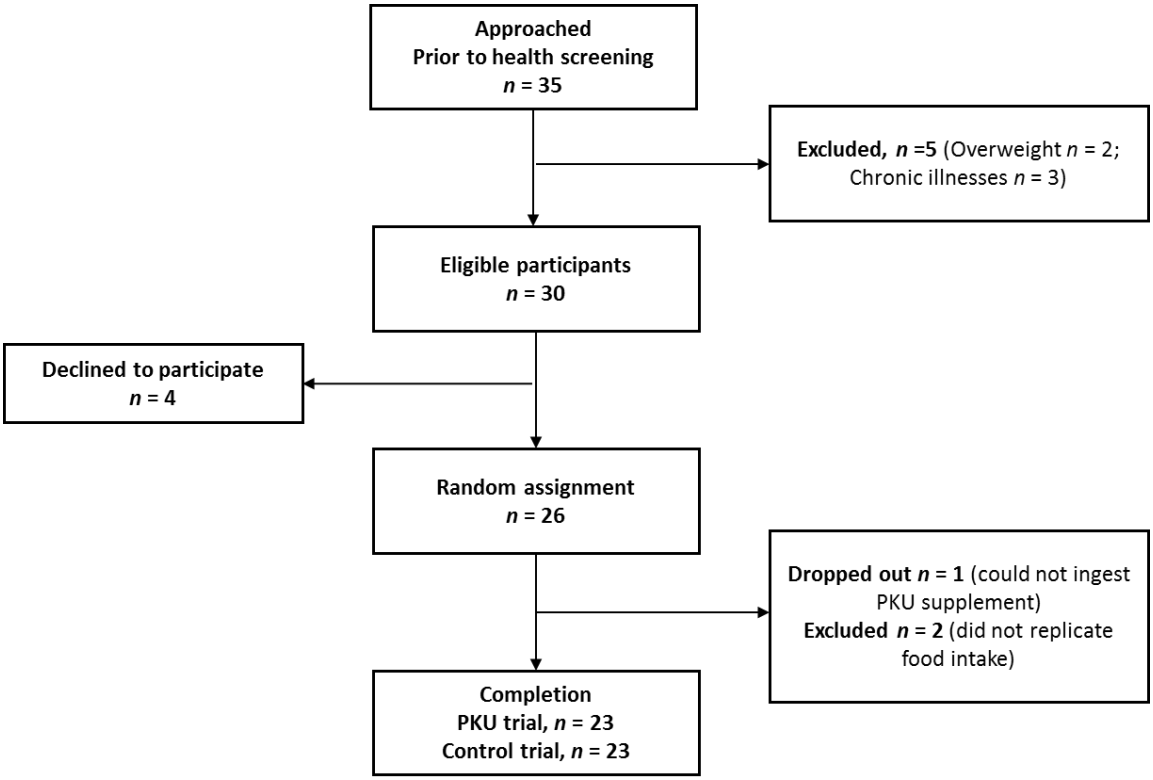


Figure 2.9: Study recruitment flow diagram

2.3.2 Appetite ratings

Responses of appetite scores during the PKU and the Control trial are presented in Figure 2.10. Responses of all appetite measures were not significantly different between the PKU and the Control trials (P>0.05, trial effects, two-way ANOVA).

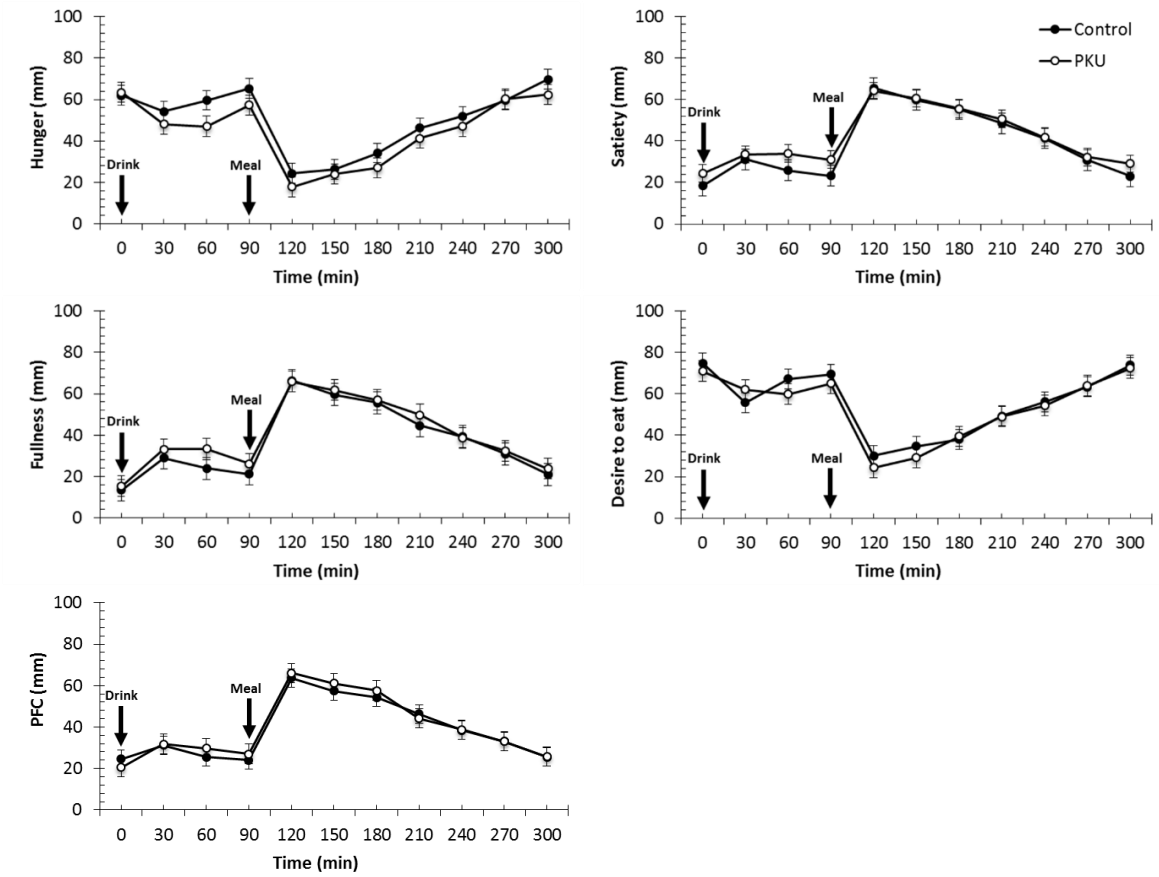


Figure 2.10: Responses of hunger, satiety, fullness, desire to eat and prospective food consumption (PFC) scores in the PKU and the Control trials (*n* = 23). Values are means ± SEM.

Time averaged appetite scores were also not significantly (*P*>0.05, paired *t*-tests) different between the PKU and the Control trials during both post-drink and post-meal periods (Table 2.2).

Table 2.2: Time-averaged subjective appetite scores (mm) during post-drink (0-90 min), post-meal (90-300 min) and the entire period (0-300 min) in the Control and the PKU trial.

	Control	**PKU**	***P* value**
Post-drink			
Hunger	58.6 ± 3.6	51.8 ± 3.5	0.12
Satiety	25.7 ± 2.5	31.7 ± 2.7	0.11
Fullness	23.2 ± 2.7	29.01 ± 3	0.17
Desire to eat	64.4 ± 3.4	63.2 ± 3.2	0.65
PFC	26.8 ± 2.7	28.4 ± 2.7	0.77

Table 2.2: Time-averaged subjective appetite scores (mm) during post-drink (0-90 min), post-meal (90-300 min) and the entire period (0-300 min) in the Control and the PKU trial.

	Control	PKU	*P* value
Post-meal			
Hunger	53.2 ± 3.5	53.9 ± 3.6	0.84
Satiety	49.8 ± 3.7	52.4 ± 3.4	0.43
Fullness	48.6 ± 3.5	51.4 ± 3.5	0.38
Desire to eat	58.8 ± 3.2	55.9 ± 3.2	0.43
PFC	48.9 ± 3.5	50.8 ± 3.5	0.26
Over 300 min			
Hunger	48.7 ± 2.9	48 ± 3.0	0.82
Satiety	40.1 ± 2.8	43 ± 2.4	0.27
Fullness	38.7 ± 2.8	41.7 ± 2.7	0.27
Desire to eat	53.8 ± 2.7	51.8 ± 2.6	0.46
PFC	39.8 ± 2.7	41.2 ± 2.7	0.36

All values are means ± SEM, *n* = 23
PFC, prospective food consumption

2.3.3 Appetite gut hormones

Responses of plasma concentrations of GLP-1 and PYY were not significantly (*P*>0.05, trial effects, two-way ANOVA) different between the two trials (Figure 2.11). Time averaged plasma concentrations of GLP-1 and PYY were also not significantly (*P*>0.05, paired *t*-tests) different between two trials (Table 2.3).

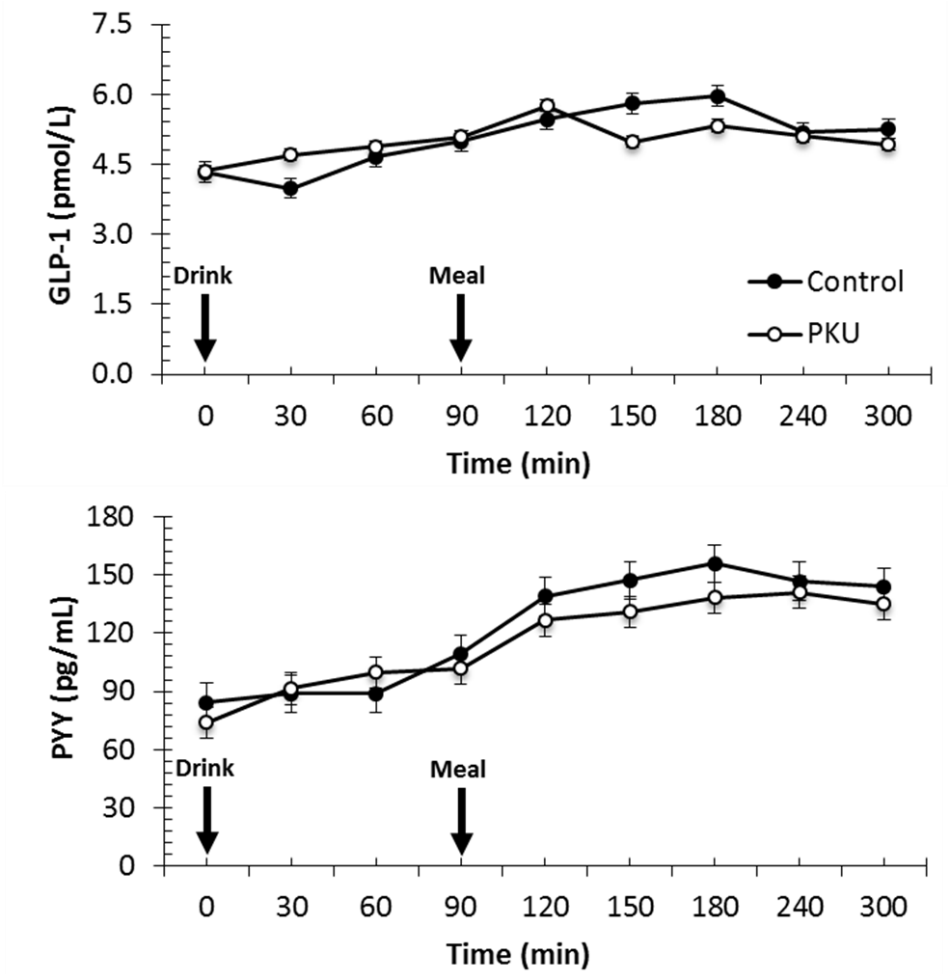


Figure 2.11: Responses of plasma concentrations of glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) in the PKU and the Control trials (*n* = 12). Values are means ± SEM.

2.3.4 Glucose and insulin responses

Responses of plasma concentrations of glucose and insulin were significantly (P<0.05, trial effects, two-way ANOVA) higher in the PKU trial than in the Control trial (Figure 2.12). Post-hoc Tukey test analysis showed that the plasma concentration of insulin was significantly (P<0.05) higher at 120 minutes in the PKU than in the Control trial, and that plasma concentrations were not significantly different between the two trials at any corresponding time point (Figure 2.12). Time averaged concentrations of insulin and glucose were significantly (P<0.05, paired *t*-tests) higher in the PKU trial than in the Control trial (Table 2.3). The differences in

time averaged plasma concentrations of insulin and glucose were significant (P< 0.05, paired t-tests) for both post-drink and post-meal periods (Table 2.3).

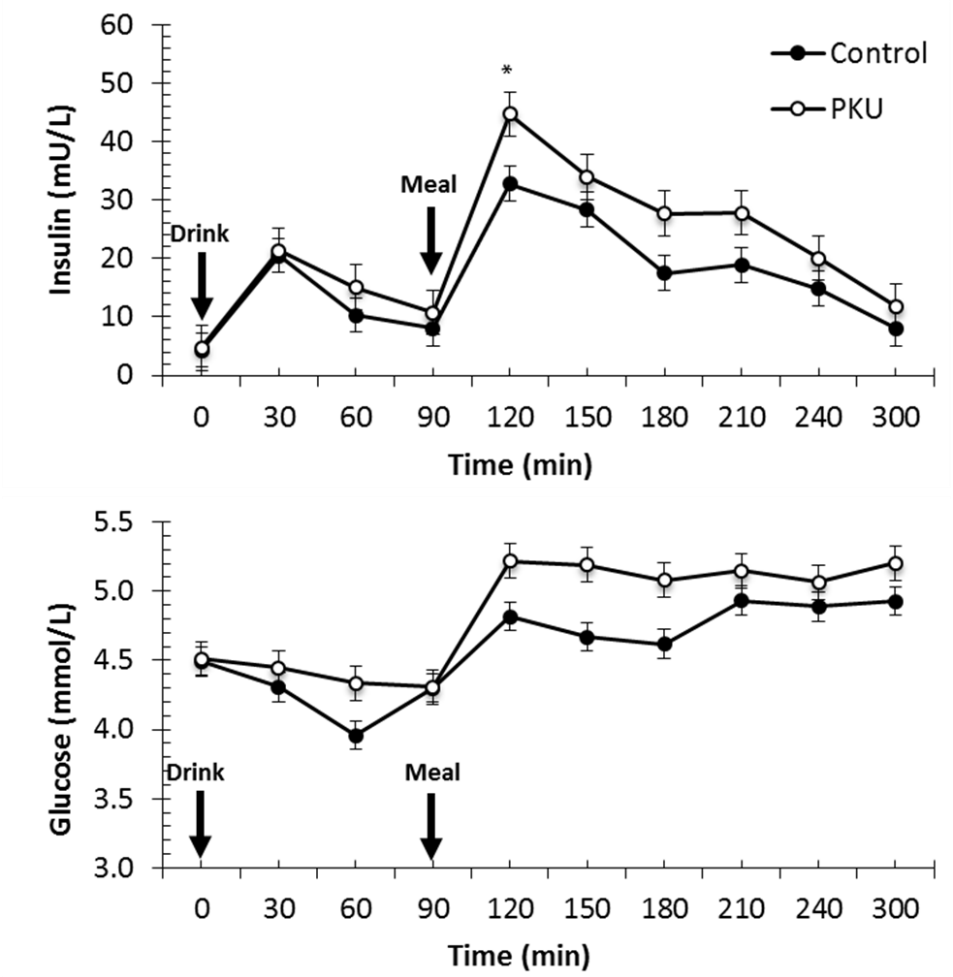


Figure 2.12: Responses of plasma concentrations of insulin and glucose in the PKU and the Control trials (n = 12). Values are means ± SEM.

2.3.5 Triglyceride (TAG) concentrations

Responses of triglyceride plasma concentrations were not significantly (P>0.05, trial effects, two-way ANOVA) different between the two trials (Figure 2.13). Time averaged plasma concentrations of triglycerides were also not significantly (P>0.05, paired t-tests) different between the two trials (Table 2.3).

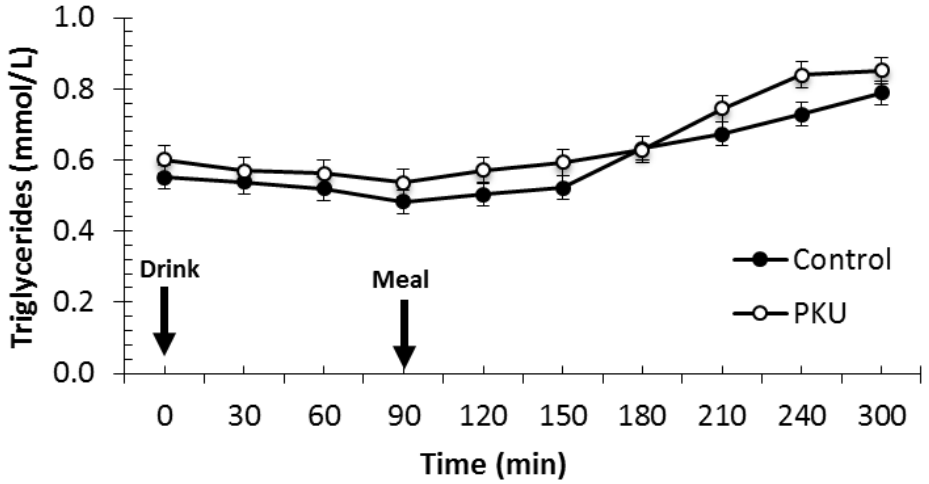


Figure 2.13: Responses of plasma concentrations of triglycerides in the PKU and the Control trials (*n* = 12). Values are means ± SEM.

Table 2.3: Time-averaged concentrations of GLP-1, PYY, glucose, insulin and triglycerides for post-drink (0-90 min), post-meal (90-300 min) and the entire period (0-300min) in the Control and the PKU trials

	Control	PKU	*P* value
GLP-1 (pmol/L)[a]			
Post-drink	4.12 ± 0.44	4.34 ± 0.46	0.09
Post-meal	5.03 ± 0.74	4.77 ± 0.52	0.64
Over 300 min	4.76 ± 0.65	4.64 ± 0.50	0.96
PYY (pg/mL)			
Post-drink	91.50 ± 17.4	93.00 ± 15.8	0.70
Post-meal	144.60 ± 16.8	133.3 ± 15.6	0.15
Over 300 min	128.7 ± 16.8	121.2 ± 15.4	0.22
Insulin (mU/L)			
Post-drink	12.70 ± 1.4	15.30 ± 2.18	0.04
Post-meal	18.60 ± 2.3	26.34 ± 3.4	0.01
Over 300 min	16.83 ± 2.0	23.03 ± 2.9	0.01
Glucose (mmol/L)			
Post-drink	4.22 ± 0.15	4.40 ± 0.12	0.02
Post-meal	4.77 ± 0.14	5.08 ± 0.22	0.02
Over 300 min	4.61 ± 0.14	4.88 ± 0.18	0.01
Triglycerides (mmol/L)			
Post-drink	0.52 ± 0.05	0.56 ± 0.04	0.45
Post-meal	0.64 ± 0.04	0.70 ± 0.06	0.41
Over 300 min	0.60 ± 0.04	0.66 ± 0.05	0.42

All values are means ± SEM, *n* = 12, [a] Statistical analysis conducted on log transformed values

GLP-1, glucagon-like peptide-1; PYY, peptide YY

2.3.6 Energy and macronutrient intake during *ad libitum* buffet dinner

During the *ad libitum* buffet dinner energy (PKU, 5.0 ± 0.3 MJ; Control, 5.3 ± 0.3 MJ), fat (PKU, 58 ± 4 g; Control, 62 ± 4 g), carbohydrate (PKU, 124 ± 9g; Control, 130. ± 9 g), and protein (PKU, 37 ± 5 g; Control, 38 ± 4 g,) intake were not significantly (P>0.05, paired t-tests) different between the PKU and the Control trials.

2.3.7 Metabolic rate and thermic effect of feeding

Resting metabolic rate (RMR) did not differ between trials (PKU, 4.14 ± 0.12 kJ; Control, 4.10 ± 0.13 kJ, P=0.4, paired t-test). Metabolic rate measured before and after drink and meal intake is illustrated in Figure 2.14. Analysis of two-way repeated measures ANOVA showed that the postprandial responses of metabolic rate were significantly different between the two trials (P<0.05, trial effect), with the value at 90 minutes post-drink intake being significantly (P<0.05) higher and nearly all values of the post-meal period significantly (P<0.05) lower in the PKU trial than in the Control trial (Figure 2.14).

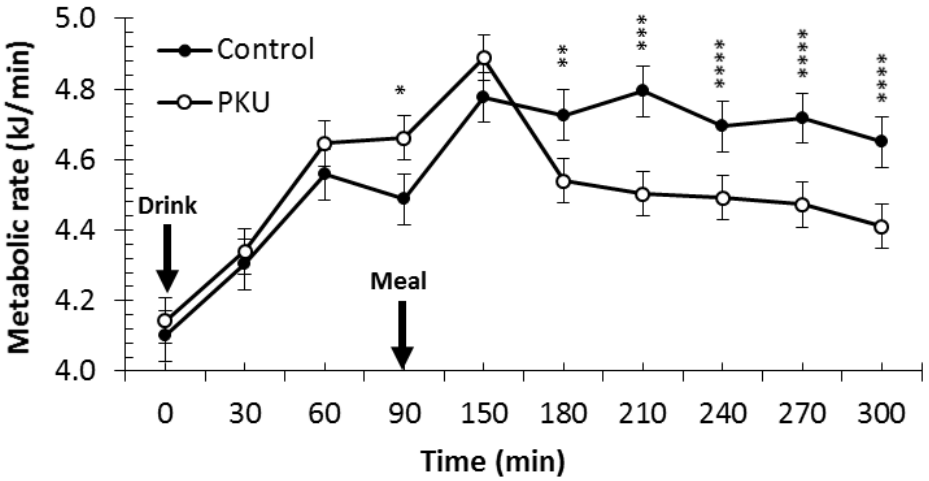


Figure 2.14: Responses of metabolic rate in the PKU and the Control trials (n = 23). Values are means ± SEM.

* P < 0.05, ** P < 0.03, *** P < 0.02, **** P < 0.001 (Tukey t-test)

Differences between the PKU and the Control trials for TEF, calculated as percentage increase in EE above RMR and the relative increase in EE (expressed as percentage of energy provided by the drink), were significant (P< 0.05, paired t-tests) only for the post-meal period (Table 2.4). The cumulative difference in energy expended above the RMR during the entire period of the experimental trials was 40.9 ± 15.8 kJ.

Table 2.4: Thermic effect of feeding calculated as percentage (%) increase in EE above RMR and as increase in EE as percentage (%) of EI in the Control and the PKU trials

	Control	PKU	*P* value
Increase above RMR (%)			
Post-drink	8.6 ± 1.1	10.1 ± 1.2	0.301
Post-meal	15.6 ± 1.2	10.3 ± 1.4	0.004
Over 300 min	13.2 ± 1.0	10.2 ± 1.5	0.056
Increase as percentage of EI			
Post-drink	6.1 ± 0.7	7.0 ± 0.7	0.35
Post-meal	6.6 ± 0.5	4.3 ± 0.5	0.001
Over 300 min	6.4 ± 0.4	4.8 ± 0.5	0.023

All values are means ± SEM, n = 23

2.3.8 Fat and CHO oxidation

Data on the time average rates of fat and CHO oxidation, the time averaged respiratory exchange ratios (RER) and the time average percentages of energy expenditure obtained from fat and carbohydrate are presented in Table 2.5. Analysis of two-way repeated measures ANOVA showed that fat and CHO oxidation rates (g/minute) were not significantly (P>0.05) different at any time point between the two trials (Figure 2.15). Following drink intake differences in the rate and the total amount of fat and CHO oxidised and the RER were not significantly (P>0.05, paired t-tests) different between the PKU and the Control trial. During the post-meal period and the entire period of the experimental trial, the rate and amount of fat oxidised

was significantly (P<0.05, paired t-tests) lower and the amount of CHO oxidised significantly (P<0.05, paired t-tests) higher in the PKU than in the Control trial (Figure 2.15 and Figure 2.16).

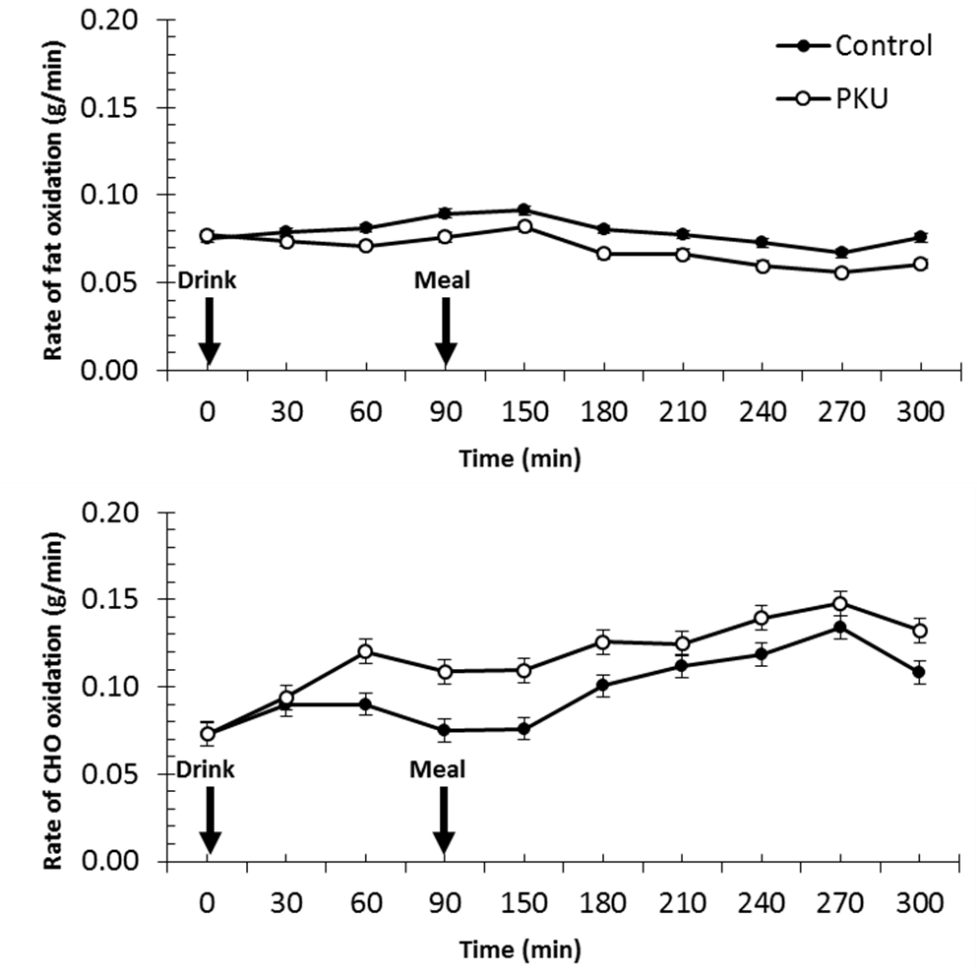


Figure 2.15: Fat and CHO oxidation rates in the PKU and the Control trials (n = 23). Values are means ± SEM.

Time averaged values of RER during the post-meal period and the entire period of the experimental trial were significantly lower (P<0.05, paired t-tests) in the Control than in the PKU trial (Table 2.5). Following drink intake, percentages of energy expenditure obtained from fat and CHO were not significantly (P>0.05, paired t-tests) different between the PKU and the Control trial (Table 2.5). During the post-meal period and the entire period of the experimental trial, the percentage

of energy expanded from fat was significantly (P<0.05, paired t-tests) lower and the percentage of energy expanded from CHO was significantly (P<0.05, paired t-tests) higher in the PKU than in the Control trial (Table 2.5).

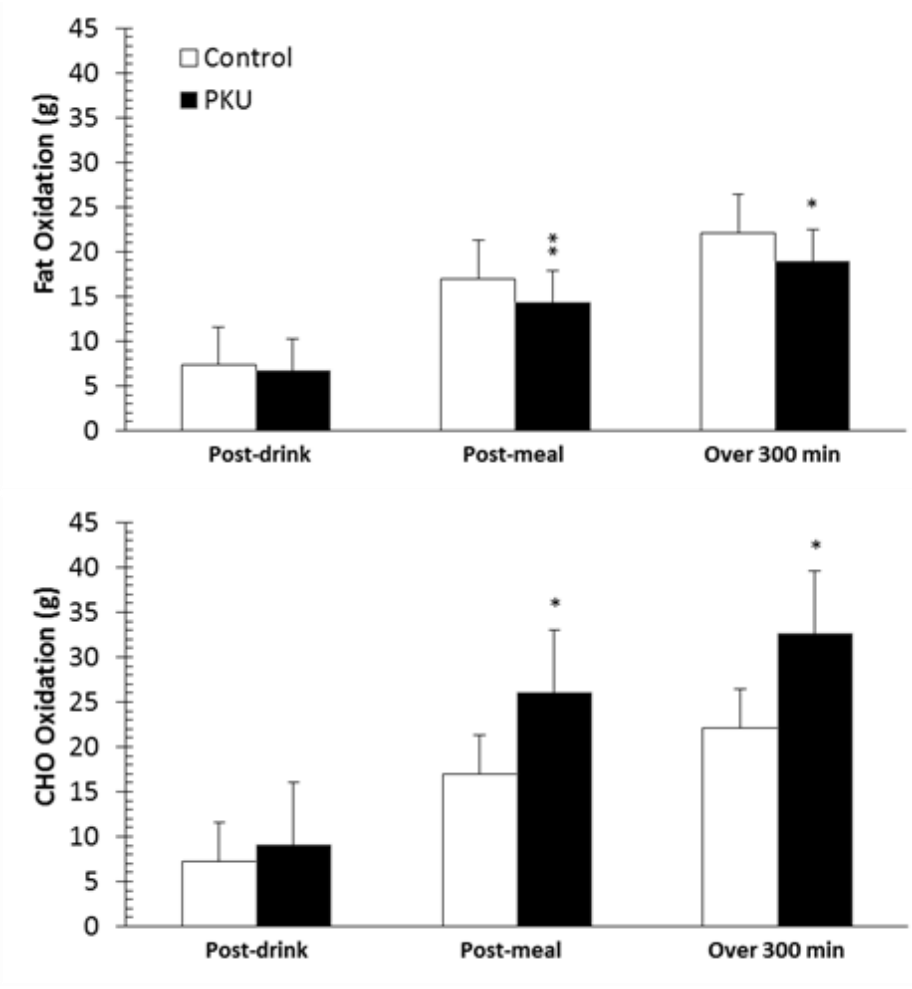


Figure 2.16: Total amount of fat and CHO oxidised during the post-drink (0-90 min) and post-meal (90-300 min) and the entire period (0-300 min) in the PKU and the Control trials (n = 23). Values are means ± SEM.
* P < 0.005; ** P < 0.001 (paired t-test).

Table 2.5: Time-averaged values of the rate of fat and carbohydrate oxidation, respiratory exchange ratio (RER), and percentage of energy expenditure obtained from fat and carbohydrate during post-drink (0-90 min), post-lunch (90-300 min), and the entire period (0-300 min) in the Control and the PKU trials

	Control	PKU	*P* value
Rate of fat oxidation (g/min)			
Post-drink	0.08 ± 0.00	0.08 ± 0.00	0.186
Post-meal	0.08 ± 0.00	0.07 ± 0.00	0.000
Over 300 min	0.08 ± 0.00	0.07 ± 0.00	0.002
Rate of CHO oxidation (g/min)			
Post-drink	0.08 ± 0.00	0.10 ± 0.01	0.155
Post-meal	0.11 ± 0.00	0.13 ± 0.00	0.006
Over 300 min	0.10 ± 0.00	0.12 ± 0.00	0.013
RER			
Post-drink	0.79 ± 0.007	0.81 ± 0.010	0.072
Post-meal	0.81 ± 0.005	0.83 ± 0.008	0.002
Over 300 min	0.80 ± 0.005	0.82 ± 0.007	0.003
EE from fat (%)			
Post-drink	70.9 ± 3.1	65.1 ± 3.9	0.176
Post-meal	67.4 ± 2.2	57.7 ± 2.9	0.001
Over 300 min	68.0 ± 2.2	59.6 ± 2.9	0.002
EE from CHO (%)			
Post-drink	30.6 ± 2.5	37.9 ± 3.9	0.079
Post-meal	34.8 ± 2.0	42.9 ± 2.9	0.002
Over 300 min	33.4 ± 1.9	41.0 ± 2.8	0.004

All values are means ± SEM, (*n* = 23)

CHO, carbohydrate; EE, energy expenditure

2.3.9 Regression of Appetite Measures on Hormone and Glucose Responses, and TEF

Regression slopes, 95% CIs and R^2 values for the regression of appetite scores on hormone and glucose concentrations obtained by considering all time points of the experimental trials of 300 minutes are presented in Table 2.6. Relations between the responses of insulin, GLP-1, PYY and responses of appetite scores showed significant correlations, while correlation for the relations between responses of glucose or TEF and responses of appetite scores were not significant.

Table 2.6: Regression analysis (Means ± SEM), 95 CIs for the means of the observed slopes and R2 values for within subject relations between appetite scores and glucose, hormone concentrations (*n* = 12) and TEF (*n* = 23)

	Slope	95% CI	R^2
Hunger *vs* glucose	-9.3 ± 4.3	-18.7, 0.01	0.2 ± 0.04
Satiety *vs* glucose	7.6 ± 4.7	-2.7, 17.9	0.1 ± 0.05
Fullness *vs* glucose	9.3 ± 5.0	-1.4, 20.0	0.2 ± 0.05
Desire to eat *vs* glucose	-7.3 ± 3.8	-15.7, 0.9	0.1 ± 0.04
PFC *vs* glucose	8.4 ± 4.3	-0.9, 17.8	0.2 ± 0.06
Hunger *vs* insulin	-0.9 ± 0.1*	-1.3, -0.6	0.3 ± 0.05
Satiety *vs* insulin	0.8 ± 0.1*	0.6, 1.1	0.3 ± 0.05
Fullness *vs* insulin	0.9 ± 0.1*	0.7, 1.2	0.3 ± 0.05
Desire to eat *vs* insulin	-0.9 ± 0.1*	-1.1, -0.7	0.3 ± 0.04
PFC *vs* insulin	0.8 ± 0.1*	0.6, 1.0	0.3 ± 0.04
Hunger *vs* GLP-1	-11 ± 4.0 *	-19.3, -2.1	0.1 ± 0.03
Satiety *vs* GLP-1	13.3 ± 4.2*	4.2, 22.4	0.1 ± 0.03
Fullness *vs* GLP-1	15.5 ± 4.3*	6.3, 24.1	0.1 ± 0.04
Desire to eat *vs* GLP-1	-11.5 ± 3.0*	-16.7, -6.0	0.1 ± 0.04
PFC *vs* GLP-1	10.2 ± 2.7*	4.4, 16.0	0.1 ± 0.04
Hunger *vs* PYY	-0.2 ± 0*	-0.3, -0.1	0.1 ± 0.03
Satiety *vs* PYY	0.3 ± 0*	0.1, 0.4	0.1 ± 0.04
Fullness *vs* PYY	0.3 ±0*	0.1, 0.5	0.2 ± 0.04
Desire to eat *vs* PYY	-0.3 ± 0*	-0.4, -0.1	0.2 ± 0.04
PFC *vs* PYY	0.2 ± 0*	0.1, 0.4	0.2 ± 0.04
Hunger vs TEF	-5.9 ± 7	-20.4, 8.6	0.1 ± 0.02
Satiety vs TEF	8.5 ± 8	-7.2, 24	0.1 ± 0.03
Fullness vs TEF	7.8 ± 8	-9.1, 24.7	0.2 ± 0.03
Desire to eat vs TEF	1.8 ± 7	-13.1, 16.7	0.1 ± 0.02
PFC vs TEF	6.3 ± 7	-7.4, 20	0.2 ± 0.03

* Mean of the regression slopes significantly different from zero, $P < 0.05$ (Student's one sample *t* test)

PFC, prospective food consumption; GLP-1, glucagon-like peptide-1; PYY, peptide YY; TEF, thermic effect of feeding

2.4 Discussion

This study reports for the first time that the consumption of a meal based on PKU special low protein foods, lacking in protein and composed of CHO and fat, diminishes the TEF and reduces postprandial fat oxidation, however it has no detrimental impact on subjective appetite measures, plasma concentrations of appetite regulating gut hormones, triglycerides and subsequent EI during *ad libitum* buffet dinner. Collectively, these data suggest that long-term adherence to a PKU dietary regimen may potentially contribute to the development of overweight and obesity. Although the trend of obesity and overweight in PKU is similar to that in general population [184, 186, 187], our data suggests that development of obesity in PKU patients may be related to wider spectrum of risk factors.

One of the study aims was to investigate whether meal based on PKU special low protein foods has a detrimental impact on appetite scores and plasma concentrations of PYY and GLP-1. Thus, study participants were investigated in a counter-balanced manner after the consumption of 2.0 MJ containing Control meal (18% of energy from protein, and 36 % and 47 % of energy from CHO and fat, respectively) and after an isocaloric PKU type meal lacking protein (54 % and 46 % energy from CHO and fat, respectively). Regardless of the differences in the macronutrient composition of the two meals, subjective appetite and satiety scores and plasma concentrations of PYY and GLP-1 during the post-meal period were not significantly different between the PKU and the Control trials. In addition, EI during the subsequent ad libitum buffet style meal consisted of approximately 5 MJ and was also not significantly different between the two trials. Thus, the hypothesis that whole protein lacking and CHO and fat based PKU type meals detrimentally affect appetite and satiety regulation cannot be supported by our findings. This, when combined with other evidence [252-254], suggests that changes in satiety and

appetite regulating hormones might be expected when the protein content of a meal becomes higher rather than lower in comparison to the protein intake provided by the habitual diet. Regardless of PKU meal based on special low protein foods having no impact on appetite hormones, the concentrations of GLP-1 after ingestion of PKU protein substitute drink tended to be higher than after milk-based drink. This most likely coincided with greater increase in plasma concentrations of amino acids, which have been reported to be related to appetite hormones and satiety responses [349].

As expected, due to higher CHO content of the PKU lunch, plasma glucose and insulin glucose responses during post lunch period were significantly higher in the PKU than the Control trial. Plasma glucose and insulin glucose responses after drink intake were also significantly higher in the PKU than in the Control trial. This difference was found regardless the glycaemic index of the PKU protein substitute drink being as low as 19% (value provided on request from the manufacturer). Therefore, significantly higher glucose and insulin concentrations in the PKU trial can be explained by protein substitute drink being composed of free amino acids and Control drink providing whole protein. It is known that ingestion of free amino acids in comparison to ingestion of equivalent amount of whole protein induces higher insulin [132] and glucose [350] responses. Thus, the finding of enhanced postprandial responses of insulin and glucose found after both consumption of PKU protein substitute drink and lunch implies that long term PKU type dietary regimen may lead to the development of insulin resistance.

The plasma triglyceride responses were not significantly different between the PKU and Control trials during either post-drink or post-lunch periods. It has been reported that the main determinant of postprandial TAG levels is the amount of fat and energy ingested with the meal [351]. Thus, not significantly different TAG concentrations

between the two trials found in this study was due to similar fat and energy content of both protein drinks and lunch meals provided during the two trials. However, studies on patients with PKU revealed that plasma concentrations of TAG and VLDL-cholesterol [352] were significantly higher, and HDL-cholesterol were significantly lower [185] than in healthy matched controls. When compared to normal-type meals, PKU-type meals are very rich in CHO, and have higher glycaemic index [104] which can be expected to enhance plasma TAG [353, 354]. Therefore, lifelong treatment with PKU-type meals may detrimentally alter lipid metabolism, and enhance atherogenic risk in patients with PKU [353-355].

This is the first study investigating how a PKU type meal modifies TEF, a metabolic factor known to contribute to the regulation of energy balance and thus development of overweight and obesity [210]. In accordance with findings that TEF of separate nutrients is highest for protein [252, 261, 334], we found that during post-meal and the entire period of the experimental trial TEF was significantly lower in the PKU than in the Control trial, and that the cumulative difference in energy expended above RMR between trials lasting for 5 hours consisted of approximately 41 kJ. While this is a small difference and TEF varies within the range of 5–15% of total daily EE, long-term consumption of meals based on PKU special low protein foods can result in diminished daily EE and induce body weight gain of approximately 1 kg in a period of a single year [220]. Thus, unfavourable attenuation of TEF induced by PKU type dietary regimen may be among the physiological mechanisms explaining the risk of obesity in patients with PKU [184, 186, 187].

Previous evidence suggests that reduced fat oxidation promotes a positive fat balance and is one of the multifactorial origins of obesity [210]. Thus, we investigated how a PKU protein substitute and meal impact postprandial fat oxidation. We found that during the post-meal and the entire period of the

experimental trial the amount of fat oxidised was significantly lower and the RER significantly higher in the PKU than in the Control trial. Diminished fat oxidation found in the PKU trial can be explained by the enhanced CHO content [252, 297] and therefore higher insulin response, and possibly lack of protein [297] in the PKU meal. Thus, regardless of the mechanisms involved in reduced fat oxidation following the PKU meal, the obtained data suggests that the increased overweight and obesity reported in patients with PKU [184, 186, 187] may, at least in part, be related to the detrimental impact of PKU type foods on fat oxidation. Thus, future studies should investigate whether fat oxidation of the PKU population differs from that of matched controls and if so, how this affects energy balance and obesity in this group of patients.

Since the required amount of amino acids in a PKU type diet is obtained from consuming PKU protein substitutes [315, 331], the design of the experimental trial aimed to mimic a realistic scenario encountered in the daily life of patients with PKU and thus also involved consumption of the typical PKU protein substitute. The PKU protein substitute drink consisted of L-amino acids (free from PHE) while the Control drink was whole protein-enriched milk. The drinks were energy and volume matched and consumed 90 minutes prior to the intake of the corresponding meal. We found that appetite scores and plasma GLP-1 and PYY responses, as well as rate of fat oxidation and RER measured for 90 minutes after the PKU protein substitute drink, were not significantly different from the responses found after the intake of the Control drink. The TEF during the post-drink period was also not different between the PKU and the Control trials. However, at 90 minutes after drink intake, metabolic rate was significantly higher in the PKU than the Control trial. This enhancement in the metabolic rate was counterbalanced quickly after the consumption of the PKU type meal. Thus, when combined, findings of the post-drink and post-meal periods,

imply that the PKU type dietary regimen may have adverse effect on energy balance due to the consumption of special low protein foods rather than the intake of the PKU protein substitute drink.

In this study responses of hunger, fullness, satiety and prospective food consumption (PFC) scores paralleled with plasma concentrations of GLP-1, PYY, and insulin. Thus, it was of interest to further explore the relations between gastrointestinal hormones, glucose, insulin and responses of appetite scores and contribute to the debate whether they can can be considered as biomarkers of appetite [264, 356-358]. Correlation of TEF as another potential regulator of appetite was also explored [236, 256, 262]. To achieve these, we used a statistical approach that emphasises on within participant relations between changes in appetite scores and changes in glucose and hormone concentrations and TEF [358] and, obtained regression slopes and R^2 values for the regression of appetite scores on glucose and each of the measured appetite hormones and TEF. As in some previous studies [357-360], we found that subjective appetite measures were significantly associated with plasma concentration of GLP-1 and PYY, and insulin but not glucose or TEF.

This study is not without limitations. Study participants were healthy men and women. We appreciate that the responses found in healthy individuals may not be applicable to patients with PKU. Therefore, future studies should investigate how measures considered in this study are impacted by meal based on special low protein foods consumed by patients with PKU. The relevance of the attenuated TEF and reduced postprandial fat oxidation for the enhanced prevalence of obesity reported in the PKU population [184, 186, 187] should be confirmed by future studies comparing how these factors are influenced by long-term adherence to meals based on PKU special low protein foods and how they differ between patients with PKU and healthy matched controls. It can be argued that the 90 minute interval

used between drink and meal intake is another limitation of this study as in clinical practice of PKU management dietitians advise protein substitute intake in very close proximity to the meal [361]. On the other hand, it has been reported that a reasonable proportion of patients with PKU fail to consume prescribed protein substitutes [123, 128, 133]. Future studies should investigate combined effect of both PKU protein substitute and meals based on special low protein foods on research outcomes of this study. The responses of gut hormones and other blood parameters were measured in a subset of the volunteers since previous research [252, 297, 359] has shown that using this number allowed identification of meaningful and statistically significant differences in plasma concentrations of PYY and GLP-1 and other blood parameters measured in this study. In addition, post-hoc power calculations revealed that in case of PYY study achieved more than 85% power.

Strength of this study related to the cross-over design which helped to reduce the large variations caused by inter-individual differences. Additionally, to further diminish the risk of intra-individual differences, female participants were required to take part while on the same phase of their menstrual cycle. This was achieved by asking all female participants to start at the very beginning of menstrual cycle phases and the second trials were conducted after 5 days wash-out. Thus, it can be said that both trials were conducted during either follicular or luteal menstrual phases. Although metabolic rate measurements might have not been affected by this [362], the menstrual phase could have induced slight changes in hormone responses, particularly in insulin levels [363, 364].

2.5 Conclusions

The main findings of this study suggest that consumption of SLPF-based meals has no detrimental impact on subjective appetite ratings, gut appetite hormones and energy intake in healthy volunteers.

Consumption of meal based on SLPF produces a lower TEF and postprandial fat oxidation than an ordinary meal in healthy volunteers. These metabolic alterations may contribute to the origins of obesity in people with PKU on contemporary dietary management.

Phenylketonuria type meal has no impact on plasma TAG compared to ordinary isocaloric type meal, but significantly increases postprandial plasma concentrations of glucose and insulin. Therefore, patients following such lifelong dietary treatment may have increased atherogenic risk.

The study results confirm the notion that GLP-1, PYY and insulin are regulators of appetite and that glucose or TEF does not contribute to appetite regulation.

Chapter Three: Contribution of Thermic Effect of Feeding and Fat Oxidation to Body Fatness and Prevalence of Obesity in Patients with PKU

3.1 Background

Phenylketonuria (PKU) is an incurable condition and the current management is based on a 'low-PHE diet' which involves restriction of dietary PHE, by reducing the natural protein intake, and concomitant supplementation with all other essential amino acids, in order to meet body requirements [102]. Therefore, protein foods rich in PHE, such as meat, cheese, poultry, eggs and milk, and plant/vegetable proteins such as potatoes and cereals are not permitted or restricted to very small amounts in the 'low-PHE diet' [315, 365]. Alternatively, manufactured special low protein foods (SLPF) are prescribed for patients with PKU to cover energy requirements and provide variety in their diet [103]. These foods are reported to be high in CHO and sometimes fat, and have high glycaemic index and provide more energy per weight unit compared to their protein containing equivalent normal foods [103-105]. The restriction level of the low-PHE diet depends on patients' tolerance to dietary PHE with the diet being more 'relaxed' in those who are above 10 years old and brain development has been completed [73, 89].

Nutritional deficiencies and malnutrition have frequently been reported in patients with PKU mainly due to the very restrictive nature of the low-PHE diet [102]. However, in recent years malnutrition, in terms of undernutrition, is not common [182, 187]. This is mainly due to the development and availability of PKU protein substitutes and SLPF for people with PKU [73, 124, 168, 182, 366]. These SPLF are central for PKU management and believed to improve patients' adherence to dietary treatment [103, 108]. Recent evidence though reports an increasing prevalence of obesity in PKU [107, 134, 184-187]. A study by Burrage and colleagues revealed that the percentages of overweight and obese PKU females were 55% and 33%, respectively [184]. In addition, previous studies [134, 192] found an increased tendency of overweight/obesity in females with PKU from the

beginning of adolescence. While the prevalence of obesity in patients with PKU is similar to that in healthy population [105, 134, 185, 186, 188], the underlying aetiology of obesity in this group of patients remains unknown.

Likewise, scarce evidence suggests that people with PKU tend to have a higher fat mass and FFM when compared to healthy individuals [107, 192, 197-202]. This infers that for a given weight and height people with PKU would be fatter and perhaps at higher risk of cardiovascular diseases [204], insulin resistance [205] and type-II diabetes [206]. However, this evidence is still not conclusive. Data obtained using skinfold-thickness, Dual-energy X-ray absorptiometry (DEXA), body impedance analysis (BIA) and total body electrical conductivity (TOBEC) showed no significant differences in measured body compartments between patients with PKU and healthy controls [107, 197-201] but the percentage of body fat was significantly higher in patients with PKU than in healthy controls when air-displacement plethysmography (ADP) technique was used [192]. Inconsistency in the findings of previous research may be explained by different body composition techniques employed and small sample size and inappropriately matched control groups [107, 192, 197-201]. Indeed all of the previously applied body composition techniques suffer from inaccuracy and imprecision bias, particularly when they are applied to people with chronic illness [195]. Hence, more studies applying gold standard techniques to measure body fatness and lean mass and considering an appropriate age-gender matched controls are needed.

Considering the increasing prevalence of obesity and limited evidence which reports a higher prevalence of adiposity in patients with PKU than in controls, more research is required to address the origins of obesity in people with PKU and whether these differ from people without the condition. Increased obesity in people with PKU may be related to either increased energy intake from consumption of high CHO/fat foods

or reduced energy expenditure, or a combination of both, leading to positive energy balance [367]. Research on PKU showed decreased levels of catecholamines, serotonin [275], while increased levels of leptin [238] in patients with PKU, particularly those with poor metabolic control, which may affect many metabolic pathways related to energy balance and energy substrate metabolism [80-82, 276, 277].

Resting metabolic rate (RMR) is the largest component (60 – 75%) of total daily energy expenditure (TDEE) [268]. Body weight and body composition are the main determinants for RMR [270, 271], with FFM being a much greater predictor of RMR than FM [210]. Obese individuals have higher RMR compared to non-obese individuals due to larger body size and higher amounts of FM and FFM [271]. When RMR values are adjusted for FFM, the difference between obese and non-obese individuals disappear [270, 278]. However, variations between individuals still exist despite the adjustment for body composition [210, 279-281]. Since there is a tendency of patients with PKU having higher FM and lower FFM [107, 192, 201, 202], it is possible that they have lower RMR. Therefore, evidence on RMR and contribution to aetiology of obesity in PKU remains unclear.

Despite its significance in nutritional management and status, very little information is available on energy metabolism of patients with PKU with RMR being measured only in two studies [201, 274]. Allen and colleagues evaluated RMR in 30 patients with PKU and found no significant difference from healthy controls, with tendency of PKU females having lower RMR than control females (4703 vs 5167 kJ/d) [201]. However, the control group was not properly matched as patients in PKU group were younger, and had significantly lower FFM, measured by BIA. Another study conducted by Quirk and others measured RMR in 36 female adolescents with PKU but did not compare to a control group [274]. Therefore, research is needed to clarify

whether RMR is different from healthy matched controls while applying better techniques to measure body composition.

Thermic effect of feeding (TEF) is another component of energy expenditure (EE) and comprises 10 – 15% of TDEE [282]. It is calculated as amount or percentage of increase in EE above RMR or proportional to energy intake after meal or food ingestion [284, 286]. Prior research showed that high protein meals produce higher TEF and postprandial fat oxidation, compared to isocaloric high CHO or fat meals [251, 252, 259, 288, 297]. In our previous study conducted on healthy individuals (Chapter 2), we investigated the effect of a 'PKU type' meal (SLPF-based meal) consumption on TEF and postprandial fat oxidation. We found that TEF measured for 5 hours was significantly lower by 40 kJ after consumption of PKU type foods. Although this small difference between the two trials, this can result in diminished daily EE, and if repeated on a day-by-day basis, this can induce weight gain of 1 kg in a single year [220, 288]. Fat oxidation was also lower after consumption of PKU type compared with the Control (normal-type) meal. Although the mechanistic link between obesity and a reduced capacity of fat oxidation is yet to be characterised [290], some studies have considered fat oxidation to be a potential regulator of energy balance and contributor to the aetiology of obesity [210, 368-370]. The results from our previous study (Chapter 2), thus suggest that reduced TEF and changes in body energy substrate oxidation may be among factors contributing to the increased risk of overweight and obesity in patients with PKU. These should be investigated by comparing TEF and, fasting and postprandial fat oxidation between patients with PKU and age-gender and BMI matched controls.

No studies so far have measured the TEF and substrate oxidation in patients with PKU and compared these against well-matched healthy controls. In addition, there is a need to clarify whether RMR is different from healthy matched controls while

applying accurate techniques to measure body composition. Contribution of these factors to body fatness and body composition of patients with PKU should also be explored.

We hypothesise that obesity and adiposity in patients with PKU are the result of alterations in components of energy expenditure and energy substrate oxidation. In addition, patients with PKU will have higher body fatness (adiposity)' compared with healthy, socio-demographic characteristics and BMI-matched controls. Thus, this is a pilot study which aims to investigate whether components of energy expenditure such as RMR and TEF, along with energy substrate oxidation and compartments of body composition are different between patients with PKU and sociodemographic and age-gender/BMI-matched healthy controls. In addition, this pilot study will allow power calculation to estimate the number of participants needed for a larger-scale study.

3.2 Methods

3.2.1 Participants

Children and adults (10 – 45 years old) with PKU attending the metabolic medicine clinics in the area of NHS Greater Glasgow and Clyde (GGC) were approached for recruitment. Participants were free from history of acute and chronic illness (other than PKU) requiring regular appointments to the doctor and/or chronic use of medication or major gastrointestinal surgery where major part of the gut has been resected as these conditions are known to impact on energy expenditure and dietary intake [371-376]. In addition, pregnant or lactating women were also excluded from participation. People with learning or mobility difficulties or those with incapacity to provide informed consent were excluded. The clinical treatment team evaluated patients' capacity to consent before they have been considered to participate in the

study. The study (Appendix B.1) was approved by the NHS South West Health Research Authority with REC reference ID: 16/SW/0288, and IRAS project ID: 205652). The study was registered at ClinicalTrials.Gov database with identifying number NCT03309345.

3.2.1.1 Identification of eligible participants with PKU

Eligible patients were identified from the clinical appointment lists held by the medical team of the metabolic medicine clinics in NHS GGC. A member of the treatment team sent an introductory letter (Appendices B.2 – B.5) about the study with the study information leaflet to those participants who were eligible to participate. This gave adequate time for the participants and their carers to consider the study prior to their subsequent clinical appointment.

On the day of their clinical appointment a member of the clinical team asked the participants if they have considered the study and they would be interested in participating. Those who expressed an interest were then introduced to the researcher at the end of their clinical appointment. The researcher discussed the study with participants who were interested in the study and answered any question they had directly after their clinical appointment and scheduled a study visit at the Metabolic Research Unit, in Human Nutrition, University of Glasgow, Glasgow Royal Infirmary. On the day of study visit, the participants completed a consent form (Appendices B.12 – B.13) at the Metabolic Research Unit. The consent form was photocopied for patient, study records and patient's medical notes. For children (10 - 15 years old), the parent/carers were asked to provide assent (Appendix B.11) in addition to participant's consent. Although we believe that the children are old enough (age 10 to 15) to consent for themselves, their competency to provide informed consent was also checked with a member of the clinical team. Participants

between 10 to 15 years old were asked to attend along with their parent/carers. The researcher explained to the participants that they have every right to drop out of the study at any point without an explanation, if they feel this is not suitable for them. To increase recruitment rate, the researcher presented the study at the PKU group sessions, cooking and social events that were organised by the local clinical team.

3.2.1.2 Healthy controls

Individual characteristics of patients with PKU in relation to gender, BMI and age were taken into consideration during recruitment of controls. Pregnant or lactating women were excluded from participation. Those with any chronic illnesses or bone injuries were also excluded. Controls were recruited by means of poster advertisement, university's email-broadcasting and word of mouth in the campus of the University of Glasgow, and other public areas (libraries, train/bus stations). Subjects who have expressed an interest to participate were sent a participant information leaflet (Appendices B.6 – B.8). Participants had as much time as they feel is necessary to decide whether they would like to participate in the study. Considering the participant is eligible and happy to participate, a study visit was organised in a similar way to that arranged for patient participants. The researcher explained to the participants that they have every right to drop out of the study at any point without an explanation, if they feel this is not suitable for them. Interested participants were asked to provide informed written consent by both children and their carers (Appendices B.14 – B.16).

3.2.2 Study design

The study utilsed a case-control design in which participants were allocated to either PKU or healthy control groups. Participants were asked to attend the Metabolic Research Unit at NLB (GRI) after 12-hour overnight fasting refraining from alcohol

consumption or smoking for at least 24 hours. Participants were instructed to avoid any planned exercise during the 48 hours prior visit to the Metabolic Research Unit. In the morning of the study visit, participants were advised to travel by a comfortable mean (own cars or taxi) and travel expenses were reimbursed. Height, body weight and handgrip strength were measured upon arrival at the unit. RMR was measured between 7:00 – 10:00 AM for duration of 20 minutes. Participants were asked to provide a baseline saliva sample followed by dose consumption of 4.53 g (for children) and 9.07 g (for adults) deuterium (D_2O) diluted in drinking water (87% of the total solution). Normal or PKU-type isocaloric (children, 1653.1 kJ; adults, 2056.4 kJ) breakfast meal was then provided based on intervention group-type (Control or PKU accordingly). Following breakfast, TEF was measured at 30, 60, 90, 120, 150 and 180 minutes, with each measurement lasting for 20 minutes (including 10 minutes rest in-between measurements). Two saliva samples were collected at 3 and 3.5 hours after ingestion of the D_2O dose. A schematic diagram of the study design is illustrated in Figure 3.1.

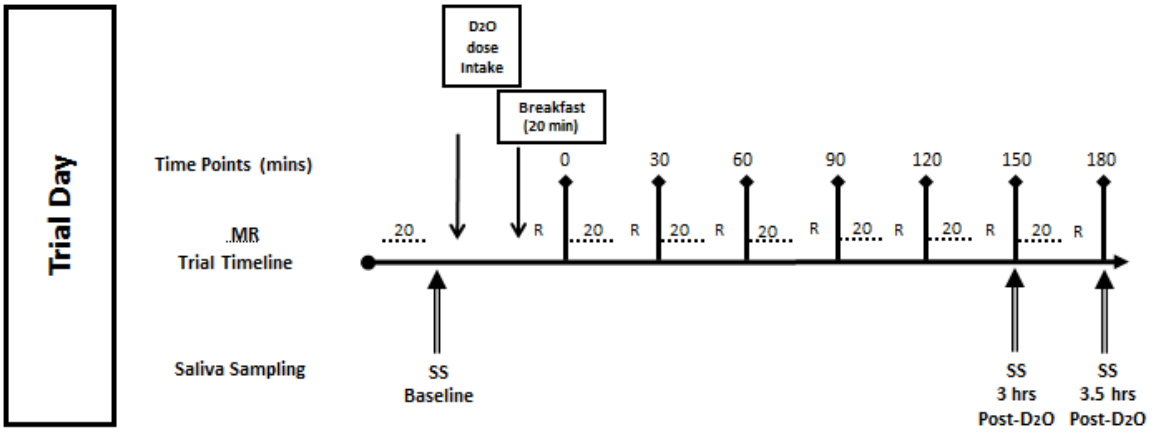


Figure 3.1: Schematic Diagram of Experimental Trial

R, in between measurements resting time (10 min); MR, metabolic rate measurement (20 minutes); SS, saliva sampling

3.2.3 Anthropometric measurements

3.2.3.1 Height

Height was measured using a Harpenden wall-mounted stadiometer. Participants were asked to stand barefoot, with their back against a fixed backboard and their arms hanging laterally by their body (Figure 3.2). The head was positioned with the line of eyesight perpendicular to the backboard. The participants were instructed to relax and a moveable headboard was lowered to the top of the head with light pressure added to compress the hair. Gentle upward pressure was applied to the lower jaw and height was measured to the nearest millimetre. The height z-scores were calculated according to the UK's 1990 growth data in which height is considered short if z-score was < - 2, or normal if z-score was > - 2 [377].

Figure 3.2: Child participant performing a height measurement on Harpenden wall-mounted stadiometer (Holtain Ltd, Crymych, Pembs, UK)

3.2.3.2 Body weight

Body weight was measured using a digital scale (Tanita - TBF-310, UK). Before measurement, participants were asked to remove extraneous clothing and jewellery. The measurement was performed while the participant wearing minimal clothing, typically lightweight shorts, a t-shirt and without shoes or socks. The participants were instructed to stand with both feet flat on the scale and their arms hanging laterally by their body. The measurements were performed to the nearest of 0.01 kg. The body mass index (BMI) was derived as weight (kg) divided by height (m) squared (kg/m^2). The weight and BMI z-scores were calculated according to the UK's 1990 growth data. Weight is considered low if z-score below < - 2, normal if between > - 2 to 1.036, and high if above 1.036. BMI is considered thin if z-score below < - 2, normal if between > - 2 to 1.036, overweight if between 1.036 and 1.645, and obese if above 1.645.

3.2.4 Body composition

3.2.4.1 Total body water

Body composition is frequently divided into two components, body fat mass and fat-free mass (muscle, bone, and water) [378]. Water is the largest chemical component of human body and is essential medium of the body's internal environment and its functioning [379]. Total body water can be measured accurately by deuterium oxide (D_2O) dilution technique. Deuterium oxide (D_2O) dilution is a well-established technique to measure total body water (TBW) from which body composition is estimated using a two-compartment model, assuming the body is composed of fat (FM) and fat-free mass (FFM).

The D_2O dilution is a reference and safe method for determining TBW [380], and has been widely used in children and clinical setting studies [381-386]. The natural

abundance of deuterium is 0.015% [387]. This means that an average adult man of 72 kg weight with 43.5 kg body water contains approximately 6.25 grams of deuterium. Deuterium oxide is a labelled water (D_2O) with stable isotopes (tracers) in which 99.8 or 99.9% of the hydrogen atoms are in the form of deuterium. This is referred to as 99.8 (or 99.9) atom% ^2H_2O or as D_2O. In normal physiological circumstances water content of FFM or lean body mass is constant and equals to 73.2% [388] in average adults or ranges between 74 to 79% in children [389-391]. Thus, FFM (kg) equals TWB (kg) divided by the constant hydration factor of lean tissue [387]. FM is calculated as the difference between FFM and body weight.

3.2.4.2 Deuterium oxide (D2O) doses preparation and storage

The children and adults study participants were given a fixed age-specific (child vs adult) dose of deuterium labelled water following guidelines from the International Atomic Energy Agency [387] in Vienna, Austria [387]. Deuterium stock solution and doses were prepared in a clean food preparation area at Scottish Universities Environmental Research Centre (SUERC), Glasgow, UK. All study D_2O doses were prepared with the same batch of a single stock of deuterium solution. A 5-litre flask was weighed with the cap on using an electronic sensitive scale of four decimal digits. The scale was then set to zero. A 3.205 litres (3205.01 g) of drinking water was added to the flask and weighed while the cap is placed on the scale. A 415.3 g of known deuterium oxide (D, 99.9%-DLM4, Cambridge Isotope Laboratories, Inc., MA, USA) was added (Deuterium solution). The solution was then shaken gently to ensure complete mixing and allowed to remain for few minutes at room temperature. Screw capped, and leak proof bottles [125-mL] (Nalgene®, Thermo Fisher Scientific, UK) were labelled with dose ID, stock batch number and date. Each bottle with the lid was weighed using an electronic sensitive scale of four decimal digits, and the weight was recorded (i.e. empty Bottle weight). A 35 or 70 g of deuterium (D_2O)

solution was added to each bottle to ingest by child or adult participant, respectively (Figure 3.3).

Figure 3.3: Deuterium (D_2O) water dose ready for ingestion

3.2.4.3 Saliva sampling and storage

Measurement of TBW using saliva is a none-invasive, less-burden and valid sampling method, and produces similar results as those obtained from other sampling methods i.e. plasma and urine [392-394]. Saliva samples were collected from the participant's mouth by using a passive cotton ball soaking sampling method (Figure 3.4). The participants were asked to transfer the cotton ball into a disposable 20-ml syringe (Omnifix®, Braun, Germany) with the plunger being removed. The plunger was then replaced into the body of the syringe and used to extract the saliva from the cotton ball into sample tubes. After collection of baseline sample, 35 or 70

g of deuterium oxide solution was given to child or adult participant, respectively, to drink orally via a plastic straw. About 50 mL of drinking water was added to the dose bottle and the participant was asked to drink it through the same straw to ensure that no labelled water was left in the bottle. Participants were fasting and had no drink for at least 30 minutes before the samples were collected. All samples were collected into a 2-mL tightly capped cryogenic tubes pre-marked with participant's ID, D_2O dose bottle ID number and collection time point, and were immediately stored in an upright freezer at -20°C until analysis.

Figure 3.4: Equipment used for saliva sampling (Cotton ball soaking method)

Gloves, Cotton balls, 20-mL syringe, 2-mL labelled cryogenic tubes

3.2.4.4 Analysis of deuterium enrichment in saliva samples and calculations of body composition

Samples were transferred into dry ice package to SUREC Isotope laboratory in Glasgow, UK, where analysis was performed. Enrichment of deuterium in saliva samples was determined using Fourier Transform Infrared (FTIR) spectrophotometer (Agilent 4500t FTIR, Malaysia). In preparation for the analysis, the saliva samples were left at room temperature for an hour to defrost and the FTIR spectrophotometer was switched on to allow warm up period. The Agilent 4500t FTIR instrument is a portable FTIR unit (Figure 3.5) which includes the sample monitoring window and DialPath Zinc selenide (ZnSe) window attached to a rotating arm with three different positions each of specific range of optical wavelengths (50 – 200 microns). The unit is connected to a computer with a specialised software (MicroLab) installed for performing the analyses and demonstrating the readings of dilution space (D_2O enrichment) in the samples.

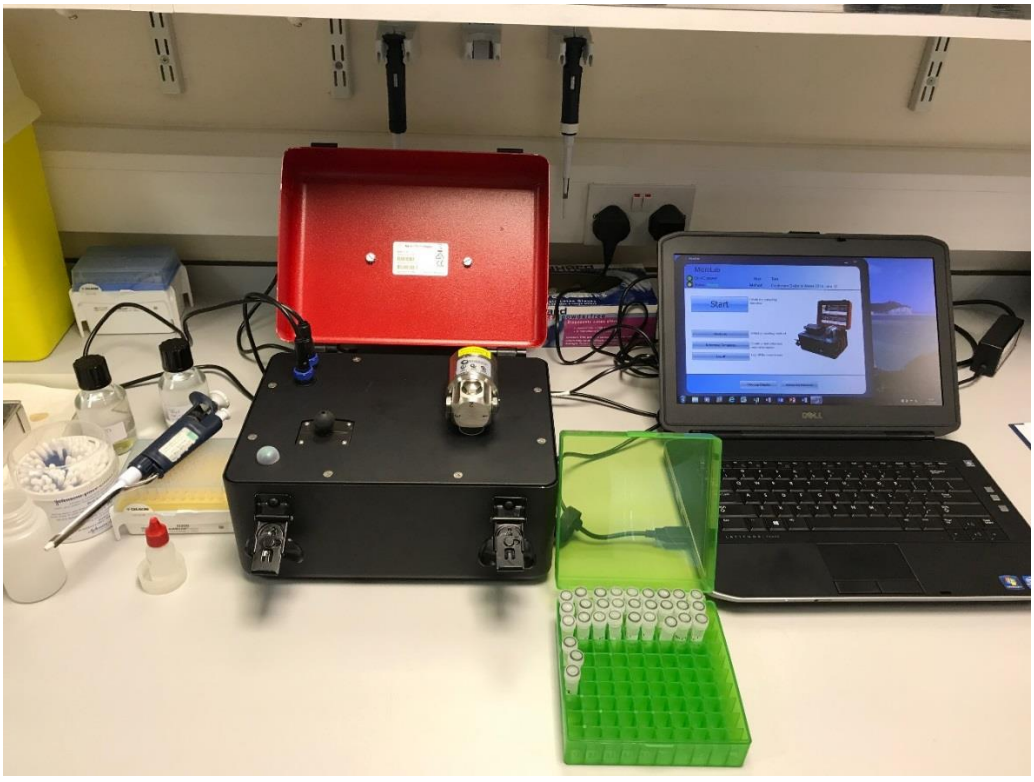


Figure 3.5: Fourier Transform Infrared (FTIR) spectrophotometer unit (Agilent 4500t FTIR, Malaysia) and analysis consumables

To allow calculation of D_2O enrichment (dilution space) in the saliva samples, two calibration standards of known deuterium dilutions were prepared from the same deuterium stock solution which was used for the entire study: standard 1, drinking water; and standard 2, diluted D_2O dose. Before each measurement, both ZnSe and DialPath windows were cleaned with methanol and dried using cotton swabs. Then, a background spectrum reading was collected to ensure accuracy of each measurement by making a baseline profile of the system conditions. Following this, standards (30 µL) or saliva samples (30 µL) were pipetted into the sample monitoring area and the accessory optical arm was then rotated, so that the DialPath ZnSe window on position number 2 (i.e. 100 microns) is facing down towards the sample monitoring area. Infrared (IR) energy passes through the sample and measurements of D_2O enrichment (dilution space) are given in mg/kg unit. The reading is then recorded, and steps are repeated for subsequent measurements of standards or samples.

Using the mean of deuterium enrichment based on the two post-dose samples, the dilution space and total body water (TBW) were calculated accordingly [387]. Raw values for body water were converted to fat-free body mass using age/gender-specific values for the hydration of fat-free tissue (Table 3.1) [FFM (kg) = TBW (kg) / age-gender-specific hydration factor] [387, 388, 391, 395]. The FM is then calculated as the difference between body weight and FFM [FM (kg) = body weight (kg) – FFM (kg)].

Table 3.1: Age-gender specific FFM (%) hydration factors* used for calculation of body composition by deuterium dilution technique

Age (years)	Boys	Girls
9-10	76.2	77
11-12	75.4	76.6
13-14	74.7	75.5
15-16	74.2	75
17-20	73.8	74.5
>20 **	73.2	

* Estimating body composition in children and the elderly. Advances in body composition assessment [391]

** Studies on body composition. III. The body water and chemically combined nitrogen content in relation to fat content [388]

The ratio of FM to FFM (kg) [396], and percentages of TBW, FM and FFM to total body weight were calculated. FMI and FFMI (kg/m^2) were also calculated by division of FM and FFM (in kg) by height (m^2) squared [396]. The calculations of FMI and FFMI are reported to exclude differences associated with height/growth, thus, provide better interpretation of nutritional status and comparison of body composition measures between study groups or participants [397].

3.2.5 Measurement of metabolic rate

3.2.5.1 Instrument (Metabolic system)

The resting metabolic rate (RMR) and thermic effect of feeding were measured by indirect calorimetry, with a computerised open-circuit ventilated hood system (*Quark RMR*®, COSMED, Italy, running *Omnia Software* version 1.6.2) (Figure 3.6). The *Quark RMR*® metabolic system consists of a clear plastic canopy [398] that is connected to the Quark RMR unit by a flexible plastic tube with a disposable antibacterial filter. It has a bi-directional turbine flowmeter consisting of an

optoelectronic reader and is connected to the back of the unit during performance of the RMR measurements. The optoelectronic reader measures infrared light interruptions (accuracy ±0.2 %) caused by the spinning blade inside the turbine. The *Quark RMR*® metabolic system uses online stream paramagnetic (accuracy ±0.1 %) and digital infrared analysers (accuracy ±0.1 %) for determination of fractional O_2 and CO_2 concentrations, respectively, in the expired air samples. The metabolic system is also provided with multisensory ambient condition module by which temperature, humidity and pressure in the testing environment can be determined.

On trial day, *Quark RMR*® system was switched on 30 minutes prior to use, and flow/volume and gas calibrations were performed before each measurement. Flow/volume calibration is performed manually using a 3-liter calibration syringe (accuracy ± 3 ml). Flows and volumes are measured by the bidirectional digital turbine, which offers a very low resistance to flow. Air passing through the helical conveyors causes the spiral rotation of the turbine rotor. The rotating blade interrupts the infrared light beamed by the three diodes of the optoelectronic reader. For gas calibration, an automatic program is initiated and steps for the gas analyser and delay time calibrations are followed. Provided gas cylinder containing precisely known (±0.02% absolute) concentrations of O_2 (16%) and CO_2 (5%) is used for the standard two-point gas calibration procedure recommended by the manufacturer.

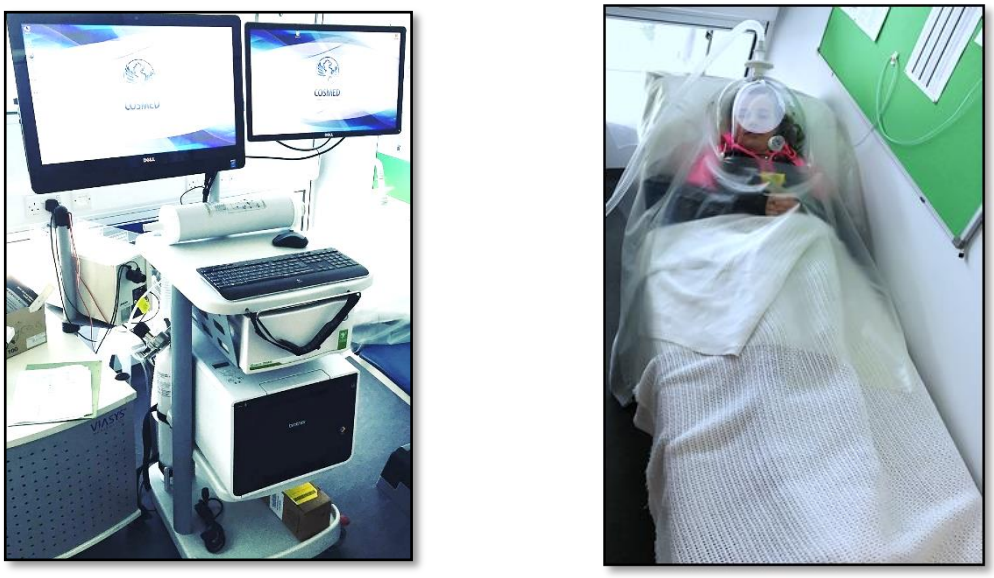


Figure 3.6: *Qurak RMR* indirect calorimetry system (COSMED®, Italy) (Left) and child participant (Right) laying under canopy during measurement of metabolic rate

An integrated programme window provided with *Omnia Software* ver. 1.6.2 is established to verify the validity of each calibration, including flow/volume and gas calibrations, (based on differences between previous and current calibration values being within acceptable range ≤±1 %). Thus, only valid calibration is accepted. In addition, ambient conditions including temperature, pressure and humidity are automatically measured during every calibration and measurement processes through integrated ambient module located in the compact housing of the metabolic cart. Step by step description of metabolic rate measurement protocol by *Quark RMR*® system is shown in Appendix B.20.

Accuracy of the *Quark RMR*® indirect calorimetry system was validated using Alcohol (99.9% absolute Ethanol) burning test [346, 347]. The validation tests were conducted on a weekly basis through the study period and according to the laboratory standard operating procedure. The difference between measured and calculated CO_2 production from Alcohol burning tests conducted during the study

period was within acceptable range ≤±5% (provided by the manufacturer) with CV of ≤5% between tests. The scientific principle of Alcohol burning test for validating the accuracy of metabolic cart system was explained in Chapter 2. Step by step description of Alcohol burning test protocol for validation of the *Quark RMR*® system is shown in Appendix B.21.

3.2.5.2 Calculations

Calculations of resting metabolic rate (RMR) and fat and CHO oxidation rates from expired gas by indirect calorimetry were descried in Chapter 2. Because metabolic rate varies with changes in body size and lean mass [234, 399, 400], RMR, fat and CHO oxidation rates were expressed per kg body weight and FFM. Thermic effect of feeding (TEF) was calculated as percentage increase in energy expenditure (EE) above RMR, and the relative increase in EE expressed as percentage of energy intake provided [284] by breakfast meal (Equations 1).

Relative increase in EE as percentage (%) of [Equation 1]:
= (TEF in kJ ×100) / EI from breakfast meal in kJ

3.2.6 Breakfast meals

Breakfast meals were selected from common local foods and special low protein foods (SLPF) used in the PKU group were obtained from popular PKU medical food providers. The PKU foods were suggested by senior metabolic dieticians based on typical breakfast-meals consumed by patients with PKU. Breakfast meal for children in the PKU group was based on SLPF and comprised of one bread slice Promin®, 48 g topped with butter Lurpak®, 10 g, and strawberry jam Tesco, 20 g, and chocolate cookies Juvela®, 30 g providing a total of 1653 kJ (Figure 2.4). Breakfast meal for adults in the PKU group consisted of two bread slices Promin®, 96 g topped with butter Lurpak®, 20 g, and strawberry jam Tesco, 40 g providing a total of 2056

kJ. In the Control group, breakfast meal for children was based on normal foods and comprised of one bread slice Warburtons, 48 g topped with butter Lurpak®, 10 g, and strawberry jam Tesco, 20 g, and Everyday Value chocolate chip cookies Tesco, 30 g providing a total of 1675 kJ (Figure 2.4).

Control breakfast meal **PKU breakfast meal**

Figure 3.7: Normal and PKU foods provided in breakfast meals of the Control and PKU trials

Energy content of child's breakfast meal: Control 1675 kJ, PKU 1653 kJ

Energy content of adult's breakfast meal: Control 1970 kJ, PKU 2056 kJ

Breakfast meal for adults in the Control group consisted of two bread slices Warburtons, 96 g topped with butter Lurpak®, 20 g, and strawberry jam Tesco, 40 g providing a total of 1970 kJ. A glass of tap water was provided with breakfast meals. The breakfast meals for each age group were isocaloric, and matched for weight content, but differed in macronutrient composition (Figure 2.5 and Figure 3.9) and (Table 3.2). In the Control group, breakfast meal provided 7 g (7% of EI) or 10 g (8% of EI) of protein for children or adults, respectively, which is within normal protein content of a typical meal [260, 401], while the meal based on SLPF, protein content was negligible and consisted only of ≤0.6 grams (≤0.5% of EI). All participants were asked to consume their entire breakfast meal within 20 minutes.

Table 3.2: Energy, fat, carbohydrate and protein provided by breakfast meals in the Control and the PKU groups.

	Children		Adults	
	Control	**PKU**	**Control**	**PKU**
Energy (kilo joules)	1675	1653	1970	2056
Fat (g)	16.2	17.3	17.4	19.7
CHO (g)	54.2	58	67.1	75.2
Protein (g)	6.6	0.4	9.7	0.6

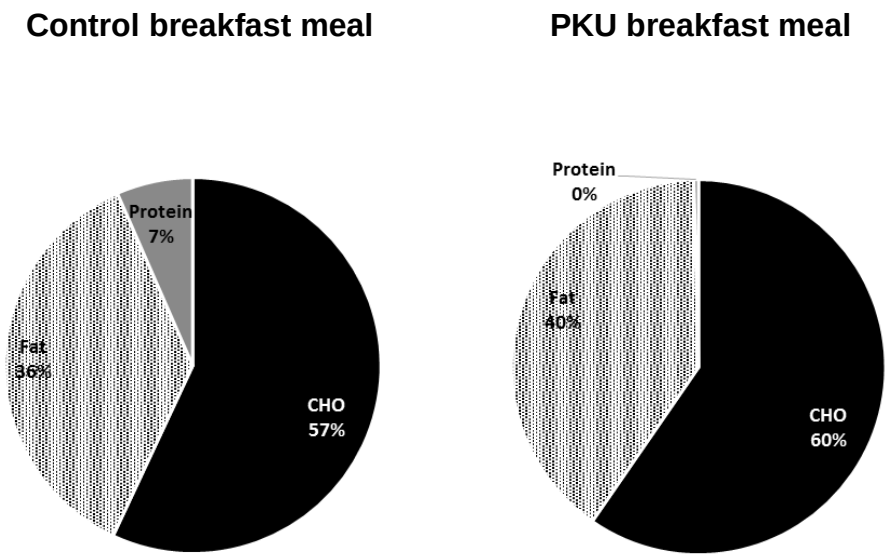


Figure 3.8: Macronutrient energy distribution of Control and PKU breakfast meals provided to children

Energy content: Control breakfast meal 1675 kJ, PKU breakfast meal 1653 kJ

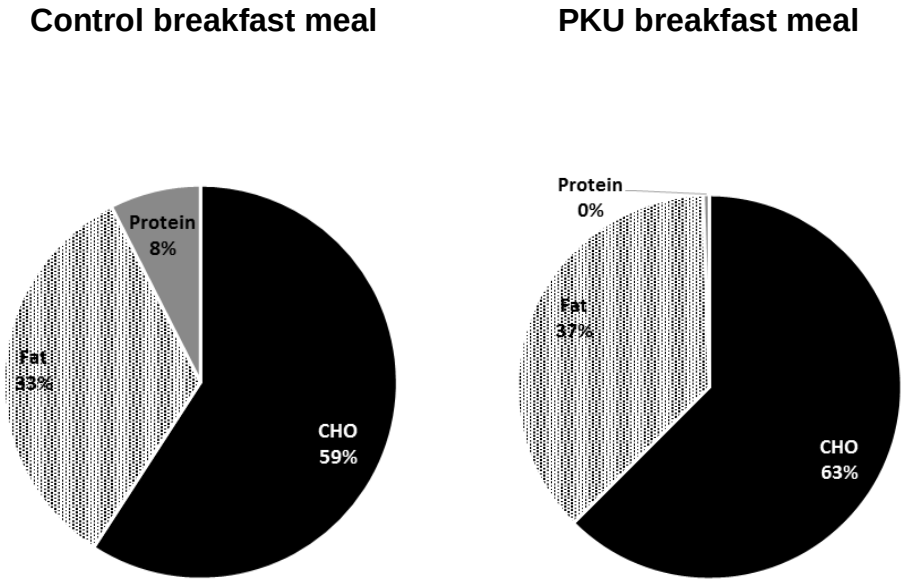


Figure 3.9: Macronutrient energy distribution of Control and PKU breakfast meals provided to adults

Energy content: Control breakfast meal 1970 kJ, PKU breakfast meal 2056 kJ

3.2.7 End of study visit

At the end of the study visit, each participant received a sandwich of his/her choice from lunch menu, a pack of crackers or chocolate cookies with drink options of orange juice, tea or coffee. The patients with PKU were free to choose from the PKU or non-PKU type foods provided in the menu. Accompanying parents/guardians were provided with lunch meal in which they had chosen prior to the study visit. Indicative, lunch menu and composition are shown in Appendices B.9 and B.10. Reasonable expenses such as travel tickets, fuel mileage or car parking were reimbursed. Participants who successfully completed the experimental trial during visit to NLB received cinema tickets with their family and siblings (~£12 value each ticket) as a gift for taking part in the study.

3.2.8 Clinical data on history of PKU disease activity and severity

Information about disease management relevant to the study (i.e. records of PHE concentrations and number of prescribed protein exchanges were collected from the patients' medical notes) (Appendix B.17). The data were obtained for one year prior to the participants' date of study visit. The data search was conducted in line with the NHS Code of Practice (Scotland, 2012 Version 2.1) and data protection laws.

PKU metabolic control

PKU metabolic control was based on the routine blood spot measurements of PHE concentrations. All PHE measurements were classified as high or within age specific treatment targets as recommended by the MRC party group (Table 3.3) [125]. The percentage of measurements with raised PHE concentrations for every participating patient in the year preceding date of study visit (participation) was calculated and this value was used as a continuous variable in the analysis.

Table 3.3: PHE treatment targets for the management of PKU*

Age	*Concertation targets (µmol/l)*
0 - 5 years	120 – 360
> 5 – 10 years	120 – 480
> 10 years	120 – 700

*Medical Research Council Working Party on Phenylketonuria (MRC 1993, UK

PKU disease severity

The severity of PKU was defined as the patient's daily tolerable amount of dietary PHE (milligrams of PHE per day). Assuming that one gram of protein contains 50 mg of PHE, the median number of prescribed protein exchanges was calculated for every participating patient in the year preceding the date of study visit (participation). The number of protein exchanges varies depending on patient tolerance to dietary PHE, which in turn is associated with the type of their genetic mutation. The cut-offs used to classify the severity of PKU were based on the MRC group party recommendations as follows: Sever PKU, < 6 g protein/d; Moderate PKU, 6-8 g protein/d; Mild PKU, > 8 g protein/d.

3.2.9 Assessment of compliance to PKU treatment

Compliance to dietary treatment of participating PKU patients was evaluated by the PKU clinical staff who in charge of managing the case using paper and pen Visual Analogue Scales (VAS) format. The PKU clinical staff was asked to place a vertical mark on the relative horizontal line corresponding to patient's quality of compliance. The lines (100-millimetre scale) were anchored by best compliance (i.e. Very compliant) and poorest compliance (i.e. Not compliant) judgmental words from the right to the left respectively (Figure 2.7) and Appendix B.19. The VAS included three questions to evaluate patient's compliance to PKU treatment in terms of (a) intake

of prescribed PKU protein substitutes, (b) intake of prescribed natural protein exchanges and (c) overall PKU treatment. The millimetres (out of 100 mm) from anchored left side to the placed vertical mark was measured and used as a score for compliance to each component of the scale. This score was then used as a continuous variable in the analysis. A higher score (out of 100) is associated with better compliance.

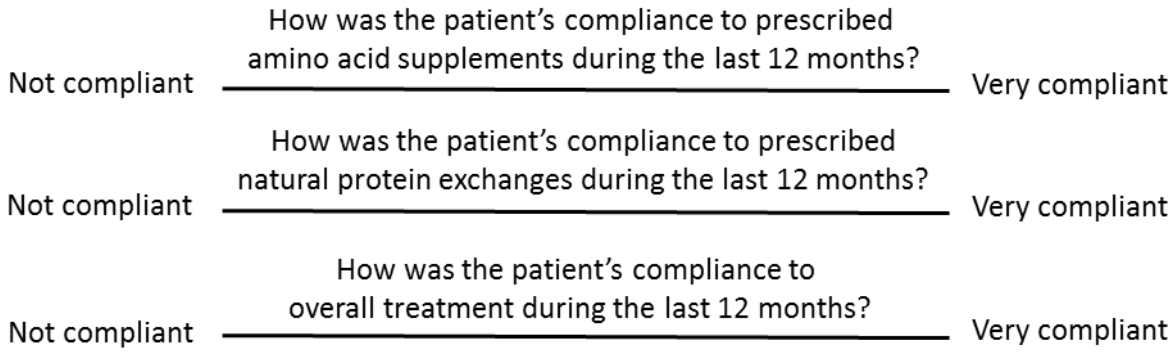


Figure 3.10: Visual analogue scale (VAS) for clinical judgement on patient's compliance to PKU treatment (100-millimetre scale)

3.2.10 Statistical analysis

Data were assessed for normality using the Anderson-Darling test. Participants' characteristics, measures of body composition, total and time average values of RMR, TEF, fat and CHO oxidation were compared using Mann-Whitney test. Age and body weight were plotted against parameters of body composition to assess relationships between variables in both study groups. The general linear model was used to examine the group effect (PKU vs Control) on individual responses (univariate) including body composition, resting metabolic rate, thermic effect of feeding, fat and CHO oxidation. Multivariate linear regression analysis was used to examine the group effect (PKU vs Control) on these responses, accounting for sex and age as covariates. As general linear regression assumes that data is normally

distributed and fitted on a line, the data was transformed using natural log Box-Cox transformation of the response variable. In the PKU patients group, general linear model was also used to examine the effect of compliance to PKU treatment on body fatness. Statistical analyses were performed using Minitab (version 17.3.1; Minitab, Inc., State College, PA).

3.3 Results

3.3.1 Participants

The study was completed by 23 participants of which 13 (Figure 3.11) were patients with PKU and 10 were healthy controls. The PKU group consisted of 7 adults (71% females) and 6 children (33% females), while the Control group consisted of 6 adults (66% females) and 4 children (25% females) (Table 3.4). Median BMI and weight z-scores were not significantly different between the two groups. However, there were more obese participants in the PKU than in the Control group (Table 3.4). Height z-scores were normal in both groups (Table 3.4).

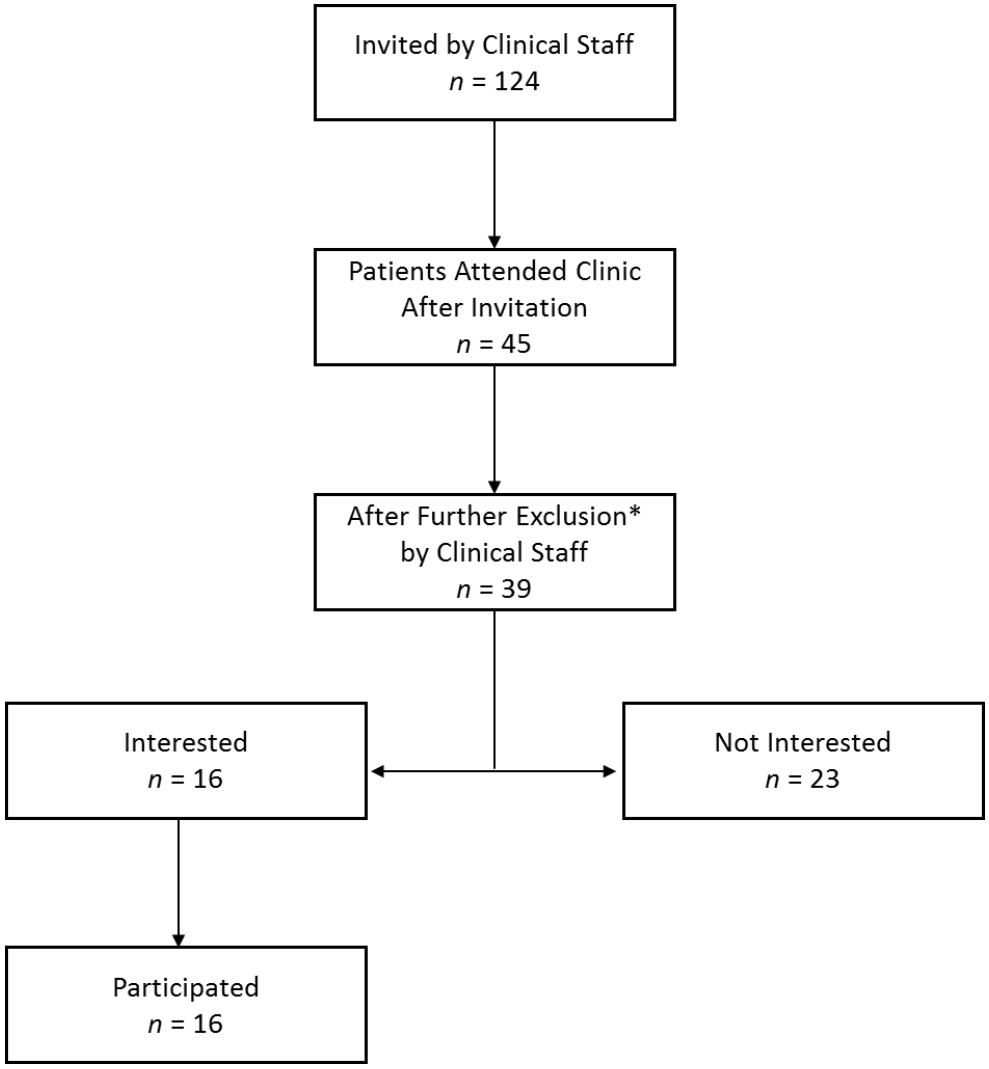


Figure 3.11: Flow diagram for PKU group study recruitment and participation

* Reasons for exclusions after further review by clinical staff included pregnancy, having chronic condition other than PKU

Table 3.4: Descriptive characteristics of participants in the Control and the PKU groups

	Control (*n*=10)	PKU (*n*=13)	*P*-value
n (F/M)	(5/5)	(7/6)	
Age (Years)	26.5 (23.2)	28.0 (24.5)	0.90
<16 years (*n)*	4	6	
≥16 years (*n)*	6	7	
Weight (kg)	67.6 (48.2)	68.7 (62.0)	0.66
Weight z-scores*	0.95 (2.20)	1.20 (2.60)	0.33
Height (cm)	166.7 (30.03)	160.7 (22.6)	0.90
Height z-scores*	0.60 (0.70)	0.32 (1.93)	0.73
Short height (%)	0	0	
Normal height (%)	100	100	
BMI z-scores*	0.90 (2.83)	1.30 (2.75)	0.43
Thin (%)m	0	0	
Normal (%)	60	38	
Overweight (%)	20	15.4	
Obese (%)	20	46.6	

Values are medians (IQR) unless indicated

* Z-scores for weight, height and BMI were calculated using British-1990 LMS growth reference data

F, females; M, males; BMI, body mass index

The PKU participants had good metabolic control during the year preceding their participation and the vast majority (77%) of them had mild PKU (Table 3.5). In addition, the patients' median compliance scores to prescribed PKU protein substitutes and overall PKU treatment were above 70 (out of 100).

Table 3.5: Disease characteristics and VAS* compliance scores to PKU treatment of the participants with PKU (*n*=13)

	% or median (IQR)
PKU Severity	
Severe PKU (%)	15.4
Moderate PKU (%)	7.6
Mild PKU (%)	77
PKU metabolic control**	5.0 (33.9)
Compliance to PKU protein substitutes score***	85.0 (18.5)
Compliance to natural protein exchanges score***	68.5 (30.5)
Compliance to overall PKU treatment score***	76.0 (29.5)

*VAS, visual analogue scale (100-millimetre-scale)

**Percentage of PHE measurements above treatment targets. Calculated from 262 PHE concertation measurements, median (IQR), 9 (30) per patient in the year prior study participation

***A higher score (out of 100) is associated with better compliance

3.3.2 Body composition

Total body water (TBW), fat mass (FM) and fat-free mass (FFM) expressed as percentages of body weight and kg per height squared (mass indices) did not differ (P>0.5, Mann-Whitney test) between the Control and PKU groups (Table 3.6). The ratios of FM to FFM were also not significantly different between the two groups (P>0.5, Mann-Whitney test) (Table 3.6). However, patients with PKU tended to have higher percentage of FM and lower percentage of FFM than healthy controls (Table 3.6).

Table 3.6: Measures of body composition of participants in the Control and the PKU groups

	Control (*n*=10)	PKU (*n*=13)	*P*-value
TBW (kg)	35.9 (22.3)	31.9 (18.3)	0.87
TBW (%)	54.8 (10.1)	47.60 (10.8)	0.14
FM (kg)	15.2 (18.1)	22.5 (31.2)	0.36
FM (%)	25.1 (12.6)	35.0 (12.7)	0.12
FMI (kg/m^2)	5.1 (4.9)	8.1 (9.3)	0.22
FFM (kg)	48.5 (31.6)	43.7 (26.0)	0.92
FFM (%)	74.9 (12.6)	65.0 (12.7)	0.13
FFMI (kg/m^2)	15.7 (6.5)	15.7 (4.7)	1
FM/FFM	0.3 (0.3)	0.5 (0.3)	0.13

All values are medians (IQR)

TBW, total body water; FM, fat mass; FFM, fat-free mass; FMI, fat mass index; FFMI, fat-free mass index; FM/FFM, ratio of fat mass to fat-free mass

On visual inspection, when FFM and FM (kg) were plotted against body weight, patients with PKU appeared to have higher FM per kg body weight than healthy controls. The relationship between FFM and body weight was less clear (Figure 3.12).

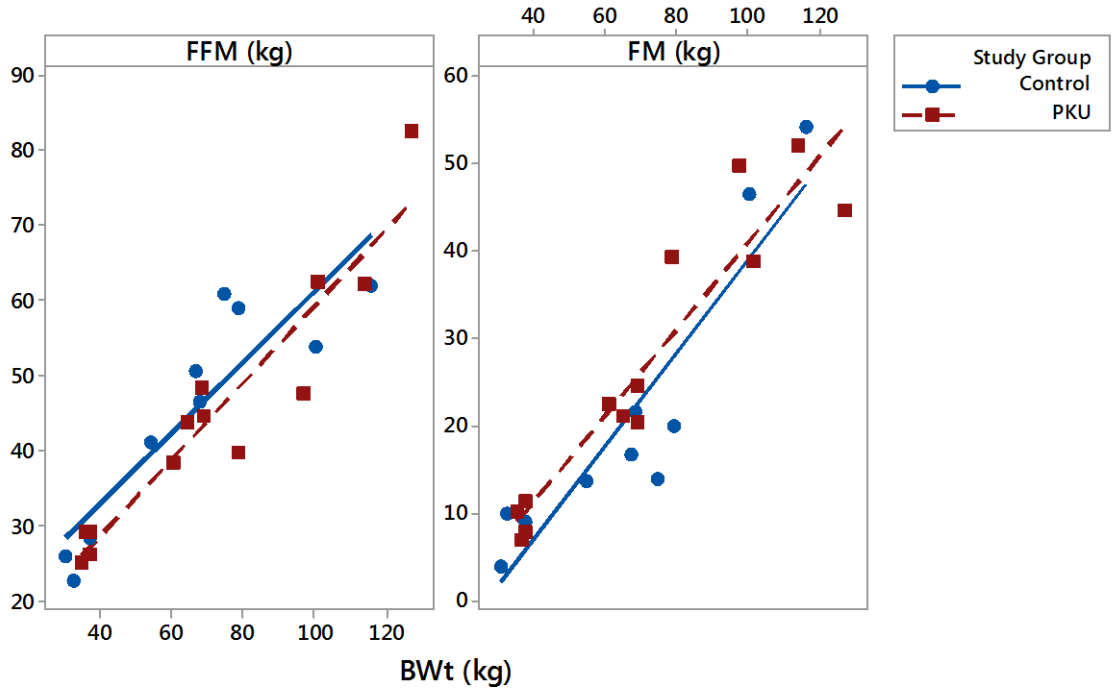


Figure 3.12: Scatterplots with regression lines of FFM and FM (kg) against body weight (kg) in 13 patients with PKU and 10 healthy controls

BWt, body weight; FFM, fat-free mass; FM, fat mass

Similarly, FFMI and FMI (kg/m^2) were plotted against age, patients with PKU appeared to have higher FMI units per year of age than healthy controls. The relationship between FFMI and age was less profound (Figure 3.13).

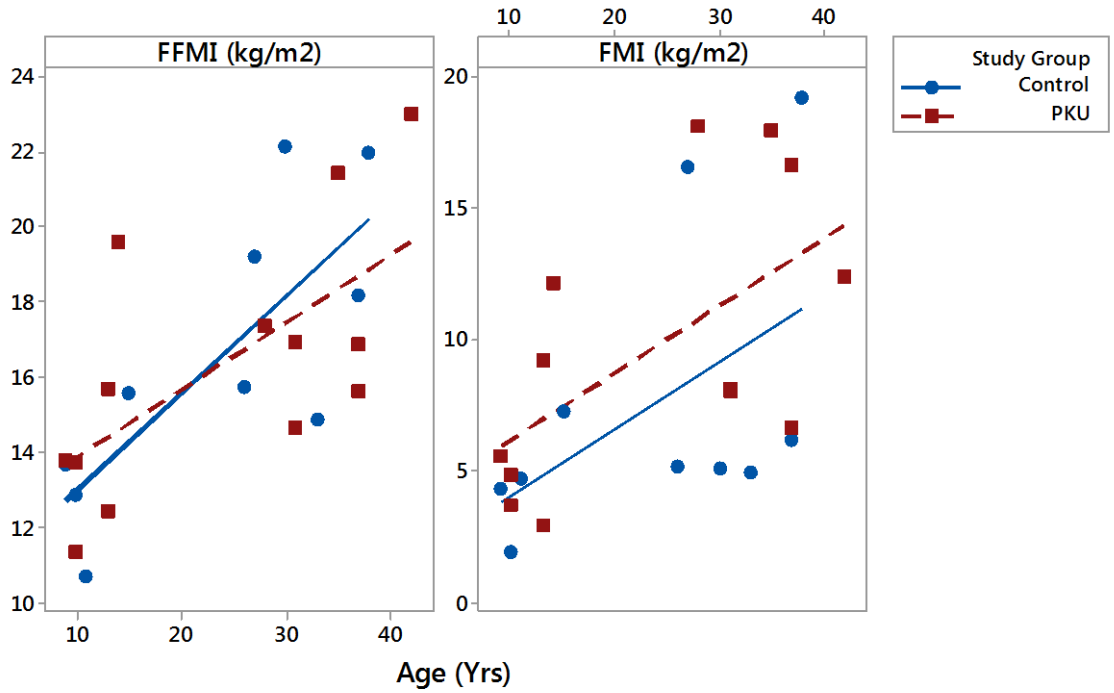


Figure 3.13: Scatterplots with regression lines of FFMI and FMI (kg/m^2) against age (years) in 13 patients with PKU and 10 healthy controls

FFMI, fat-free mass index; FMI, fat mass index

3.3.3 Metabolic rate and thermic effect of feeding

Metabolic rate measured before and after breakfast meal, expressed per kg body weight and FFM, is illustrated in Figure 3.14. Raw fasting values of resting metabolic rate, median (IQR), did not differ (*P*=0.8, Mann-Whitney test) between the Control 4.03 (1.83) kJ/min and the PKU 4.37 (1.83) kJ/min groups.

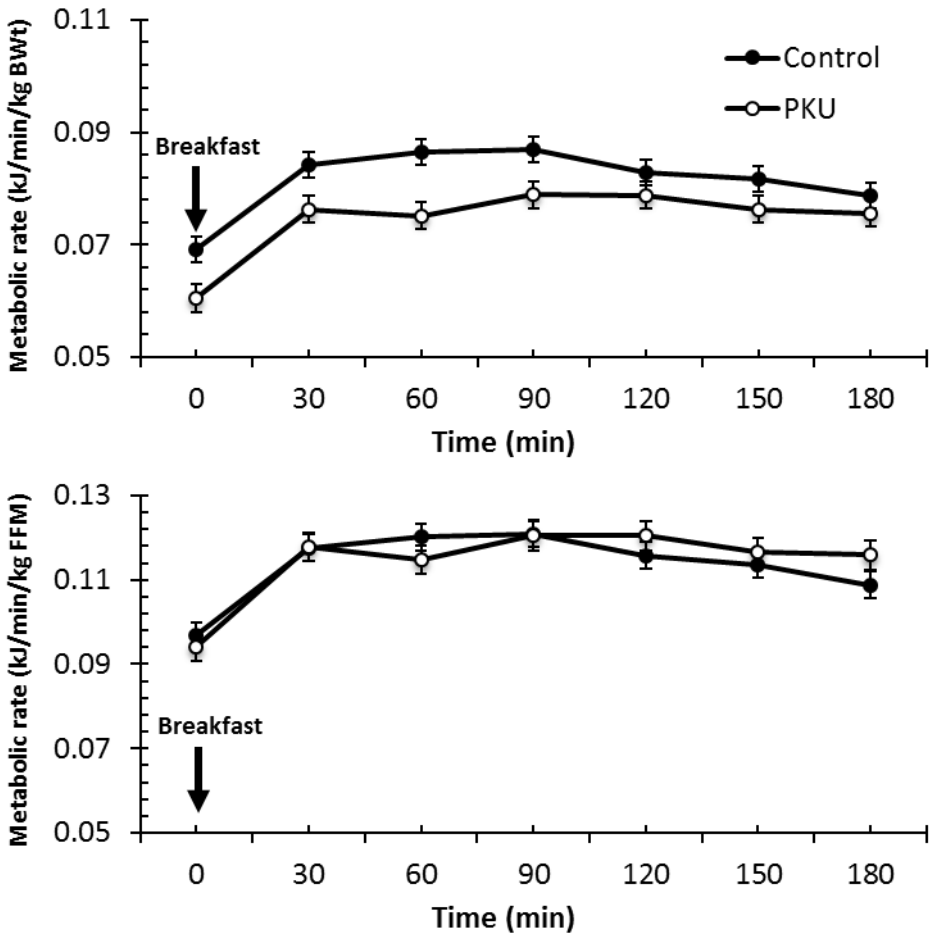


Figure 3.14: Responses of metabolic rate during 180 min experimental trials in the PKU (n = 13) and the Control groups (n = 10) expressed per kg body weight and FFM. Values are means ± SEM.

BWt, body weight; FFM, fat-free mass

The fasting metabolic rate was also not significantly different between the two groups when expressed either per kg body weight or kg of FFM (Table 3.7).

Table 3.7: Resting metabolic rate (kJ/min) expressed per kg body weight and FFM in the Control and the PKU groups

	Control (*n*=10)	PKU (*n*=13)	*P*-value
RMR/min/kg BWt	0.065 (0.024)	0.059 (0.024)	0.27
RMR/min/kg FFM	0.093 (0.025)	0.104 (0.041)	1

All values are medians (IQR)

RMR, resting metabolic rate; BWt, body weight; FFM, fat-free mass

On visual inspection, a positive relationship was observed similarly in both groups when FFM and FM (kg) were plotted against resting metabolic rate (kJ/min) (Figure 3.15).

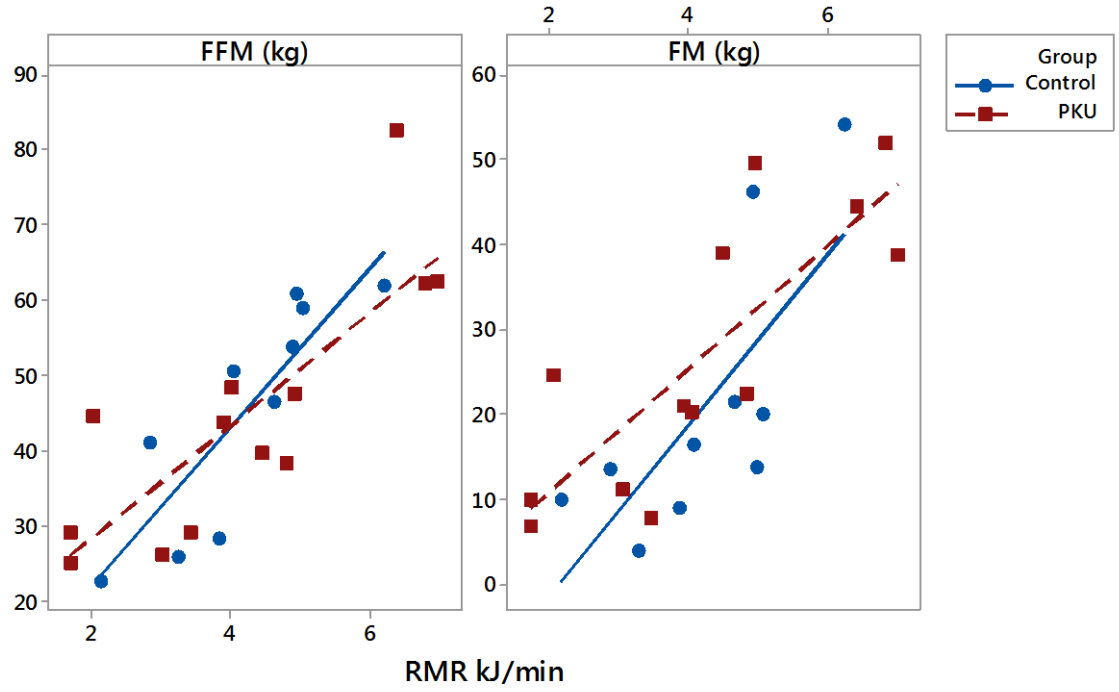


Figure 3.15: Scatterplots with regression lines of FFM and FM (kg) against RMR (kJ/min) in 13 patients with PKU and 10 healthy controls

FFMI, fat-free mass index; FMI, fat mass index; RMR, resting metabolic rate

Differences between the PKU and the Control groups for TEF, calculated as percentage increase in EE above RMR and the relative increase in EE (expressed as percentage of energy provided by breakfast meal), were not significant (P>0.5, Mann-Whitney test) (Table 3.8).

Table 3.8: Thermic effect of feeding calculated as percentage (%) increase in EE above RMR and as increase in EE as percentage (%) of EI in the Control and the PKU groups

	Control (*n*=10)	PKU (*n*=13)	*P*-value
TEF as increase above RMR (%)	13.6 (28.2)	10.9 (51.8)	1
TEF increase as percentage of EI	6.7 (8.6)	5.8 (7.9)	1

All values are medians (IQR)

TEF, thermic effect of feeding; RMR, resting metabolic rate; EI; energy intake

3.3.4 Fat and CHO oxidation

Fat and CHO oxidation rates measured before and after breakfast meal, expressed per kg body weight and FFM, are illustrated in Figure 3.16 and Figure 3.17, respectively. Fasting values of fat oxidation rate, median (IQR), did not differ (*P*=0.8, Mann-Whitney test) between the Control 0.067 (0.065) g and the PKU 0.071 (0.061) g groups. Similarly, fasting values of CHO oxidation rate, median (IQR), were not significantly different (*P*=0.7, Mann-Whitney test) between the Control 0.09 (0.078) g and the PKU 0.083 (0.076) g groups.

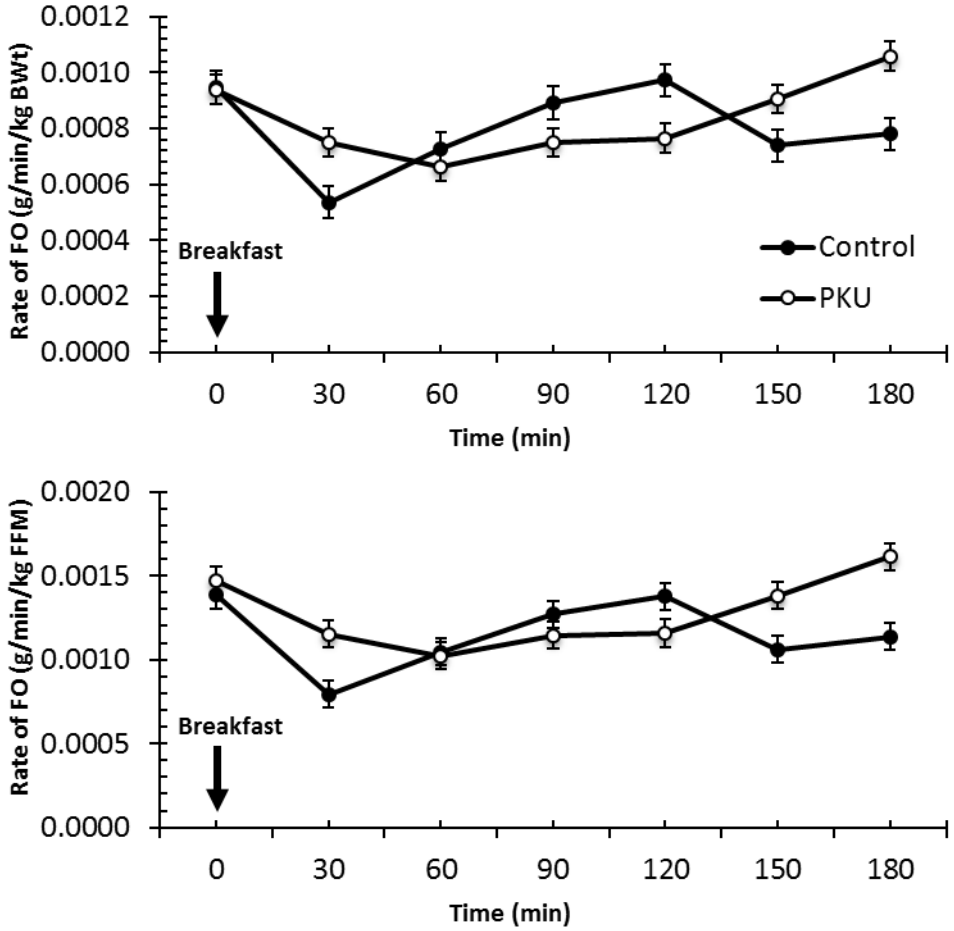


Figure 3.16: Responses of fat oxidation rate during 180 min experimental trials in the PKU (*n* = 13) and the Control groups (*n* = 10) expressed per kg body weight and FFM. Values are means ± SEM.

FO, fat oxidation; BWt, body weight; FFM, fat-free mass

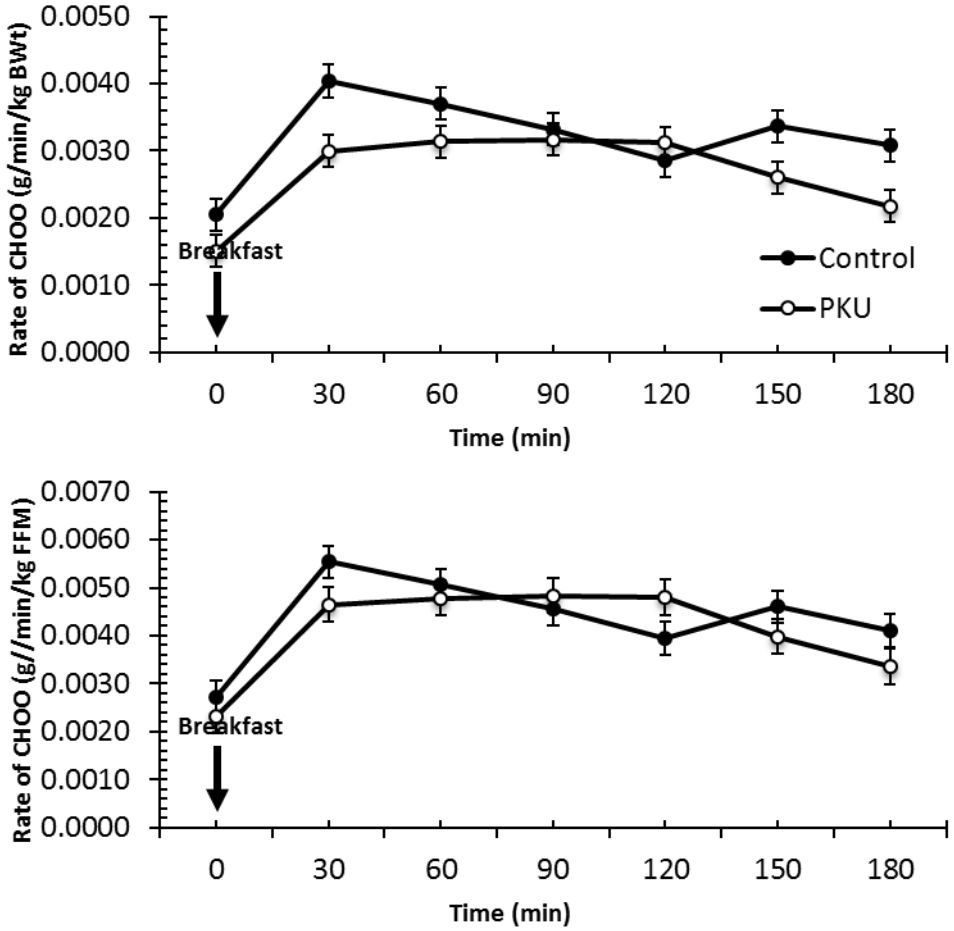


Figure 3.17: Responses of CHO oxidation rate during 180 min experimental trials in the PKU (*n* = 13) and the Control groups (*n* = 10) expressed per kg body weight and FFM. Values are means ± SEM.

CHOO, carbohydrate oxidation; BWt, body weight; FFM, fat-free mass

The total amount of oxidised fat and CHO (expressed per kg body weight and FFM) did not differ (P>0.5, Mann-Whitney test) between the PKU and the Control groups (Table 3.9). The percentages of energy expenditure obtained from fat and CHO were also not significantly (P>0.5, Mann-Whitney test) different between the two groups (Table 3.9).

Table 3.9: Total amount of oxidised fat and CHO (expressed per kg body weight and FFM), and time average percentages of energy expenditure obtained from fat and CHO during 180 min experimental trials in the Control and the PKU groups

	Control (*n*=10)	PKU (*n*=13)	*P*-value
Total FO g	10.65 (4.80)	9.70 (6.80)	0.90
Total FO/kg BWt	0.16 (0.10)	0.17 (0.10)	0.87
Total FO/kg FFM	0.23 (0.18)	0.24 (0.16)	0.60
Total CHOO g	37.60 (10.90)	35.00 (16.60)	0.40
Total CHOO /kg BWt	0.55 (0.37)	0.56 (0.31)	1.00
Total CHOO /kg FFM	0.80 (0.41)	0.80 (0.42)	0.64
EE from Fat (%)	44.10 (20.33)	42.70 (17.84)	0.90
EE from CHO (%)	55.90 (20.33)	57.35 (17.84)	0.06

All values are medians (IQR)

FO, fat oxidation; BWt, body weight; FFM, fat-free mass; CHOO, carbohydrate oxidation; EE (%), average percentage of energy expenditure from fat and CHO

3.3.5 Group effect on FFM, FM, RMR, TEF, fat and CHO oxidation

General linear model analysis revealed that there was no group effect on body fatness and fat-free mass, resting metabolic rate, thermic effect of feeding, and total grams of oxidised fat and CHO (Table 3.10).

Table 3.10: Univariate general linear model analysis* for the group effect on body fatness and fat-free mass, resting metabolic rate, thermic effect of feeding, total fat and CHO oxidation

Response	*P-value*	R^2	β̂
FM (%)	0.13	10.7%	0.23 (PKU)
FMI	0.25	6.3%	0.31 (PKU)
FFM (%)	0.18	8.3%	-0.09 (PKU)
FFMI	0.96	0.01%	-0.004 (PKU)
FM/FFM	0.14	10.1%	0.32 (PKU)
RMR /kg BWt	0.25	6.2%	-0.14 (PKU)
RMR/kg FFM	0.67	0.9%	-0.05 (PKU)
TEF as increase above RMR (%)	0.34	4.3%	0.46 (PKU)
TEF increase as percentage of EI	0.58	1.5%	0.22 (PKU)
Total FO/kg BWt	0.76	0.5%	0.06 (PKU)
Total FO/kg FFM	0.43	3.0%	0.15 (PKU)
Total CHOO/kg BWt	0.43	3.0%	-0.13 (PKU)
Total CHOO/kg FFM	0.76	0.4%	-0.04 (PKU)
EE from fat (%)	0.48	2.4%	0.14 (PKU)
EE from CHO (%)	0.61	1.2%	-0.05 (PKU)

*Data were transformed using natural log Box-Cox transformation

FM, fat mass; FFM, fat-free mass; FMI, fat mass index; FFMI, fat-free mass index; FM/FFM, ratio of fat mass to fat-free mass; RMR, resting metabolic rate; BWt, body weight; EI, energy intake; FO, fat oxidation; CHOO, carbohydrate oxidation; EE (%) percentage of energy expenditure

After accounting for age and sex as confounders, multivariate linear regression analysis revealed that there was no group effect on fat-free mass, resting metabolic rate, thermic effect of feeding, and total grams of oxidised fat and CHO (Table 3.11). However, there was a tendency of PKU patients to have positive higher percentage of absolute body fatness (kg) (*P*=0.08) and its ratio to fat-free mass (*P*=0.09) (Table 3.11).

Table 3.11: Multivariate general linear model analysis* for the group effect (adjusted for age and sex) on body composition, resting metabolic rate, thermic effect of feeding, total fat and CHO oxidation

Response	*P-value*	R^2	β̂
FM (%)	0.08	45.6%	0.21 (PKU)
FMI	0.18	46.9%	0.30 (PKU)
FFM (%)	0.13	43.9%	-0.08 (PKU)
FFMI	0.95	56.0%	-0.004 (PKU)
FM/FFM	0.09	45.8%	0.30 (PKU)
RMR/kg BWt	0.18	47.5%	-0.13 (PKU)
RMR/kg FFM	0.70	13.1%	-0.04 (PKU)
TEF as increase above RMR (%)	0.34	24.4%	0.43 (PKU)
TEF increase as percentage of EI	0.58	16.5%	0.21 (PKU)
Total FO/kg BWt	0.76	10.7%	0.06 (PKU)
Total FO/kg FFM	0.45	11.3%	0.14 (PKU)
Total CHOO/kg BWt	0.37	46.1%	-0.12 (PKU)
Total CHOO/kg FFM	0.78	30.2%	-0.03 (PKU)
EE from fat (%)	0.51	9.2%	0.13 (PKU)
EE from CHO (%)	0.65	9.9%	-0.049 (PKU)

*Data was transformed using natural log Box-Cox transformation

FM, fat mass, FFM, fat-free mass, FMI, fat mass index; FFMI, fat-free mass index; FM/FFM, ratio of fat mass to fat-free mass; RMR, resting metabolic rate; BWt, body weight; EI, energy intake; FO, fat oxidation; CHOO, carbohydrate oxidation; EE (%), percentage of energy expenditure

3.3.6 Effect of compliance to PKU treatment on body fatness

In univariate regression analysis, using the general linear model, poor compliance to natural protein exchanges and overall PKU treatment was associated with high body fatness (Table 3.12). Compliance to prescribed natural protein exchanges and overall PKU treatment explained 39% and 33% of the variations in body fatness, respectively (Table 3.12). Similarly, compliance to prescribed PKU protein substitutes tended (*P*=0.06) to negatively associate with the body fatness.

The effect of compliance to overall PKU treatment on body fatness was lost after accounting for age as a confounder in the multivariate linear regression analysis (Table 3.12). Still, compliance to prescribed natural protein substitutes tended (*P*=0.06) to negatively associate with the body fatness despite the adjustment to age (Table 3.12).

Table 3.12: General linear regression* for the effect of compliance to PKU treatment on percentage of body fat mass before (Univariate) and after adjustment for age (Multivariate)

Response	*P-value*	R^2	β
Univariate			
Compliance to prescribed PKU protein substitutes	0.06	27%	-0.007
Compliance to prescribed natural protein exchanges	0.02	39%	-0.007
Compliance to prescribed overall PKU treatment	0.04	33%	-0.007
Multivariate			
Compliance to prescribed PKU protein substitutes	0.21	39%	-0.004
Compliance to prescribed natural protein exchanges	0.07	50%	-0.006
Compliance to prescribed overall PKU treatment	0.16	42%	-0.005

*Data was transformed using natural log Box-Cox transformation

3.4 Discussion

This is a pilot study aimed to test for first time the hypothesis that patients with PKU have higher body fatness and, that obesity and adiposity in patients with PKU is the result of alterations in components of energy expenditure and energy substrate oxidation. The study found that FM and FFM were not different between patients with PKU and healthy controls. TEF and, postprandial fat and CHO oxidation rates in patients with PKU were also found to be not significantly different from healthy controls. In addition, fasting values of RMR and fat oxidation in patients with PKU were similar to healthy controls. Thus, our pilot data suggests that increasing

prevalence of overweight and obesity in patients with PKU [107, 134, 156, 184-187] may be due to other factors such as increased energy intake and/or reduced energy expenditure due to sedentary lifestyle or lower physical activity. However, these findings should be confirmed using a larger sample size.

This is the first study to use a reference method deuterium dilution technique to measure body composition in patients with PKU. FM and FFM were not different from healthy controls. This is contrary to previous studies which have reported higher body fatness and lower lean mass in patients with PKU. However, this could be explained by less accurate techniques being used to measure body composition e.g. skinfold thickness and air-displacement plethysmography. These studies have not consistently adjusted for differences in body size, age or gender between groups [192, 201, 202]. However, our findings were in accordance with previous research conducted to measure body composition in patients with PKU using DEXA, TOBIC and BIA [107, 197-200]. PKU tended to have higher percentage of body FM and ratio of FM to FFM. By all means, data obtained on body composition diffrencenies between patiens with PKU and healthy controls should be interpreted with caution since sample size in this pilot study was not big enough.

In our study, most of the PKU participants had mild PKU type in which a considerable amount of natural protein (up to 14 g/day) is allowed in their diet, compared to patients with severe type of PKU (< 5 g/day) [102, 114]. These patients may not totally depend on SLPF for their treatment [99, 103]. The PKU participants in this study also exhibited good compliance to their prescribed PKU protein substitutes which was associated with reduced FM in the PKU group. Jani and colleagues assessed body composition using DEXA in 86 patients with PKU, 94%

of whom had poor metabolic control [107]. The study reported that total protein and intake of PKU protein substitutes were positively associated with FFMI, and that high intake of natural protein was associated with low ratio of FMI to FFMI in adult patients with PKU [107]. Furthermore, a recent study conducted on 37 patients with PKU showed that intake of prescribed natural protein exchanges and PKU protein substitutes explained 44.5% of variations in percentage of body FM [402]. This is in accordance with our study, where compliance to prescribed natural protein exchanges explained about 40% of variation in percentage body fat mass. However, our findings with regard to association between compliance to prescribed PKU protein substitutes and body fatness did not reach significance despite the tendency observed. Belanger-Quintana et al. reported that patients with severe PKU type were more likely to become overweight than those with other types of PKU severity [134]. Indeed, a study by Albersen and colleagues measured body composition in 20 patients with severe PKU using air-displacement (BodPod) technique and showed that these patients had significantly higher percentage of body FM than healthy matched controls [192]. Another study found lower lean body mass (estimated from skinfold thickness) in 37 children with severe PKU compared to healthy matched controls [202]. Thus, not significantly different body composition found in our study between PKU and Control groups may be due to most of patient participants being on relaxed diet with good adherence to intake of prescribed PKU protein substitutes. Unfortunately, data on dietary intake were not included in this study. Future studies should aim to evaluate dietary intake and its association to body composition variables in patients with PKU.

Our finding that the resting metabolic rate was not different between patients with PKU and healthy controls is consistent with previous evidence of RMR being similar between PKU children and healthy matched controls [201]. The metabolic control

and phenotypes of PKU children in Allen et al.'s study were not reported. Energy expenditure regulating neurotransmitters and hormones were reported to significantly deteriorate in patients with poor metabolic control, but were comparable to healthy controls in those with good metabolic control [80-82, 238-240, 276, 277]. A study by Burrage and others showed that the prevalence of overweight or obesity was higher by 32% in non-compliant compared to compliant patients with PKU [184]. In addition, two previous studies reported positive association between BMI and PHE concentrations [133, 186]. Most of our PKU participants had mild PKU type and had good metabolic control, at least during the year prior their participation in the study. Thus, our data when combined with results from other studies imply that metabolic rate may be different in patients with poor, but not with good metabolic control or those with more severe PKU type.

The study was also first of its type in which TEF, and postprandial fat and CHO oxidation rates were measured in patients with PKU and healthy controls after consumption of isocaloric group-type based breakfast meal. PKU participants were provided with SLPF-based breakfast meal whereas healthy controls were provided with isocaloric normal food-based breakfast meal before which TEF, and postprandial fat and CHO oxidation rates were measured for the following three hours (180 minutes). TEF, and postprandial fat and CHO oxidation rates were not different between the PKU and the Control groups. These findings did not confirm the hypothesis generated from our previous study (Chapter 2) which suggests that diminished TEF and postprandial fat oxidation are among factors contributing to the prevalence of overweight and obesity in PKU. However, compared to our previous study (Chapter 2) conducted on healthy individuals, the differences in whole natural protein and CHO between the two trial lunch meals were 21 g (18% of EI) and 19.5 g (18% of EI), respectively, while in this study the differences in whole natural protein

and CHO between the group-based breakfast meals were only 9 g (8% of EI) and 8 g (4% of EI), respectively. Therefore, finding no differences in TEF, postprandial fat and CHO oxidation may be related to the macronutrient composition of study breakfast meals being relatively low to pick a significant effect. Indeed, the difference in protein content between meals in the previous experimental studies was as much as 70% of EI [253, 254, 256], which is far above the recommended protein intake (10-15% of EI) [260, 261]. Our data suggests that patients with PKU, mainly of mild severity type, have no altered TEF and postprandial fat and CHO oxidation. In addition, the data suggests that small differences in macronutrient composition of isocaloric meals have no impact on TEF and, postprandial fat and CHO oxidation.

As all studies, this study has some limitations. Due to difficulties in recruitment and limited number of patients with PKU within Greater Glasgow and Clyde area, we were able to approach 39 patients who met our inclusion criteria. Recruiting control children and adults with PKU were most challenging issues during this study. It should be emphasised that PKU is a rare condition in which the estimated incidence of PKU in Scotland is 1 per 8000 livebirths [20]. Thus, due to the limited number of patients with this rare condition in the study area, we recruited children and adults including both males and females. This might have produced negative false results of the study, although the fact that baseline characteristics of study participants were not significantly different between the two groups. To overcome heterogeneity issue in our sample, general linear regression analysis was used to assess the group effect on the study outcomes (responses) while accounting to the differences in age and gender factors between the PKU and the Control groups. In addition, parameters of body composition were expressed as kg of body weight divided per height squared (FFMI and FMI) to account for the variations in growth between study

participants. Other variables such as RMR, TEF, fat and CHO oxidation were also expressed per kg body weight and FFM. These calculations are believed to eliminate variations in study outcomes [397] due to variability of the sample and not achieving strictly matching controls when entered in the multivariate analysis model. The wide variability range of age in the study groups was a result of aiming to investigate the study outcomes in lean and overweight/obese participants. Data on puberty was not available to investigate whether this had impact on study outcomes or not. In addition, it should be noted that our PKU sample may not be the best representation of PKU population. The patients' metabolic control in this study was good (median % raised PHE was only 5), and their compliance scores to PKU protein substitutes and natural protein exchanges were quite adequate. In addition, most our PKU participants had mild PKU type while it has been reported that severe PKU is the most prevalent disease severity type of PKU, compromising about 55% of PKU severity types worldwide, and 68% in some European countries [16, 52]. Therefore, to obtain a more accurate understanding of factors contributing to aetiology of obesity in PKU, the study outcomes should be performed in a large-scale study considering different types of PKU severity and quality levels of metabolic control (within PKU group comparisons) to evaluate body composition and probably components of energy expenditure and metabolism (energy and nutrient balance). Data on other factors contributing to obesity aetiology such as energy intake [209, 210] and physical activity energy expenditure [209, 300-303] were not included in this thesis.

As this was a pilot stay, obtained data was utilised to calculate number of participants required to conduct study which achieves 85%. Post-hoc power calculation revealed that to detect 1% difference in FM at 0.85 power, sample size of 21 participants would be required for each group. Similar calculations were

conducted on the relative increase in EE, expressed as percentage of energy provided by the ingested food, and revealed that 19 participants are needed to detect 2% difference at power of 0.85.

Regardless difficulties in recruitment, our study demonstrates that it is feasible to conduct metabolic investigations on patients with PKU including children and adults. Yet, it should be mentioned that data from three PKU participants were excluded. Two children with PKU were not able to stay still under the canopy during the RMR measurement as per our indirect calorimetry protocol. Another adult with PKU admitted that he could not sleep well during the night before the experimental trial and had to drink some coffee few hours before attending our metabolic laboratory. This is not surprising since it has been reported that patients with PKU may present with anxiety, poor mood and executive skills [309-311]. The rest of participants, however, showed good compliance to our study protocol.

3.5 Conclusion

In conclusion, the data suggest that presence of PKU has no effect on body composition, TEF, postprandial fat and CHO oxidation. However, the obtained data should be interpreted with caution and requires confirmation from studies conducted on a larger sample size.

Chapter Four: Micronutrient Status of Children with PKU and Factors Associated with Micronutrient Deficiencies

Some of the results from this chapter have been presented in the following meeting:

H. Alfheeaid, J. Jones, B. Cochrane, P. Robinson, D. Malkova, K. Gerasimidis, 2015, Micronutrient Status of Children with PKU, *Symposium on Stratified Medicine in Paediatrics*, Glasgow University, Queen Elizabeth University Hospital, Glasgow, UK

4.1 Background

Blood PHE levels in PKU patients are controlled via adherence to a low-PHE diet. This diet restricts dramatically natural dietary protein to a tolerance level, specific to each individual patient [315, 365]. As the low-PHE diet is a very restrictive dietary regime (≤ 5 g/d protein in most severe cases), PKU patients are prescribed micronutrient-enriched PKU protein substitutes to achieve their essential daily requirements of amino acids and micronutrients [102]. These PKU protein substitutes are designed to meet the nutritional requirements of patients of different age groups and PKU severity [121].

Micronutrient deficiencies are frequently reported in patients with PKU [102]. Vitamin B12, folate and trace elements, such as selenium and zinc are amongst the most commonly reported deficiencies in PKU patients [117]. The exact reason for these micronutrient deficiencies still remains elusive and it is believed to be associated with poor compliance to PKU protein substitutes and the composition of the first protein substitutes available to the patients. PKU protein substitutes underwent several modifications during the last two-decades. In early years, the protein substitutes did not contain micronutrients and in some occasions, vitamins and minerals were prescribed separately to the patients [117]. Later on, most available PKU protein substitutes became enriched with micronutrients [102] and have been the main source of vitamins and trace elements for people with PKU. However, it still yet remains unclear whether the prevalence of micronutrient deficiencies changed in relation to changes in the composition of PKU protein substitutes. In addition, factors predicting micronutrients deficiencies require further investigation.

Limited evidence is available on predictors of micronutrient status in PKU patients [121, 155]. Previous studies suggested that micronutrient status in PKU patients is

associated with PHE levels, a biomarker commonly used to reflect metabolic control [320]. Poor metabolic control reflects low adherence to dietary treatment [321] and commonly intake of PKU protein substitutes, hence low micronutrient intake [121]. It is interesting though, that studies reporting low levels of selenium and zinc tend to show high or normal levels of other micronutrients such as folic acid, and vitamins B12 and E [121, 155, 189, 313]. Recent studies have also reported an association between poor PHE control and suboptimal micronutrient status [121, 155]. If poor adherence to dietary treatment was assumed to be the cause of reported deficiencies, it would be expected that other micronutrients would also be deficient. Indeed, studies reported that high adherence to low-PHE diet could be a risk factor for developing micronutrient deficiencies [155, 322, 323]. PKU phenotype (PKU severity) and PHE tolerance showed significant association to increased or reduced levels of some micronutrients [155]. However, these previous studies looked individually at potential factors associated with micronutrient status, but none have explored how these factors together may explain micronutrient deficiencies in this group of patients, and particularly in patients with serial micronutrient assessments. In addition, since nutritional needs and dietary compliance vary with age, the association between micronutrients status and age should also be investigated [121]. Optimal micronutrient profile for the PKU protein substitutes has not yet been defined [102], thus exploring predictors of micronutrient status may assist with identifying micronutrient issues within subgroups and determine micronutrient requirements and the development of stratified nutritional products in patients with PKU. Therefore, there is a need to explore predictors such as age, gender, metabolic control, PKU severity and body weight status in relation to micronutrient status, and to explore potential micronutrient interaction in this group of patients.

This study aimed to evaluate the micronutrient status of children with PKU and explore the contribution of factors associated with micronutrient deficiencies using a large retrospective dataset of patients with serial measurements.

4.2 Methods and subjects

4.2.1 Study design and eligible participants

Eligible participants were all paediatric patients (≤16 years old) with PKU who attended the metabolic medicine clinic at Yorkhill Royal Hospital for Sick Children (RHSC), Glasgow, United Kingdom between 1990 and 2013. Patients who attended the clinic prior to 1990 were excluded due to incomplete collection of data. All patients were diagnosed at birth via the national newborn screening program and were followed up in the same single centre until discharge to adult services. Patients' demographics and clinical data were collected through case note review (Table 4.1).

4.2.2 Blood samples and micronutrient measurements

Micronutrient measurements were available from non-fasting blood samples collected for the routine annual screening of micronutrient status. These were vitamins A, B12, D, E, serum folate, and erythrocyte folate and the trace elements copper, selenium, zinc and serum ferritin as a biomarker of iron stores. Blood samples collected for non-routine purposes (from patients >1 to younger than 4 years old) were excluded. The blood micronutrient concentrations were compared to the laboratory age and gender-specific reference ranges (Appendix C.1) and were classified as "below", "within" or "above" normal ranges (NR). Similarly, routine vitamin D screening was initiated in 2006 and their results were classified into "deficient", "insufficient" or "adequate" level status. Vitamin D blood concentrations were considered deficient if below 25 (nmol/L), insufficient between 25-50 (nmol/L),

and adequate if above 50 (nmol/L) as per local reference ranges. Routine vitamin screening was initiated in patients over 4 years of age from since 1996. To compare results of PKU patients with the prevalence of micronutrients deficiencies in the healthy population, blood micronutrient levels were also compared against the results (95% CI) from the UK's National Diet and Nutrition Survey (NDNS 2008-2009) in the general healthy population. The NDNS results were available for children 11- 16 years old (Appendix C.2) and allowed comparison for all measured micronutrients in this study except for copper.

Table 4.1: Cumulative number of blood samples, anthropometric, nutritional, and biochemical measurements, and demographics of paediatric patients with PKU who attended the metabolic medicine clinic at the RHSC between 1979 and 2013 (original pre-screened data)

		Patients n (%)	**Blood samples or measurements n (%)**
N		170	1,045
Sex	M	84 (49.41)	583 (55.88)
	F	86 (50.59)	462 (44.22)
Follow-up (Micronutrients) years			
	1979 – 1985	10	60
	1986 – 1990	20	70
	1991 – 1995	37	70
	1996 – 2000	67	202
	2001 – 2005	79	280
	2006 – 2010	97	252
	2011 – 2013	64	111
Anthropometry			
	Height		5,220
	Weight		5,410
	BMI		5,209

Table 4.1: Cumulative number of blood samples, anthropometric, nutritional, and biochemical measurements, and demographics of paediatric patients with PKU who attended the metabolic medicine clinic at the RHSC between 1979 and 2013 (original pre-screened data)

		Patients n (%)	Blood samples or measurements n (%)
Nutrition			
Number of prescribed protein exchanges			4749
Biochemistry			
PHE measurements			39,318
Micronutrients (total)			5,634
Vitamins			
	Vitamin A		617
	Vitamin B12		623
	Vitamin E		616
	Vitamin D		125
	Folate (Serum)		521
	Folate (RBC)		479
Trace elements			
	Copper		669
	Selenium		691
	Zinc		656
	Ferritin		637

RBC, red blood cells

4.2.3 PKU metabolic control

Metabolic control was based on the routine, blood spot measurements of phenylalanine (PHE) concentrations. Two different approaches were used to define metabolic control in this study. First, all PHE measurements were classified as high

or within age specific treatment targets, as recommended by the MRC party group (Table 4.2) [125]. The percentage of measurements with raised PHE concentrations for every patient in a year was calculated and this value was used as a continuous variable in further analysis. In the second approach, the percentage of measurements with raised PHE concentrations for each year were ranked and then grouped into quartiles. Measurements within the first quartile were considered to represent best metabolic control whereas those within the fourth quartile indicated worst metabolic control. Both approaches were used to explore the association between metabolic control and micronutrient status.

Table 4.2: MRC[a] recommendations for PHE treatment targets for the management of PKU

Age	PHE concentration targets (µmol/l)
0 - 5 years	120 – 360
> 5 – 10 years	120 – 480
> 10 years	120 – 700

[a] Recommendations on the dietary management of phenylketonuria. Report of Medical Research Council Working Party on Phenylketonuria [125]

4.2.4 PKU severity and PHE tolerance

The severity of PKU was defined as the patient's daily tolerable amount of dietary PHE (milligrams of PHE per day). Assuming that one gram of protein contains 50 mg of PHE, the median number of prescribed protein exchanges was calculated for every patient, the year prior to each micronutrient screening. The number of protein exchanges varied depending on patient tolerance to dietary PHE which in turn is associated with their genetic mutation type. The cut-offs used to classify the severity of PKU were based on the MRC group party recommendations (Table 4.3).

Table 4.3: PKU severity classes based on daily tolerable dietary PHE from prescribed number of protein exchanges

Prescribed protein exchanges (g/d)	Equivalent tolerable dietary PHE (mg/d) [a]	PKU severity class
< 6	< 300	Severe PKU
> 6-8	> 300-400	Moderate PKU
> 8-12	> 400-600	Mild PKU

[a] 50 mg of tolerable dietary PHE = 1 protein exchange (1 gram protein)

4.2.5 Anthropometry

Anthropometric measurements, height and weight, recorded in the year prior to micronutrient screening were used to generate annual median body mass index (BMI) z-scores according to the UK's 1990 reference data. These median values for each year were then classified into thin, normal, overweight and obese classes according to established cut-offs (Table 4.4) [403].

Table 4.4: Body mass index (BMI) centiles, z-score equivalents and classification

BMI centiles	BMI SDS (z-score)	BMI classes
< 2nd	< -2	Thin
25th to 85th	-2 to 1.036	Normal
85th to 95th	1.036 to 1.645	Overweight
> 95th	> 1.645	Obese

UK's 1990 growth reference centiles [377]

4.2.6 Age at testing

Measurements were grouped into four age bands (1 year or less, 4 to 5, 6 to 10 and 11 to 16 years) to predict changes in micronutrient status with increasing age. These

bands were based on the age groups in which PHE treatment targets are set to different ranges [125]. However, screening for micronutrients is not routinely performed for patients who are between > 1 to < 4 years, thus measurements within this age range were excluded.

4.2.7 Patient adherence to guidelines for biochemical, anthropometric and dietetic monitoring

The annual number of biochemical, anthropometric and dietetic monitoring assessments was calculated, for each patient, the year preceding micronutrient screening. Patient' adherence was represented as the percentage of these assessments against national recommendations (Table 4.5).

Table 4.5: Summary of MRC[a] recommendations for frequency of biochemical, anthropometric and dietetic monitoring assessments for the management of PKU

Recommendations		
Frequency of PHE monitoring assessments		
	Age	*Frequency*
	< 6 months	Weekly
	6 months – 4 years	Fortnightly
	> 4 years	Monthly
Frequency of growth and nutrition monitoring assessments		
	Age	*Frequency*
	< 12 months	Every 2 – 3 months
	1 – 4 years	Every 3 – 4 months
	> 4 years	Every 6 months;
		Annually for micronutrients[b]

[a] Recommendations on the dietary management of phenylketonuria. Report of Medical Research Council Working Party on Phenylketonuria [125]

[b] A consensus document for the diagnosis and management of children, adolescents and adults with phenylketonuria, the National Society for Phenylketonuria (NSPKU) [404]

4.2.8 Blood analysis

Micronutrient blood analysis was carried out at the Scottish Trace Element and Micronutrient Reference Laboratory (STEMRL), Royal Infirmary, Glasgow, and the Departments of Clinical Biochemistry and Haematology, Yorkhill Hospital using accredited methods. Liquid chromatography (LC) tandem-mass spectrometry was used to measure PHE concentrations from routine dried blood spots and vitamin D (25HCC) levels in serum. Plasma copper, selenium and zinc levels were determined using inductively coupled plasma mass spectrometry (ICP-MS). Serum vitamin A and vitamin E levels (tocopherol) were measured by high performance liquid chromatography (HPLC) with UV detection. Levels of erythrocyte and serum folate and vitamin B12 were measured according to laboratory standard procedure.

4.2.9 Statistical analyses

Descriptive statistics were performed to present demographics and disease characteristics. Micronutrient measurements were categorised as below, within or above NR. Predictors of micronutrient status were explored only for those micronutrients which were above or below the laboratory normal ranges (NR) for more than 10% of the measurements. Measurements indicative of overweight and obesity were merged together to form one class in order to improve statistical power in subgroup analysis. Similarly, moderate and mild severity of PKU were also combined and compared against severe PKU.

Predictors of micronutrient status included sex, age at testing, year the measurement took place, metabolic control [(a) percentage of measurements with raised PHE concentrations, (b) quartiles of measurements with raised PHE concentrations], PKU severity and annual median of BMI z-scores. Categorical data was compared using Chi-square or Fisher's exact test. For continuous variables,

differences according to the micronutrient status were explored using two-sample *t* or Mann-Whitney test. A heatmap was constructed to visualise micronutrient status pattern using a supervised approach in R package. Univariate binary logistic regression analyses were conducted to examine associations between micronutrient status and each potential predictor. The strength of association between predictors and micronutrient status was measured by odds ratio and associated 95% CI. The process of selection and inclusion of variables in the multivariate model was based on univariate logistic regression analysis. Associated predictors with *P* values ≤0.05 were entered in a multivariate model and their independent association was tested using forward stepwise elimination. The models included only those predictors with *P* value ≤0.05 as independent predictors of micronutrient status, and were entered in descending order starting from the most significant (with the highest R^2 value) to the least significant (with the lowest R^2 value) predictor. Vitamin B12 in the low-PHE diet is sourced via a micronutrient-enriched PKU protein substitutes [323, 405], thus it could be used as a predictor to check patients' adherence to the intake of the protein substitutes as indicated previously [155]. Vitamin B12 status was used as a predictor in the regression analysis of deficient micronutrients (those showing low concentrations of more than 10% of the measurements) to examine association with adherence to the intake of PKU protein substitutes. Furthermore, association between levels of a micronutrient with another was examined to predict potential interactions between micronutrients. Since the PKU control is frequently reported to deteriorate with age, linear fit regression was conducted to examine how PKU control (as a percentage of raised PHE concentration measurements) and PKU severity (as annual median PHE tolerance) were explained by age. In addition, one-way ANOVA test was used to check whether or not the average age significantly varied within groups of follow up

years. Statistical significance was considered when *P* values ≤ 0.05. Analyses were carried out using Minitab ® (v 17.1.0) statistical software package.

4.3 Results

4.3.1 Study Participants and blood samples

From the 170 paediatric PKU patients who attended the metabolic clinic between 1979 and 2013, were entered in the database, 89 did not meet the inclusion criteria and were removed from further analysis. Thus, 81 patients (48% of initial participant cohort), were included in this study all of whom were prescribed on a low-PHE diet. From these patients, a total of 538 blood samples were collected for their annual micronutrient screening. Micronutrient assessment performed for non-routine purposes (i.e. age 1 to 4 years; n= 19) were excluded, as were samples from patients older than 16 years (n= 7). Therefore, 512 blood samples were brought forward and included in this study (49% of total samples) (Table 4.6).

Both genders were equally represented in the study sample (Table 4.6). Two thirds of micronutrient assessments were available from children between 4 – 10 years (61%); 31% from children between 11-16 years and 8% from children ≤1 year. Median age at last blood sample collection was 13 years (Q1-Q3= 6.7-15.2). Forty-one patients (50%) had at least one micronutrient assessment in all four age groups; 55 (68%) and 69 (85%) in any two and three age groups respectively. The median number of blood samples collected per patient during the entire study period was 7 (Q1-Q3= 3-9). The majority (62%) of the included samples were collected between 2001 and 2010 (Table 4.6). Analysis by one way-ANOVA showed that average age at testing (when samples were collected) was significantly (*P*<0.001) higher in follow-up years after 2001, and between 1996 - 1995 compared to earlier years.

Table 4.6: Number of blood samples collected in respective follow-up years, status and number of anthropometric, nutritional, and biochemical measurements and demographics of paediatric PKU patients who attended the metabolic medicine clinic at the RHSC between 1990 – 2013

		Patients n (%)	Blood samples / Measurements n (%)
N		81	512
Sex	M	40 (49.38)	265 (51.76)
	F	41 (50.61)	247 (48.24)
Age (Years)			
	≤ 1	37	40 (7.81)
	4 – 5	59	103 (20.12)
	6 – 10	65	208 (40.63)
	11 – 16	46	161 7(31.45)
Follow-up Years (Micronutrients)			
	1990 – 1995	26	26 (5.08)
	1996 – 2000	38	79 (15.43)
	2001 – 2005	47	170 (33.20)
	2006 – 2010	51	147 (28.71)
	2011 – 2013	51	90 (17.58)
Anthropometry			
Annual median Height z-score (SD)*			
Total [d]: 1,726	Short < -2	6	11 (2.16)
	Normal > -2	81	499 (97.84)
Median weight z-score (SD)*			
Total [d]: n=1,734	Low < -2	4	13 (2.55)
	Normal -2 to 1.036	74	387 (75.88)
	High >1.036	31	110 (21.57)
Median BMI z-score (SD)*			
Total [a]: n=1,726	Thin [b]	3	8 (1.57)

Table 4.6: Number of blood samples collected in respective follow-up years, status and number of anthropometric, nutritional, and biochemical measurements and demographics of paediatric PKU patients who attended the metabolic medicine clinic at the RHSC between 1990 – 2013

		Patients n (%)	**Blood samples / Measurements n (%)**
	Normal [b]	68	351 (68.82)
	Overweight [b]	32	85 (16.67)
	Obese [b]	25	66 (12.94)
PKU severity*			
Total [a]: n=1,638	Severe PKU [c]	65	363 (74.08)
	Moderate PKU [c]	19	67 (13.67)
	Mild PKU [c]	20	60 (12.24)
Biochemistry			
Quartiles [d] of measurements with raised PHE concentrations *			
Total [a]: n=13,376	1st Quartile (0 – 4.35%)	38	
	2nd Quartile (>4.35 – 20%)	52	
	3rd Quartile (>20 – 50%)	52	
	4th Quartile (>50 – 100%)	37	

* Annual median measurements per patient per year in which micronutrients were screened

[a] Total number of measurements used to calculate annual medians

[b] Classified BMI z-scores were according to UK's established cut offs (Cole et al., 1995)

[c] Severity classification was based on patient's PHE tolerance (mg/d). The classes cut-offs were according the hospital's clinical management practice

[d] Quartiles of measurements with high PHE concentrations were calculated from percentages of measurements with raised PHE concentrations for every patient and each year

4.3.2 Micronutrient status

Trace elements were the most frequently measured micronutrients, while vitamin D was the least measured micronutrient (Figure 4.1). A substantial proportion of

vitamins B12 (28%), E (55%) and folate (serum, 46%; RBC, 35%) measurements was above the normal ranges (NR) (Figure 4.1). Vitamin D levels were insufficient in 15% of the measurements. Unlike vitamins, trace element measurements showed the opposite trend and some were low in patients with PKU. Plasma selenium, zinc, and copper levels were below the NR in 44%, 15% and 9% of the measurements, respectively. On visualisation, there was no obvious pattern or clustering of the status of the various micronutrients tested (Figure 4.2).

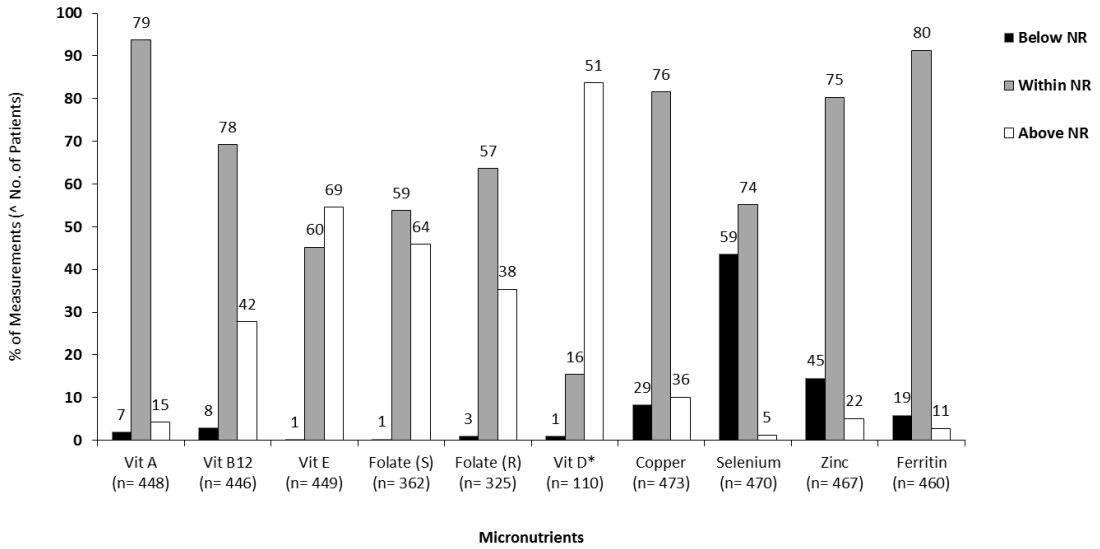


Figure 4.1 Status of micronutrient measurements of PKU patients who attended metabolic medicine clinic at RHSC 1990 – 2013, compared with laboratory normal ranges (NR)

Total micronutrient measurements= 4010

* Vitamin D measurements were classified into deficient ■, insufficient ▒ and adequate □

n= number of total micronutrient measurements

Folate (S), folate levels in serum; Folate (R), folate levels in red blood cells

Blood micronutrient results (95% CI) from the UK's NDNS were available for children aged above 11 years old. Compared to these, nearly half of the vitamin B12 measurements from the PKU children in this study were above the 97.5% centile and none of them were below the 2.5th (Table 4.7). Folate was low in 8% and 15% of serum and RBC measurements in PKU children. Approximately one-third of

selenium and zinc measurements in the PKU children were below 2.5th centile, whilst the vast majority of vitamin A, E, D and ferritin measurements were within the centile range (2.5th to 97.5th range) (Table 4.7).

Table 4.7: Micronutrient status of PKU patients over 11 years old compared to blood micronutrient results (2.5th to 97.5th centile range ''CR'') of UK NDNS[a]

		Total no. measurements	Below CR n (%)[b]	Within CR n (%)[b]	Above CR n (%)[b]
Vitamins					
	Vitamin A	149	1 (0.67)	146 (97.99)	2 (1.34)
	Vitamin B12	154	0	78 (50.65)	76 (49.35)
	Vitamin E	150	2 (1.33)	148 (98.67)	0
	Vitamin D	46	0	40 (86.96)	6 (13.04)
	Folate (Serum)[c]	112	9 (8.04)	97 (86.61)	6 (5.36)
	Folate (RBC)[c]	99	15 (15.15)	81 (81.82)	3 (3.03)
Trace elements					
	Copper	N/A			
	Selenium	150	46 (30.67)	96 (64.00)	8 (5.33)
	Zinc	145	34 (23.45)	110 (75.86)	1 (0.69)
	Ferritin	156	0	155 (99.36)	1 (0.64)

[a] UK National Diet and Nutrition Survey (NDNS 2008-2009)

[b] Micronutrient concentrations were considered within CR when measurements fall between lower 2.5 and upper 97.5 centile values. Measurements below lower 2.5 or above upper 97.5 centiles were classified as below or above CR, respectively.

[c] Folate NDNS data was available on March 2015

[d] No NDNS centails available for copper

CR, centile range; RBC, red blood cells

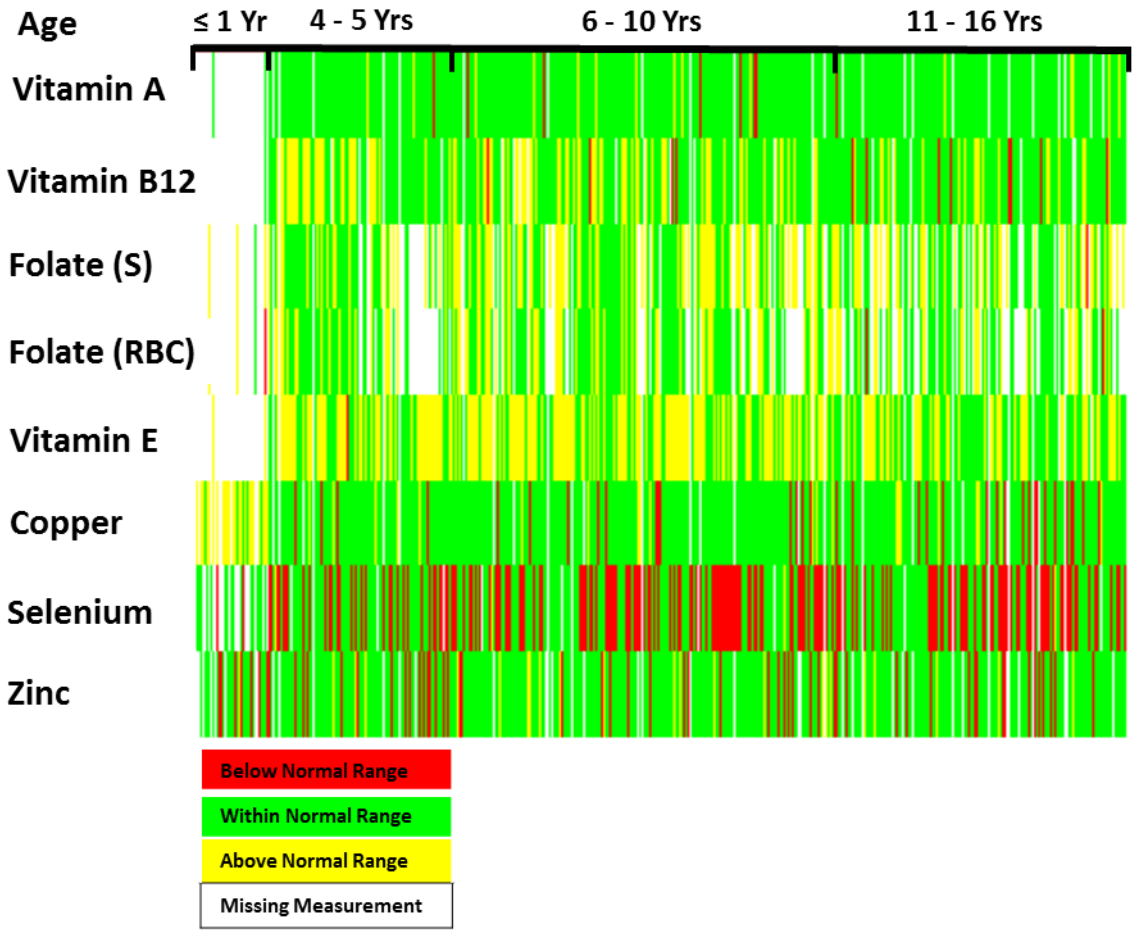


Figure 4.2: Heatmap for status of individual measures of micronutrients across age groups of PKU patients who attended metabolic medicine clinic at RHSC 1990 – 2013[a]

[a] Measurements compared to laboratory reference ranges

RBC, red blood cells

4.3.3 PKU metabolic control

Indices of metabolic control were calculated for the 512 micronutrient screening years using a total of 13,376 PHE measurements. The upper quartile (4th Quartile), representing poorest metabolic control, included years in which more than 50% of PHE measurements were above the treatment targets; while the lowest quartile (1st Quartile), representing best metabolic control, included years in which 95% or more of the PHE measurements were within treatment targets (Table 4.6). Linear regression analysis revealed that there was a significant (P<0.01; R^2, 2.06%; β, 1.072) correlation between age increase and proportion of measurements with raised PHE concentrations.

4.3.4 PKU severity and PHE tolerance

The large majority (75%) of collected blood samples were from patients with severe PKU; 13% and 12% were from patients with moderate and mild PKU, respectively (Table 4.6). There was a significant (P<0.001; R^2, 2.95%; β, 0.137) association between the median PHE tolerance (i.e. number of protein exchanges) with age increase.

4.3.5 Anthropometry

Annual median BMI z-score values per micronutrient screening year were calculated from a total of 1,726 measurements. Seventy percent of these measurements (derived from n=68 patients) were classified as normal and the remaining 30% were overweight or obese (derived from a total of 57 patients) (Table 4.6). The median of BMI z-score (median per year) was 0.42 (Q1-Q3= -0.19-1.17). The median for height and weight z-scores (median per year) were -0.26 (Q1-Q3= -0.83-0.2) and 0.28 (Q1-Q3= -0.60-0.94), respectively.

4.3.6 Patient adherence to guidelines for biochemical, anthropometric and dietetic monitoring

The annual number of PHE monitoring assessments in all age groups was almost twice the recommended number of assessments per year advised by the MRC party group (Table 4.8). Patients adhered to the recommended frequency of PHE monitoring assessments (% of the recommended frequency) in the vast majority of follow-up years. Similarly, the annual number of anthropometric and dietetic monitoring assessments for all age groups exceeded the recommended number of assessments per year. The patients adhered to the recommended frequency of anthropometric and dietetic monitoring assessments (% of the recommended frequency) in almost all follow-up years as shown in Table 4.8.

Table 4.8: Annual number (median and quartiles) of biochemical, anthropometric and dietetic monitoring assessments, and achieved recommended frequency (%recommended frequency per year) during follow-up years for all patients and according to each age group

	Age	Recommended frequency (per year)[a]	Patients (n)	Median (% Rec)	Q1 (% Rec)	Q3 (% Rec)	Achieved/Adherence n (%)
PHE measurements							
	≤ 6 months	Weekly[c]	36	41.5 (2500)	22 (1225)	68 (4600)	36 (100)
	6 months to 1 year	Fortnightly[c]	4	51 (193.1)	26 (91.3)	67 (206.1)	3 (75)
	≥ 4 years	Monthly	80	20 (166.7)	13 (108.3)	33 (275)	368 (77.97)
	All		81	21 (175)	13 (108.3)	35 (306.3)	407 (79.49)
Dietetic reviews [d]							
	≤ 1 year	4 - 6	37	7 (650)	4 (400)	11 (1025)	34 (100)
	≥ 4 years	2	80	3 (150)	3 (100)	4 (200)	415 (91.01)
	All		81	3 (150)	2 (100)	4 (200)	449 (91.36)

Table 4.8: Annual number (median and quartiles) of biochemical, anthropometric and dietetic monitoring assessments, and achieved recommended frequency (%recommended frequency per year) during follow-up years for all patients and according to each age group

	Age	Recommended frequency (per year)[a]	Patients (n)	Median (% Rec)	Q1 (% Rec)	Q3 (% Rec)	Achieved/Adherence n (%)
BMI measurements[e]							
	≤ 1 year	4 – 6	37	6 (500)	3.25 (300)	8 (800)	40 (100)
	≥ 4 years	2	80	3 (150)	3 (150)	4 (200)	433 (92.13)
	All		81	3 (150)	3 (150)	4 (200)	473 (92.75)

[a] Medical Research Council Working Party on Phenylketonuria (MRC 1993), UK

[b] Percentages calculated as [number of blood spots (or follow-ups) obtained from a patient in a year ÷ yearly recommended number of blood spots (or follow-ups) x 100]

[c] Up to the measurement date from birth

[d] Calculated from number of protein prescriptions

[e] Calculation of percentage compliance with dietetic and BMI reviews was based on the minimum number of yearly follow-up

4.3.7 Predictors of micronutrient status in PKU patients

4.3.7.1 Gender

Vitamin E status was associated (P=0.014 x^2-test) with gender, with a higher percentage of measurements, above the NR found for females (Figure 4.3). The status of other micronutrients did not show any gender effect (Figure 4.3).

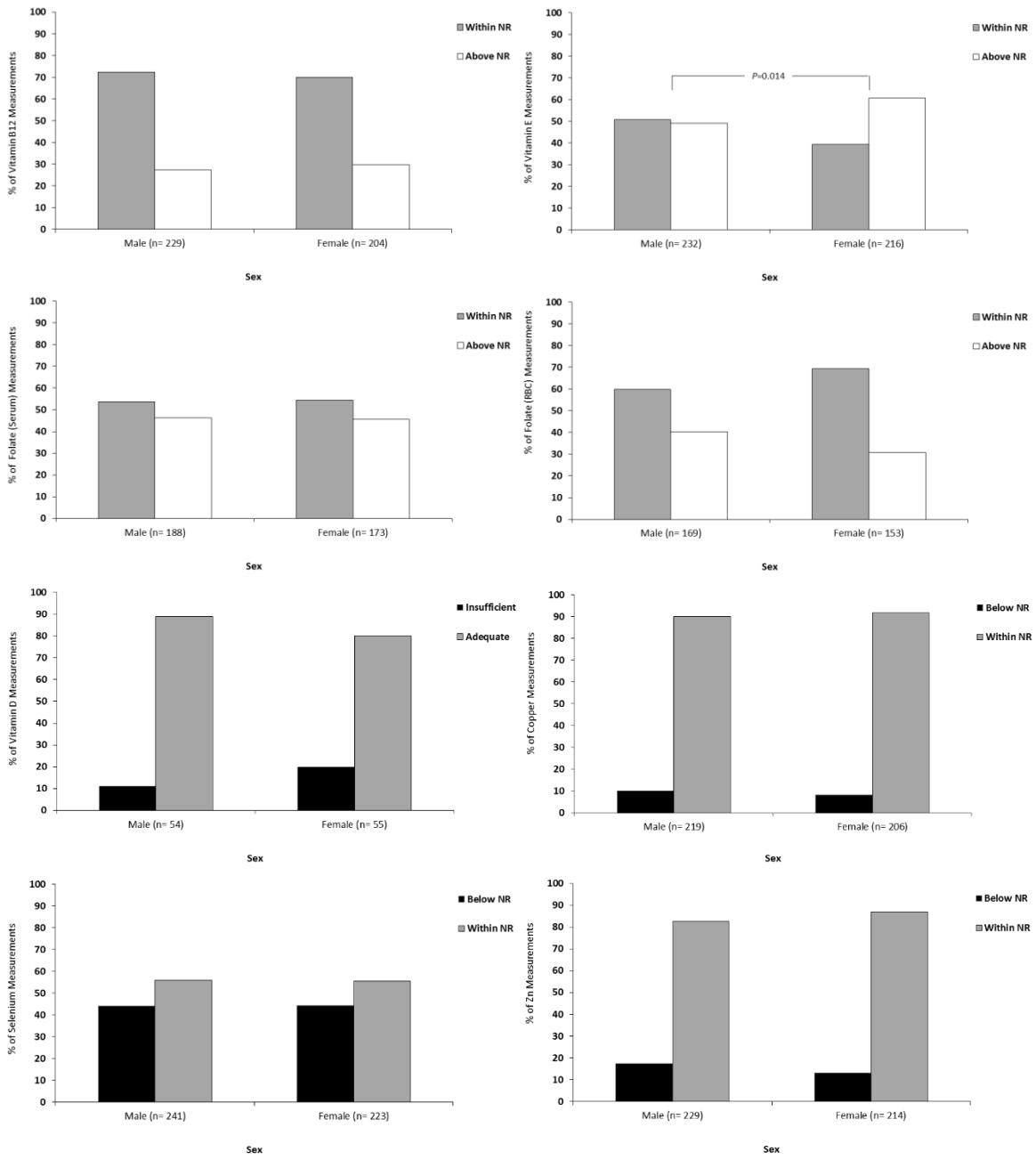


Figure 4.3: Status of vitamin B12, E and D, folate, copper, selenium and zinc measurements of male and female patients with PKU who attended metabolic medicine clinic at RHSC 1990 – 2013, according to gender

RBC, red blood cells

4.3.7.2 Age

Younger age (in yeas) was associated with vitamin B12 and E measurements above NR, while low copper measurements were associated with older age (Table 4.9).The same was found when status of vitamins B12 and E were compared between age categories (Figure 4.4). Low copper and selenium status were more

common in children older than 6 years when compared to younger age groups. Similarly, the proportion of low zinc measurements was significantly higher in children aged between 4 - 5 years than in those aged 6 - 10 years old (Figure 4.4).

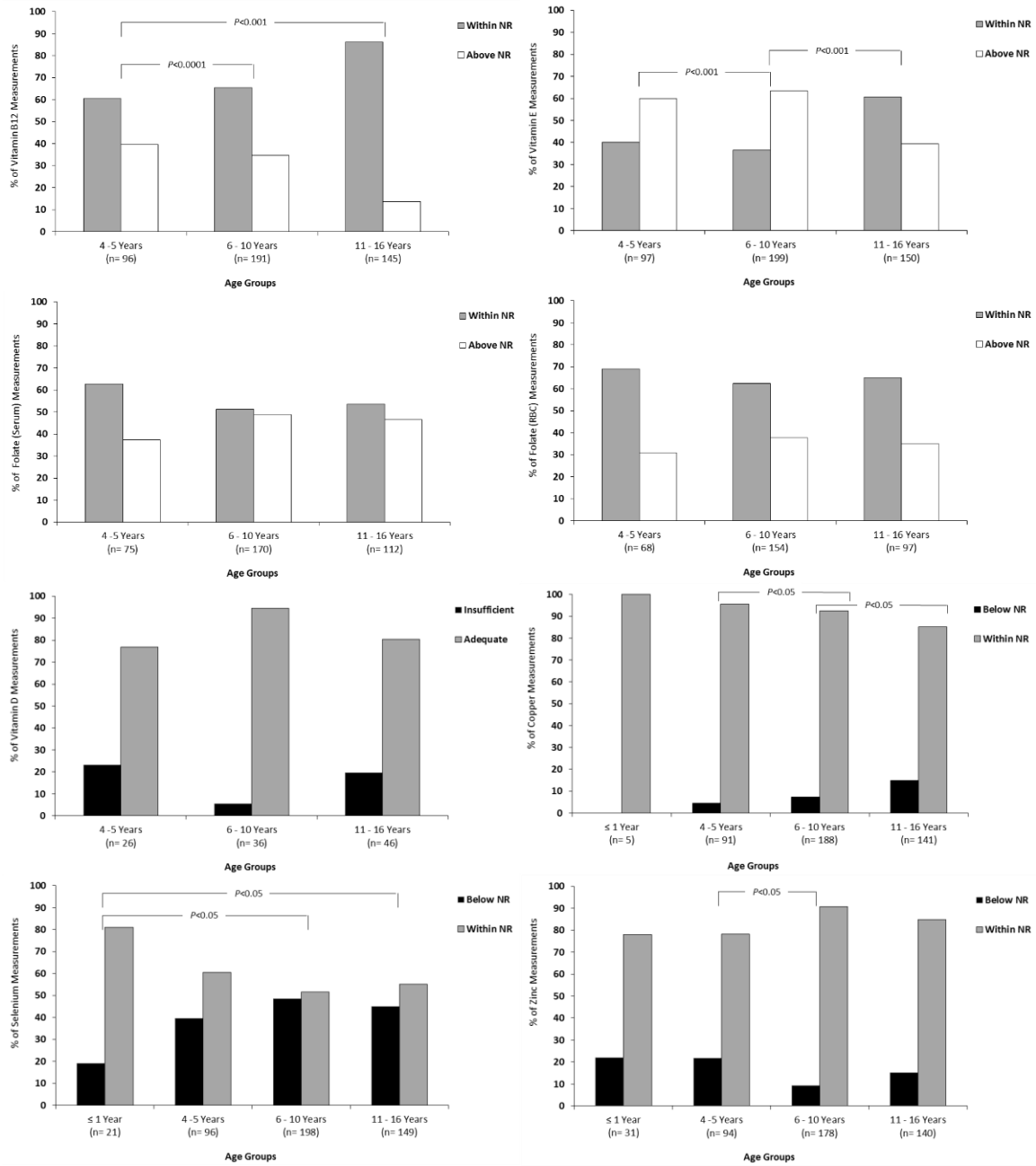


Figure 4.4: Status of vitamin B12, E and D, folate, copper, selenium and zinc measurements by age groups of PKU patients who attended metabolic medicine clinic at RHSC 1990 – 2013

* Vitamin screening has never been introduced in clinical practice for infants under 1 year old.

All p-values were from Fisher's exact test

RBC, red blood cells

4.3.7.3 PKU Metabolic control

A. Percentage of measurements with raised PHE concentrations:

Measurements of vitamins B12 (P<0.01), E (P<0.001) and folate (serum P<0.001; RBC P<0.005) above the NR were associated with better metabolic control (low median percentage of measurements with raised PHE concentrations) than those with values within NR (Table 4.9). Low measurements of copper were also (P<0.05) associated with better metabolic control than those with values within NR. In contrast, low measurements of selenium were significantly (P<0.001) associated with poorer metabolic control (high median percentage of measurements with raised PHE concentrations) than those with values within NR (Table 4.9).

B. Quartiles of measurements with raised PHE concentrations:

Quartiles of measurements with raised PHE concentrations correspond to metabolic control, with a decrease in quality of metabolic control from the lower to upper quartile. Measurements which had the poorest metabolic control (in the upper quartiles), were found to have a significantly lower proportions of vitamin B12, vitamin E and RBC folate measurements above the NR. On the contrary, the number of serum folate measurements above NR was significantly higher in measurements with good metabolic control (Figure 4.5). Compared to the lower quartiles, the number of low selenium measurements was significantly higher in third and fourth quartiles of measurements with raised PHE concentrations. On the contrary, the number of low copper measurements was highest in the first quartile. However, a significant difference was only reported between the number of low copper measurements in the first quartile and the third quartile (Figure 4.5). Vitamin D and zinc status did not differ significantly between quartiles of measurements with raised PHE concentrations.

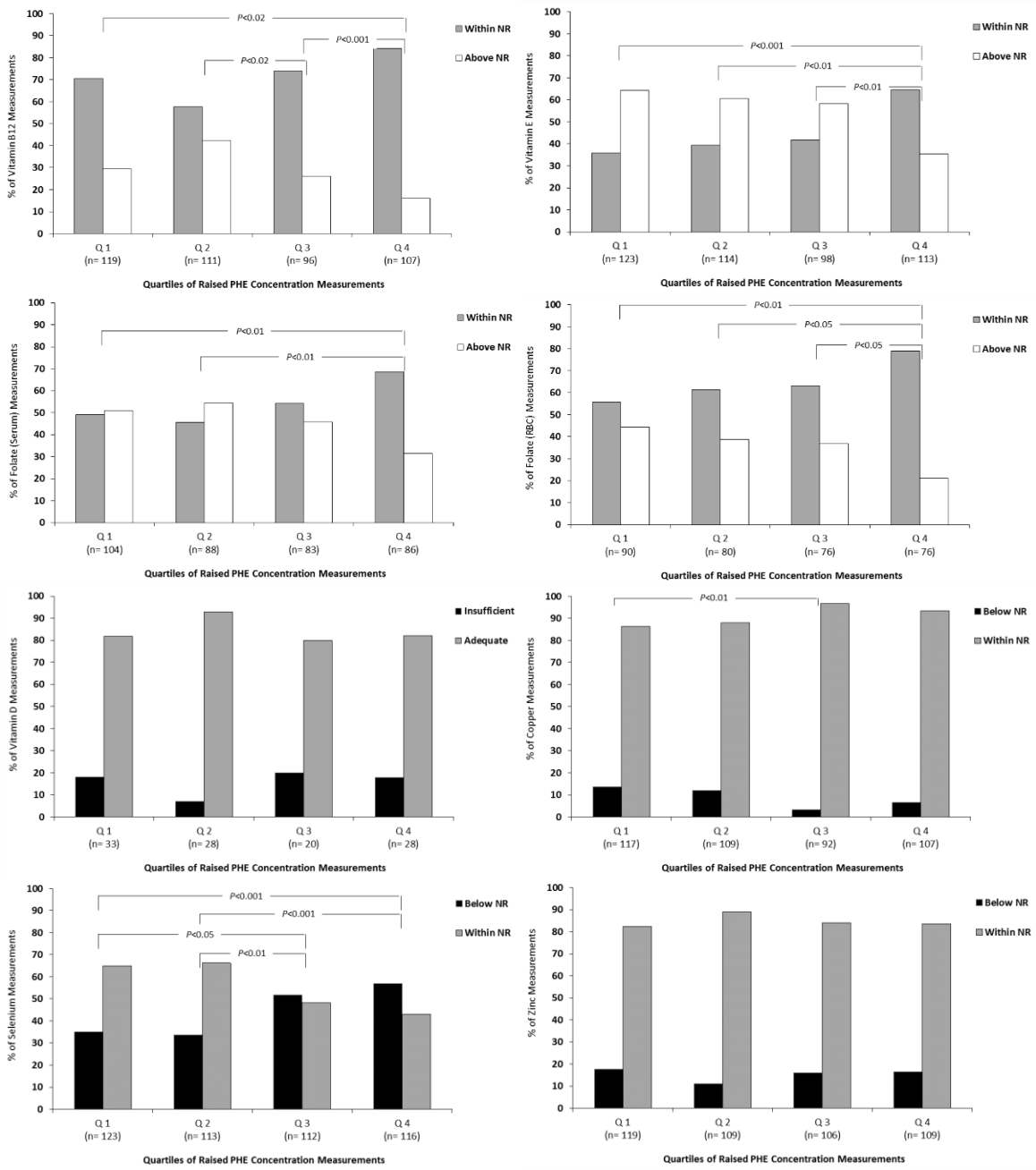


Figure 4.5: Status of vitamin B12, E and D, folate, copper, selenium and zinc measurements within quartiles[a] of measurements with raised PHE concentrations of PKU patients who attended metabolic medicine clinic at RHSC 1990 – 2013

[a] Quartiles of measurements with raised PHE concentrations were calculated from percentages of measurements with raised PHE concentrations for every patient in each year

All yielded p-values were form Fisher's exact test: exact p-value

Q1, first (lower) quartile; Q2, second quartile; Q3, third quartile; Q4, forth (upper) quartile

RBC, red blood cells

4.3.7.4 PKU severity and PHE tolerance

Moderate or mild PKU were associated with a significantly higher proportion of high vitamin E ($P<0.05$) and low copper measurements ($P<0.001$). The percentage of low selenium measurements was significantly higher ($P<0.01$ x^2-test) in severe PKU (Figure 4.6). No significant differences were found between PKU severity classes for vitamin B12, vitamin D, folate and zinc.

Low selenium status was associated ($P<0.001$) with low PHE tolerance (low median protein exchanges) (Table 4.9). In contrast, high PHE tolerance was associated with low copper status ($P<0.001$) (Table 4.9). No statistical differences were found between PHE tolerance levels with the status of vitamins B12, E, D, folate and zinc.

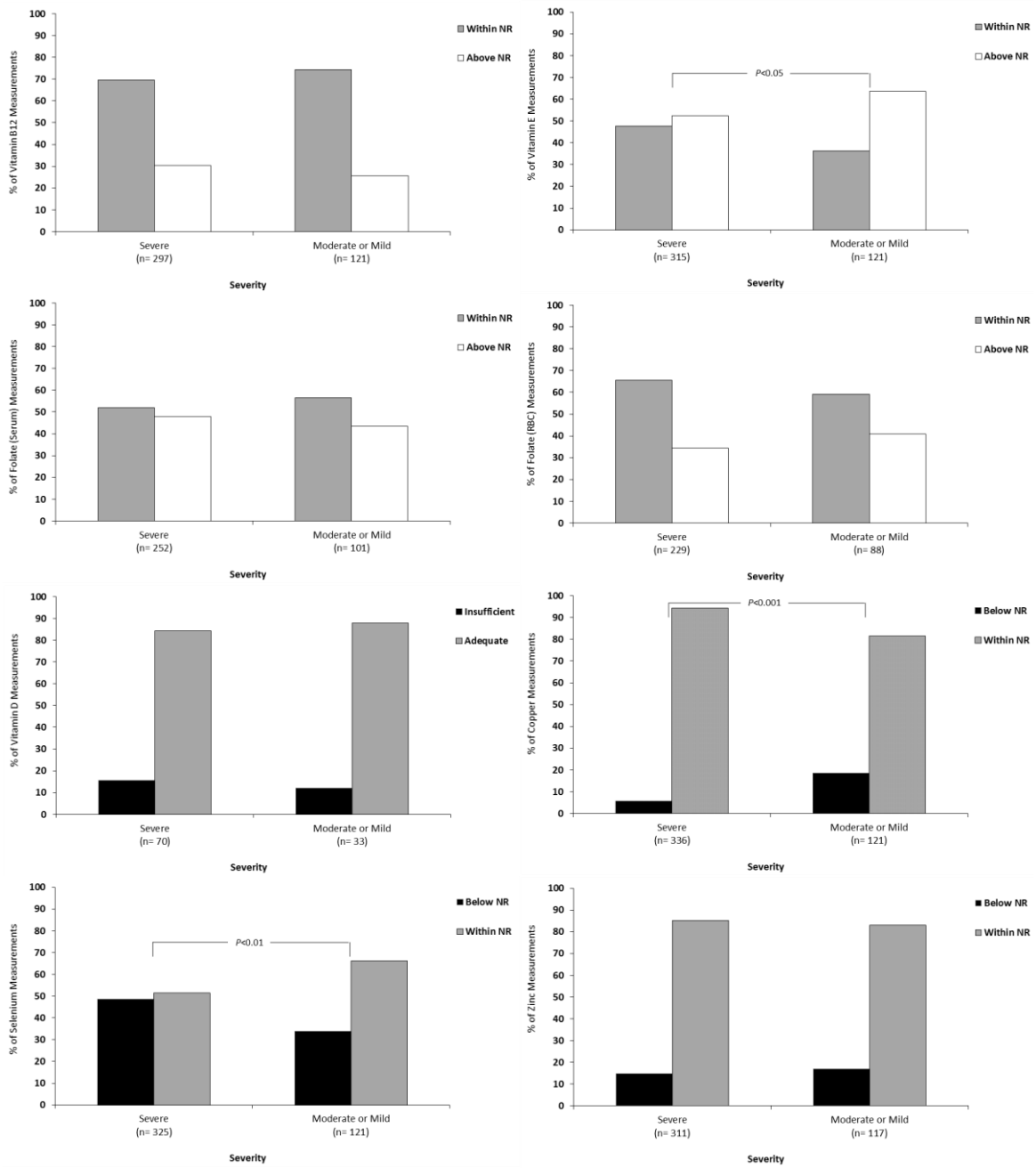


Figure 4.6: Status of vitamin B12, E and D, folate, copper, selenium and zinc within classes* of disease severity for PKU patients who attended metabolic medicine clinic at RHSC 1990 - 2013

*PKU severity classes were based on median annual PHE tolerance level (mg/d). The PKU severity cut-offs from PHE tolerance were as follow: Severe PKU <300 mg/d, moderate PKU > 300 - 400 mg/d and > 400 - 600 mg/d for mild PKU

RBC, red blood cells

4.3.7.5 Anthropometry

BMI, neither as a z-score (Table 4.9) nor as classes (Figure 4.7) was associated with the status of any micronutrient studied.

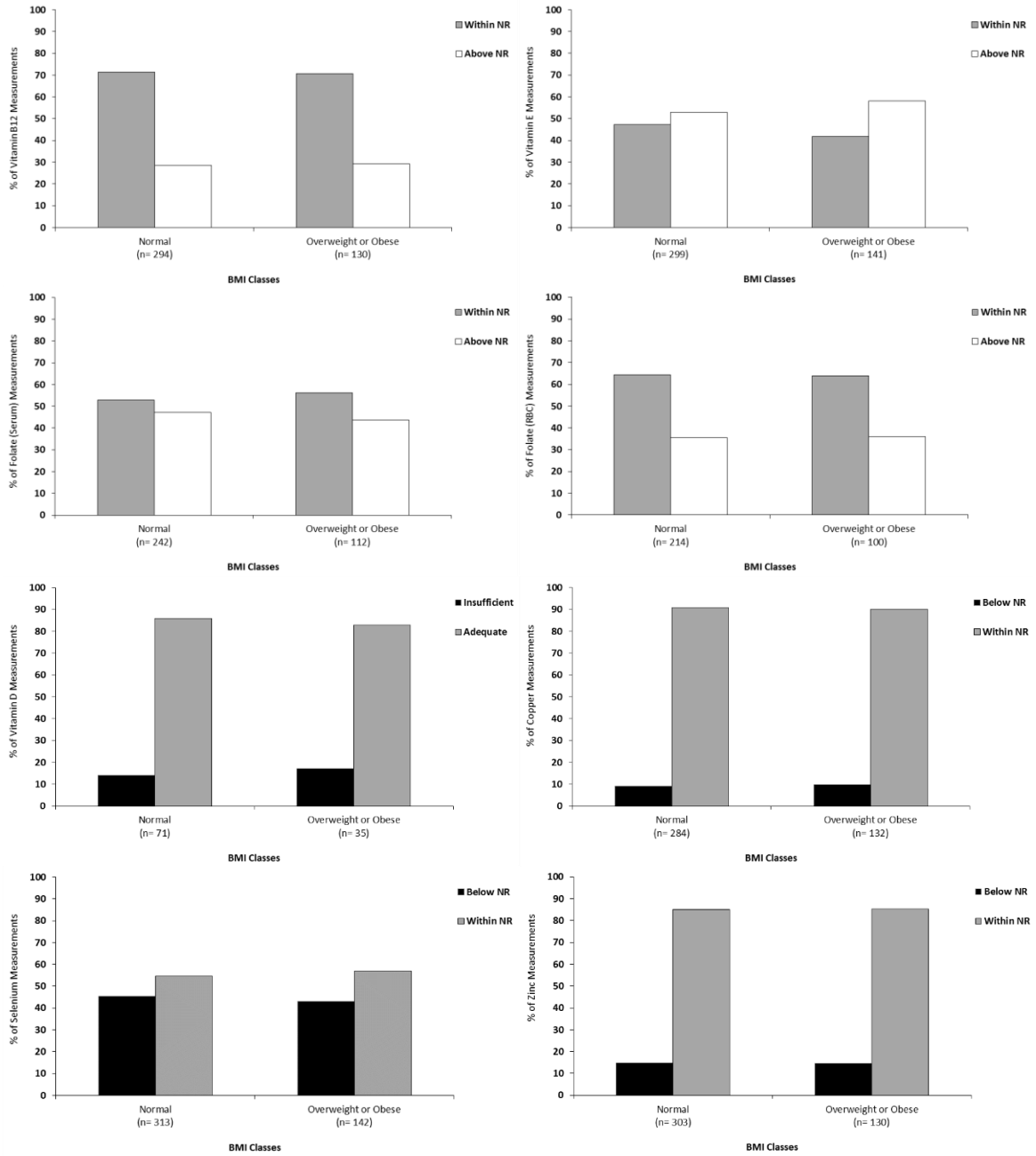


Figure 4.7: Status of vitamin B12, E and D, folate, copper, selenium and zinc measurements within classes of BMI * for PKU patients who attended metabolic medicine clinic at RHSC 1990 – 2013

* Classified BMI z-scores were according to UK's established cut offs (Cole et al., 1995)

RBC, red blood cells

4.3.7.6 Temporal changes in micronutrient status

Compared with micronutrient assessments performed prior to 2006, the proportions of vitamin B12 measurements above the NR were significantly lower in the period between 2006 and 2013 (Figure 4.8). Similarly, the proportion of vitamin E and serum folate measurements above the NR was significantly less between 2001 and 2005 compared to other follow-up periods. The proportions of copper and zinc measurements below NR were significantly high during 2010 to 2013. However, the proportions of selenium measurements below NR were significantly declined after 2006 (Figure 4.8).

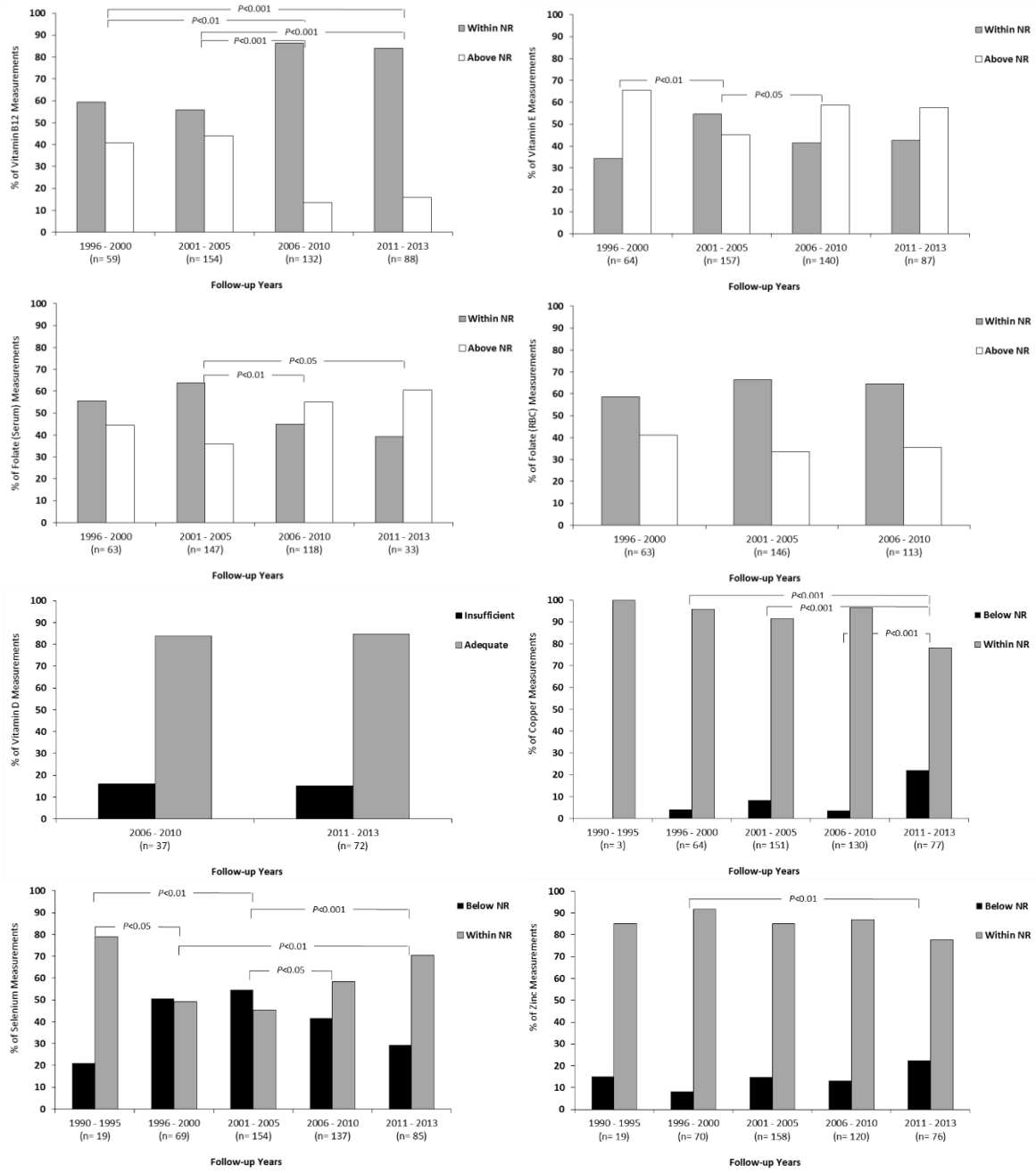


Figure 4.8: Status of vitamin B12, E and D, folate, copper, selenium and zinc measurements during follow-up years * for PKU patients who attended metabolic medicine clinic at RHSC 1990 – 2013

* Vitamin screening was initiated in patients over 3 years old from 1996. Vitamin screening has never been introduced in clinical practice for infants under 1 year old. Vitamin D was not assessed until 2006 for all patients.

All p-values were from Fisher's exact test

RBC, red blood cells

Table 4.9: Micronutrient status predictors: age at testing, percentage of measurements with raised PHE concentrations, median PHE tolerance, median BMI z-score within status classes of vitamins B12, E, Folate, D, and trace element measurments of Copper, Selenium and Zinc in PKU patients

Predictor		Micronutrient status	B12	E	D[a]	Folate (Serum)	Folate (RBC)	Micronutrient status	Cu	Se	Zn
Age (years)[b]	Median	Within NR	9.8	9.9	13.2	8.8	9.0	Below NR	11.5	9.0	7.7
	Q1		6.7	6.4	5.0	6.1	6.1		6.9	6.3	4.3
	Q3		12.9	13.5	15.1	11.7	11.4		13.8	11.6	12.3
	Median	Above NR	7.9	8.2	9.0	9.1	8.9	Within NR	8.8	8.6	8.7
	Q1		5.4	6.1	6.2	6.6	6.4		6.1	5.3	5.9
	Q3		9.9	10.1	13.2	11.4	11.7		11.6	12.2	11.6
	P-value		<0.001	<0.001	NS	NS	NS		<0.05	NS	NS
Percentage of Measurements with raised PHE concentrations[c]	Median	Within NR	28.6	21.8	23.6	23.3	21.5	Below NR	7.7	29	21
	Q1		5.9	2.3	0	4.0	5		0	6.0	2.1
	Q3		69.0	57.5	80	61.0	55.6		25.0	58.6	55.0
	Median	Above NR	12.5	11.5	14.0	12.5	10.2	Within NR	20	13.3	18.2
	Q1		2.1	2.8	3.0	2.0	0		4.0	2.3	4.0
	Q3		33.3	31.0	52.0	36.0	32.0		53.0	40.0	50.0
	P-value		<0.0001	<0.01	NS	<0.01	<0.001		<0.05	<0.001	NS
Median PHE tolerance (mg/d)[d]	Median	Within NR	250	225	200	250	200	Below NR	350	200	250
	Q1		150	150	150	150	150		219	150	193.5
	Q3		350	300	500	350	350		412.5	300	400
	Median	Above NR	250	225	200	250	200	Within NR	250	250	250
	Q1		150	150	150	150	150		150	150	150
	Q3		350	350	387.5	350	350		300	350	350
	P-value		NS	NS	NS	NS	NS		<0.001	<0.001	NS
Median BMI z-score (SD)[e]	Mean	Within NR	0.53	0.44	0.57	0.54	0.56	Below NR	0.53	0.52	0.47
	SD		1.00	1.04	0.99	0.98	0.98		1.05	0.97	1.08
	Mean	Above NR	0.52	0.61	0.46	0.53	0.47	Within NR	0.52	0.52	0.51
	SD		1.02	1.01	1.01	1.03	1.06		1.02	1.08	1.00
	P-value		NS	NS	NS	NS	NS		NS	NS	NS

[a] Vitamin D classes: insufficient (in Within NR row) and adequate (in Over NR row)

[b] Median age at date of micronutrient screening

Table 4.9: Micronutrient status predictors: age at testing, percentage of measurements with raised PHE concentrations, median PHE tolerance, median BMI z-score within status classes of vitamins B12, E, Folate, D, and trace element measurments of Copper, Selenium and Zinc in PKU patients

Predictor	Micronutrient status	B12	E	D[a]	Folate (Serum)	Folate (RBC)	Micronutrient status	Cu	Se	Zn

[c] Median of percentages of measurements with raised PHE concentrations

[d] Median of PHE tolerance (mg/d) measurements

[e] Mean value of BMI z-score measurements

B12, vitamin B12; E, vitamin E; D, vitamin D; RBC, red blood cells; Cu, copper; Se, selenium; Zn, zinc

4.3.8 Regression analysis on predictors of micronutrient status

Multivariate logistic regression analysis showed that age increase and percentage of measurements with raised PHE concentrations were independent predictors of high status of vitamins B12 and E (Table 4.10). Mild/moderate PKU severity and adherence with the recommended dietetic monitoring assessments were also independent predictors of high vitamin E and B12 status, respectively. Female gender was a predictor of high vitamin E status. However, there was a significant (P<0.02) interaction between gender and adherence with the recommended PHE monitoring assessments in the multivariate model for vitamin E. The significant association between adherence with the recommended PHE monitoring assessments and status of both vitamins B12 and E; as well as the association between mild/moderate PKU severity with status of vitamin E, both of which were revealed in the univariate analysis, were lost in the multivariate model (Table 4.11 and Table 4.12).

When looked at trace elements, mild/moderate PKU severity and age increase were independent predictors of low copper status (Table 4.10). Mild/moderate PKU severity, metabolic control (% raised PHE concentrations) and compliance to PKU protein substitutes (based on vitamin B12 status) were independent predictors of low selenium status. The univariate analysis showed that compliance to PKU protein substitutes was a predictor of low zinc status, but this association was lost when we accounted for other confounders in the multivariate analysis (Table 4.12).

Table 4.10: Predictors of blood micronutrients status in PKU patients based on multivariate logistic analysis with P, R2, and β̂ values, 95% CI and odds ratio

Predictor/Micronutrient	Vitamin B12 (Response: ANR)				Vitamin E¥ (Response: ANR)				Copper (Response: BNR)				Selenium (Response: BNR)			
	P-v	R^2	β̂	OR (95% CI)	*P-v*	R^2	β̂	OR (95% CI)	*P-v*	R^2	β̂	OR (95% CI)	*P-v*	R^2	β̂	OR (95% CI)
Age	0.000	7.94%	-0.13	0.88 (0.82, 0.94)	0.000	8.84%	-0.12	0.89 (0.84, 0.95)	0.025	7.72%	0.11	1.12 (1.01, 1.24)		5.88%		
Sex (Female)					0.002		0.63	1.87 (1.24, 2.82)								
% of raised PHE concentrations	0.006		-0.01	0.99 (0.98, 0.99)	0.000		-0.01	0.98 (0.98, 0.99)					0.022		0.01	1.01 (1.00, 1.02)
Severity (Mild/Moderate)					0.335		0.24	1.28 (0.77, 2.10)	0.000		1.27	3.55 (1.79, 7.10)	0.022		-0.59	0.5525 (0.33, 0.92)
BMI Z-scores																
PHE monitoring assessments (Adherent)	0.089		0.53	1.71 (0.90, 3.22)	0.550		0.16	1.17 (0.70, 1.96)								
Dietetic monitoring assessments (Adherent)	0.034		1.18	3.26 (0.94, 11.24)												
Compliance to PKU protein substitute*													0.001		-0.79	0.45 (0.28, 0.73)

¥ Interaction was found between sex and achieved PHE monitoring assessments in the module, interaction *P*< 0.02

* Based on vitamin B12 status [406]

ANR, above normal reference ranges; BNR, below normal reference ranges

Table 4.11: Predictors of blood vitamin status in PKU patients based on univariate logistic analysis with P, R2, and β̂ values, 95% CI and odds ratio

Predictor/Micronutrient	**Vitamin B12 [406]**				**Vitamin E [406]**				**Folate (Serum) [406]**				**Folate (RBC) [406]**			
	P-v	R^2	β̂	OR (95% CI)	*P-v*	R^2	β̂	OR (95% CI)	*P-v*	R^2	β̂	OR (95% CI)	*P-v*	R^2	β̂	OR (95% CI)
Age	0.000	4.78%	-0.16	0.85 (0.80, 0.91)	0.000	3.76%	-0.13	0.87 (0.83, 0.93)	0.847	0.01%	0.01	1.00 (0.95, 1.07)	0.790	0.02%	-0.01	0.99 (0.92, 1.06)
Sex (Female)	0.583	0.06%	0.12	1.12 (0.74, 1.71)	0.014	0.97%	0.47	1.59 (1.10, 2.32)	0.907	0.00%	-0.02	0.97 (0.65, 1.48)	0.074	0.76%	-0.42	0.66 (0.41, 1.04)
% of raised PHE concentrations	0.000	2.86%	-0.01	0.99 (0.98, 0.99)	0.000	4.60%	-0.02	0.98 (0.98, 0.99)	0.000	2.83%	-0.01	0.99 (0.98, 0.99)	0.000	3.34%	-0.02	0.98 (0.97, 0.99)
Severity (Mild/Moderate)	0.335	0.18%	-0.23	0.79 (0.49, 1.28)	0.033	0.76%	0.46	1.59 (1.03, 2.45)	0.448	0.12%	-0.18	0.83 (0.53, 1.33)	0.290	0.27%	0.27	1.31 (0.79, 2.18)
BMI Z-scores	0.925	0.00%	-0.01	0.99 (0.80, 1.22	0.096	0.46%	0.15	1.17 (0.97, 1.40)	0.976	0.01%	-0.01	0.99 (0.81, 1.23)	0.445	0.14%	-0.10	0.91 (0.73, 1.15)
PHE monitoring assessments (Adherent)	0.000	2.42%	0.99	2.71 (1.50, 4.92)	0.029	0.78%	0.51	1.66 (1.05, 2.63)	0.806	0.01%	0.06	1.06 (0.64, 1.77)	0.865	0.01%	-0.05	0.95 (0.53, 1.70)
Dietetic monitoring assessments (Adherent)	0.002	1.99%	1.59	4.91 (1.47, 16.35)	0.165	0.32%	0.48	1.62 (0.82, 3.22)	0.120	0.50%	0.61	1.84 (0.84, 4.06)	0.340	0.22%	0.49	1.64 (0.58, 4.70)

ANR, above normal reference ranges

Table 4.12: Predictors of blood trace elements status in PKU patients based on univariate logistic analysis with P, R2, and β values, 95% CI and odds ratio

Predictor/Micronutrient	Copper (BNR)				Selenium (BNR)				Zinc (BNR)			
	P-v	R^2	β	OR (95% CI)	*P-v*	R^2	β	OR (95% CI)	*P-v*	R^2	β	OR (95% CI)
Age	0.008	2.69%	0.13	1.14 (1.03, 1.25)	0.398	0.11%	0.02	1.02 (0.97, 1.07)	0.379	0.20%	-0.03	0.97 (0.91, 1.04)
Sex (Female)	0.522	0.16%	-0.22	0.80 (0.41, 1.56)	0.929	0.00%	0.02	1.02 (0.70, 1.50)	0.200	0.43%	-0.34	0.71 (0.42, 1.20)
% of raised PHE concentrations	0.051	1.46%	-0.01	0.99 (0.98, 1.01	0.000	2.16%	0.01	1.01 (1.00, 1.02)	0.788	0.02%	0.01	1.00 (0.99, 1.01)
Severity (Mild/Moderate)	0.000	5.75%	1.33	3.80 (1.92, 7.51)	0.005	1.28%	-0.61	0.54 (0.35, 0.84)	0.560	0.09%	0.17	1.19 (0.66, 2.10)
BMI Z-scores	0.980	0.00%	0.01	1.01 (0.73, 1.38)	0.987	0.00%	0.01	1.00 (0.84, 1.20)	0.794	0.02%	-0.03	0.96 (0.74, 1.25)
PHE Monitoring Assessments (Adherent)	0.933	0.00%	-0.03	0.96 (0.43, 2.18)	0.892	0.00%	-0.03	0.97 (0.62, 1.52)	0.614	0.07%	0.17	1.18 (0.60, 2.32)
Dietetic Monitoring Assessments (Adherent)	0.972	0.00%	-0.02	0.98 (0.28, 3.37)	0.306	0.17%	-0.36	0.70 (0.35, 1.39)	0.574	0.09%	0.30	1.35 (0.46, 3.97)
Compliance to PKU protein substitute*	0.57	1.48%	-0.81	0.44 (0.17, 1.09)	0.000	%2.30	-0.80	0.45 (0.29, 0.70)	0.009	%2.06	-0.89	0.41 (0.20, 0.83)

* Based on vitamin B12 status [406]

BNR, below normal reference ranges; ANR, above normal reference ranges

4.3.9 Association of deficient micronutrients with status of other micronutrients

Deficiency for one nutrient was not associated with deficiency of another. In some instances, normal concentrations for one micronutrient were associated with excess in another (Figure 4.9). Normal selenium status was significantly (*P*<0.001) associated with excess of vitamin B12 status (Figure 4.9). Low zinc status was significantly (*P*<0.05) associated with deficiency of copper whereas normal zinc status was significantly associated with excess status of vitamin B12 (*P*<0.05) and E (*P*<0.05) (Figure 4.10). No association was found between copper and selenium, or copper and vitamin status (data not presented).

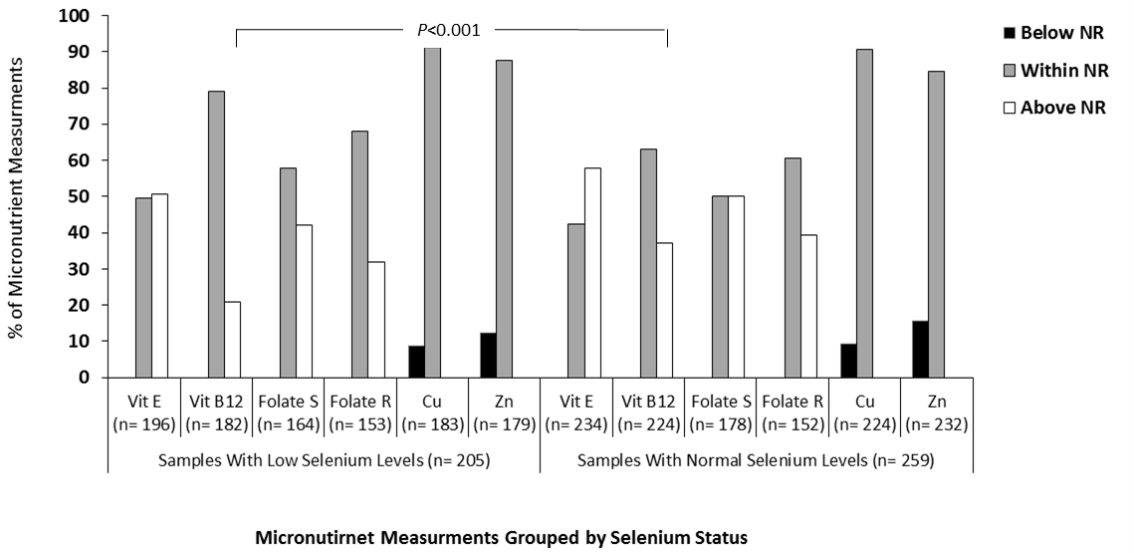


Figure 4.9: Status of micronutrient measurements according to selenium status (low/normal) in blood samples of PKU patients who attended metabolic medicine clinic at RHSC between 1990 – 2013, compared to laboratory normal ranges

n= number of micronutrient measurements

Folate S, serum folate; folate R, folate in red blood cells

All p-values were from Fisher's exact test

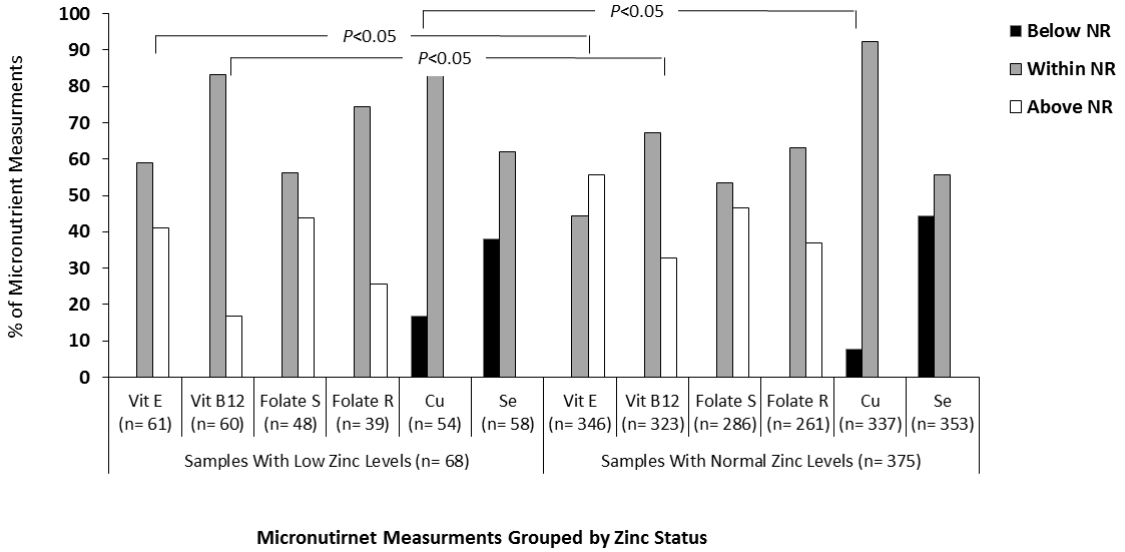


Figure 4.10: Status of micronutrient measurements according to zinc status (low/normal) in blood samples of PKU patients who attended metabolic medicine clinic at RHSC between 1990 – 2013, compared to laboratory normal ranges

n= number of micronutrient measurements

Folate S, serum folate; folate R, folate in red blood cells

All p-values were from Fisher's exact test

4.4 Discussion

In this study, we present a retrospective analysis of the micronutrient status of a large cohort of patients with PKU followed during their disease course. In addition, we explored plausible predictors which might associate with patients' micronutrient status. Only one study [121] have assessed longitudinally the blood micronutrient status and investigated associated predictors in children with PKU on dietary treatment. In this study, we presented data on 512 micronutrient screenings (including 4,010 micronutrient measurements) from 81 paediatric patients with PKU. The main findings of this study were in accordance with previously published studies [10, 121, 155, 189, 322, 407-410] indicating that compared to normal reference ranges, patients with PKU have high measurements of vitamin B12, E and folate, yet low selenium and zinc measurements. Interestingly, a higher proportion of PKU children had low selenium and zinc measurements, and high vitamin B12

measurements compared with the UK NDNS population. These results suggest that the micronutrient deficiencies seen in patients with PKU are specific to their condition and not a reflection of the epidemiology in the general UK population. In contrast to previous research, the strength of this study is that we were able to explore predictors of micronutrient status deficiency using a large dataset of longitudinal measurements. Poor metabolic control, low number of prescribed protein exchanges (more severe PKU), and low adherence to PKU protein substitutes were predictors associated with low selenium status. On the other hand, low zinc status was solely predicted by low adherence to PKU protein substitutes. Nevertheless, collectively these predictors explained a small proportion of (5.8 – 8.8 %) variation in the micronutrient levels in patients with PKU. Both selenium and zinc showed reduced concentrations in blood samples of patients with high or normal status of vitamins E, B12 and folate. The results suggest that selenium and zinc deficiencies reported in this group of patients may be due to other factors which we were unable to measure in this retrospective study. These factors may include low bioavailability of those nutrients from the PKU protein substitutes as suggested by others [121, 155, 322, 405] but which have never been investigated within nutrient balance studies.

Low selenium status in patients with PKU has been reported in the literature for several years now [117, 121, 155, 189, 314, 316, 328]. It has also been reported that selenium dietary intake in this group of patients does not correlate with corresponding plasma concentrations which suggests that the low selenium in plasma is not attributed solely to suboptimal intake [315]. Selenium content of PKU protein substitutes is well above the micronutrient limits recommended by ECD of foods for special medical purposes [102, 121]. Evans et al calculated the median selenium intake using prescribed PKU protein substitutes for each age group, and

found that the intake exceeded the RNI by 200% [121]. However, low selenium status was present in 31% of their blood samples contradicting intake with blood-based status. As the prescribed PKU protein substitutes in this study were similar to those in Evans et al study, it could be assumed that the selenium intake of each corresponding age group would have been similar with our population. Despite this, selenium was low in 44% of our total blood samples. In addition, when compared to selenium levels in healthy population (using UK NDNS 2008-2009 dataset), selenium measurements were low in 30% of the blood samples in this study. Although the association was statistically significant, low adherence to PKU protein substitutes contributed very little to low selenium status, and that 45% of blood samples with good adherence to intake of PKU protein substitutes had still low selenium concentrations. These results suggest that despite high selenium provision, blood concentrations remained low in children with PKU.

High proportions of vitamin E, vitamin B12 and folate measurements over the NR in blood samples with low selenium status were reported in this study. If low selenium status was a consequence of the PKU dietary treatment, then these levels would not have been expected in the group of patients who had high or normal levels of vitamins B12, E and folate. Thus, dietary compliance may not be the only contributing factor to the low selenium status of PKU children observed in this study. Data from our study showed that low selenium status was also associated with a lower number of prescribed protein exchange (PHE tolerance). Gut absorption of selenium is improved when integrated with high biological value natural protein, such as that of animal source [411]. In PKU patients treated with BH4, more natural protein is permitted in their diet (up to 30 g/d) and have a lower prescription of micronutrient-enriched PKU protein substitutes. In this population, one study reported that out of 29 PKU patients prescribed BH4, only one patient exhibited low

selenium status [155]. Another study found a significantly lower serum selenium in adult patients with PKU taking protein substitutes compared to patients with HPA taking or not the PKU protein substitutes but with less restriction on intake of foods containing natural protein [322]. These results suggest that restricted intake of natural protein and probably reduced bioavailability from the PKU protein substitutes, may explain the low selenium status in patients with PKU.

Only one study has tried to evaluate bioavailability of micronutrients from the PKU protein substitutes in patients with PKU. Stroup and colleagues conducted a cross-over study [146] to investigate the impact of protein composition on micronutrient bioavailability by comparing two different PKU protein substitutes. One was based on PHE free L-amino acids while the other was based on whole GMP whey protein which is naturally free from PHE. The study [146] revealed that L-amino acids based PKU protein substitutes have significantly high dietary acid load compared to substitutes based on GMP resulting in increased urinary excretion of studied micronutrients i.e. calcium and magnesium. Yet, the effect of L-amino acids on selenium bioavailability was not examined in that study [146], but it is likely that high dietary acid load of commonly used L-amino acids based PKU protein substitutes may also affect selenium bioavailability and contribute to decreased concentrations of this trace element in patients with PKU. Macronutrient composition in ingested food may also affect selenium bioavailability. A study by Moreda-Piñeiro and colleagues [412] reported a negative correlation between selenium bioavailability and high proportions of protein in food [412]. This suggests that high proportions of protein equivalents (L-amino acids) (40 – 88% of total macronutrient composition) in PKU protein substitutes may reduce selenium bioavailability and thus lead to low selenium status in patients with PKU. However, this previous study [412] on selenium bioavailability was conducted on in-vitro condition using foods composed

of intact protein rather than PHE-free L-amino acids. Selenium is known antioxidant, and important for maintaining optimal growth and cardiac functioning [413, 414]. Although no study reported clinical manifestations of selenium deficiency in patients with PKU, sub-clinical symptoms may happen. While selenium intake in this group of patients, and bioavailability from PKU protein substitutes are yet to be evaluated, frequent screening for its biomarkers in the body is recommended.

In contrast to the other micronutrients evaluated in this study, low copper status was associated with good metabolic control. Although zinc tended to show a similar association, this effect was not significant. In a similar study by Evans et al, a significant correlation was found between good metabolic control and low levels of zinc in plasma [121]. Higher proportions of low copper measurements were observed in blood samples with low zinc status which is intriguing. Bioavailability of copper and zinc is decreased when both consumed in excess doses or in absence of natural protein intake [415]. This means that patients with good dietary compliance may have reduced levels of copper and zinc when their intake is attained via the micronutrient-enriched PKU protein substitutes. Indeed, new micronutrient-enriched PKU protein substitutes were introduced after 2002 (PKU cooler, PKU express and PKU gel from Vitaflo International), replacing vitamin and mineral tablets prescribed alongside PKU protein substitutes. Long-term supplementation of vitamin and minerals in isolation of the PKU protein substitutes reportedly associates with poor compliance [416]; hence many patients favoured the new protein substitutes compared to those implemented prior to 2002. This would explain the decline in zinc and copper status seen after the introduction of new micronutrient-enriched PKU protein substitutes following 2002.

Consistent with previous research [121, 155], substantial proportions of folate (in serum and erythrocytes) measurements were found high in this study. This is

probably due to the high supplementation of folic acid in PKU protein substitutes [102, 121] which aimed to ensure sufficient supply for pregnant women and various age groups [102, 155, 405]. Erythrocyte (RBC) folate provides a better measure for long-term folate status [417]. Our data showed that the number of erythrocyte folate measurements above NR tended to decrease during last follow-up years which may be associated with introduction of the new PKU protein substitutes after 2002. Folic acid content was reduced in the new protein substitutes (400 mg/d) compared to the old supplements (740 mg/d). Yet, the folic acid content of the current PKU protein substitutes is 300 – 120 % RNI of children between 1 – 16 years old. Excessive folic acid intake was linked with cognitive impairment and development of certain forms of cancers [418]. Thus, micronutrient profile of PKU protein substitutes should be reviewed to ensure that adequate intake does not exceed significantly the recommended dietary values.

In general, vitamin measurements in this study were within or well-above NR, which is consistent with previous research [408-410]. This may be related to the high provision of micronutrients from PKU protein substitutes. However, there is a little evidence on clinical symptoms of vitamin deficiency in patients with PKU, mostly related to vitamin B12 [177, 319]. Thus, in the light of our findings and previously available evidence [408-410] unnecessary costs on blood vitamin screenings could be avoided. In agreement with this, the recent guidelines [15] on PKU management, recommend that vitamin screening should be performed only when clinically indicated and in case of complete or partial termination of PKU protein substitutes intake alongside a restricted natural protein intake [117].

Associations between age and blood micronutrient status were observed in this study. Suboptimal selenium and copper concentrations, and a lower proportion of vitamin B12 and E measurements over the NR were noted in children of 5 years old

or above. At about age of 5 years, children start school and form their own taste preferences [419], thus may consume PKU protein substitutes less consistently and increase consumption of other foods in parallel [89]. Furthermore, our study showed that adherence to PKU protein substitutes declined with increasing age. This is reflected by loss of metabolic control with age which was also reported in previous studies [155, 420, 421].

The micronutrient status of patients with PKU varied across follow-up periods. Improved selenium, and high vitamin E and folate concentrations were noted in nutritional screenings performed after 2005. In contrast, there was a significant decline in proportions of vitamin B12 above NR during the same period. As overall metabolic control remained unchanged during follow-up years, the changes in the status of these micronutrients must have been independent of metabolic control and may be associated with the composition of PKU protein substitutes. After 2002, there was a noticeable shift towards the use of new micronutrient-enriched PKU protein substitutes among patients attending our metabolic clinic. Therefore, intake of vitamin B12, E, folate and selenium may have increased after 2005 due to constant provision of these micronutrients via their prescribed PKU protein substitutes compared to previous periods in which protein substitutes without micronutrients were being prescribed. However, it has been reported that efficacy of vitamin B12 absorption is reduced at higher intakes [422], which may explain the decrease proportions of this vitamin above NR in the years following 2005.

This study has its own limitations. First, since dietary micronutrient intake was not directly assessed in this study, contribution of intake from total diet to body micronutrient pool would be very minimal [314, 315] considering that PKU protein substitutes are the main source of micronutrients in the low-PHE diet [102]. Thus, we assessed adherence to intake of PKU protein substitutes by using vitamin B12

status in blood samples collected for micronutrient screening. In the low-PHE diet, vitamin B12 is sourced via the micronutrient-enriched PKU protein substitutes [117], in which its supply has been reported to positively associate with the compliance to intake of these protein substitutes [155, 323, 405, 423]. Thus, blood vitamin B12 status can be used as a proxy for compliance to intake of micronutrient-enriched protein substitutes in PKU. Unlike most studies [155, 156, 316, 317], we used percentage PHE measurements above treatment targets, but not annual median/mean PHE concentrations, to assess metabolic control quality. This approach, when compared to the use of mean/median PHE concentrations, is less likely to be affected by fluctuations in PHE concentrations and number or frequency of PHE measurements over the study period. Thus, allow a better understanding of how changes in metabolic control may associate to micronutrient imbalances and deficiencies in PKU. A second limitation could be that PKU severity classification in this study was not based on initial disease mutation of the patients although this is not commonly done in clinical practice. Hence, PKU severity was based on their tolerance to protein exchanges (tolerable PHE mg/day). The length of follow-up and the number of micronutrient screenings varied between patients, however, the vast majority of children provided samples in more than one age group and half of them provided samples in all four age groups. As data in this study was collected retrospectively, some patients, more likely those with severe PKU or with poor adherence to dietary treatment, may have not attended some clinical/dietetic follow-ups. Thus, some data may be missed. However, patients in this study exhibited good adherence to clinical and dietetic follow-up, which in turn resulted in a large dataset to explore predictors of micronutrient status. The RHSC is the sole referral hospital in the West of Scotland, thus all children diagnosed with PKU in this geographical region were included in this study, representing 50% of all children diagnosed with PKU in Scotland. Another strength of this study is that we compared

micronutrient blood levels of children with PKU to the epidemic of micronutrient deficiencies in healthy population, using data from a large national diet and nutrition survey (UK's NDNS 2008-2009). Although the NDNS results used for this comparison was limited for measurements obtained from patients over 11 years old, the findings provided valuable information on the specific epidemiology of selenium and zinc deficiencies in PKU compared to the ones occurring in the general population.

As commonly happens in paediatric clinical practice, blood samples were obtained in non-fasting state. However, this may only have had effect on plasma zinc levels [121, 424, 426]. Plasma or serum micronutrient concentrations is most widely used indicator of micronutrient status in clinical research [117, 426, 427]. Alternative biomarkers including functional markers can provide a more sensitive index for status of trace elements in the body [425]. These biomarkers include glutathione peroxidase (GSHP) enzymes or selenoproteins for selenium status; erythrocyte cooper-zinc superoxide dismutase (-SOD) activity for status of cooper; or erythrocyte metallothionein for assessment of zinc status [425, 428]. However, best biomarker for status of selenium, zinc or coper is yet to be identified [426-428]. In this study, measurements of plasma selenium, zinc and cooper concentrations were conducted using ICP-MS which is a gold standard technique for determination of these trace elements. The ICP-MS method is well-established and very stable across laboratories in the UK.

4.5 Conclusions

This study found that a high proportion of children with PKU had deficient levels of selenium and zinc but at the same time many of these patients had high levels of other micronutrients including vitamin B12, E and folate.

The data presented in this study suggests that the micronutrient deficiencies seen in this group of patients is specific to PKU and not a reflection of the epidemiology in the general UK population.

Poor metabolic control, severe PKU, and low adherence to PKU protein substitutes predicted low selenium status, while zinc status was solely predicted by low adherence to PKU protein substitutes. However, these predictors explained a small percentage of the variation in the status of selenium and zinc in patients with PKU. Therefore, selenium and zinc deficiencies reported in patients with PKU may be due to other factors which we were unable to measure in this retrospective study such as low bioavailability of those nutrients from the PKU protein substitutes.

The bioavailability of the trace elements used in PKU protein substitutes should be evaluated in further research.

Chapter Five: General Discussion and Conclusions

5.1 General discussion

PKU is an incurable condition and the current management is based on a 'low-PHE diet'. This requires restriction of dietary PHE, by reducing the natural protein intake, and concomitant supplementation with all other essential amino acids in order to meet body protein requirements [102]. Therefore, protein foods rich in PHE, such as meat, cheese, poultry, eggs and milk, and plant/vegetable proteins such as potatoes and cereals are not permitted or restricted to very small amounts in the 'low-PHE diet' [315, 365]. Manufactured special low protein foods (SLPF) are prescribed for patients with PKU to cover energy requirements and provide variety in their diet [103]. These foods are high in CHO and sometimes fat, and have high glycaemic index and provide more energy per weight unit compared to their protein-containing equivalent normal foods [103-105]. As the low-PHE diet is a very restrictive therapeutic regime, PKU protein substitutes, prescribed to patients with PKU, are enriched with vitamins and trace elements to supply essential daily requirements [102, 117].

Advancement in PKU management, including dietary practices, led to nutritional problems that had never been reported before but become more frequent in the recent years. Overweight and obesity, rather than undernutrition [174, 182, 187], became increasingly reported in patients with PKU, with some studies suggesting higher prevalence in female than male patients [107, 134, 184-187]. Overall trend of overweight and obesity in patients with PKU is similar to that in general population [105, 134, 185, 186, 188]. However, data regarding adiposity and body composition in PKU is inconsistent [107, 197-200] with some studies showing high tendency of patients with PKU having higher FM and lower FFM compared to healthy controls [107, 192, 201, 202].

Therefore, one of the aims of this thesis was to contribute to the debate about the topic of obesity in patients with PKU and specifically looking at their adiposity. In the cross-sectional study (Chapter 3) which was conducted on 13 patients with PKU patients and 10 healthy controls, body composition was determined using deuterium dilution, a reference technique to measure FFM and FM. It was found that FFM and FM were not significantly different between patients with PKU and healthy controls. These findings were in accordance with previous studies using DEXA, TOBIC and BIA techniques [107, 197-200] but in contrast to other studies, using skinfold thickness and air-displacement plethysmograph [192, 201, 202], which showed higher FM and lower FFM in patients with PKU compared to healthy controls. In addition, the percentage of obese and overweight in our PKU sample was 40% which is comparable to previous studies conducted on PKU population [184, 186, 188]. However, the data on prevalence of obesity and overweight in PKU compared to general population is inconsistent and varies from country to country [105, 169, 429, 430].

Most of the patients in our study had mild PKU type and showed good metabolic control and adherence to PKU dietary treatment including intake of protein substitutes. Patients with mild PKU-type may not depend much on SLPF for their treatment [99, 103] because a higher amount of natural protein is allowed in their diet compared to patients with severe PKU type [102, 114]. Previous studies showed that total protein and intake of PKU protein substitutes were positively associated with higher FFM and lower ratio of FMI to FFMI [107]. Therefore, protein intake in PKU patients investigated in this thesis might not have been different from protein intake of control participants, which may explain the finding of no significant difference in body composition between groups. However, body composition of

patients with other types of PKU severity or levels of metabolic control can be expected to be different from general population.

Among the aims of this thesis was the investigation of possible mechanisms of obesity in PKU patients. Therefore, the first and second experimental chapters have aimed to test the hypothesis that obesity in PKU patients is related to PKU dietary management, and specifically to reduced TEF and attenuated postprandial fat oxidation, and effects on appetite hormones. Our hypothesis was that low protein, high CHO/fat meals, in the form of SLPF will have a detrimental impact on appetite and satiety [252-254], leading to an enhanced EI, and attenuation of TEF [261, 289] and postprandial fat oxidation [252, 261, 297]; factors known to contribute independently to increased obesity risk [210].

Thus, the experimental study presented in second chapter was conducted on 23 healthy individuals to investigate the impact of PKU protein substitutes and SLPF-based meals on appetite ratings, gut appetite hormones, thermic effect of feeding (TEF) and fat oxidation. This mechanistic study was conducted in cross-over manner in which each participant was asked, in one occasion, to consume PKU protein substitute drink followed by SLPF-based lunch meal, and in another occasion, participants were asked to consume whole-protein milk drink followed by a normal type lunch meal. Appetite, metabolic rate, fat oxidation measurements and blood collections were conducted for the duration of five hours (300 minutes) followed by serving an *ad libitum buffet* dinner to measure the spontaneous energy and macronutrient intake. We found that SLPF-based meal diminished TEF and postprandial fat oxidation, but had no impact on appetite or spontaneous energy and macronutrient intake. These measures were not different following ingestion of PKU protein substitute or whole-protein milk drink. The cumulative difference in energy expended above the RMR during the entire period of the experimental trials

was 41 kJ. Although this small difference between the two trials, this can result in diminished daily EE, and if repeated on a day-by-day basis, this can induce weight gain of 1 kg in a single year [220, 288]. Thus, our data suggests that unfavourable alterations in TEF and fat oxidation may be among mechanistic factors contributing to the development of obesity in PKU. The finding of no effect of SLPF meals on appetite, gut appetite hormones and spontaneous energy intake measured at *ad libitum buffet* meal does not necessarily imply that PKU has no impact on habitual energy intake as these responses were tested in healthy individuals but not yet in patients with PKU. In the above study, we allowed a 90 minutes time interval between intake of protein substitute and SLPF-based meal which allowed investigating separate impact on research outcomes on both, protein substitute and SLPF-based meal. It should be appreciated that clinical guidelines for PKU management advise that protein substitute intake should be in very close proximity to the meal [361]. Future studies, therefore, should investigate combined effect of both PKU protein substitute and SLPF-based meals on research outcomes of this study.

As the study presented in chapter two was conducted in healthy volunteers, there was a need to confirm contribution of reduced TEF and postprandial fat oxidation to prevalence of obesity in patients with PKU. In addition, it was important to understand how these factors are influenced by long-term adherence to SLPF-based meals and how they differ between patients with PKU and healthy matched controls. Therefore, we conducted a case-control study in which we have measured TEF, and postprandial fat and CHO oxidation for three hours (180 minutes) in patients with PKU and compared to healthy controls following consumption of group-type based breakfast meals. During experimental trials, PKU participants were provided with SLPF-based breakfast meal whereas healthy controls were provided

with an isocaloric normal food-based breakfast meal. The findings indicated that TEF and, postprandial fat and CHO oxidation rates in patients with PKU were not different from healthy controls. This finding was in contradiction with results obtained by the mechanistic study conducted on healthy individuals (Chapter 2). However, it is important to note that in the clinical study, differences in protein content of the meals given to PKU patients and control participants were noticeably smaller than in the mechanistic study.

Some data obtained in this thesis should be interpreted with caution. Firstly, data on cross sectional body composition and results comparing TEF and fat and CHO oxidation (Chapter 3) were obtained only on 13 PKU and 10 Control participants. Thus, the outcomes may be different when this ongoing study reaches required sample size. Indeed, it was found that some measures of body composition such as FM (%) and ratio of FM to FFM tended (P= 0.08 and 0.09, respectively) to be higher in PKU patients. With a bigger sample size, we may observe a significant effect which we were not able to detect in this pilot study. Required numbers were not achieved primarily due to the very small number of patients with this rare condition [20], as well as time constraints. Post-hoc power calculation revealed that to detect 1% difference in FM at 0.85 power, sample size of 21 participants would be required for each group. As a result, recruitment will be extended and led by another researcher to achieve the predicted sample size, so that these preliminary results can be confirmed or rejected. It is important to note that finding no differences in TEF, postprandial fat and CHO oxidation may be related to the difference in proportions of macronutrient composition of study breakfast meals between groups being relatively small. In addition, in the meal consumed by participants in the Control group, energy provide by proteins was low and consisted of approximately 9% of total EI [260, 261, 401]. As TEF and postprandial fat oxidation depend on the

amount of protein in the meal consumed [261], the low protein content of the breakfast meal consumed by the Control participants may have led to the non-significant effects in TEF and postprandial fat oxidation between the two groups. Indeed, in previous experimental studies reporting significantly higher TEF and postprandial fat oxidation, the difference in protein content between test meals was as much as 70% of total EI [253, 254, 256], which is far above the recommended protein intake (i.e. 15% of EI) for healthy population [260, 261].

Finally, the PKU group mainly consisted of patients with mild PKU type and had good metabolic control which may not be representative of the PKU population. Energy expenditure regulating neurotransmitters and hormones severely deteriorate in patients with poor metabolic control compared to healthy controls or those with good metabolic control [80-82, 238-240, 276, 277]. This may have contributed to the similar values obtained on fasting RMR and fat oxidation between the PKU and the Control groups. Thus, similar studies are needed in patients with other types of PKU severity and variable levels of metabolic control.

Micronutrient imbalance is another issue that has been noted in the literature in patients with PKU and with an aetiology which is based mostly on anecdotal evidence. Micronutrient deficiencies, particularly selenium and zinc, exist in PKU, despite the fact that patients with PKU being prescribed with protein substitutes enriched with micronutrients in amounts exceeding the UK's RNI for healthy population [121] and limits set by ECD of foods for special medical purposes [102]. However, studies reporting deficient blood concentrations of selenium and zinc, simultaneously reported high concentrations of vitamin B12 and folate in PKU patients prescribed with micronutrient-enriched protein substitutes [121, 155, 189]. Factors associated with such micronutrient imbalances in patients with PKU have rarely been investigated [155, 169, 315-319]. Most of these studies looked

individually at potential factors associated with micronutrient status, but none have explored how these factors together may explain micronutrient deficiencies in this group of patients. Therefore, the last study of this thesis evaluated micronutrient status of children with PKU and explored separately and collectively, the contribution of factors associated with micronutrient deficiency using a large retrospective dataset with serial measurements obtained from 81 patients who provided a total of 512 blood samples for their routine annual micronutrient screening.

In accordance to previous research [121, 155, 189], we found high vitamin B12, E and folate blood concentrations, but simultaneously deficient blood concentrations of selenium and zinc in our PKU patients who were prescribed with micronutrient-enriched PKU protein substitutes. In our PKU sample, poor metabolic control, severe PKU, and low adherence to PKU protein substitutes predicted low selenium status, while deficient zinc status was solely predicted by low adherence to PKU protein substitutes. However, these predictors explained a small variation in status of plasma selenium and zinc. In addition, when measurements in our study were compared to results from the general UK population, the findings indicated that selenium and zinc deficiencies were specific to PKU condition and not a reflection of the epidemiology in the general UK population. Therefore, our data suggests that selenium and zinc deficiencies reported in patients with PKU may be due to other factors which we were unable to measure in this retrospective study such as low bioavailability of those nutrients from the PKU protein substitutes.

Studies in PKU reported that selenium and zinc blood concentrations do not correlate to their dietary intake [315]. Findings from our study showed that selenium and zinc deficiencies were present even in blood samples with good adherence to micronutrient-enriched PKU protein substitutes. A previous study [146] conducted on patients with PKU showed that protein substitutes in the form of L-amino acids,

compared to whole GMP-based protein substitutes, reduce micronutrient bioavailability by increasing dietary acid load which promotes urinary excretion of nutrients. This study [146], however, did not investigate the impact of dietary acid load caused by the L-amino acids from the PKU protein substitutes on selenium or zinc bioavailability. It is likely that the effect may also be associated with the decreased concentrations of selenium and zinc in patients with PKU. Therefore, research is needed to evaluate the bioavailability of selenium and zinc from the PKU protein substitutes, and probably compare to healthy controls using these micronutrient-enriched PKU protein substitutes to examine whether if PKU as a condition has effect on micronutrient status or not.

The length of follow-up and number of micronutrient screenings varied between study participants. However, the vast majority of children in this study provided blood samples in more than one age group and half of them provided blood samples in all four age groups. In addition, patients in this study exhibited good adherence to clinical and dietetic follow-up, which in turn resulted in a large data to explore predictors of micronutrient status. The study measurements were collected from all children diagnosed with PKU in the West of Scotland representing 50% of PKU population in Scotland.

Unlike most studies [155, 156, 316, 317], we used percentage PHE measurements above treatment targets, but not the median/mean PHE concentrations, to assess the association between metabolic control and micronutrient status. This approach, when compared to the use of mean/median PHE concentrations, is less likely to be affected by fluctuations in PHE concentrations and number or frequency of PHE measurements over the study period. Thus, allows better understanding of how changes in metabolic control may contribute to micronutrient imbalance and deficiency in PKU.

Because dietary micronutrient intake from total diet or PKU protein substitutes was not directly measured in the study presented in chapter four, we assessed adherence to intake of PKU protein substitutes by using vitamin B12 status in blood samples. In the low-PHE diet, vitamin B12 is sourced via the micronutrient-enriched PKU protein substitutes, and its supply has been reported to positively associate with the compliance to intake of these protein substitutes [323, 405, 423]. Thus, blood vitamin B12 status can be used as a proxy for compliance to intake of micronutrient-enriched protein substitutes in PKU.

5.2 Conclusions

The following conclusions can be drawn from this thesis:

- Consumption of SLPF-based meals has no detrimental impact on subjective appetite ratings, gut appetite hormones and *at libitum* energy intake.

- Presence of PKU has no effect on body composition under conditions of good metabolic control or in patients with mild PKU type. This finding should be considered as preliminary and to be confirmed in studies meeting power calculations.

- Consumption of meals based on SLPF, measured in healthy individuals, produces a lower TEF and postprandial fat oxidation in comparison to consumption of an ordinary meal. These metabolic alterations may contribute to the origins of obesity in people with PKU on contemporary dietary management. When PKU patients were compared to healthy controls, TEF and postprandial fat and CHO oxidation were not different.

- High proportions of children with PKU had deficient levels of selenium and zinc, although they simultaneously presented increased levels of other micronutrients, including vitamin B12, E and folate.

- The micronutrient deficiencies in this group of patients are specific to PKU and not a reflection of the epidemiology in the general UK population.

- Poor metabolic control, severe PKU, and low adherence to PKU protein substitutes predicted low selenium status, while deficient zinc status was solely predicted by low adherence to PKU protein substitutes.

- Predictors of micronutrient status considered in this thesis explained only a small variation in status of selenium and zinc in patients with PKU. Therefore, our data suggests that selenium and zinc deficiencies reported in this group of patients may be due to other factors which we were unable to measure in this retrospective study such as low bioavailability of those nutrients from the PKU protein substitutes.

- The bioavailability of the trace elements used in PKU protein substitutes should be evaluated in further research, and probably compare to healthy controls using these micronutrient-enriched PKU protein substitutes to examine whether if PKU as a condition has effect on micronutrient status or not.

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Appendices

Appendix A.1

University of Glasgow | College of Medical, Veterinary & Life Sciences

College of Medical, Veterinary & Life Sciences
Ethics Committee for Non-Clinical Research Involving Human Subjects

APPLICATION FORM FOR ETHICAL APPROVAL

NOTES:

THIS APPLICATION FORM SHOULD BE TYPED NOT HAND WRITTEN.

ALL QUESTIONS MUST BE ANSWERED.
"NOT APPLICABLE" IS A SATISFACTORY ANSWER WHERE APPROPRIATE.

PROJECT CODE:

Project Title

Impact of Low Phenylalanine Diet on Appetite, Appetite Hormones, and Thermogenesis

Has this application had been submitted previously to this or any other ethics committee? No
If Yes, please state the title and reference number.

Is this project from a commercial source, or funded by a research grant of any kind? No

If yes,

a) Has it been referred to Research & Enterprise?

Has it been allocated a project Number? No

b) Give details and ensure that this is stated on the Informed Consent form.

Insurance Restrictions.

The University insurance cover is restricted in certain, specific circumstances, e.g. the use of hazardous materials, work overseas and numbers of participants in excess of 5000. All such projects must be referred to Research and Enterprise before ethical approval is sought.

Date of submission: 14.05.2014

Name of all person(s) submitting research proposal
Dr Dalia Malkova[1], Dr Konstantinos Gerasimidis[2], and Hani Alfheeaid[3]

Position(s) held:
[1] Dalia Malkova, Senior Lecturer,
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Name of Principal Researcher (if different from above e.g., Student's Supervisor)

Position held

Undergraduate student project No

Postgraduate student project Yes

PhD degree being undertaken

1. Describe the purposes of the research proposed. Please include the background and scientific justification for the research. Why is this an area of importance?

Phenylketonuria (PKU) is an autosomal recessive genetic disorder characterised by the absence or deficiency of phenylalanine hydroxylase enzyme (PHA), which inhibits the hydroxylation of the amino-acid phenylalanine (Phe) to tyrosine (Chan et al.) (Alves et al., 2012). This condition results in elevated phenylalanine (Phe) levels in the body. Untreated PKU patients are at high risk of mental retardation, seizures and other neurological symptoms (Huttenlocher, 2000, Acosta et al., 2004). The incidence of PKU worldwide is 1 in 10,000 births while in Europe it is 1 in 8,700 births (Hanley, 2013). PKU is more prevalent in white people, particularly those of Celtic descent (Scottish, Irish and Welsh) (NHS, 2012). Our recent study on 135 PKU patients revealed that overweight and obesity are prevalent among this group of patients (Nurus Sa'adah et al., 2014). A previous study from USA reported that the percentage of overweight and obese PKU females was 1.8 and 2.1 higher than in normal population (Burrage et al., 2012). The findings of these studies imply that positive energy balance (intake vs expenditure) prevails in PKU patients but which exact component is up or down-regulated remains thus far unknown.

PKU is an incurable condition and the sole treatment is a low-phenylalanine diet. This means that protein foods rich in Phe, such as meat, cheese, poultry, eggs and milk are not permitted in the diet. Instead, the diet is supplemented with artificial amino acid substitutes and specialised low protein foods which contain no phenylalanine and are enriched with micronutrients (Lammardo et al., 2013). Although these dietary approaches are very effective at controlling levels of phenylalanine (Lammardo et al., 2013), they may impact on appetite regulation and daily energy intake due to modifying effect of appetite regulating hormones and food induced thermogenesis.

It is well established that diets based on high protein intake attenuates appetite and thus have been often advocated to obesity prevention and treatment (Gokmen-Ozel et al., 2011). Additionally, markers of appetite and satiety are influenced by both quantity and quality of consumed proteins (Bayham et al., 2014, Westerterp-Plantenga et al., 1999). As the amount and type of protein in PKU is altered, there is a need to investigate the effect of PKU-type meals/supplements on markers of appetite and satiety. It is possible that the prevalence of overweight and obesity among PKU patients could be linked to alteration in their appetite and satiety resulting in increased daily energy intake.

Alterations in type and amount of protein in PKU diets can also diminish enhancement in postprandial energy expenditure (thermogenesis) seen after meal or drink consumption. There is convincing evidence that protein-rich meals exert a higher thermic effect as compared to fat-rich and carbohydrate-rich meals, thus enhancing daily energy expenditure (Crovetti et al., 1998, Westerterp, 2004). Diet-induced thermogenesis enhances satiety and subsequently decreases energy intake (Halton and Hu, 2004). It has been found from previous studies that increased diet-induced thermogenesis has been directly associated with reduced hunger and increased fullness sensation (Luscombe et al., 2003, Lejeune et al., 2006, Raben et al., 2003). Furthermore, thermic response to protein ingestion may vary according to different protein sources (Veldhorst et al., 2008). Thus, studying the impact of PKU diet on thermogenesis is required. This study aims to test the hypothesis that PKU diet alters appetite and appetite regulating hormones and diminishes thermogenesis due to absence of normal intake of protein and/or consumption of supplements and foods based on specific amino acids.

In particularly we will try to answer the following questions:

1. How ingestion of PKU-type meals/supplements alters appetite and appetite hormones?
2. How postprandial energy expenditure is altered after ingestion of PKU-type meals/supplements

2. Describe the design of the study and methods to be used. Include sample size and the calculation used to determine this. Statistical advice should be obtained if in doubt.

Study Design:

This will be a single blind cross-over randomised controlled trial. Participants will undertake two experimental tests which will involve appetite, appetite hormone, gastric emptying, and thermogenesis measurements after intake of supplement in the fasted state and after consumption of lunch. Wash-out period between the two experiments will be at least seven days. In one trial (PKU trial) the participants will consume a proprietary PKU amino acid supplement (PKU cooler 20, Vitaflo®) and a dose of oral paracetamol (1,000 mg in 100 ml of water) and be asked to consume it within 5 minutes. They will then be asked to consume a low phenylalanine-type lunch after 180 minutes. In another trial (Control) the supplement will be based on milk powder and lunch on foods normally consumed by healthy individuals. The counterbalanced approach for the allocation of trials will be used to eliminate impact of the trial order. In both experimental trials, blood samples will be collected and data on appetite will be obtained in the fasted state and at 30, 60, and 90 minutes after the supplement ingestion, and at 120, 150, 180, 210, 240, 270 and 300 minutes after lunch. Three hours post lunch the participants will be offered an *ad libitum* buffet meal and the amount of food ingested will be recorded. Participants will record of all foods and drinks consumed and refrain from physical activity for 2 days preceding their first day experimental trail and will be asked to replicate this prior to the subsequent visit. Metabolic rate at rest and after consumption of supplements and lunch will be measured for duration of 20 minutes every half-hour. For experimental trials, participant will visit the metabolic unit after 12-hour overnight fast and having refrained from alcohol consumption for at least 24 hours.

Methods:

Participants:

Participants should be healthy adults from ages between 18 – 45 years. They should have their weight stable and not have been aiming to reduce their weight or been on a special diet for at least the previous 4 months. Pregnant or lactating women will be excluded from participation.

Anthropometric measurements:

Height

Height will be measured using a Stadiometer (Invicta Plastics Ltd, Leicester, UK). Subjects will stand barefoot, with their back against a fixed backboard and their arms hanging laterally by their body. The head will be positioned with the line of eyesight perpendicular to the backboard. Subjects will be instructed to relax and a moveable headboard will be lowered to the top of the head with light pressure added to compress the hair. Gentle upward pressure will be applied to the lower jaw and height will be measured to the nearest 0.01 m.

Body mass and body fatness measurement

Bioelectrical impedance analysis (TANITA-TBF-310, Cranela, UK) will be used to measure body fat percentage and body mass. All subjects will be weighed in private wearing minimal clothing, typically lightweight shorts and a t-shirt, whist standing with both feet flat on the balance and their arms hanging laterally by their body. All footwear, substantial jewellery and accessories, e.g. watches, and items within pockets will be removed prior to any measurement. Body mass will be measured using the same balance scales

throughout all experimental studies. Measurements on the female volunteers will be conducted by a female experimenter. BMI will be calculated using the formula with the use of the following formula: BMI = kg/m² [weight in kilograms/ height in meters²].

Blood collection, plasma preparation and analyses.

On arrival to the metabolic suit, participants will be asked to lay comfortably in a supine position for the duration of 10 minutes and then a venous blood sample will be collected, via an intravenous cannula, in K3EDTA tube (BD, vacutainer System, Franklin Lakes, NJ USA). Tubes containing blood samples will immediately be placed on ice and then centrifuged for 15 minutes at 3,000 rpm (Hettich D-78532 Universal 320 R Centrifuge, Tuttiligen, Germany). Plasma will be dispensed in 0.5 mL aliquots into labelled sterilised micro-centrifuge cap tubes (red micro-centrifuge tubes with cap, FR74073, Fischer Scientific, UK) and kept at -80 ^{o}C until analyses. For analysis, plasma samples will be taken out of the -80 ^{o}C freezer and allowed to defrost. The defrosted samples will be used for analysis within the same day. Prior to analysis, samples will be spun for a few seconds to ensure that the plasma is mixed and free from sediment. A single analyser run, or single plate will be used for each subject and each sample will be analysed in duplicate, with the average of the two samples being taken. Insulin will be determined using a commercially available enzyme-linked immunoassay (ELISA) (Mercodia AB, Uppsala, Sweden) and will be analysed on a spectrophotometer (SpectraMax M2, Molecular Devices, Sunnyvale, CA, USA). Commercially available kits will be used to determine glucose and TAG. Ghrelin, PYY, GLP-1, and CCK will be measured according to the manufacturer's instructions using Millipore ELISA kit (Merck, Millipore, Bioscience Division, UK); Cholecystokinin (CCK) analysed using ELISA kit (Phoenix, Pharmaceuticals, Inc., Burlingame, CA, USA). Plasma samples will be kept and analysed at the Biochemistry Laboratory of the Royal Infirmary Academic campus (Level 3, New Lister Building of the Glasgow Royal Infirmary). Access to the freezers and the Biochemistry Laboratory is strictly controlled (card assesses). Plasma spares will be kept until data has been published and then destroyed.

Measurement of metabolic rate

Metabolic rate and energy substrate (fat and CHO) oxidation will be measured by means of computerised open-circuit ventilated hood system (Oxycon Pro, Jaeger GmbH, Hoechberg, Germany). The rate of oxygen (O_2) consumption and rate of carbon dioxide (CO_2) production will be recorded every minute for the duration of 30 minutes in the fasting state, and every half-hour for same for the duration following breakfast and lunch consumption.

Measurement of food intake during *at libitum* dinner

All food and drink items (excluding water) will be weighed before and after *at libitum dinner* using standard electronic kitchen scales (accurate to ±1 g). The macronutrient and energy intake will be calculated over the whole experimental trial using the dietary software Windiets 2005 (The Robert Gordon University, Aberdeen, Scotland, UK).

Appetite measurements

Participants will be asked to express their feeling of hunger, satiety, fullness, prospective food consumption and desire to eat on Visual Analogue Scale (VAS) line of 100 mm (Flint et al., 2000) by placing a vertical mark on the horizontal line at a point, which corresponds to their feelings at that time. The lines were anchored by negative respective feeling words (I am not hungry at all) on the left and by positive feeling words (never been hungrier) on the right respectively. Quantification of the measurement will be made by measuring distance from the left end of the line to the participant's mark point.

Gastric emptying
Participants will be asked to consume 1,000 mg of paracetamol. Appearance of paracetamol in plasma will be measured as an indirect marker for the rate of gastric emptying (Näslund, 2000). Recommended doses of paracetamol for adults are: 500 mg-1 g every 4-6 hours up to a maximum of 4 g daily. Thus 1000 mg is a safe dose.

Dietary assessment and analysis
Electronic scales will be provided to subjects and they will be instructed to weight the food they prepare. They will also be asked to be as descriptive as possible when describing their food and/or drink intake i.e. details like brand name, supermarket from which food was purchased, type of food (i.e. wholemeal, wholegrain etc.) will be provided were possible. Data from food diaries will be entered in and calculated using the dietary software Windiets 2010 (The Robert Gordon University, Aberdeen, Scotland, UK).

Power calculation
25 subjects at 85 % power and at 5% significance level will allow to detect a difference of ~ 2/3 of standard deviation between trials for appetite measures and appetite hormones. This number of participants will allow to detect a difference of ~ 300 KJ in relation to energy intake during *at libitum* buffet. A 10% in number of participants was added in case of drop-outs.

Statistical analysis
Data will be assessed for normality of distribution and descriptive analysis will be carried out to reveal the mean ± SD. Statistical analysis will be carried out using the two-way ANOVA, followed by post hoc analyses for any significant interactions (pre vs. post x treatment) detected within the model. Mean values of the variable of interest will be used in the model. In addition, paired or 2-sample-tests will be used to examine the magnitude of change (Δ) that may occur from the pre- to post supplementation trials between the experimental groups (PKU and normal), when difference is detected using the post hoc analysis. Independent sample t-tests will be used to examine pre- supplementation differences between the two treatments and ensure matching of groups was successful. ANCOVA will be carried out in cases where baseline differences are detected and pre-supplementation values will be used as covariates. All statistical analysis will be carried out using Minitab® 17.1.0. version for Windows®. Statistical significance will be set at $P \leq 0.05$.

3. Describe the research procedures as they affect the research subject and any other parties involved. It should be clear exactly (i) what will happen to the research participant, (ii) how many times and (JF III and Kirk) in what order.

Recruitment and consent
The researcher will go through the information sheet with potential participants. As part of the screening process, each participant will be asked to fill in Health Screening Questionnaire. Only those who were in generally good health will be involved in the study. It will be explained that their participation is voluntary, that they may withdraw at any point if they wish, and that the data collected will be anonymised. Participants will be asked to sign consent.

PKU and Placebo supplement, and lunches
Participants during PKU trial will be asked to consume one pouch of amino acid supplement for breakfast (174 mls supplemented drink, PKU cooler 20, Vitaflo®; 20 g protein, 9.4 g carbohydrates, 0.7 g Fat). This food supplement is widely used by PKU patients in the UK. Participants in placebo trial will receive 174 mls of milk which will contain similar amounts of macronutrients. The lunch will contain cheese sandwich, green salad, cake or muffin and fruit juice. Type of these foods in lunch will be based on type of the trial either free-phenylalanine or normal foods. Weight of foods in lunch will be matched in both trials.

Preparation for experimental trials
Participants will be asked to record dietary intake for 2 days prior to the pre-intervention experimental trial, replicating this diet 2 days prior the post-intervention experimental trial. Prior to both experimental trials, participants will be asked to avoid consumption of coffee and alcohol for the duration of 24 hours. In the morning of the experimental trials they will be advised to travel by taxi and expenses will be reimbursed. Tests will be conducted according to the 'Code of Practice for Conducting Experiments on Non-Patient Human Volunteers (including Handling and Disposal of Human Blood, Urine and Sputum), re-approved by the University Ethics Committee on October 26, 2001.

Experimental trials
On the day of the experimental test the participants will come to the metabolic investigation suite at ~ 08:00. The participants will be allowed 10 minutes to relax and acclimatize. Following measurements of body composition and resting metabolic rate (RMR), a cannula will be inserted in an antecubital vein, and a baseline blood sample will be collected and appetite questionnaires will be completed. Following this, the participants will be provided either a PKU supplemented formula (PKU trial) or milk based drink (Placebo trial) and a dose of oral paracetamol (1000 mg in 100 ml of water). Participants will be asked to consume it within 5 minutes. Blood samples will be collected and data on appetite will be obtained at 30, 60, 120 and 180 minutes. Either PKU-type (PKU trial) or normal (Placebo trial) lunch will be provided after then. Further appetite questionnaires will be completed and blood samples will be obtained at 30, 60, 90 and 180 minutes. Metabolic rate will be measured for the duration of 30 minutes after supplement and lunch. Three hours post-lunch, an *ad libitum* buffet meal will then be offered and the amount of food ingested will be recorded.

4. How will potential participants in the study be (i) identified, (ii) approached and (JF III and Kirk) recruited? Give details for cases and controls separately if appropriate.

We will be recruiting 25 healthy adults who aged between 18 to 45 years. Participants will be required to be free of any medical condition at the time of testing. Participants will be recruited by means of an advertisement by university's email-broadcasting and word of mouth in the campus of the University of Glasgow, and other public areas. Subjects who will be willing to participate will be screened by means of a detailed medical history to exclude chronic illness, eating disorders, and major gastrointestinal operations. Considering the participant meets the inclusion criteria and following explanation of the study, is interested to participate, an information sheet, a medical questionnaire and a consent form will be sent via email. The participant will then need to take their time to read thoroughly, sign and return all documentation via email. Once the investigator verifies the compatibility of the participant with the study requirements via all returned documentation, the participant will be contacted to arrange a 1st visit to the laboratory. The investigator will explain to the participant that they have every right to drop out of the study at any point without an explanation, if they feel this is not suitable for them.

5. What are the ethical considerations involved in this proposal? You may wish, for example, to comment on issues to do with consent, confidentiality, risk to subjects, etc.

There are no major ethical considerations involved in this study. Some subjects may experience mild discomfort during the placement of and/or sampling of blood from a catheter placed in a vein on the dorsum of the heated hand. In our experience, this is minimal because: the catheter size is small (20 G); the catheter is indwelling, allowing for multiple sampling; while it is safely secured in place with adhesive tape, there is sufficient 'play' to allow sampling without 'pulling' on the vessel (i.e. the catheter can slide easily within the vessel); upon withdrawal of the catheter at the end of the experiment, firm pressure is maintained over the site to prevent any leakage from the vessel into the surrounding interstitium which could lead to local oedema and bruising. Importantly, if a vessel cannot be readily cannulated or if the subject is nonetheless not comfortable with proceeding, the experiment is halted. No more than 80 ml of blood will be sampled for each test i.e 80 ml during trials 1 and 2. Blood will be handled, stored and disposed of according to standard health and safety procedures.

All subjects will complete a medical questionnaire and provide their written consent with the option to withdraw from participation at any point. All medical questionnaires and consent forms which will contain private information such as participant name will be treated as confidential and will be stored in a locked file cabinet to which only primary investigators related to the study will have access. Data will be stored on the principal investigators university desktop.

Blood samples will be coded according to each subject's code. Samples will be placed in freezer bags which will be labeled with the name of the principal investigator and will be placed in the freezer located in the biochemistry laboratory. Labeled freezer bags containing samples will only be accessed by the investigator that has created the label and/or the PI of the study.

6. Outline the reasons why the possible benefits to be gained from the project justify any risks or discomforts involved.

Risk and discomfort to subjects is considered minimal. The benefits lay potentially in the participant gaining insight in the research process. Information on dietary intakes will be made available to study participants. Participants also will receive anthropometric data so they will benefit from the study personally.

7. Who are the investigators (including assistants) who will conduct the research? What are their qualifications and experience?
Dr Dalia Malkova and Dr K. Gerasimidis have extensive experience in conducting in carrying out human studies, without incident. Dr Malkova has ~15 years of experience in conducting human metabolic studies and has ~10 years' experience in venepuncture and cannulation. Dr Gerasimidis has extensive experience in carrying out dietary assessment. A PhD researcher Hani Alfheeaid (MSc in Clinical Nutrition, Glasgow Uni.) is working under the supervision of Dr Gerasimidis and Dr Malkova and he will be trained in all testing procedures involved.

8. Are arrangements for the provision of clinical facilities to handle emergencies necessary? If so, briefly describe the arrangements made.
The risks associated with the procedures involved in this study are extremely small. Test will be conducted according to the Code of Practice for Conducting Experiments in Non-Patient Human Volunteers.
In the event of an incident that is not an emergency; participants will not be permitted to leave the laboratory until they have fully recovered. Participants will be encouraged to contact their local GP. Participants will be told that one of the Principal Investigators will conduct a follow-up by telephone at the end of the same day. Participants will also be provided with 24-hour contact numbers for both principal investigators.

9. In cases where subjects will be identified from information held by another party (e.g., a doctor or hospital), describe how you intend to obtain this information. Include, where appropriate, which Multi Centre Research Ethics Committee or Local Research Ethics Committee will be applied to.

N/A

10. Specify whether subjects will include students or others in a dependent relationship and, where possible, avoid recruiting students who might feel to be, or be construed to be, under obligation to volunteer for a project. This is most likely to be when a student is enrolled on a course where the investigator is a teacher. In these circumstances, the recruitment could be carried out by one of the other investigators or a suitably qualified third party.
Some students may be recruited but will be under no pressure from staff to participate in the study. Steps will be taken to avoid the recruitment of students in a dependent relationship with the academic involved in the study.

11. Specify whether the research will include children or participants with mental illness, disability or handicap. If so, please explain the necessity of involving these individuals as research subjects and include documentation of the suitability of those researchers who will be in contact with children (e.g., Disclosure Scotland or membership of the PVG Scheme).
N/A

12. Will payment or other incentive, such as a gift or free services, be made to any research subject? If so, please specify, and state the level of payment to be made and/or the source of the funds/gift/free service to be used. Please explain the justification for offering an incentive.
We will give £50 vouchers to those who complete the study to compensate for the time spent in participating in the study.

13. Please give details of how consent is to be obtained. A copy of the proposed consent form, along with a separate information sheet, written in simple, non-technical language MUST ACCOMPANY THIS PROPOSAL FORM.

Once subjects contact primary investigator indicating their interest for participation, the investigator will send an information sheet to subjects via email, in order to give the opportunity to subjects to discuss with their families, friends make sure that the study is right for them. Once they have done so, they will be asked to contact the investigators via email or telephone and a time for their first screening test will be arranged. Each subject will be provided with a consent form outlining the testing procedures, which asks them for their written consent to participate in the project with the option to withdraw at any time (see enclosed copy). A verbal explanation will also be given and any queries answered. If there is some doubt of the subject's eligibility for the study, the subject will be excluded. Information on propionate supplementation will be given in the information sheet.

14. Comment on any cultural, social or gender-based characteristics of the subjects which have affected the design of the project or may affect its conduct.

Participants will be healthy adults and aware that they have been invited to participate in this study on this basis.

15. Please state (i) who will have access to the data and (ii) what measures will be adopted to maintain the confidentiality of the research subjects and to comply with data protection requirements. For example, will the data be anonymised, how will it be stored, how will access be restricted, and for how long will it be retained?

The information obtained will be anonymised and individual information will not be passed on to anyone outside the study group. The results of the trials will not be used for selection purposes. Data will be stored as hard copy data in a locked file cabinet to which only the principal investigator will have access. Data will also be stored in an electronic form both in the principal investigator's C drive and on an external hard disk. Data will be stored for 10 years in compliance with the University's Code of Good Practice in Research.

In regard to (ii) above, please clarify (tick one) how the data will be stored:

(a) in a fully anonymised form (link to subject broken)

(b) in a linked anonymised form (data +/- samples linked to subject identification number but subject not identifiable to researchers) **(Yes)**

(c) in a form in which the subject could be identifiable to researcher.

16. To your knowledge, will the intended group of research subjects be involved in other research? If so, please justify.

The participants will be asked not to be involved in any other research at the time of participation.

17. Proposed starting date:

May 2014

Expected completion date:

June 2016

18. Please state location(s) where the project will be carried out.

Subject testing will be carried out at the new research facility located in the metabolic investigation suite at Human Nutrition section, New Lister Building, Glasgow Royal Infirmary.

19. Please state briefly any precautions being taken to protect the health and safety of researchers and others associated with the project (as distinct from the research subjects), e.g., where blood samples are being taken.
Standard blood sampling procedures using hospital guidelines will be used in the study. The researchers will always work with participants with at least one other person in the same room at the same time.
All samples will be handled according to the 'Code of Practice for Conducting Experiments on Non-Patient Human Volunteers (including Handling and Disposal of Human Blood, Urine and Sputum).

20. Please state all relevant sources of funding or support for this study.
The student who is a graduate in clinical nutrition has a secured PhD scholarship from the Government of Saudi Arabia (represented by Royal Saudi Arabian Embassy in London) which pays tuition and bench fees, and monthly stipend.

21a). Are there any conflicts of interest related to this project for any member of the research team? This includes, but is not restricted to, financial or commercial interests in the findings. If so, please explain these in detail and justify the role of the research team. For each member of the research team please complete a declaration of conflicts of interest below.

There are no conflicts of interest

Researcher Name: Dr Dalia Malkova conflict of interest Yes / **No**
If yes, please detail below

Researcher Name: Dr Konstantinos Gerasimidis conflict of interest Yes / **No**
If yes, please detail below

Researcher Name: Hani Alfheeaid conflict of interest Yes / **No**
If yes, please detail below

Researcher Name: _____ conflict of interest Yes / No
If yes, please detail below

21b). If there are any conflicts of interest, please describe these in detail and justify conducting the proposed study.

N/A

22. How do you intend to disseminate the findings of this research?
Appropriate individual feedback will be provided to participants via email and the overall findings of the proposed research will be presented, to all participants upon successful completion of the proposed studies. Finally, the findings of the proposed research will target publication in a high impact journal and may be presented in conferences in the form of poster or oral presentation.

I confirm that have read the University of Glasgow's Data Protection Policy. [http://www.gla.ac.uk/services/dpfoioffice/policiesandprocedures/dpa-policy/]

Please initial box ✓

Name __ Dalia Malkova _____ Date 07.07.2014
(Proposer of research)
Please type your name on the line above.

For student projects:

I confirm that I have read and contributed to this submission and believe that the methods proposed and ethical issues discussed are appropriate.

I confirm that the student will have the time and resources to complete this project.

Name _____ Date _____
(Supervisor of student)
Please type your name on the line above.

Please upload the completed and signed form, along with other required documents by logging in to the Research Ethics System at - https://frontdoor.spa.gla.ac.uk/login/

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Appendix A.2

VOLUNTEER INFORMATION SHEET

IMPACT OF LOW PHENYLALANINE DIET ON APPETITE, APPETITE HORMONES AND THERMOGENESIS

You are being invited to take part in a research study. Before you decide whether or not to take part, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. Thank you for reading this.

What is the purpose of the study?

Low-phenylalanine diets are commonly prescribed to people with phenylketonuria (PKU), an inborn disease which causes accumulation of amino acid phenylalanine (Phe) in the blood. High blood Phe levels can cause mental, behavioural, neurological, and physical problems. Thus, low-phenylalanine diets help patients to manage their condition but it is not clear whether they have impact on appetite, energy intake and changes in body weight. This is important to explore as prevalence of obesity in this population is rising high. We aim to find out the effect of PKU-type meals on appetite, appetite biomarkers, and post-meal energy expenditure. We will recruit 25 healthy adults and ask them to participate in two experimental trials. On one occasion they will be asked to consume PKU-supplemented drink followed by PKU type-lunch and on another occasion the supplement and lunch will be based on normally consumed foods. Series of blood samples will be taken and appetite will be assessed during both experiments. Both experimental trials will finish with consuming an ‘all you can eat buffet’.

Why have I been chosen?

You have been chosen because you are a healthy adult aged between 18-45 years. Your body weight has been stable and you were not been aiming to reduce your weight or been on a special diet at least for the last 4 months. You are not pregnant or lactating woman.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part, you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part, you are still free to withdraw at any time and without giving a

reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?

In the first instance, you will be asked to attend for a **screening session** to ensure that you meet the inclusion criteria to take part in the study. Before enrolling in the study you will be asked to attend for a screening visit in which we will discuss with you and complete confidential questionnaires regarding your health, measure your height and weight and calculate body mass index. This is also a good opportunity for you to ask questions. If you match the inclusion criteria, you will be randomly allocated to participate in this study and start with the PKU or normal food intervention.

You will be asked to undertake two experimental trials, each lasting for ~8 hours. The time interval between the two experiments will be at least seven days. You will come to the metabolic suite of the Royal Infirmary, Glasgow, at ~8:30am, after a 12-hour fast. Body weight and body fat measurements will be conducted and then an intravenous peripheral cannula will be placed on the right or left forearm, the part of the arm between the wrist and the elbow, by a trained phlebotomist. This will be used to draw blood samples throughout the experimental trial. Baseline blood sample will be obtained after ~10 minutes of rest and then you will undergo 30 minutes of an indirect calorimetry measurement. This simply involves lying under canopy for collection of air that you breath out, which allows determination of energy expenditure during rest. Following this, you will consume a PKU supplemented drink or milk with a dose of oral paracetamol (1000 mg in 100 ml of water) and after 3 hours you will have lunch. You will be asked to undergo indirect calorimetry after breakfast and lunch. Blood samples will be obtained and appetite measurements will be conducted every 30 minutes during the experimental trial. In total, no more than 80 ml of blood will be collected. To understand what 80 mls of blood relate to, it is important to have in mind that blood donors have 1 pint (568 mls) of blood taken on each visit. Thus, 80 mls is 7 times less than what a blood donor would give. A scale from 1-100 will also be used to assess how hungry you are feeling throughout the day. The experimental trial will finish with consuming an ‘all you can eat’ buffet. You will be asked to remain at a resting position throughout the protocol. Reading material and a TV and video will be provided. Both experimental trials will be identical and will last for 8 hours.

Experimental supplements and meals

In one experimental trial, you will receive free-phenylalanine drink (Vitaflo, UK) and low phenylalanine lunch, which will include low protein bread (Juvela, UK), no protein vegan cheese (Viotros, UK), low protein crackers (Vitaflo, UK), and low protein cake (Juvela, UK). In the other experiment, you will receive 174 mls of milk and lunch based on normal food. You will be asked to consume drinks within 5 minutes and lunch within 25 minutes.

What else do I have to do?

Other than the specific tasks described above, we ask you to maintain your usual lifestyle (i.e. don’t change your diet or exercise habits) for the duration of this study. You will be asked to record your food intake throughout the day before the first

experimental test and to replicate the food intake prior to the second one. We also ask you to avoid alcohol, and any planned exercise 2 days prior to each main trial. We will ask you not to drink or eat any food 12 hours prior the experimental trials and not to take any multivitamin or supplement 24 hours before you attend.

What are the possible disadvantages and risks of taking part?

You may experience mild discomfort during the placement of and/or sampling of blood from a catheter placed in a vein. In our experience, this is minimal because the catheter size is small (20 G) and it is safely secured in place with adhesive tape. No more than 80 ml of blood will be sampled for each experimental trial. Blood will be handled, stored and disposed of according to standard health and safety procedures.

Side effects associated with PKU-type supplement

No symptoms were reported from ingestion of PKU-type drinks or foods. However, these foods or drinks may taste differently from normal foods.

What are the possible benefits of taking part?

There may be no direct benefits to you but as a result of being involved in this study you will receive health information about yourself including body composition (e.g. your body muscle) and accurate measurements of your basal metabolic rate. You will also receive information on your habitual intake of energy, and nutrients. We will provide you with feedback about the main study findings and also about your own results and would be delighted to explain our findings and discuss their implications with you.

Will my taking part in this study be kept confidential?

All information that is collected about you during the course of the research will be kept strictly confidential. Any information about you that leaves the University will have your name and address removed so that you cannot be recognised from it.

What will happen to the results of the research study?

The results of this study will be presented at leading national and international conferences and published in leading scientific journals. At no point you will be personally identified in the dissemination of the results.

Who is organising and funding the research?

The study is a part of a funded PhD project

Who has reviewed the study?

The study details have been reviewed by the College of Medical, Veterinary & Life Sciences Ethics Committee for Non-Clinical Research Involving Human Subjects.

Contact for Further Information

Any questions about the procedures used in this study are encouraged. If you have any doubts or questions, please ask for further explanations by contacting:

Hani Alfheeaid
PhD Researcher
Human Nutrition, School of Medicine
College of Medical, Veterinary & Life Sciences
University of Glasgow
Room 3.84, Level 3,
New Lister Building
Glasgow Royal Infirmary
10-16 Alexandra Parade
G31 2ER
Mobile: 07533 260 555
E-mail:
h.alfheeaid.1@research.gla.ac.uk

Dr Dalia Malkova
Senior Lecturer
Human Nutrition, School of Medicine
College of Medical, Veterinary & Life Sciences
University of Glasgow
Room 3.09, Level 3,
New Lister Building
Glasgow Royal Infirmary
10-16 Alexandra Parade
G31 2ER
Tel: 0141 201 8690
E-mail: Dalia.Malkova@glasgow.ac.uk

You will be given a copy of this information sheet and a signed consent form to keep for your records.

Appendix A.3

Participant ID	

HEALTH SCREEN FOR STUDY VOLUNTEERS

(Impact of Low Phenylalanine Diet on Appetite, Appetite Hormones and Thermogenesis)

Name: …… Age: ……

It is important that volunteers participating in research studies are currently in good health and have had no significant medical problems in the past. This is to ensure (i) their own continuing well-being and (ii) to avoid the possibility of individual health issues confounding study outcomes.

Please complete this brief questionnaire to confirm fitness to participate:

At present, do you have any health problem for which you are:

(a)	On medication, prescribed or otherwise	yes [ ]	no [ ]
(b)	Attending your general practitioner	yes [ ]	no [ ]
(c)	On a hospital waiting list	yes [ ]	no [ ]

In the past two years, have you had any illness which required you to:

(a)	Consult your GP	yes [ ]	no [ ]
(b)	Attend a hospital outpatient department	yes [ ]	no [ ]
(c)	Be admitted to hospital	yes [ ]	no [ ]

Have you ever had any of the following:

(a)	Convulsions/epilepsy	yes [ ]	no [ ]
(b)	Asthma	yes [ ]	no [ ]
(c)	Eczema	yes [ ]	no [ ]
(d)	Diabetes	yes [ ]	no [ ]
(e)	A blood disorder	yes [ ]	no [ ]
(f)	Head injury	yes [ ]	no [ ]
(g)	Digestive problems	yes [ ]	no [ ]
(h)	Hearing problems	yes [ ]	no [ ]
(i)	Problems with bones or joints	yes [ ]	no [ ]
(j)	Disturbance of balance/co-ordination	yes [ ]	no [ ]
(k)	Numbness in hands or feet	yes [ ]	no [ ]
(l)	Disturbance of vision	yes [ ]	no [ ]
(m)	Thyroid problems	yes [ ]	no [ ]
(n)	Kidney or liver problems	yes [ ]	no [ ]
(o)	Chest pain or heart problems	yes [ ]	no [ ]
(p)	Any other health problems	yes [ ]	no [ ]

For female volunteers only

(a) Are you pregnant or think that you might be pregnant yes [ ] no [ ]

(b) Do you take the contraceptive pill or other hormone-based contraceptives yes [ ] no [ ]

(c) Are you postmenopausal yes [ ] no [ ]

(d) Are you receiving Hormone Replacement Therapy (HRT)
yes [ ] no [ ]

5. Have any of your immediate family ever had any of the following: (if yes please give details including age of first diagnosis)

(a) Any heart problems yes [ ] no [ ]

(b) Diabetes yes [ ] no [ ]

(c) Stroke yes [ ] no [ ]

(d) Any other family illnesses yes [ ] no [ ]

6. Do you currently smoke yes [ ] no [ ]
Have you ever smoked yes [ ] no [ ]

If so, for how long did you smoke and when did you stop? ……

How many units of alcohol do you typically drink in a week? …….

If you know your body weight (kg) please write here	
If you know your height (cm) please write here	

For female volunteers only:
When did you finish your last period? / /
How many days does it normally last? ……
How many days do you normally have between periods? ……

If YES to any question, please describe briefly if you wish (e.g. to confirm whether problem was short-lived, insignificant or well controlled.) (Use a separate sheet if necessary)

……
……
……
……
……
……

Name and address of GP
……
……
……

Blood pressure measured at screening……..mm Hg

Appendix A.4

Participant ID	

Centre Number:
Project Number:
Subject Identification Number for this trial:

CONSENT FORM

Title of Project: Impact of Low Phenylalanine Diet on Appetite, Appetite Hormones, and Thermogenesis

Name of Researcher(s): Dr Dalia Malkova, Dr Konstantinos Gerasimidis, and Hani Alfheeaid

Please initial box

I confirm that I have read and understand the information sheet dated 15/05/2014 for the above study and have had the opportunity to ask questions. ☐

I understand that my participation is voluntary and that I am free to withdraw at anytime, without giving any reason, without my legal rights being affected. ☐

I agree to take part in the above study. ☐

Name of subject	Date	Signature
Name of Person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

(1 copy for subject; 1 copy for researcher)

College of MVLS Ethics Committee 2785 May 2014

Appendix A.5

Participant ID		Trial Type Code		Measurement time		

Visual Appetite Scale (VAS)

Please answer the following questions by placing a vertical mark through the line for each question. Regard the end of each line as indicating the most extreme sensation you have ever felt and mark how you feel **NOW.**

1. How **hungry** do you feel (now)?

I am not hungry ——— **Never been hungrier**

2. How **satisfied** do you feel (now)?

I am not satisfied at all ——— **I cannot eat another bite**

3. How **full** do you feel (now)?

Not at all full ——— **Totally full**

4. How **much** do you think you **can eat** (now)?

A lot ——— **Nothing at all**

5. How strong is your **desire to eat** ((now)?

Not at all ——— **Very**

Appendix A.6

Open Buffet Lunch Menu

		Please Tick [✓] to Choose
Appetizer		Green Salad
Main Course **2 Selections**	**Choose Two ¥**	☐ Beef Lasagne ☐ Lamb Moussaka ☐ Rich Beef Bolognese Pasta and Focaccia Crostoni ☐ Fajita Chicken with Pitta Bread ☐ Indian Chicken & Pilau Rice ☐ Roasted Butternut Squash Lasagne (V)* ☐ Vegetable & Paneer Korma with Pilau Rice (V)*
Side Dishes	**Choose One**	☐ Cheddar Cheese Chips ☐ Vinegar Chips ☐ Salted Chips
	Choose One	☐ Grape/Orange ☐ Grape/Apple
Dessert	**Choose One**	☐ Chocolate Cheese Cake ☐ Raspberry/Strawberry Cheese Cake ☐ Strawberry Tarts

¥ Depends on market availability
* Suitable for vegetarians

Appendix A.7

University of Glasgow | School of Medicine

GLUCOSE Hexokinase Method (GLUC-HK)
(Randox Laboratories Ltd. Cat. #GL1611)

A developed method for micro-volume assay of plasma glucose concentrations on microtiter plates 96-wells

Hani Alfheeaid / Dr Dalia Malkova

A. Prepare bench for analysis
   1. Disinfect the bench with alcohol
   2. Obtain the following:
      a. 2 – 20 µl pipettes
      b. 20 – 200 µl pipettes
      c. 200 – 1000 µl pipettes
      d. Multichannel pipette 50 - 300 µl
      e. 20 µl white tips
      f. 200 µl filtered and unfiltered tips
      g. 300 µl unfiltered tips
      h. 1000 µl blue tips
      i. Microtiter Plate (Corning® clear untreated 96 well plate)
      j. Plate sealer
   3. Print the plate layout with standards (calibrators), QC and samples IDs

B. Preparation of samples (PLASMA) EDTA
      a. Defrost samples at least 30 minutes before start
      b. Thoroughly vortex samples for at least 10 seconds before moving to centrifuge
      c. Spin samples in centrifuge for ~ 30 seconds before pipetting into plate (this is to remove precipitate or particulate matter). Be careful not to disturb the pellet when removing from the centrifuge, or when plating the samples.

C. Preparation of Quality controls (QC)
      a. Get the lower serum QC (Green lid) from Randox (Cat. No. HN 1530) and higher serum QC (Red lid) from Randox (Cat. No. HN 1530) [NOTE: the quality control can be different depending on the type of sample. Consultation is recommended here.]
      b. Add the required volume of distilled water (5 ml) (as per accompanying instruction manual) into the bottle to dilute
      c. Vortex the control for at least 10 seconds
      d. Record the control concentration normal ranges which can be obtained from the Randox accompanying CD

e. Pipette ~ 50 µl of the prepared quality control into small Eppendorf tubes to be stored in freezer -80 °C for future analyses

D. Preparation of analysis reagent mixture:
   a. Pour whole Enzyme reagent (R1b) bottle into Buffer (R1a) bottle; rinse smaller bottle x5 times to ensure complete dilution
   b. Mix thoroughly by inversion

   * The working reagent is stable for 3 months at +2 to +8°C or for 2 weeks at +15 to +25°C, protected from light.

E. Leave A1 and A2 wells as blank
F. Pipette 2.9 µl of the accompanying standard (ready for use) into wells C1-C2 and D1-D2. The aim of pipetting more than 1 duplicated wells of the same standard is to obtain a good average for its OD to be used later for calculation of the samples concentrations.
G. Pipette 2.9 µl of the Low QC into the assigned wells (in duplicate)
H. Pipette 2.9 µl of the High QC into the assigned wells (in duplicate)
I. Pipette 2.9 µl of the samples into the assigned wells according to the planned plate layout
J. Gently tap the plate on the bench to ensure the samples are being positioned into the bottom of the wells
K. Pipette 290 µl of the prepared reagent mixture into the wells [standards, QCs and samples], (reverse pipetting is recommended). DO NOT ADD to A1 and A2
L. Gently tap the plate on the bench to ensure the samples are being positioned into the bottom of the wells
M. Cover the plate with plastic sealer
N. Incubate on the plate shaker (700-900 rpm) for 10 minutes at room temperature (18–27°C)
O. Read plate within 30 minutes (remove the sealer just before reading), at both 340 nm and 380 nm. Randox has recommends to calculate only at 340 nm reading for this bi-chromatic measurement

Calculations:
Average (A1 and A2) blank wells – OD for standards or samples= Final OD reading
∴ Concentrations=

$$Standard\ concentration \times \frac{final\ OD\ reading\ for\ sample}{final\ OD\ reading\ for\ standard}$$

Appendix A.8

University of Glasgow | School of Medicine

TRIGLYCERIDES (TRIGS)
(Randox Laboratories Ltd. Cat. #TR212) GPO-PAP Method

A developed method for micro-volume assay of plasma TAG concentrations on microtiter plates 96-wells

Hani Alfheeaid / Dr Dalia Malkova

P. Prepare bench for analysis
   4. Disinfect the bench with alcohol
   5. Obtain the following:
      a. 2 – 20 µl pipettes
      b. 20 – 200 µl pipettes
      c. 200 – 1000 µl pipettes
      d. Multichannel pipette 50 - 300 µl
      e. 20 µl white tips
      f. 200 µl unfiltered tips
      g. 300 µl unfiltered tips
      h. 1000 µl blue tips
      i. Microtiter Plate (Corning® clear untreated 96 well plate)
      j. Plate sealer
   6. Print the plate layout with standards (calibrators), QC and samples IDs

Q. Preparation of samples (PLASMA) EDTA
   a. Defrost samples at least 30 minutes before start
   b. Thoroughly vortex samples for at least 10 seconds before moving to centrifuge
   c. Spin samples in centrifuge for ~ 30 seconds before pipetting into plate (this is to remove precipitate or particulate matter). Be careful not to disturb the pellet when removing from the centrifuge, or when plating the samples.

R. Preparation of Quality controls (QC)
   a. Get the lower serum QC (Green lid) from Randox (Cat. No. HN 1530) and higher serum QC (Red lid) from Randox (Cat. No. HN 1530) [NOTE: the quality control can be different depending on the type of sample. Consultation is recommended here.]
   b. Add the required volume of distilled water (5 ml) (as per accompanying instruction manual) into the bottle to dilute
   c. Vortex the control for at least 10 seconds
   d. Record the control concentration normal ranges which can be obtained from the Randox accompanying CD
   e. Pipette ~ 50 µl of the prepared quality control into small Eppendorf tubes to be stored in freezer -80 °C for future analyses

S. Preparation of analysis reagent mixture:
    a. Reconstitute one vail of Enzyme reagent (R1b) with Buffer (R1a) bottle; rinse smaller bottle x5 times to ensure complete dilution
    b. Mix thoroughly by inversion

    *The working reagent is stable for 21 days at +2 to +8°C or for 3 days at +15 to +25°C, protected from light.
T. Leave A1 and A2 wells as blank
U. Pipette 5 µl of the accompanying standard (ready for use) into wells B1-B2, C1-C2, and D1-D2. The aim of pipetting more than 3 duplicated wells of the same standard is to obtain a good average for its OD to be used later for calculation of the samples concentrations.
V. Pipette 5 µl of the QC into the assigned wells (in duplicate)
W. Pipette 5 µl of the samples into the assigned wells according to the planned plate layout
X. Pipette 5 µl of the prepared reagent mixture into the blank wells
Y. Gently tap the plate on the bench to ensure the samples are being positioned into the bottom of the wells
Z. Add 150 µl of the prepared reagent mixture to each well including blank
AA. Cover the plate with plastic sealer
BB. Incubate on the plate shaker (700-900 rpm) for 10 minutes at room temperature (18–27°C)
CC. Read plate within 60 minutes (remove the sealer just before reading), at both 500 nm and 546 nm. Randox has recommended to calculate only the 500 nm reading for this bi-chromatic measurement

Calculations:
Enzyme reagent optical density (EROD): Average A1, A2 – Average B1, B2
OD for standards or samples (Final OD reading)= OD of standards or samples – EROD
∴ Concentrations=

$$Standard\ concentration \times \frac{final\ OD\ reading\ for\ sample}{final\ OD\ reading\ for\ standard}$$

Appendix A.9

University of Glasgow

Measurement of Resting Energy Expenditure

Indirect Calorimetry

(*Oxycon Pro* Indirect Calorimetry) SOP [*Hani v.3.2*]

Energy Expenditure can be measured with direct calorimetry by putting the subject in a calorimeter and measuring the amount of heat produced by the body mass. Direct calorimetry is very expensive and impractical and therefore is not commonly performed in the clinical setting. Energy Expenditure can also be measured indirectly with a metabolic cart. Gas exchange is analysed to determine the volume of air passing through the lungs, the uptake of oxygen ($\dot{V}O_2$) and the amount of Carbon Dioxide expelled to atmosphere (CO_2 output – $\dot{V}O_2$). These measurements allow the Resting Energy Expenditure (REE) and the Respiratory Quotient (RQ) to be calculated.
The RQ represents the ratio of carbon dioxide exhaled to the amount of oxygen consumed by the individual. RQ assists in the interpretation of the REE results.

Optimal conditions for REE testing (during and after the measurement):

- The subject should fast for at least 12 hours prior to the measurement. Smoking is refrained for at least 2 hours before the measurement.
- Testing conditions should optimally remain quiet throughout the procedure, and a normal room temperature must be maintained.
- A steady state should be achieved during the test defined as a 5-minutes period when the average minute $\dot{V}O_2$ and $\dot{V}CO_2$ changes by less than 10% and the average RQ changes by less than 5%.
- The subject should refrain from moving during the test.
- Medications recently taken should be noted (particularly stimulants or depressants).
- A test of minimum 15-20 minutes is recommended to assure stable, interpretable measurements.
- The first 5 minutes of data acquisition should be discarded when performing the test.
- Before starting a REE test, the system must be calibrated Triple V flow transducer and the gas analysers by performing their calibrations accordingly.

- After turning the device on, the system must be warmed up for at least 30 minutes before beginning calibration or testing procedures.
- Regular calibration is necessary to assure the system is acquiring reliable measurements.

General notes for using *Oxycon Pro* Indirect Calorimetry Cart

- Hood, tube, flow sensor, twin tube line, Triple-V and turbine must be cleaned and disinfected (using Ethanol 70%) after each trial/every subject. Tube edges MUST be blocked with tissues after it is dried to prevent contamination and be ready for the following trial.
- When gas cylinder is replaced, check gas concentration values from the setting options window for gas calibration screen and change if they are different, values to be found on info card on the new cylinder
- Do not expose the *Oxycon Pro* to sunlight or heaters
- Record last values for both volume and gas calibrations

Pre-measurement preparations:

- Check gas cylinder, gas pressure preferably to be > 300 bsl (Open gas cylinder now: just to the limit when you hear the gas going through tube)
- Make sure that hood, tube, flow sensor, twin tube line, Triple-V and turbine cleaned, disinfected and dried prior to start (preferably one day before the trial) *
- Switch on *Oxycon Pro* PC (Enter password: 12345) [If the "Hood" measurement is used for highly accurate studies, CareFusion recommends a warm-up time of 2 hours].
- WAIT 15 minutes for warming up time (automatically done by *Oxycon Pro* when PC is started)
- Check ambient conditions (It is preferable to press the play bottom F1 again just to ensure new reading) and save
- WAIT 5 minutes (this will allow *Oxycon Pro* to adjust to the new ambient conditions)
- Do volume calibration ‘’If ambient conditions were manually edited, do both calibrations immediately’’ (Repeat volume calibration when differences in Ergebnisse high & low (Gain Ex and Gain In) flow values are ±1% or more)
- WAIT 15 minutes

- Do gas calibration ‘’If ambient conditions were edited, do both calibrations immediately’’ (Repeat gas calibration when differences in C, perc gas and D.time values are ±1% or more)
- Calibrations should be repeated several times (especially gas) when *Oxycon Pro* was not used for long periods > 4days or gas cylinder was changed.

During REE measurement:

- Keep subject away from any electronic/radio field at least 1.5 meters distance from other objects including *Oxycon Pro* itself
- Allow a distance of at least 2 meters between subject vs *Oxycon Pro* vs mobile phones (any device with radio waves)
- Centre the subject’s head in the plastic hood
- Place the ref. O_2 transparent tube at the subject’s neck and the tube’s slot to the top
- Measurements should be recorded after the minute 5 or over from time of placing the plastic canopy on the subject (Measurements of first 5 minutes were usually omitted in several published studies)
- $FeCO_2$ value must be between 0.4 – 0.8%, if not, adjust air flow rate (Range: 25-125 l/min); Go to tools and canopy Flow (the default value is 40 l/min)
- Both calibrations must be done before each test (Volume then gas calibration); even before pre-drift measurements
- RECORD NEW values of both calibrations on the provided last calibration record sheet.

After measurements:

- CLOSE gas cylinder
- Clean and disinfect flexible tube with 70% liquid ethanol
- Hang the tube in the metabolic room to dry
- Clean and disinfect *Oxycon Pro* unit and all parts (TripleV, turbine and hood) with disinfection wipes

Appendix A.10

University of Glasgow

Alcohol (Ethanol) Burning Indirect Calorimetry System Validation Test

(*Oxycon Pro* Indirect Calorimetry) SOP [*Hani v.1.2*]

To check the accuracy of the system, tests with the combustion of 99.9% ethanol periodically are performed. By combustion of 1 mol of ethanol, 3 mol of O_2 is consumed and 2 mol of CO_2 is produced:

$C_2H_5OH + 3\ O_2 = 2\ CO_2 + 3\ H_2O$
Molecule weight of Ethanol: 46.08

46.08 g Ethanol = 3 mol O_2 = 67.23 l O_2
1 g Ethanol= 67.23/46.08 = 1.459 l O_2

46.08 g Ethanol= 2 mol CO_2 = 44.82 l CO_2
1 g Ethanol = 44.82/46.08 = 0.973 l CO_2

Thus, RQ = 0.973/1.459 = 0.667

Alcohol Burning Test Steps for *Oxycon Pro* system:

1. The Hood system is prepared and calibrated as for a normal experiment.
2. Make sure that alcohol burning Kit (COSMED, Italy) is cleaned and prepared according to the manufacturer's manual and safety instructions
3. Fill the ceramic vessel with a known quantity of ethanol (during this operation is extremely important to deliver the exact quantity of liquid; avoid air bubbles and be sure that all the amount of liquid is poured into the vessel), weighed with 2 decimals
4. The tube for ref. O_2 is placed at the floor, 20-30 cm. from the burning kit and the burner is placed beside the kit.
5. Connect the alcohol burning metalic base to *Oxycon Pro* mixing chamber as normally done for real REE measurement
6. Start the experiment, and as soon as the background zeroing is finished, press F1.
7. Take the glass cap of the alcohol burner, light the burner.
8. After a while, be sure that the $FeCO_2$ is between 0.4-0.8%, if not, adjust the air flow.

9. After 20-30 min. the experiment should be stopped, the glass cap must be removed (do not touch the glass cap or metal container, they are VERY hot) and the burning must be stopped.
10. The weight of the ceramic vessel after burning must be written down.
11. The measured data should be saved and the average of the CO_2-production, the O_2-consumption, RER and EE need to be calculated. Use the automatic calculation method and input the value 0 for gram N in urine.

- Do not use the first 5 minutes for the mean values.
- Make a print out in Standard mode.

The amount of ethanol combusted per minute is calculated, together with the theoretic CO_2-combustion and O_2-consumption in ml./min. Compared with the measured values a CO_2- and an O_2-factor is calculated for each *Oxycon Pro* system.

- Alcohol tests are performed approximately once a week, in the periods where projects are going on.
- The tests should always be done after reparations or if the gas cylinder has been changed.

Appendix B.1

Body Composition, Energy Intake and Expenditure in People with Phenylketonuria (Research Proposal)

ABBREVIATIONS

Complete as required, all abbreviations used in the protocol should be defined upon first mention and added to this table

PKU	Phenylketonuria
PAH	Phenylalanine hydroxylase
BH4	Tetrahydrobiopterin
PHE	Phenylalanine
TYR	Tyrosine
CHO	Carbohydrates
TEE	Total energy expenditure
REE	Resting energy expenditure
BMR	Basal metabolic rate
PA	Physical activity
PAEE	Physical activity energy expenditure
PAL	Physical activity level
NLB	New Lister Building at Glasgow Royal Infirmary
GRI	Glasgow Royal Infirmary
GGC	Greater Glasgow and Clyde
D_2O	Deuterium oxide, also referred as deuterium water
DIT	Diet Induced Thermogenesis
BMI	Body mass index
kg	Kilograms
°C	Celsius temperature degree
$\dot{V}O_2$	Rate of oxygen consumption
$\dot{V}CO_2$	Rate of carbon dioxide production
SD	Standard deviation
ANCOVA	Analysis of covariance
SUERC	Scottish Universities Environmental Research Centre
gm	Gram
ml	Millilitre
in	Inches
FM	Fat mass
FFM	Fat free mass
TBW	Total body water
TOBEC	total body electrical conductivity
DEXA	dual-energy x-ray absorptiometry
BIA	body impedance analysis
ADP	air-displacement plethysmography
HR	Heart rate
HRmax	Maximal heart rate
COMA	Committee on Medical Aspects of Food and Nutrition Policy

Body Composition, Energy Intake and Expenditure in People with Phenylketonuria
(Research Proposal)

Background

PKU is an autosomal recessive disease characterised by the dysfunction of the enzyme phenylalanine hydroxylase (PAH) or absence of cofactor Tetrahydrobiopterin (BH4) [1]. This enzyme is required to hydrolyse amino acid phenylalanine to tyrosine. Inability of the body to hydrolyse phenylalanine (PHE) results in elevation of its levels in the blood and in brain. Untreated PKU patients are at high risk of mental retardation, seizures and other neurological symptoms [2]. PKU is a rare disorder and its prevalence ranges between one in 4,000 to one in 10,000 births [3].

PKU is an incurable condition and the current management is based on a 'PKU-type diet' which involves restriction of dietary PHE, by reducing the natural protein intake, and concomitant supplementation with all other essential amino acids, in order to meet body requirements [4]. Therefore, protein foods rich in PHE, such as meat, cheese, poultry, eggs and milk, and plant/vegetable proteins such as potatoes and cereals are not permitted or restricted to very small amounts in the 'PKU-type diet' [5, 6]. PKU control is monitored through blood levels of PHE, and monitoring is more frequent in patients below 4 years than in older patients [7]. The restriction level of the 'PKU diet' depends on patients' tolerance to dietary PHE with the diet being more 'relaxed' in those who are above 10 years old and brain development has been completed [8, 9].

Nutritional deficiencies and malnutrition have frequently been reported in patients with PKU mainly due to the very restrictive nature of the 'PKU-type diet' [4]. However, in recent years malnutrition, in terms of undernutrition, is not common. This is mainly due to the development and availability of 'PKU-type' nutritional supplements and foods for people with PKU [8, 10-12]. These special foods are believed to improve patients' compliance to a PKU diet and provide energy to their diet [12-17]. Recent evidence though reports an increasing prevalence of obesity in PKU [18-20]. A study from USA revealed that the percentages of overweight and obese PKU females were 55% and 33%, respectively [18]. Similarly an audit in our paediatric hospital on 135 PKU patients, showed similar patterns of overweight and obesity prevalence [21]. While the prevalence of obesity in PKU patients is similar to that in healthy population, the underlying aetiology of obesity in this group of patients remains unknown.

Likewise, scarce evidence suggests that people with PKU tend to have a higher fat mass and lower lean mass when compared to healthy individuals [22-28]. This infers that for a given body mass index (BMI) people with PKU will be fatter and perhaps at higher risk of cardiovascular diseases. However, the cumulative evidence is still inconsistent. Data obtained using skinfold-thickness, Dual-energy X-ray absorptiometry (DEXA), body impedance analysis (BIA) and total body electrical conductivity (TOBEC) showed no significant differences in measured body compartments between patients with PKU and healthy controls [22-26] but the percentage of body fat was significantly higher in patients with PKU than in healthy controls when air-displacement plethysmography (ADP) technique was used [27]. Inconsistency in the findings of previous research may be explained by the different

body composition techniques employed and small sample size, unmatched case-control groups [22-27]. Indeed all of the previously applied body composition techniques suffer from inaccuracy and imprecision bias, particularly when they are applied in people with chronic illness [29]. Hence, more studies applying gold standard techniques to measure body fatness and lean mass and considering an appropriate age-gender matched controls are needed.

Considering the increasing prevalence of obesity and evidence which reports a higher prevalence of adiposity in people with PKU than in controls, more research is required to address the origins of obesity in people with PKU and whether these are differ from people without the condition. Increased obesity in people with PKU may be related to: a) either a sedentary lifestyle. b) Increased energy intake from consumption on high fat/sugary food or c) a combination of both, leading to positive energy balance.

In our recent study [ClinicalTrials.gov/NCT02440932] in healthy people, we investigated the effect of consumption of a 'PKU type' diet on appetite hormones, energy intake and postprandial metabolic rate [4, 6]. While we found that the concentration of appetite hormones, subjective appetite and satiety scores, and energy intake at *ad libitum* buffet type meal were not significantly different after consumption of a 'PKU type diet' compared with a normal UK style diet, we found that postprandial metabolic rate was significantly lower by 41 kJ after PKU type foods. Fat oxidation, another potential regulator of energy balance and contributor to the aetiology of obesity, was also lower after PKU type compared with the control diet. These results are interesting and suggest that one of the causes of the increased risk of overweight and obesity in PKU patients can be related to reduction in postprandial metabolic rate (diet induced thermogenesis-DIT), a component of daily energy expenditure, and changes in body energy substrate metabolism.

No studies so far have measured the individual components of TEE in patients with PKU (i.e. Physical Activity Energy Expenditure ''PAEE'', Resting Metabolic Rate ''RMR'', DIT and intake) and compared these against well matched healthy controls. Previous studies have shown a negative correlation between elevated PHE plasma levels (uncontrolled PKU) with depression, anxiety, and poor mood [30, 31]. Patients with PKU have also been shown to have poor executive skills [32] and quality of life and less social interaction, thus they might be less likely to pursue an active lifestyle, engage in exercise and have social interaction [32-34]. More research is now required to assess all components of energy balance in patients with PKU, particularly energy intake and expenditure from basal body metabolic needs and physical activity. Such studies will allow us to characterise comprehensively all components of energy balance and understand better the underlying causes of obesity in patients with PKU. Whether PKU, as a condition, is associated with increased adiposity will be explored too by exploring associations between body compositions, body nutrient substrate metabolism with markers of disease (e.g. PKU severity, phenylalanine metabolic control). However, the latter is a secondary outcome of this study and any findings and generated hypotheses will need to be tested formally in subsequent appropriately designed intervention studies.

Hypothesis

- Higher risk of obesity and adiposity in people with PKU is the result of alterations in the energy balance components and energy substrate oxidation.
- People with PKU will have higher body fatness (adiposity)' compared with healthy, socio-demographic characteristics and BMI matched controls.

Primary aim

To investigate whether dietary intake and components of energy expenditure such as BMR, PAEE and DIT are different between people with PKU and sociodemographic and BMI matched healthy controls.

Secondary aims

1- To investigate which components of energy balance (i.e. intake, BMR, PAEE and DIT) are associated with obesity in people with PKU people and whether these differ to those components in healthy controls.

2- To investigate body composition aspects in people with PKU and sociodemographic and - BMI matched controls.

3- To investigate correlations between daily energy expenditure, energy intake, and body composition with disease and patients' characteristics.

Participants and Methods

A. Study setting

Recruitment of eligible PKU participants about the study will take place at the Royal Hospital for Children, the Glasgow Royal Infirmary, and Queen Elizabeth Hospital outpatient clinics. The experimental procedures of the study will be conducted at the Metabolic Research Unit, Level 3, Human Nutrition Department, New Lister Building, Glasgow Royal Infirmary, Glasgow, UK.

B. Participants

Children and adults (10 – 45 years old) with PKU attending the metabolic medicine clinics in the area of NHS Greater Glasgow and Clyde (GGC) will be approached for recruitment. Participants will be free from history of acute and chronic illness (other than PKU) requiring regular appointments to the doctor and/or chronic use of medication or major gastrointestinal surgery where major part of the gut has been resected as these conditions are known to impact on energy expenditure and dietary intake [35-39]. Pregnant or lactating women will also be excluded from participation. People with learning or mobility difficulties or those with incapacity to provide informed consent will be excluded too. The clinical treatment team will evaluate patients' capacity to consent before considering them to participate in the study.

Inclusion Criteria:

Children and adults with PKU between 10 to 45 years old
Free from history of any acute and chronic illness (other than PKU)

Exclusion Criteria:

- Patients with learning or mobility disabilities
- Patients who are deemed not competent to provide informed consent as judged by the clinical staff
- Pregnant or lactating women
- Participants who are not able to read, comprehend or communicate in English

Identification of eligible participants with PKU

Eligible patients will be identified from the clinical appointment lists held by the medical team of the metabolic medicine clinics in NHS GGC. A member of the treatment team (Mrs Barbara Cochrane or other members of the primary management clinical teams) will send an introductory letter about the study and the study information leaflet to those participants who are eligible to participate. This will give adequate time for the participants and their carers to consider the study prior to their subsequent clinical appointment.

On the day of their clinical appointment Mrs Barbara Cochrane or other member of the clinical team will ask the participants if they have considered the study and they would be interested in participating. Those who express an interest will then be introduced to the research student at the end of their clinical appointment. If the participants are interested in participating, the researcher will discuss the study with them and answer any question they might have directly after their clinical appointment and schedule a study visit at the Metabolic Research Unit, in Human Nutrition, University of Glasgow, Glasgow Royal Infirmary. On the day of study visit, the participants will complete a consent form at the Metabolic Research Unit. The consent form will be photocopied for patient, study records and patient notes. For children (10 - 15 years old), the parent/carers will be asked to provide assent in addition to participant's consent.

Although we believe that the children are old enough (age 10 to 15) to consent for themselves, their competency to provide informed consent will also be checked with a member of the clinical team. Participants between 10 to 15 years old will be asked to attend along with their parent/carers. The researcher will explain to the participants that they have every right to drop out of the study at any point without an explanation, if they feel this is not suitable for them. To increase recruitment rate, the researchers will present the study at the PKU group sessions, cooking and social events that are organised by the local clinical team.

Healthy controls

Gender, BMI and age matched healthy people will be recruited as a control group. Pregnant or lactating women will be excluded from participation. Those with any chronic illnesses or bone injuries will also be excluded. Controls will be recruited by means of poster advertisement and by university's email-broadcasting and word of

mouth in the campus of the University of Glasgow, and other public areas (libraries, train/bus stations). Subjects who have expressed an interest to participate will be sent a participant information leaflet. Considering the participant is eligible and happy to participate, a study visit will be organised in a similar way with patients with PKU. The researcher will explain to the participants that they have every right to drop out of the study at any point without an explanation, if they feel this is not suitable for them.

Children control group

Healthy control children will be recruited by advertisement poster and word of mouth in the broader campus of the University of Glasgow. Those who express an interest in participating will contact the researchers who will arrange a meeting at the metabolic research unit to discuss the study. Participant information sheet might be sent to potential participants by email or by post, prior to this meeting taking place. Participants will have as much time as they feel is necessary to decide whether they would like to participate in the study. Should the participants express an interest to participate informed written consent will be obtained by both the child and his carer. Age-specific information sheet will be provided to the children.

C. Study design and a brief summary of experimental procedures

This will be case-control study design. Participants will attend the Metabolic Research Unit at NLB (GRI) after 12-hour overnight fasting refraining from alcohol consumption for at least 24 hours. Any planned exercise should be avoided during the 48 hours prior visit to the Metabolic Research Unit. In the morning of starting the study (day 0), participants will be advised to travel by a comfortable mean (own cars or taxi) and travel expenses will be reimbursed. Height, body weight and handgrip strength will be measured upon arrival at the unit. Basal metabolic rate (BMR) will be assessed between 7:00 – 10:00 AM for a duration of 20 minutes. Participants will be asked to provide baseline saliva sample followed by consumption does of 8 g deuterium water (D_2O) diluted in drinking water. Normal or PKU-type isocaloric (children, 395 kcal; adults, 490 kcal) breakfast meal will then be provided based on intervention group-type (Control or PKU accordingly). Following breakfast, DIT will be measured at 30, 60, 90, 120, 180 min, with each measurement lasting for 20 minutes (including 10 minutes rest in-between measurements). Two saliva samples will be collected at 3 and 3.5 hours after ingestion of D_2O. Following this, participants will be asked to perform a moderate incremental intensity exercise for 20 minutes using a treadmill while wearing facemask to collect expired gases. Participants will then receive a training session on use of completion of estimated food records and use of a physical activity accelerometer equipment. In addition, PKU participants (and their parents if ≤15 years old) will be asked to complete quality of life (QoL) questionnaires during this visit. Food intake and physical activity diaries will be handed to all participants during this session. Participants will be required to wear the accelerometer equipment on their right hip for seven consecutive days excluding bedtime and keep a record of non-wear times. During these days, they will also prospectively record estimated food and beverages intake over four days including one weekend day. At the end of each day, a researcher will call participants (5 – 10 mints/call) to cross-check data completion of both physical activity and food intake diaries.

Arrangements will be made in relation to the collection of dietary records and accelerometer readings at the end of the trial. Collected data on physical activity and estimated food intake will be reviewed by the researcher and participants will be contacted to clarify any incomplete data set. A schematic diagram of the study design is illustrated in Figure 1. Detailed descriptions on measurement methods are presented under separate subheadings below.

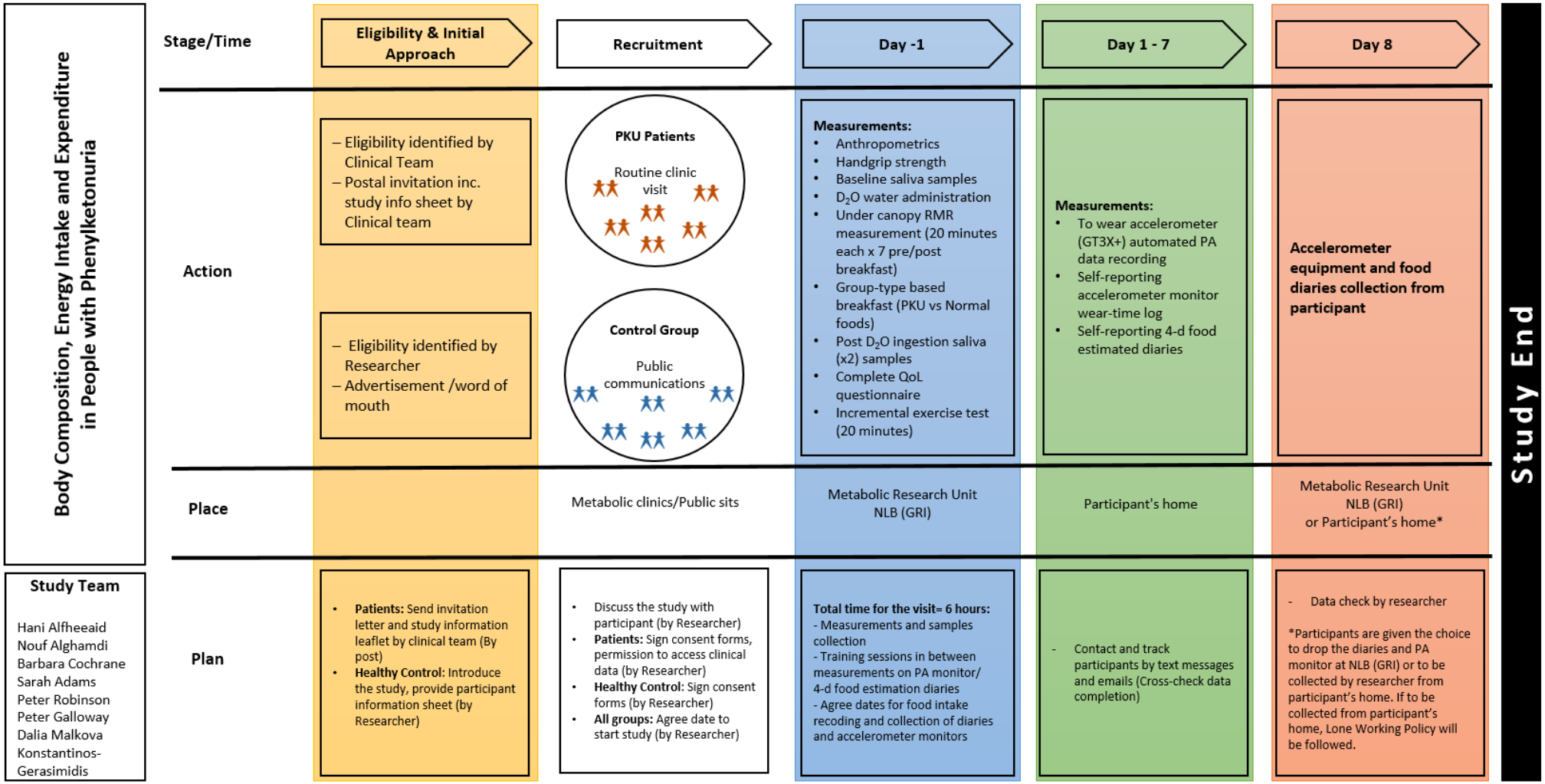

Figure 1. Schematic diagram of the study design

D. Measurement methods

i. Anthropometric measurements

Height

Height will be measured using a wall-mounted stadiometer. Subjects will stand barefoot, with their back against a fixed backboard and their arms hanging laterally by their body. The head will be positioned with the line of eyesight perpendicular to the backboard. Subjects will be instructed to relax and a moveable headboard will be lowered to the top of the head with light pressure added to compress the hair. Gentle upward pressure will be applied to the lower jaw and height will be measured to the nearest 0.01 m.

Body mass

Body mass will be measured using (TANITA-TBF-310, UK) balance scale. All subjects will be weighed in private wearing minimal clothing, typically lightweight shorts and a t-shirt, whilst standing with both feet flat on the balance and their arms hanging laterally by their body. All footwear, substantial jewellery and accessories, e.g. watches, and items within pockets will be removed prior to any measurement. Body mass index (BMI) will be calculated using the following formula: BMI = kg/m² [weight in kilograms/ height in meters²].

ii. Handgrip strength

Grip strength will be measured by using a handgrip dynamometer as a measure of strength and physical capacity. The dynamometer will be adjusted for hand size and participants will be asked to perform a maximal contraction for few seconds with the non-dominant hand. The test will be repeated three times (with a pause of about 10-20 seconds between measurements) and the highest value achieved will be recorded. If the difference between any two measures is more than 3 kgs., the test will be repeated once more after a rest period.

iii. Body composition determination, saliva samples collection, storage and analysis

Body composition will be determined using the deuterium water (D_2O) dilution technique [40]. This method is safe and very accurate for determining body composition, and has been widely used in children and clinical setting studies [41-46]. The principle of this technique assumes that normal physiological circumstances water content of lean body mass is constant and equals to 73.2% in average adults or ranges between 75 to 77% in children [47, 48]. Thus, values of TBW, can be used to calculate fat free mass (FFM) and fat mas (FM). Participants will be provided a dose of 8 g of D_2O [49] diluted in drinking water (80% of total ingested volume) during their visit to the Metabolic Research Unit at NLB (GRI). D2O doses will be prepared into plastic dose bottles and labelled with a unique code for each participant. Doses will be administered under close supervision and drinking straws will be used to avoid potential spillage. Participants will be asked to provide a baseline saliva samples (in the fasted state) and another samples will be obtained at 3 and 3.5 hrs after ingestion

of the D_2O. Samples will be collected into code marked 2 mL dry cryovials and will be stored in freezers at -20 °C of the research laboratory at Level 3, NLB (GRI). Suitable collection equipment will be provided to each participant. Access to the freezers and the laboratory is strictly controlled (card accessed). Upon completion of the study, all samples will be sent to the accredited laboratory at Scottish Universities Environmental Research Centre (SUERC), Glasgow, for measurements of TBW by means of Fourier Transform Infrared Spectrometry (FTIR). Saliva sample spares will be kept until data has been published and then destroyed.

iv. Metabolic rate measurement

Metabolic rate will be measured by means of computerised open-circuit ventilated hood system (Quark RMR®, COSMED, Italy). Participants will be asked to lay supine, still, and awake during the test. Once comfortable, a clear plastic canopy (weight, 550 gm; measures, 19.6 x 12.99 x 9.44 inc) will be placed on the participant's head and rate of oxygen consumption ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) will be recorded every 30 seconds for total duration of 20 minutes with 10 minute break (Fig. 2). These values will be used to calculate ratios of respiratory exchange and energy expenditure by using indirect calorimetry equations described by [50]. Volume and gas calibrations of the apparatus will be performed before each measurement.

Figure 2: Quark RMR® indirect calorimeter, COSMED, Italy

v. Breakfast meal and end of experimental trials lunch

During their visit to the metabolic research unite, participants in both groups will be asked to consume a breakfast after their BMR (baseline) is measured. Participants in PKU group will receive a breakfast based on PKU-type foods (low-PHE bread, low-protein jam, low-protein butter, low-protein cookies). These foods are widely used by patients with PKU in the UK. Participants in control group will receive a similar breakfast based on normal foods with calories content and weight being matched between the groups. Following last saliva sample collection, each participant will be provided a drink of their choice, water or a glass of orange juice (free of protein). At the end of the visit, each participant will receive a sandwich of his/her choice from lunch menu, a pack of crackers or chocolate cookies with drink options of orange juice,

tea or coffee. The PKU patients will be free to choose from the PKU or non-PKU type foods provided in the menu. Accompanying parents/guardians will be provided with lunch meal in which they had chosen prior to their visit. Indicative, breakfast meal and lunch menu composition are shown in tables 1 and 2.

Table 1: Energy and macronutrient composition, and servings of breakfast meal for PKU and Control groups

	Manf.	Serving	kcal	Protein	PHE	CHO	Fat		Manf.	Serving	kcal	Protein	PHE	CHO	Fat
PKU-type foods (PKU Group)								**Normal foods (Control Group)**							
Breakfast Meal (10 – 15years)															
L P Fresh Sliced Bread	Promin	1 Slice (48 g)	123.3	0.14	7.4	24.8	2	Toastie Sliced (White)	Warburtons	1 Slice (48 g)	113	4.7	235	20.8	0.9
Slightly Salted Butter	Lurpak	10 g	70.6	0.05	2.5	0.06	7.8	Slightly Salted Butter	Lurpak	10 g	70.6	0.05	2.5	0.06	7.8
Strawberry Jam	Tesco	20 g	51.8	0.10	5	12.7	0	Strawberry Jam	Tesco	20 g	51.8	0.10	5	12.7	0.02
Chocolate Chip Cookies	Juvala	3 Cookies (30 g)	149.4	0.12	5.4	20.4	7.4	Chocolate Chip Cookies	Tesco	3 Cookies (31.8 g)	165	1.8	90	20.7	7.5
Total			395.1	0.4	20.3	58.0	17.3	**Total**			400.4	6.6	332.5	54.2	16.2
Breakfast Meal (17– 45 years)															
L P Fresh Sliced Bread	Promin	2 Slices (96 g)	246.7	0.3	14.8	49.7	4.1	Toastie Sliced (White)	Warburtons	2 Slice (96 g)	226	9.4	470	41.6	1.8
Slightly Salted Butter	Lurpak	20 g	141.2	0.1	5	0.1	15.6	Slightly Salted Butter	Lurpak	20 g	141.2	0.1	5	0.1	15.6
Strawberry Jam	Tesco	40 g	103.6	0.2	10	25.4	0.0	Strawberry Jam	Tesco	40 g	103.6	0.2	10	25.4	0
Total			491.5	0.6	29.8	75.2	19.7	**Total**			470.8	9.7	485	67.1	17.4

Table 2: Energy and macronutrient composition, and servings in the lunch menu for study participants

	Manf.	Serving	kcal	Protein	PHE	CHO	Fat		Manf.	Serving	kcal	Protein	PHE	CHO	Fat
PKU-type foods								**Normal foods**							
Cheese Sandwich	Violife/ Promin	146 g	389.2	0.3	14.8	59.7	15.6	Cheese Sandwich	Tesco LM/ Warburtons	146 g	378	22.4	1120	41.7	12.8
								Chicken Salad Sandwich	Tesco	157 g	361	25.1	1255	34.7	12.6
								Smoked Ham & Cheddar Sandwich	Tesco	170 g	445	24.7	1235	38.8	20.4
								Prawn Mayonnaise Sandwich	Tesco	180 g	370	17	850	41.4	14.3
								Ploughmans Sandwich (V)	Tesco	180 g	419	16.7	835	43.5	18.6
One option of sides								*One option of sides*							
Crackers	Vitaflo	1 packet (40 g)	177	0.2		30.8	5.8	Crackers	Ritz Cheese Crackers	11 Crks. (40 g)	201	4	200	24	9.8
Chocolate Chip Cookies	Juvela	3 Cookies (30 g)	149.4	0.12	5.4	20.4	7.4	Chocolate Chip Cookies	Tesco	3 Cookies (31.8 g)	165	1.8	90	20.7	7.5
								Chips Lightly Salted/Cheddar	Tesco	30 g	154	2.1	105	15.5	9.0
One option of drinks								*One option of drinks*							
Orange Juice (Everyday Value)	Tesco	200 ml	78	0.6		18	0	Orange Juice	Tropicana	300 ml	154	2.4	120	30	0
Tea*		5 g	0	0		0	0	Tea		5 g	0	0	0	0	0
Coffee*		20 g	0	0		0.1	0	Coffee		20 g	0	0	0	0.1	0
Optional additions								*Optional additions*							
ProZero milk (optional)	Vitaflo	40 g	26	0	0	3.2	1.5	Semi skimmed milk (optional)	Tesco	40 g	20	1.4	70	1.9	0.72
Sugar (optional)		3 g	12	0	0	3	0	Sugar (optional)		3 g	12	0	0	3	0

vi. Measurements of PA and PAEE

Several techniques are available to measure energy expenditure of PA. One of the most commonly used methods in research is accelerometery which provides valuable information about both real life patterns and energy expenditure of PA [51-53]. Accelerometers are small wearable activity monitors that record acceleration of physical activity throughout wearing time [52]. Accelerometers collect data on intensity, duration and frequency of participant's physical activity which can then be used to categorise PA into intensity patterns and calculate PAEE [54]. Gold standard energy expenditure measurement techniques such as doubly labelled water (DLW) would be a good choice if the TEE was the interest of measure not/rather than an individual energy expenditure component such as PAEE [53]. Furthermore, using mobile indirect calorimetry would not provide information on physical activity life style but only structured activities (in lab activities) [54].

ActiGraph GT3X+® accelerometer (weight, 27g; measures, 1.5 x 1.44 x 0.70 inc) equipment will be used to measure and record acceleration of participant's physical activity. ActiGraph GT3X+ is a solid-state triaxle accelerometer which is enabled to measure motion data on three axes (Vertical, Axis 1; Horizontal, Axis 2; Perpendicular, Axis 3). ActiGraph accelerometer has been validated to measure PAEE and categorise patterns of PA in several age groups including children [55-58].

Participants will be required to wear snugly, but comfortably, the accelerometer GT3X+ on their right hips either above or beneath clothing with the Axis 1 upward facing (Fig. 3). The participants will have to wear them for seven consecutive days which represent habitual lifestyle. Participants must wear the accelerometer during waking hours and keep record of non-wear times. They should record the time they put the monitor on in the morning and when taking it off in the evening in their activity diaries. They also have to keep notes of time and reason if they take the monitor off during the day. Participants will be allowed to remove the monitor just before bedtime or when willing to swim or shower. As was mentioned before, participants/parents will receive a full training session on how to wear accelerometers and use the physical activity monitoring diary which includes instruction sheet. These will be provided during their visit to NLB (GRI).

Figure 3: ActiGraph GT3X+ accelerometer worn on waistband (left) and directions of measured motion axes (right)

The monitors will be prepared in advance to automatically record PA data starting from 7:00 AM of the following day (day 1 after the visit to Metabolic Research Unit) and cease at 7:00 AM in the morning of day 8. This will allow PA data collection of complete seven days. The raw data (counts/minute) will be imported by accompanying and well cited ActiLife software® v 6.13.2 after completion of assessment period for each participant. The software allows data cleaning using

pre-set algorithms developed from literature to validate wear-time which will be corrected according to PA monitoring diaries. The software also provides data scoring tools with a selection of pre-set cut-off points and algorithms, developed from the literature (including 14 published cut-offs and algorithms), respectively, to score data into PA levels and calculate PAEE from the obtained raw data. The choice of which cut-offs and algorithms to be used is based on studied age group and the aim of the study (e.g. assessment of sedentary vs vigorous PA). Each accelerometer will be calibrated before handing in to participants.

Data from counts will be used to obtain energy expenditure of waking hours. Taking into consideration sleeping time and that energy expenditure during sleep is 95% of RMR [59], daily TEE will be obtained. Regardless availability of algorithms, individual regression equations will be used to precisely calculate energy expenditure of waking hours and PAEE [54, 60]. Therefore, individual calibration curves for counts against $\dot{V}O_2/\dot{V}CO_2$ will be established during rest, and continuous incremental exercise. Basically, each participant will conduct walking/ running test with starting speed of 2 km/h being increased by 1 km/h every 4 minutes. The test will be stopped when heart rate (HR) just reaches 80 % of maximal heart rate (HRmax) defined as 220 – age in years. During this incremental test, participants will wear both ActiGraph GT3X+ accelerometer and age-appropriate face mask (Fig. 4 and Table 3), connected to indirect calorimetry equipment (Quark RMR®, COSMED, Italy) to measure rate of oxygen consumption ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$). Values of $\dot{V}O_2/\dot{V}CO_2$ (ml/minute) will be recorded during last minute of each stage. Data obtained will then be used to establish curves for accelerometer counts against VO_2 and VCO_2. The individual relationships between counts obtained from accelerometer and VO_2 and VCO_2 values will be used to calculate PAEE and TEE. All tests will be conducted under safety instructions provided from manufactures and lab guidelines.

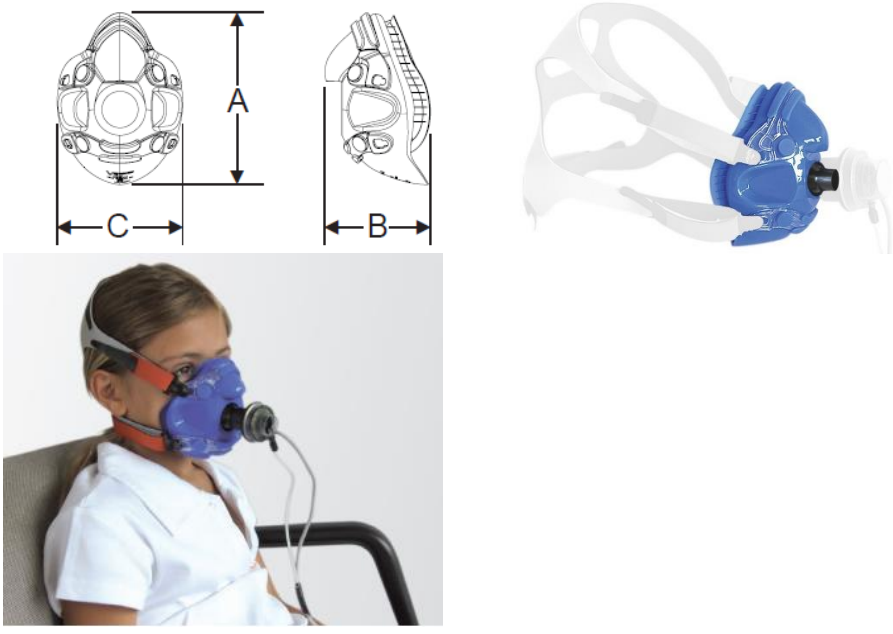

Figure 4. Sectional drawing and figure for illustration of wear during incremental exercise (calibration) test (below) for COSMED® silicone face mask used to collect expired gases in exercise mode (Source: COSMED®, Italy)

Table 3: Weight and dimensions of COSMED® silicone face mask used to collect expired air during exercise

Characteristic	Mask Face Pieces Sizes			
	Large	Medium	Small	Extra Small
A (inches)	5.9	5.6	5.2	4.8
B (inches)	3.7	3.4	3.2	3.1
C (inches)	4.2	4.2	4.0	3.9
Deadspace (ml)	143	125	99	88
Weight (gm)	128	118	100	89

vii. Dietary analyses

Food intake diaries will be provided to all participants. Participants will be asked to record detailed description of food and/or drink intake, time and place of consumption. They will also record details on food brand, preparation and cooking methods. Portion sizes should be estimated using household measures or natural unit sizes (e.g. slices of bread) are commonly used for this. Participants will be asked to record their intake for four days including one weekend day. Parents/guardians will assist their children to compete the food records. Participants will receive a full training session on how to estimate intake and how to use the provided food diaries during their visit to NLB (GRI). In addition, a researcher will interview each participant over the phone, when convenient, to cross-check data completion during days in which food estimation diaries will be recorded. Data from food diaries will be compared to reference UK photographic atlas of food portion sizes to estimate weight of food intake. Food and energy intake will then be calculated using the dietary software Windiets® 2010 (The Robert Gordon University, Aberdeen, Scotland, UK).

viii. Quality of life assessment

PKU participants' quality of life will be assessed by means of validated PKU-specific Health-related Quality of Life Questionnaires (PKU-QOL) developed by Merck Seroo S.A – Geneva – April 2010, Mapi Research Trust ©. The questionnaires cover the physical, emotional, and social impacts of PKU and its treatment on patients' lives and come along with a software to calculate PKU-QoL scores. These questionnaires are available for the following age groups: Child PKU-QOL (9-11 years), Adolescent PKU-QOL (12-17 years) and Adult PKU-QOL (≥ 18 years). They also enable the evaluation of the health-related quality of life of children by their parents using additional form of the questionnaires (Parent PKU-QOL). Elements in the questionnaires are divided into sections assessing patients' health, PKU diet and PHE-free supplements, patient's/ parent's daily life with PKU, and patient's/parent's general feeling about PKU. The recall period focuses on the past seven days for all sections except for 'patient's/parent's general feeling' where the recall period is 'in general'. Items has a 5-point Likert-type intensity or frequency response scale with an additional "Does not apply" or "I don't…/My child doesn't" response to some questions. Each participant in the PKU group will be asked to complete relevant questionnaire during their visit to the Metabolic Research Unit, NLB (GRI).

E. Clinical data on history of disease activity

Once the participant has given consent, information about disease management relevant to the study (i.e. records of PHE levels, weight and height status, and number of protein exchanges and dietary compliance, quantity and types of prescribed PKU foods and amino acid supplements) will be collected from the patients' medical notes. The data will be obtained for one year prior to the participants' intended day of study start. Only the direct research team will have access to the participants' medical notes and the search will be conducted in line with the NHS Code of Practice (Scotland) 2012 Version 2.1 and current data protection laws.

F. Participants' confidentiality

Participants, when consented, will be allocated an alphabetic code that will be used to label samples and data, to protect their identity throughout the study procedures and data analysis. Participants' name, address and contact details will be kept in a separate file locker which is placed in locked office (card-access) at Human Nutrition Department, Level 3, NLB (GRI). Indirect calorimetry data collected during resting and exercise and dietary intake will be kept in ID coded files in file locker. ID marked saliva samples will be stored in secured freezers of the Biochemistry laboratory at Level 3, NLB (GRI) and in a similar way at SUERC.

G. Dissemination of Results

We intend to disseminate the study design, protocols, and findings from this study in national and international scientific conferences and publications in peer-reviewed journals, PhD students' thesis and clinical trials registration domains. We may also present some of the findings in the annual PKU days for people with PKU and their relatives. All reports will be anonymised and the participants will not be able to be identified by the presented results. Summary results will be made available to the study participants upon request.

H. Data Retention

Anonymous electronic data will be stored in the University of Glasgow safe networks and it will be backed up every fortnight according to the university IT arrangements. Electronic data will be held for 10 year of study completion.

I. Statistical analysis

Data will be assessed for normality of distribution using Anderson-Darling test and descriptive analysis will be carried out to reveal the mean ± SD. Independent *t* test or Mann-Whitney test will be used to examine differences in body composition parameters (FM, FFM and TWB) and TEE, calculated percentages of energy balance [(energy intake/TEE) x 100], components of energy expenditure, dietary intake, percentages of estimated average energy requirements recommended by Committee on Medical Aspects of Food and Nutrition Policy (COMA), physical activity level (PAL) cut-offs and PAEE variables between the two groups, respectively, with normal or non-normal distribution in the independent groups. Pearson's correlation coefficient or Spearman's rho will be used to investigate the relationship, respectively, of variables with normal or non-normal distribution between measured energy expenditure in the patient group with currently available

predictive equations developed from healthy population. Similarly, PKU related clinical data of participating patients will be utilised to investigate correlations of energy expenditure components, and energy intake and parameters of body composition. In addition, PAL cut-offs, calculated percentages of energy balance and percentages of estimated average energy requirements recommended by COMA will be utilised to compare between participants of different body weights within PKU group. All statistical analysis will be carried out using Minitab® 17.2.1 version for Windows®. Statistical significance will be set at $P \leq 0.05$.

J. Sample size and power calculations

A proper power calculation is difficult to perform due to absence of previous pilot data. Assuming a mean difference in daily physical activity of 200 kcals between PKU patients and matched controls and a pooled SD of 350 kcals, 32 participants will be needed in each group to achieve a power of 80% with a significance level of 0.05. Power calculation will be repeated after recruitment of 10 participants per group and the research team will inform the Research Ethics Committee should amendment be required.

Estimation of recruitment and completion rate is difficult to make but our extensive experience working with children with chronic conditions such as (Crohn's disease, coeliac disease, juvenile idiopathic arthritis) suggests that patients and their carers are usually very motivated to participate. In a previous similar study, recruitment and completion rate was approximately 75% and we believe that having the support of the clinical team will overcome any barriers with regard to achieving our target sample size. Should we fail to achieve the required number of participants locally, we will extend the study to our neighboring clinical service in Edinburgh which have already expressed an interest in participating.

K. Participant compensation and rewards

Reasonable expenses such as travel tickets or fuel millage will be reimbursed. Participants who successfully complete the experimental trial during visit to NLB (day 1) will receive cinema tickets with their family and siblings (~£12 value each ticket) as a gift for taking part in this study. In addition, participants will receive £50 Amazon vouchers once they complete successfully the study and return the accelerometer, and food and activity diaries. This reward/incentive will encourage participation to the study and partially compensate for the time the participants and their carers (when appropriate) allocated to this.

L. Research fund

The study will be funded as part of a PhD student of Hani Alfheeaid and Nouf Alghamdi by the Government of Saudi Arabia through the Embassy of the Kingdom of Saudi Arabia in London.

M. Research team

Academic Research Staff

The research students, Hani Alfheeaid (Nutritionist) and Nouf Alghamdi (Dietitian) will be supervised by an academic team with different expertise in the field of energy balance, exercise physiology and nutritional assessment (Dr Dalia Malkova) and in

clinical nutrition (Dr Konstantinos Gerasimidis). Both PhD students have MSc degree in Human Nutrition with Clinical Nutrition specialisation from the University of Glasgow. The third year PhD student Hani Alfheeaid has very good experience in conducting metabolic study on humans and extensive knowledge on indirect calorimetry and dietary assessment. PhD students will also receive all required training including preparation of D_2O water doses and, handling urine and saliva samples. All researchers have received Good Clinical Practice certificates.
Both students are able to perform independently all research activities. However, for the purposes of this study, Hani Alfheeaid will measure the resting metabolic rate (RMR) and diet induce thermogenesis (DIT) for both PKU and control groups using indirect calorimetry. Nouf Alghamdi will measure physical activity energy expenditure (PAEE) and analyses dietary intake for all the participants. Other measurements (e.g. height, weight, hand grip strength and saliva sample collection) will be carried out interchangeably.

Analysis of deuterium enrichment in saliva samples will be conducted at the SUREC's biochemistry lab, Glasgow under supervision of Professor Thomas Preston who has extensive experience on stable isotope biochemistry analysis.

Clinical Research Staff

The research students will have also the support from medical staff at Royal Hospital for Children and Glasgow Royal Infirmary including the clinical dietitians Mrs Barbara Cochrane and Ms Sarah Adams along with clinical biochemist consultant Dr Peter Gallowy.

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Appendix B.2

Invitation Letter (Patients with Phenylketonuria)

Dear (child's and parents' or participant's name)

Research is very important to understand the cause and improve future treatment in people with chronic conditions such as Phenylketonuria.

Your doctors and dietitians work together with researchers from the University of Glasgow to find out more about the nutrition and health of people with Phenylketonuria. The study (Body Composition, Energy Intake and Expenditure in People with Phenylketonuria) has been approved by an NHS research ethics committee that it is safe for you or your child to participate. Herewith, we have enclosed a participant information leaflet for you to read carefully. If you agree, a researcher will meet you after the end of your next clinical appointment at the metabolic clinic to answer any questions you may have and consider participation in the study.

If there is anything you would like to discuss, please do not hesitate to contact us.

Thank you.

Yours sincerely,

Barbara Cochrane
Sarah Adams
Peter Robinson

Appendix B.3

Version #3
Date: 19/10/2016

Participant Information Sheet for Children with Phenylketonuria (10 – 15 years)

Study Title: Body Composition, Energy Intake and Expenditure in People with Phenylketonuria

We would like to invite you to take part in a study in people with a condition called Phenylketonuria (PKU). We very much hope you will consider being part of our study, but before you choose to participate, we would like from you to know what the study involves. Please take time to read the following information carefully; you can discuss it with your carers/friends if you wish. Please take your time to decide whether or not you would like to take part.

What is the purpose of the study?

PKU is an illness which can increase the level of a food ingredient called phenylalanine in the blood. High blood phenylalanine levels can cause serious problems to children with PKU. A diet low in phenylalanine helps children with PKU to manage their condition but the effects of this diet on other aspects of their health are unclear. We would therefore like to investigate whether the diet, physical activity and body energy needs are different between children with PKU and children of the same age, gender and body-size but without the condition.
This study is being done as part of the studies of two mature students (Nouf Alghamdi and Hani Alfheeaid) with the help of the PKU treatment teams at 3 different hospitals in Glasgow.

Why have I been invited?

You have been invited because we need to compare children who have PKU with children who do not have this condition. You have PKU but otherwise are healthy and aged between 10-15 years.

Do I have to take part?

No, it is entirely up to you and your parent/carer to decide whether you would like to take part in the study or not. If you choose to take part, we will ask you to sign a form stating that you are happy to do so. We will do this on the same day you will be visiting our lab for the study. If you decide to take part but then change your mind this is fine too. You are absolutely free to drop out from the study at any time you wish without giving a reason and it will not in any way affect how the doctors and nurses look after you now or in the future.

What will happen to me if I take part?

The study day (it will take 4 to 6 hours):

√ Prior to the study visit, you will be advised to stop doing exercise for 48 hours.

√ On the study day, we will invite you to our laboratory with your parent/carer in the morning after an overnight fast (i.e. without having had breakfast).

√ Height and body weight will be measured upon arrival at the laboratory.

√ Then, your hand grip strength will be measured by using a special device.

√ A saliva (spit) sample will then be collected. To do this test, we will ask you to chew a piece of clean white cotton for 60 seconds.

√ We will then ask you to drink a small amount of special water. This special type of water does not look or taste any different to tap water and it is needed to measure your body muscle stores.

√ Then, we will ask your permission to the amount of energy you use while you rest. For this assessment, you will be asked to lie down, relax, remain as still as possible for 20 minutes and breathe normally whilst a clear plastic hood (like an astronaut's helmet) will be placed over your head. The machine we use in this test is similar to Photo 1

√ A PKU breakfast will then be offered. Following this breakfast, the energy that your body uses up will be measured 5 times; each will last for 20 minutes. We then will ask you to provide another two saliva samples.

√ You will then be asked to walk on an automatic walking belt (like the ones people use in the gym) increasing your speed slowly over a period of 25 minutes. During this test, we will ask you to wear a face mask (see Photo 2) and a tiny step counter (the size of a matchbox) around your waist.

√ At the end of the visit, we will offer you a light lunch of your choice.

√ We are interested to know more about your everyday life. Therefore, during the visit, we will ask you to complete a survey about your life with PKU. This will take about 25 minutes.

On the days following the study visit, you will be asked to do the following:

√ To wear the step counter on your right hip for 7 consecutive days (see Photo 3). This is a small activity device (worn using a special belt) that counts your steps. We will ask you to write down when you take off the step counter (before bedtime, or to have a shower or swim).

√ We will ask you to write down what you eat and drink during 4 days, with one of being a weekend day. We will provide you with food and drink intake diaries and the researchers will advise you and your parent/carer on how to complete them.

√ After your carer permission, the researcher will phone you for 5 to 10 minutes to check how you are getting on with wearing the step counter and writing down your food and drink intake.

√ Once you have finished writing your diaries, you will be able to choose whether to come with your parent/carer to our laboratory to return the diaries and the step counter or to ask and allow us to come to your home and collect them from you.

Photo 1

Photo 2

Photo 3

What are the possible disadvantages and risks of taking part?

There are absolutely no risks of participating in the study.

√ You may feel a bit uncomfortable wearing the plastic helmet, the mask and step counter while we are measuring how much energy your body uses up. However, these are all safe and have been designed for children and previously tested in other studies similar to this one. We will also try these on you and make sure you are happy and you have no problem before starting.

√ You may feel embarrassed about providing saliva but do not worry you will be ensured privacy.

√ You may also be worried about your ability to complete the exercise test. Please be sure that this test will only involve walking at a light and moderate speed on a walking belt.

√ Writing down your food and drink intake and wearing the step-counter may be not comfortable or you may even forget to do them at all. But do not worry about it because at the end of each day, a researcher will call you for 5 – 10 minutes to check on your progress. Your parent/carer will help you too.

Will my taking part in this study be kept confidential?

Yes, no one will know about you joining the study apart from the researchers and the people organising the study.

Will I receive anything for taking part?

You and your family will receive free cinema tickets when you successfully completed the first day of the study. A £50 Amazon voucher will be happily given to you when you have successfully completed the entire study.

The study researchers:

Nouf Alghamdi
PhD Researcher
Mobile: xxxxx xxx xxx

Hani Alfheeaid
PhD Researcher
Mobile: xxxxx xxx xxx

Thank you for reading this Information Sheet

The Study Timeline

4 to 6 hours

Procedure	Duration	Photo
Stop exercising for 48 hours before the lab visit		
Fast overnight		
Height and body weight measurements	5 minutes	
Hand grip strength will be measured by using a special device. Testing is done by grasping the device and squeezing it as hard as you can	5 minutes	
A saliva (spit) sample will be collected. We will ask you to chew a piece of clean cotton to help with saliva collection	1 minute	
Drink a small amount of special water	5 minutes	
Measure the amount of energy you use while you rest	20 minutes	
Breakfast will then be provided to you	15 minutes	
Following breakfast, the energy that your body consumes will be measured 5 times in total	Each will last for 20 minutes	
Provide another two saliva samples	2 minutes	
Walk on an automatic walking belt. During this test, you will be wearing a face mask and a tiny step counter around your waist	25 minutes	  
At the end of the visit, your will receive a light lunch of your choice		

Appendix B.4

Version #3
Date: 19/10/2016

Parent/Guardian Information Sheet for Children with Phenylketonuria

Study Title: Body Composition, Energy Intake and Expenditure in People with Phenylketonuria

We would like to invite your child to take part in a study in people with Phenylketonuria (PKU) in the future. We very much hope you will consider your child being part of our study, but before you decide, we would like from you to know what the study involves. Please read the following information carefully; you can discuss it with your child/friends if you wish. Please take your time to decide whether or not you would like for your child to take part. Several of the measurements will be fun.
.

What is the purpose of the study?

PKU is an illness which can increase the level of a food ingredient called phenylalanine in the blood. High blood phenylalanine levels can cause serious problems to children with PKU. A diet low in phenylalanine helps children with PKU to manage their condition but the effects of this diet on other aspects of their health are unclear. We would therefore like to investigate whether the diet, physical activity and body energy needs are different between children with PKU and children of the same age, gender and body-size but without the condition.
This study is being done as part of the studies of two mature students (Nouf Alghamdi and Hani Alfheeaid) with the help of the PKU treatment teams at 3 different hospitals in Glasgow.

Why has my child been invited to take part in this study?

Your child has been invited because we need to compare children who have PKU with children who do not have this condition. Your child has PKU but otherwise is healthy and aged between 10-15 years.

Does my child have to take part?

No, it is entirely up to you to decide whether you would like your child to take part in the study or not. If you choose for your child to take part and your child doesn't mind, we will ask you to provide assent in addition to child's consent (stating that your child is happy to take part and you don't mind him/her participating). We will do these on the same day you and your child will be visiting our lab for the study. If you or your child change your mind about taking part this is fine too. Your child is absolutely free to withdraw from the study at any time you or your child wish without giving a reason and it will not in any way affect how the doctors and nurses look after your child now or in the future.

What does my child have to do if my child and I agree to take part?

The study day (it will take 4 to 6 hours):

√ Prior to the study visit, your child will be advised to stop doing exercise for 48 hours

√ On the study day, we will invite your child to come with you to our laboratory in the morning after an overnight fast (i.e. without having had breakfast).

√ Your child's Height and body weight will be measured upon arrival at the laboratory.

√ Then your child's hand grip strength will be measured by using a special device

√ A saliva (spit) sample will then be collected. To do this test, we will ask your child to chew a piece of clean white cotton for 60 seconds.

√ We will then ask your child to drink a small amount of special water. This special type of water does not look or taste any different to tap water and it is needed to measure your child's body muscle stores.

√ Then, we will ask your child's permission to the amount of energy your child's use while he/she rests. For this assessment, your child will be asked to lie down, relax, remain as still as possible for 20 minutes and breathe normally whilst a clear plastic hood (like an astronaut's helmet) will be placed over his/her head. The machine we use in this test is similar to Photo 1

√ A PKU Breakfast will then be offered to your child. Following this breakfast, the energy that your child's body uses up will be measured 5 times; each will last for 20 minutes. We then will ask your child to provide another two saliva samples.

√ Your child will then be asked to walk on an automatic walking belt (like the ones people use in the gym) increasing his/her speed slowly over a period of 25 minutes. During this test, we will ask your child to wear a face mask (see Photo 2) and a tiny step counter (the size of a matchbox) around his/her waist.

√ At the end of the visit, we will offer you and your child a light lunch of his/her choice.

√ We are interested to know more about your and your child's everyday life. Therefore, during the visit, we will ask you to complete a questionnaire about your daily life having a KU child and we will also ask your child to complete the same questionnaire adapted to his/her age. This will take about 25 minutes.

On the days following the study visit, your child will be asked to do the following:

√ To wear the step counter on his/her right hip for 7 consecutive days (see Photo 3). This is a small activity device (worn using a special belt) that counts your child's steps. We will ask you to help your child to write down when he/she takes off the step counter (before bedtime, or to have a shower or swim).

√ We will ask you to help your child to write down what he/she eats and drinks during 4 days, with one of being a weekend day. We will provide your child with food and drink intake diaries and the researchers will advise you and your child on how to complete them.

√ After your permission, the researcher will phone your child for 5 to 10 minutes to check how he/she is getting on with wearing the step counter and writing down his/her food and drink intake.

√ Once your child has finished writing his/her diaries, you will be able to choose whether to come to our laboratory to return to us your child's diaries and the step counter or to ask and allow us to come to your home and collect them from you.

Photo 1

Photo 2

Photo 3

What are the possible disadvantages and risks of taking part?

There are absolutely no risks of participating in the study.

√ Your child may feel a bit uncomfortable wearing the plastic helmet, the mask and step counter while we are measuring how much energy his/her body uses up. However, these are all safe and previously tested in other studies similar to this one. We will also try these on your child and make sure your child is happy and has no problem before starting.

√ Your child may feel embarrassed about providing saliva but do not worry he/she will be ensured privacy.

√ Your child may also be worried about his/her ability to complete the exercise test. Please be sure that this test will only involve walking at a light and moderate speed on a walking belt.

√ Your child may be feel not comfortable from writing down his/her food and drink intake and wearing the step counter or he/she may even forget to do them at all. But do not worry about it because at the end of each day, a researcher will call you and your child when convenient for 5-10 minutes to check on his/her progress.

Will my child taking part in this study be kept confidential?

Yes, no one will know about your child joining the study apart from the researchers and the people organising the study.

Will my child receive anything for taking part?

Your and his/her family will receive free cinema tickets when you successfully completed the first day of the study. A £50 Amazon voucher will be happily given to your child when he/she successfully completed the entire study.

Nouf Alghamdi
PhD Researcher
Mobile: xxxxx xxx xxx
E-mail: n.alghamdi.1@research.gla.ac.uk

Hani Alfheeaid
PhD Researcher
Mobile: xxxxx xxx xxx
E-mail: h.alfheeaid.1@research.gla.ac.uk

Thank you for reading this Information Sheet.

The Study Timeline

4 to 6 hours

Procedure	Duration	Photo
Stop exercising for 48 hours before the lab visit		
Fast overnight		
Height and body weight measurements	5 minutes	
Hand grip strength will be measured by using a special device. Testing is done by grasping the device and squeezing it as hard as you can	5 minutes	
A saliva (spit) sample will be collected. We will ask you to chew a piece of clean cotton to help with saliva collection	1 minute	
Drink a small amount of special water	5 minutes	
Measure the amount of energy you use while you rest	20 minutes	
Breakfast will then be provided to you	15 minutes	
Following breakfast, the energy that your body consumes will be measured 5 times in total	Each will last for 20 minutes	
Provide another two saliva samples	2 minutes	
Walk on an automatic walking belt. During this test, you will be wearing a face mask and a tiny step counter around your waist	25 minutes	  
At the end of the visit, your child will receive a light lunch of your choice		

Appendix B.5

Version #3
Date: 19/10/2016

Participant Information Sheet for Adults with Phenylketonuria

Study Title: Body Composition, Energy Intake and Expenditure in People with Phenylketonuria

We would like to invite you to take part in a study in people with a condition called Phenylketonuria (PKU). We very much hope you will consider being part of our study, but before you choose to participate, we would like from you to know what the study involves. Please take time to read the following information carefully; you can discuss it with your relatives/friends if you wish. Please take your time to decide whether or not you would like to take part.

What is the purpose of the study?

PKU is an illness which can increase the level of a food ingredient called phenylalanine in the blood. High blood phenylalanine levels can cause serious problems to adults with PKU. A diet low in phenylalanine helps adults with PKU to manage their condition but the effects of this diet on other aspects of their health are unclear. We would therefore like to investigate whether the diet, physical activity and body energy needs are different between adults with PKU and adults of the same age, gender and body-size but without the condition.
This study is being done as part of the studies of two mature students (Nouf Alghamdi and Hani Alfheeaid) with the help of the PKU treatment teams at 3 different hospitals in Glasgow.

Why have I been invited?

You have been invited because we need to compare adults who have PKU with adults who do not have this condition. You have PKU but otherwise are healthy and aged more than 15 years. If you are currently pregnant or lactating you are not suitable to participate.

Do I have to take part?

No, it is entirely up to you to decide whether you would like to take part in the study or not. If you choose to take part, we will ask you to sign a form stating that you are happy to do so. We will do this on the same day you will be visiting our lab for the study. If you decide to take part but then change your mind this is fine too. You are absolutely free to drop out from the study at any time you wish without giving a reason and it will not in any way affect how the doctors and nurses look after you now or in the future.

What will happen to me if I take part?

The study day (it will take 4 to 6 hours):

√ Prior to the study visit, you will be advised to stop doing exercise for 48 hours and refrain from drinking alcohol for 24.

√ On the study day, we will invite you to our laboratory in the morning after an overnight fast (i.e. without having had breakfast).

√ Height and body weight will be measured upon arrival at the laboratory.

√ Then, your hand grip strength will be measured by using a special device.

√ A saliva (spit) sample will then be collected. To do this test, we will ask you to chew a piece of clean white cotton for 60 seconds.

√ We will then ask you to drink a small amount of special water. This special type of water does not look or taste any different to tap water and it is needed to measure your body muscle stores.

√ Then, we will ask your permission to measure the amount of energy you use while you rest. For this assessment, you will be asked to lie down, relax, remain as still as possible for 20 minutes and breathe normally whilst a clear plastic hood (like an astronaut's helmet) will be placed over your head. The machine we use in this test is similar to Photo 1

√ A PKU breakfast will then be offered. Following this breakfast, the energy that your body uses up will be measured 5 times; each will last for 20 minutes. We then will ask you to provide another two saliva samples.

√ You will then be asked to walk on an automatic walking belt (like the ones people use in the gym) increasing your speed slowly over a period of 25 minutes. During this test, we will ask you to wear a face mask (see Photo 2) and a tiny step counter (the size of a matchbox) around your waist.

√ At the end of the visit, we will offer you a light lunch of your choice.

√ We are interested to know more about your everyday life. Therefore, during the visit, we will ask you to complete a survey about your life with PKU. This will take about 25 minutes.

On the days following the study visit, you will be asked to do the following:

√ To wear the step counter on your right hip for 7 consecutive days (see Photo 3). This is a small activity device (worn using a special belt) that counts your steps. We will ask you to write down when you take off the step counter (before bedtime, or to have a shower or swim).

√ We will ask you to write down what you eat and drink during 4 days, with one of being a weekend day. We will provide you with food and drink intake diaries and the researchers will advise on how to complete them.

√ After your permission, the researcher will phone you to talk to you for 5 to 10 minutes to check how you are getting on with wearing the step counter and writing down your food and drink intake.

√ Once you have finished writing your diaries, you will be able to choose whether to come to our laboratory to return the diaries and the step counter or to ask and allow us to come to your home and collect them from you.

Photo 1

Photo 2

Photo 3

What are the possible disadvantages and risks of taking part?

There are absolutely no risks of participating in the study.

√ You may feel a bit uncomfortable wearing the plastic helmet, the mask and step counter while we are measuring how much energy your body uses up. However, these are all safe and previously tested in other studies similar to this one. We will also try these on you and make sure you are happy and you have no problem before starting.

√ You may feel embarrassed about providing saliva but do not worry you will be ensured privacy.

√ You may also be worried about your ability to complete the exercise test. Please be sure that this test will only involve walking at a light and moderate speed on a walking belt.

√ Writing down your food and drink intake and wearing the step-counter may be not comfortable or you may even forget to do them at all. But do not worry about it

because at the end of each day, a researcher will call you for 5 – 10 minutes to check on your progress.

Will my taking part in this study be kept confidential?

Yes, no one will know about you joining the study apart from the researchers and the people organising the study.

Will I receive anything for taking part?

You and your family will receive free cinema tickets when you successfully completed the first day of the study. A £50 Amazon voucher will be happily given to you when you have successfully completed the entire study.

Nouf Alghamdi
PhD Researcher
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Hani Alfheeaid
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Mobile: xxxx xxx xxx
E-mail: h.alfheeaid.1@research.gla.ac.uk

Thank you for reading this Information Sheet.

The Study Timeline

4 to 6 hours

Procedure	Duration	Photo
Stop exercising for 48 hours before the lab visit		
Fast overnight		
Height and body weight measurements	5 minutes	
Hand grip strength will be measured by using a special device. Testing is done by grasping the device and squeezing it as hard as you can	5 minutes	
A saliva (spit) sample will be collected. We will ask you to chew a piece of clean cotton to help with saliva collection	1 minute	
Drink a small amount of special water	5 minutes	
Measure the amount of energy you use while you rest	20 minutes	
Breakfast will then be provided to you	15 minutes	
Following breakfast, the energy that your body consumes will be measured 5 times in total	Each will last for 20 minutes	
Provide another two saliva samples	2 minutes	
Walk on an automatic walking belt. During this test, you will be wearing a face mask and a tiny step counter around your waist	25 minutes	  
At the end of the visit, you will receive a light lunch of your choice		

Appendix B.6

Version #3
Date: 19/10/2016

Participant Information Sheet for Healthy Control Children (10-15 years)

Study Title: Body Composition, Energy Intake and Expenditure in People with Phenylketonuria

We would like to invite you to take part in a study in people with a condition called Phenylketonuria (PKU). We very much hope you will consider being part of our study, but before you choose to participate, we would like from you to know what the study involves. Please take time to read the following information carefully; you can discuss it with your carers/friends if you wish. Please take your time to decide whether or not you would like to take part.

What is the purpose of the study?

PKU is an illness which can increase the level of a food ingredient called phenylalanine in the blood. High blood phenylalanine levels can cause serious problems to children with PKU. A diet low in phenylalanine helps children with PKU to manage their condition but the effects of this diet on other aspects of their health are unclear. We would therefore like to investigate whether the diet, physical activity and body energy needs are different between children with PKU and children of the same age, gender and body-size but without the condition.
This study is being done as part of the studies of two mature students (Nouf Alghamdi and Hani Alfheeaid) with the help of the PKU treatment teams at 3 different hospitals in Glasgow.

Why have I been invited?

You have been invited because we need to compare children who have PKU with children who do not have this condition. You do not have PKU, you are otherwise healthy and aged between 10-15 years.

Do I have to take part?

No, it is entirely up to you and your parent/carer to decide whether you would like to take part in the study or not. If you choose to take part, we will ask you to sign a form stating that you are happy to do so. We will do this on the same day you will be visiting our lab for the study. If you decide to take part but then change your mind this is fine too. You are absolutely free to drop out from the study at any time you wish without giving a reason.

What will happen to me if I take part?

The study day (it will take 4 to 6 hours):

√ Prior to the study visit, you will be advised to stop doing exercise for 48 hours.

√ On the study day, we will invite you to our laboratory with your parent/carer in the morning after an overnight fast (i.e. without having had breakfast).

√ Height and body weight will be measured upon arrival at the laboratory.

√ Then, your hand grip strength will be measured by using a special device.

√ A saliva (spit) sample will then be collected. To do this test, we will ask you to chew a piece of clean white cotton for 60 seconds.

√ We will then ask you to drink a small amount of special water. This special type of water does not look or taste any different to tap water and it is needed to measure your body muscle stores.

√ Then, we will ask your permission to measure the amount of energy you use while you rest. For this assessment, you will be asked to lie down, relax, remain as still as possible for 20 minutes and breathe normally whilst a clear plastic hood (like an astronaut's helmet) will be placed over your head. The machine we use in this test is similar to Photo 1

√ A breakfast will then be offered. Following this breakfast, the energy that your body uses up will be measured 5 times; each will last for 20 minutes. We then will ask you to provide another two saliva samples

√ You will then be asked to walk on an automatic walking belt (like the ones people use in the gym) increasing your speed slowly over a period of 25 minutes. During this test, we will ask you to wear a face mask (see Photo 2) and a tiny step counter (the size of a matchbox) around your waist.

√ At the end of the visit, we will offer you a light lunch of your choice.

On the days following the study visit, you will be asked to do the following:

√ To wear the step counter on your right hip for 7 consecutive days (see Photo 3). This is a small activity device (worn using a special belt) that counts your steps. We will ask you to write down when you take off the step counter (before bedtime, or to have a shower or swim).

√ We will ask you to write down what you eat and drink during 4 days, with one of being a weekend day. We will provide you with food and drink intake diaries and the researchers will advise you and your parent/carer on how to complete them.

√ After your carer permission, the researcher will phone you for 5 to 10 minutes to check how you are getting on with wearing the step counter and writing down your food and drink intake.

√ Once you have finished writing your diaries, you will be able to choose whether to come with your parent/carer to our laboratory to return the diaries and the step counter or to ask and allow us to come to your home and collect them from you.

Photo 1

Photo 2

Photo 3

What are the possible disadvantages and risks of taking part?

There are absolutely no risks of participating in the study.

√ You may feel a bit uncomfortable wearing the plastic helmet, the mask and step counter while we are measuring how much energy your body uses up. However, these are all safe and have been designed for children and previously tested in other studies similar to this one. We will also try these on you and make sure you are happy and you have no problem before starting.

√ You may feel embarrassed about providing saliva but do not worry you will be ensured privacy.

√ You may also be worried about your ability to complete the exercise test. Please be sure that this test will only involve walking at a light and moderate speed on a walking belt.

√ Writing down your food and drink intake and wearing the step-counter may be not comfortable or you may even forget to do them at all. But do not worry about it because at the end of each day, a researcher will call you for 5 – 10 minutes to check on your progress. Your parent/carer will help you too.

Will my taking part in this study be kept confidential?

Yes, no one will know about you joining the study apart from the researchers and the people organising the study

Will I receive anything for taking part?

You and your family will receive free cinema tickets when you successfully completed the first day of the study. A £50 Amazon voucher will be happily given to you when you have successfully completed the entire study.

The study researchers:

Nouf Alghamdi
PhD Researcher
Mobile: xxxxx xxx xxx

Hani Alfheeaid
PhD Researcher
Mobile: xxxxx xxx xxx

Thank you for reading this Information Sheet.

The Study Timeline

4 to 6 hours

Procedure	Duration	Photo
Stop exercising for 48 hours before the lab visit		
Fast overnight		
Height and body weight measurements	5 minutes	
Hand grip strength will be measured by using a special device. Testing is done by grasping the device and squeezing it as hard as you can	5 minutes	
A saliva (spit) sample will be collected. We will ask you to chew a piece of clean cotton to help with saliva collection	1 minute	
Drink a small amount of special water	5 minutes	
Measure the amount of energy you use while you rest	20 minutes	
Breakfast will then be provided to you	15 minutes	
Following breakfast, the energy that your body consumes will be measured 5 times in total	Each will last for 20 minutes	
Provide another two saliva samples	2 minutes	
Walk on an automatic walking belt. During this test, you will be wearing a face mask and a tiny step counter around your waist	25 minutes	  
At the end of the visit, you will receive a light lunch of your choice		

Appendix B.7

Version #3
Date: 19/10/2016

Parent/Guardian Information Sheet for Healthy Control Children

Study Title: Body Composition, Energy Intake and Expenditure in People with Phenylketonuria

We would like to invite your child to take part in a study in people with Phenylketonuria (PKU) in the future. We very much hope you will consider your child being part of our study, but before you decide, we would like from you to know what the study involves. Please read the following information carefully; you can discuss it with your child/friends if you wish. Please take your time to decide whether or not you would like for your child to take part. Several of the measurements will be fun.

What is the purpose of the study?

PKU is an illness which can increase the level of a food ingredient called phenylalanine in the blood. High blood phenylalanine levels can cause serious problems to children with PKU. A diet low in phenylalanine helps children with PKU to manage their condition but the effects of this diet on other aspects of their health are unclear. We would therefore like to investigate whether the diet, physical activity and body energy needs are different between children with PKU and children of the same age, gender and body-size but without the condition.
This study is being done as part of the studies of two mature students (Nouf Alghamdi and Hani Alfheeaid) with the help of the PKU treatment teams at 3 different hospitals in Glasgow.

Why has my child been invited to take part in this study?

Your child has been invited because we need to compare children who have PKU with children who do not have this condition. Your child does not have PKU and aged between 10-15 years.

Does my child have to take part?

No, it is entirely up to you to decide whether you would like your child to take part in the study or not. If you choose for your child to take part and your child doesn't mind, we will ask you to provide assent in addition to child's consent (stating that your child is happy to take part and you don't mind him/her participating). We will do these on the same day you and your child will be visiting our lab for the study. If you or your child change your mind about taking part this is fine too. Your child is free to withdraw from the study at any time you or your child wish without giving a reason.

What does my child have to do if my child and I agree to take part?

The study day (it will take 4 to 6 hours):

√ Prior to the study visit, your child will be advised to stop doing exercise for 48 hours

√ On the study day, we will invite your child to come with you to our laboratory in the morning after an overnight fast (i.e. without having had breakfast).

√ Your child's Height and body weight will be measured upon arrival at the laboratory.

√ Then your child's hand grip strength will be measured by using a special device

√ A saliva (spit) sample will then be collected. To do this test, we will ask your child to chew a piece of clean white cotton for 60 seconds.

√ We will then ask your child to drink a small amount of special water. This special type of water does not look or taste any different to tap water and it is needed to measure your child's body muscle stores.

√ Then, we will ask your child's permission to measure the amount of energy your child's use while he/she rests. For this assessment, your child will be asked to lie down, relax, remain as still as possible for 20 minutes and breathe normally whilst a clear plastic hood (like an astronaut's helmet) will be placed over his/her head. The machine we use in this test is similar to Photo 1

√ A breakfast will then be offered to your child. Following this breakfast, the energy that your child's body uses up will be measured 5 times; each will last for 20 minutes. We then will ask your child to provide another two saliva samples.

√ Your child will then be asked to walk on an automatic walking belt (like the ones people use in the gym) increasing his/her speed slowly over a period of 25 minutes. During this test, we will ask your child to wear a face mask (see Photo 2) and a tiny step counter (the size of a matchbox) around his/her waist.

√ At the end of the visit, we will offer you and your child a light lunch of his/her choice.

On the days following the study visit, your child will be asked to do the following:

√ To wear the step counter on his/her right hip for 7 consecutive days (see Photo 3). This is a small activity device (worn using a special belt) that counts your child's steps. We will ask you to help your child to write down when he/she takes off the step counter (before bedtime, or to have a shower or swim).

√ We will ask you to help your child to write down what he/she eats and drinks during 4 days, with one of being a weekend day. We will provide your child with food and drink intake diaries and the researchers will advise you and your child on how to complete them.

√ After your carer permission, the researcher will phone your child for 5 to 10 minutes to check how he/she is getting on with wearing the step counter and writing down his/her food and drink intake.

√ Once your child has finished writing his/her diaries, you will be able to choose whether to come to our laboratory to return your child's diaries and the step counter or to ask and allow us to come to your home and collect them from you.

Photo 1

Photo 2

Photo 3

What are the possible disadvantages and risks of taking part?

√ There are absolutely no risks of participating in the study.

√ Your child may feel a bit uncomfortable wearing the plastic helmet, the mask and step counter while we are measuring how much energy his/her body uses up. However, these are all safe and previously tested in other studies similar to this one. We will also try these on your child and make sure your child is happy and has no problem before starting.

√ Your child may feel embarrassed about providing saliva but do not worry he/she will be ensured privacy.

√ Your child may also be worried about his/her ability to complete the exercise test. Please be sure that this test will only involve walking at a light and moderate speed on a walking belt.

√ Your child may be feel not comfortable from writing down his/her food and drink intake and wearing the step counter or he/she may even forget to do them at all. But

do not worry about it because at the end of each day, a researcher will call your child when convenient for 5-10 minutes to check on his/her progress.

Will my child taking part in this study be kept confidential?

Yes, no one will know about your child joining the study apart from the researchers and the people organising the study.

Will my child receive anything for taking part?

Your and his/her family will receive free cinema tickets when your successfully completed the first day of the study. A £50 Amazon voucher will be happily given to your child when he/she successfully completed the entire study.

Nouf Alghamdi
PhD Researcher
Mobile: xxxxx xxx xxx
E-mail: n.alghamdi.1@research.gla.ac.uk

Hani Alfheeaid
PhD Researcher
Mobile: xxxxx xxx xxx
E-mail: h.alfheeaid.1@research.gla.ac.uk

Thank you for reading this Information Sheet.

The Study Timeline

4 to 6 hours

Procedure	Duration	Photo
Stop exercising for 48 hours before the lab visit		
Fast overnight		
Height and body weight measurements	5 minutes	
Hand grip strength will be measured by using a special device. Testing is done by grasping the device and squeezing it as hard as you can	5 minutes	
A saliva (spit) sample will be collected. We will ask you to chew a piece of clean cotton to help with saliva collection	1 minute	
Drink a small amount of special water	5 minutes	
Measure the amount of energy you use while you rest	20 minutes	
Breakfast will then be provided to you	15 minutes	
Following breakfast, the energy that your body consumes will be measured 5 times in total	Each will last for 20 minutes	
Provide another two saliva samples	2 minutes	
Walk on an automatic walking belt. During this test, you will be wearing a face mask and a tiny step counter around your waist	25 minutes	  
At the end of the visit, your child will receive a light lunch of your choice		

Appendix B.8

Version #3
Date: 19/10/2016

Participant Information Sheet for Healthy Control Adults

Study Title: Body Composition, Energy Intake and Expenditure in People with Phenylketonuria

We would like to invite you to take part in a study in people with a condition called Phenylketonuria (PKU). We very much hope you will consider being part of our study, but before you choose to participate, we would like from you to know what the study involves. Please take time to read the following information carefully; you can discuss it with your relatives/friends if you wish. Please take your time to decide whether or not you would like to take part.

What is the purpose of the study?

PKU is an illness which can increase the level of a food ingredient called phenylalanine in the blood. High blood phenylalanine levels can cause serious problems to adults with PKU. A diet low in phenylalanine helps adults with PKU to manage their condition but the effects of this diet on other aspects of their health are unclear. We would therefore like to investigate whether the diet, physical activity and body energy needs are different between adults with PKU and adults of the same age, gender and body-size but without the condition.

This study is being done as part of the studies of two mature students (Nouf Alghamdi and Hani Alfheeaid) with the help of the PKU treatment teams at 3 different hospitals in Glasgow.

Why have I been invited?

You have been invited because we need to compare adults who have PKU with adults who do not have this condition. You do not have PKU, you are healthy and aged more than 15 years. If you are currently pregnant or lactating you are not suitable to participate.

Do I have to take part?

No, it is entirely up to you to decide whether you would like to take part in the study or not. If you choose to take part, we will ask you to sign a form stating that you are happy to do so. We will do this on the same day you will be visiting our lab for the study. If you decide to take part but then change your mind this is fine too. You are absolutely free to drop out from the study at any time you wish without giving a reason.

What will happen to me if I take part?

The study day (it will take 4 to 6 hours):

√ Prior to the study visit, you will be advised to stop doing exercise for 48 hours and refrain from drinking alcohol for 24.

√ On the study day, we will invite you to our laboratory in the morning after an overnight fast (i.e. without having had breakfast).

√ Height and body weight will be measured upon arrival at the laboratory.

√ Then, your hand grip strength will be measured by using a special device.

√ A saliva (spit) sample will then be collected. To do this test, we will ask you to chew a piece of clean white cotton for 60 seconds.

√ We will then ask you to drink a small amount of special water. This special type of water does not look or taste any different to tap water and it is needed to measure your body muscle stores.

√ Then, we will ask your permission to measure the amount of energy you use while you rest. For this assessment, you will be asked to lie down, relax, remain as still as possible for 20 minutes and breathe normally whilst a clear plastic hood (like an astronaut's helmet) will be placed over your head. The machine we use in this test is similar to Photo 1

√ A PKU breakfast will then be offered. Following this breakfast, the energy that your body uses up will be measured 5 times; each will last for 20 minutes. We then will ask you to provide another two saliva samples.

√ You will then be asked to walk on an automatic walking belt (like the ones people use in the gym) increasing your speed slowly over a period of 25 minutes. During this test, we will ask you to wear a face mask (see Photo 2) and a tiny step counter (the size of a matchbox) around your waist.

√ At the end of the visit, we will offer you a light lunch of your choice.

On the days following the study visit, you will be asked to do the following:

√ To wear the step counter on your right hip for 7 consecutive days (see Photo 3). This is a small activity device (worn using a special belt) that counts your steps. We will ask you to write down when you take off the step counter (before bedtime, or to have a shower or swim).

√ We will ask you to write down what you eat and drink during 4 days, with one of being a weekend day. We will provide you with food and drink intake diaries and the researchers will advise on how to complete them.

√ After your permission, the researcher will phone you to talk to you for 5 to 10 minutes to check how you are getting on with wearing the step counter and writing down your food and drink intake.

√ Once you have finished writing your diaries, you will be able to choose whether to come to our laboratory to return the diaries and the step counter or to ask and allow us to come to your home and collect them from you.

Photo 1

Photo 2

Photo 3

What are the possible disadvantages and risks of taking part?

There are absolutely no risks of participating in the study.

√ You may feel a bit uncomfortable wearing the plastic helmet, the mask and step counter while we are measuring how much energy your body uses up. However, these are all safe and previously tested in other studies similar to this one. We will also try these on you and make sure you are happy and you have no problem before starting.

√ You may feel embarrassed about providing saliva but do not worry you will be ensured privacy.

√ You may also be worried about your ability to complete the exercise test. Please be sure that this test will only involve walking at a light and moderate speed on a walking belt.

√ Writing down your food and drink intake and wearing the step-counter may be not comfortable or you may even forget to do them at all. But do not worry about it because at the end of each day, a researcher will call you for 5 – 10 minutes to check on your progress.

Will my taking part in this study be kept confidential?

Yes, no one will know about you joining the study apart from the researchers and the people organising the study.

Will I receive anything for taking part?

You and your family will receive free cinema tickets when you successfully completed the first day of the study. A £50 Amazon voucher will be happily given to you when you have successfully completed the entire study.

Nouf Alghamdi
PhD Researcher
Mobile: xxxxx xxx xxx
E-mail: n.alghamdi.1@research.gla.ac.uk

Hani Alfheeaid
PhD Researcher
Mobile: xxxxx xxx xxx
E-mail: h.alfheeaid.1@research.gla.ac.uk

Thank you for reading this Information Sheet.

The Study Timeline

4 to 6 hours

Procedure	Duration	Photo
Stop exercising for 48 hours before the lab visit		
Fast overnight		
Height and body weight measurements	5 minutes	
Hand grip strength will be measured by using a special device. Testing is done by grasping the device and squeezing it as hard as you can	5 minutes	
A saliva (spit) sample will be collected. We will ask you to chew a piece of clean cotton to help with saliva collection	1 minute	
Drink a small amount of special water	5 minutes	
Measure the amount of energy you use while you rest	20 minutes	
Breakfast will then be provided to you	15 minutes	
Following breakfast, the energy that your body consumes will be measured 5 times in total	Each will last for 20 minutes	
Provide another two saliva samples	2 minutes	
Walk on an automatic walking belt. During this test, you will be wearing a face mask and a tiny step counter around your waist	25 minutes	  
At the end of the visit, you will receive a light lunch of your choice		

Appendix B.9

Participant ID

Body Composition, Energy Intake and Expenditure in People with Phenylketonuria

(PKU Lunch Menu)

		Please Tick [✓] to Choose							
		Participant	Accompany	PKU Lunch Menu	Serving	Kcal (g)	Protein (g)	CHO (g)	Fat (g)
Sandwich		☐	☐	Cheese Sandwich (Violife cheese/ Promin bread)	146 g	389.2	0.3	59.7	15.6
Sides	Choose One	☐	☐	Crackers (Vitaflo)	1 packet (40 g)	177	0.2	30.8	5.8
		☐	☐	Chocolate Chip Cookies (Juvela)	3 Cookies (30 g)	149.4	0.12	20.4	7.4
Drinks	Choose One	☐	☐	Orange Juice (Tesco)	200 ml	78	0.6	18	0
		☐	☐	Tea*	5 g	0	0	0	0
		☐	☐	Coffee*	20 g	0	0	0.1	0

* Sugar and ProZero milk will be provided separately

Appendix B.10

Participant ID

Body Composition, Energy Intake and Expenditure in People with Phenylketonuria

(Normal Lunch Menu)

		Please Tick [✓] to Choose								
		Participant	Accompany	Normal Lunch Menu	Manf.	Serving	kcal	Protein (g)	CHO (g)	Fat (g)
Sandwich	Choose One	☐	☐	Cheese Sandwich	Tesco LM/ Warburtons	146 g	378	22.4	41.7	12.8
		☐	☐	Chicken Salad Sandwich	Tesco	157 g	361	25.1	34.7	12.6
		☐	☐	Smoked Ham & Cheddar Sandwich	Tesco	170 g	445	24.7	38.8	20.4
		☐	☐	Prawn Mayonnaise Sandwich	Tesco	180 g	370	17	41.4	14.3
		☐	☐	Ploughmans Sandwich (V)	Tesco	180 g	419	16.7	43.5	18.6
Sides	Choose One	☐	☐	Crackers	Ritz Cheese Crackers	11 Crks. (40 g)	201	4	24	9.8
		☐	☐	Chocolate Chip Cookies	Tesco	3 Cookies (31.8 g)	165	1.8	20.7	7.5
		☐	☐	Chips Lightly Salted/Cheddar	Tesco	30 g	154	2.1	15.5	9.0
Drinks	Choose One	☐	☐	Orange Juice	Tropicana	300 ml	154	2.4	30	0
		☐	☐	Tea*		5 g	0	0	0	0
		☐	☐	Coffee*		20 g	0	0	0.1	0

V= Suitable for vegetarians

* Sugar and semi skimmed milk will be provided separately

Appendix B.11

Participant ID	

ASSENT FORM [PKU Copy]

To be completed by the child/parent

Researcher: (…Hani A. Alfheeaid / Nouf A. Alghamdi…), School of Medicine, University of Glasgow

Body Composition, Energy Intake and Expenditure of People with Phenylketonuria

Please circle

Please circle your answers:
Have you read or has somebody else explained this project to you? Yes /No
Do you understand what this project is about? Yes /No
Have you asked all the questions you want? Yes/No
Have you had your questions answered in a way that you understand? Yes / No
Do you understand that it's OK to stop taking part at any time? Yes/No
Are you happy to take part? Yes/No
If any answers are 'no' or you don't want to take part, please don't sign your name.

If you do want to take part, please sign your name below

Child or Parent name _____

Signature _____

Date _____

The person who explained this project to you should sign here:

Print Name _____

Signature _____

Date _____

Appendix B.12

Participant ID	

CONSENT FORM (Parent/Carer) [PKU Copy]

Researcher: (…Hani A. Alfheeaid / Nouf A. Alghamdi…), School of Medicine, University of Glasgow

Body Composition, Energy Intake and Expenditure in People with Phenylketonuria

Please Initial BOX

1. I confirm I have read and understood the study information sheet (Ver.__ dated_____) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my and my child's participation is voluntary and that I and my child are free to withdraw at any time without giving any reason and without my child's medical care or legal rights being affected. ☐

3. I understand that data from my child's medical notes, but only that part relevant to this study, will be looked at by the researchers of this study and possibly by the NHS Greater Glasgow & Clyde R&D office for evaluation purposes. I give permission for these individuals to have access to my child's records. ☐

4. If I or my child withdraw from the study, I am happy for the researchers to retain and use any collected data and samples. ☐

5. I agree my child understands this study and is happy to participate ☐

6. I am happy to for my child's GP to be informed about him/her taking part in this study ☐

7. I agree for my child to take part in the above study ☐

Name of Participant

Name of Parent/Carer	Date	Signature

Name of person taking consent	Date	Signature

Appendix B.13

Participant ID	

PARTICIPANT CONSENT FORM [PKU Copy]

Researcher: (…Hani A. Alfheeaid / Nouf A. Alghamdi…), School of Medicine, University of Glasgow

Body Composition, Energy Intake and Expenditure in People with Phenylketonuria

Please Initial BOX

1. I confirm I have read and understood the study information sheet (Ver.___ dated_____) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, and without medical care or legal rights being affected. ☐

3. I understand that data from my medical notes (only relevant to this study), will be looked at by the researchers of this study and possibly by the NHS Greater Glasgow & Clyde R&D office for evaluation purposes. I give permission for these individuals to have access to my records. ☐

4. If I withdraw from the study, I am happy for the researchers to retain and use any collected data and samples. ☐

5. I am happy to inform my GP about me taking part in this study ☐

6. I agree to take part in the above study ☐

Name of Participant	Date	Signature

Name of Person taking consent	Date	Signature

Appendix B.14

Participant ID	

ASSENT FORM [Control Copy]

To be completed by the child/parent

Researcher: (…Hani A. Alfheeaid / Nouf A. Alghamdi…), School of Medicine, University of Glasgow

Body Composition, Energy Intake and Expenditure of People with Phenylketonuria

Please circle

Please circle your answers:

Have you read or has somebody else explained this project to you? Yes/No
Do you understand what this project is about? Yes/No
Have you asked all the questions you want? Yes/No
Have you had your questions answered in a way that you understand? Yes / No
Do you understand that it's OK to stop taking part at any time? Yes/No
Are you happy to take part? Yes/No

If **any** answers are '**no**' or you don't want to take part, please don't sign your name.

If you **do** want to take part, please sign your name below

Child or Parent name _____

Signature _____

Date _____

The person who explained this project to you should sign here:

Print Name _____

Signature _____

Date _____

Appendix B.15

Participant ID	

CONSENT FORM (Parent/Carer) [Control Copy]

Researcher: (…Hani A. Alfheeaid / Nouf A. Alghamdi…), School of Medicine, University of Glasgow

Body Composition, Energy Intake and Expenditure in People with Phenylketonuria

Please Initial BOX

1. I confirm I have read and understood the study information sheet (Ver.___ dated_____) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my and my child's participation is voluntary and that I and my child are free to withdraw at any time without giving any reason and without my child's legal rights being affected. ☐

3. If I or my child withdraw from the study, I am happy for the researchers to retain and use any collected data and samples. ☐

4. I understand that my child's information may be looked at by representatives of the study Sponsor, NHS GG&C, where it is relevant to him/her taking part in the study. ☐

5. I agree my child understands this study and is happy to participate ☐

6. I agree for my child to take part in the above study ☐

Name of Participant

Name of Parent/Carer	Date	Signature

Name of person taking consent	Date	Signature

Appendix B.16

Participant ID	

PARTICIPANT CONSENT FORM [Control Copy]

Researcher: (…Hani A. Alfheeaid / Nouf A. Alghamdi…), School of Medicine, University of Glasgow

Body Composition, Energy Intake and Expenditure in People with Phenylketonuria

Please Initial BOX

1. I confirm I have read and understood the study information sheet (Ver.___ dated_____) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, and without legal rights being affected. ☐

3. I understand that my information may be looked at by representatives of the study Sponsor, NHS GG&C, where it is relevant to my taking part in the study.. ☐

4. If I withdraw from the study, I am happy for the researchers to retain and use any collected data and samples. ☐

5. I agree to take part in the above study ☐

Name of Participant	Date	Signature
Name of Person taking consent	Date	Signature

Appendix B.17

Body Composition, Energy Intake and Expenditure in People with Phenylketonuria

Case Report Form (PKU Group)

I. Participant

ID:..... Test Date: / /

Gender: M/F DOB: / / SIMD Rank: SIMD quintile:

Height: cm Weight: kg BMI: kg/m² BMR: kJ Impedance:

Fat: % Fat Mass: kg Fat Free Mass: kg Total Body Water: kg

Grip strength: Ask to use non-dominant hand. Allow 10 – 20 seconds between measurements 1st to 3rd. If the **difference** in scores is over 3 kgs, measure 4th **after rest** period

* Record the result of each measurement to the nearest kilogram

1st (kg)	2nd (kg)	3rd (kg)	Repeated 4th (kg)

II. Deuterium Dose

Did the participant fast overnight? Yes ☐ / No ☐

If not, how long was the fast before taking the dose? hrs

Dose bottle number:

Dose weight: gm

Drinking water: gm

Pre-ingestion weight (Dose + Container + Straw + Added water): gm

Post-ingestion weight (Dose + Container + Straw): gm

Was the dose consumed correctly? Yes ☐ / No ☐

If not, what was the weight of the non-consumed water? gm

Was the container rinsed 1 x 50 ml water?

The same straw was used Yes ☐ / No ☐

Notes:

III. Sample collection times (Actual)

Time of baseline saliva sample hrs/minutes

Time of post-does [at 3.0 hrs] saliva sample hrs/minutes

Time of post-does [at 3.5 hrs] saliva sample hrs/minutes

PKU related clinical data

Weight Status

Number of weight measurements during last 12 months:

Measurement Date	Weight (kg)
/ /	
/ /	
/ /	
/ /	
/ /	
/ /	

If more measurements were recorded, use the additional note sheet

Growth Status

Number of height measurements during last 12 months:

Measurement Date	Height (cm)
/ /	
/ /	
/ /	
/ /	
/ /	
/ /	

If more measurements were recorded, use the additional note sheet

Dietary management

Is the patient on/off diet?

Number of protein exchange prescriptions during last 12 months:

Is the patient compliant to overall treatment? Yes ☐ / No ☐

Is the patient compliant to amino acid supplements? Yes ☐ No ☐ Sometimes ☐

Is the patient compliant to protein exchanges? Yes ☐ No ☐ Sometimes ☐

Prescription Date	Number of protein exchanges
/ /	
/ /	
/ /	
/ /	
/ /	
/ /	

If more prescriptions were recorded, use the additional note sheet

Prescriptions of special PKU foods and supplements

Date	Food/Supplement	Quantity	Date	Food/Supplement	Quantity
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If more prescriptions were recorded, use the additional note sheet

PHE Status

PKU type:

Number of PHE measurements during last 12 months:

Test Date	Result	Test Date	Result	Test Date	Result	Test Date	Result
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/ /		/ /		/ /		/ /	
/ /		/ /		/ /		/ /	
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Additional Notes

Appendix B.18

Body Composition, Energy Intake and Expenditure in People with Phenylketonuria

Case Report Form (Control Group)

I. Participant

ID:..... Test Date: / /
Gender: M/F DOB: / / SIMD Rank: SIMD quintile:
Height: cm Weight: kg BMI: kg/m² BMR: kJ Impedance:
Fat: % Fat Mass: kg Fat Free Mass: kg Total Body Water: kg

Grip strength: Ask to use non-dominant hand. Allow 10 – 20 seconds between measurements 1st to 3rd. If the **difference** in scores is over 3 kgs, measure 4th **after rest** period
* Record the result of each measurement to the nearest kilogram

1st (kg)	2nd (kg)	3rd (kg)	Repeated 4th (kg)

II. Deuterium Dose

Did the participant fast overnight? Yes ☐ / No ☐
If not, how long was the fast before taking the dose? hrs

Dose bottle number:
Dose weight: gm
Drinking water: gm
Pre-ingestion weight (Dose + Container + Straw + Added water): gm
Post-ingestion weight (Dose + Container + Straw): gm

Was the dose consumed correctly? Yes ☐ / No ☐
If not, what was the weight of the non-consumed water? gm
Was the container rinsed 1 x 50 ml water?

The same straw was used Yes ☐ / No ☐
Notes:

III. Sample collection times (Actual)

Time of baseline saliva sample hrs/minutes
Time of post-does [at 3.0 hrs] saliva sample hrs/minutes
Time of post-does [at 3.5 hrs] saliva sample hrs/minutes

Additional Notes

Appendix B.19

Body Composition, Energy Intake and Expenditure in People with Phenylketonuria

Visual Analogue Scale for Clinical Judgement on Patient's Compliance to PKU Treatment

Patient Study ID:

Please answer the following questions by placing a vertical mark through the line for each question. Regard the end of each line as indicating the best compliance and mark how you evaluate the patient's compliance over the last 12 months

1. How was the patient's compliance to prescribed amino acid supplements during the last 12 months?

Not compliant _____ **Very compliant**

2. How was the patient's compliance to prescribed natural protein exchanges during the last 12 months?

Not compliant _____ **Very compliant**

3. How was the patient's compliance to overall treatment during the last 12 months?

Not compliant _____ **Very compliant**

Appendix B.20

University of Glasgow

Measurement of Resting Energy Expenditure

Indirect Calorimetry

(*Quark RMR* Indirect Calorimetry) SOP [*Hani v.1.3*]

Energy Expenditure can be measured with direct calorimetry by putting the subject in a calorimeter and measuring the amount of heat produced by the body mass. Direct calorimetry is very expensive and impractical and therefore is not commonly performed in the clinical setting. Energy Expenditure can also be measured indirectly with a metabolic cart. Gas exchange is analysed to determine the volume of air passing through the lungs, the uptake of oxygen ($\dot{V}O_2$) and the amount of Carbon Dioxide expelled to atmosphere (CO_2 output – $\dot{V}O_2$). These measurements allow the Resting Energy Expenditure (REE) and the Respiratory Quotient (RQ) to be calculated.
The RQ represents the ratio of carbon dioxide exhaled to the amount of oxygen consumed by the individual. RQ assists in the interpretation of the REE results.

Optimal conditions for REE testing (during and after the measurement):

- The subject should fast for at least 12 hours prior to the measurement. Smoking is refrained for at least 2 hours before the measurement.
- Testing conditions should optimally remain quiet throughout the procedure, and a normal room temperature must be maintained.
- A steady state should be achieved during the test defined as a 5-minute period when the average minute $\dot{V}O_2$ and $\dot{V}CO_2$ changes by less than 10% and the average RQ changes by less than 5%.
- The subject should refrain from moving during the test.
- Medications recently taken should be noted (particularly stimulants or depressants).
- A test of minimum 15-20 minutes is recommended to assure stable, interpretable measurements.
- The first 5 minutes of data acquisition should be discarded when performing the test.
- Before starting a REE test, the system must be calibrated using the ID18 turbine and the analysers by performing the Ergo calibration.

- After turning the device on, the system must be warmed up for at least 30 minutes before beginning calibration or testing procedures.
- Turn on Hood blower during warm up period (the green switch at the back of the unit).
- Regular calibration is necessary to assure the system is acquiring reliable measurements.

A1. Flowmeter (flow/volume) turbine calibration

Flow/volume calibration is performed using a 3-liter calibration syringe.

Note: *If an anti-bacterial filter is used during testing, you should also use one when performing the turbine calibration.*

The turbine flowmeter does not require daily calibration since it is not affected by pressure, humidity and/or temperature. However, regular calibration should still be performed as well as the recommended maintenance procedures (see System maintenance chapter of the device user's manual).

Recommended Calibration Frequency (for RMR):

Each week, if the flowmeter is changed, if the ambient conditions (temperature, humidity and pressure) change significantly and in all the cases you suspect that the measurements are not reliable anymore.

Note: *Since ventilation is very low during REE testing (normally <10 litres/min), the turbine calibration should be performed with very slow manoeuvres (each manoeuvre lasting between 10-15 seconds).*

Flowmeter (Flow/volume) Turbine Calibration Steps:

1. **Start** *Omnia Software*
2. In the Calibration panel, **press the Flowmeters calibration** tile.
3. Begin with the syringe piston pushed all the way in.
4. **Press** the Start button
5. Select the device and the flowmeter.
6. Wait until that the red dot in the lower right part of the screen becomes green.
7. Move the piston in and out for 6 inspiratory and expiratory strokes (2 strokes for ID18 turbine ''5-6 seconds each''). Movements must be constant and circle lines are close to each other.

The graph on the left will display the F/V graph. On the right, you will see the current stroke and, at the end of the calibration manoeuvre, the inspiratory and expiratory gains, and the measured volumes with their relative errors (both inspiratory and expiratory) before and after the calibration. Relative errors must not exceed 0.5%. If relative errors is above 0.5%, repeat calibration.
8. During the manoeuvre, in order to restart the calibration, press **Redo**, to end the calibration press **Stop**, to cancel it press **Abort**.
9. At the end of the calibration, press **Accept**. New values will be stored.

A2. Gas analyser calibration

The *Omnia Software* allows to automatically calibrate the zero, gain and delay of the gas sensors. It is strongly recommended to perform these calibrations prior to each test.

Note: *Before using the device, you must allow for the required warm-up period (10 minutes).*

Recommended Calibration Frequency (for RMR):

Each day or if the ambient conditions (temperature, humidity and pressure) change significantly

Before Starting Calibration, Chick connections:

- In order to calibrate the analysers, the sampling line should be disconnected from the rear and be connected to the front panel of the Quark unit.
- The cylinders used for the calibration must be properly connected to the rear panel of the Quark and the output pressure should be set according the ranges reported on the rear panel of the Quark (5 – 7 bar).
- Before starting the calibration, the reference values should be correct (see the Setting Reference Values section in Quark RMR user's manual).

Note: *Cellular phones should be turned off to eliminate potential electrical interferences.*
Note: *If a "gas absent" error appears during the calibration, please check that the cylinder's residual pressure is above 10 bar and that the cocks are open and output gas pressure is between 5-7 bar.*

To start the calibration, **press** Gas calibration in the Calibration panel. Another panel will open, in which you can **select** which gas calibration must be performed.

Gas (Analyser) Calibration Steps

1. **Start** *Omnia Software*
2. To start a calibration, **press** the corresponding calibration tile in the Gas calibration panel.
3. **Press** Start.
4. **Select** the device.
5. Check the warnings, the cylinder gas concentrations and the O_2 span.
6. In case, edit the gas concentrations values according to the cylinder's certificate of analysis and the O_2 span according to your needs.
7. Press **OK.**
8. Wait till the end of the calibration.
9. During the manoeuvre, press **Redo** in order to restart, press **Abort** to cancel.

At the end of the manoeuvre, the calibration results are shown in numerical and graphical format. The bars near each parameter show their current value and their previous one.

10. Press **OK** to accept the check, **Cancel** to abort and **Factory Setting** to restore the factory settings.

If a value is out of range, the corresponding field is highlighted. Repeat the calibration and, if the problem persists please contact the COSMED support.

Important: *The gas calibration graph is the determinant for the quality of the calibration and thus the following measurements and data. Make sure that the measured gas lines are matching reference lines during calibration on the graph. No curves or fluctuations from the reference lines should appear. If so happened, please press factory setting and redo the calibration.*

Sampling line checks and maintenance (Permapure)

- Do not bend, squash or deform the sampling line. Any "kink" in the sample line will reduce the internal lumen of the line and affect accuracy of measurement.
- Do not keep the sampling line open to the atmosphere, particularly in crowded or smoky environments. Keep the sampling line in sealed plastic bag in a dark cool and dry place.
- If saliva enters the tube it should be replaced immediately.
- Periodically grease the O-ring on the connector to ease fitting to optical flowmeter.

- Replace the sampling line every 100 exercise tests or 200 PFT tests or every 6 months. In any case, sampling line will become discoloured (brownish) with age and may cause calibration to fail.

Note: *ALWAYS replace sample line as the first step in troubleshooting a failed gas calibration.*

B1. Starting REE measurement (Canopy mode)

1. Do not measure a comatose or incapacitated patient with the canopy in a place without a source of back-up power to assure continuous operation of the flow generator in the event of a power failure.
2. Constant attention by a qualified individual is required whenever a patient is mechanically ventilated. Some equipment malfunctions require immediate action. A malfunction may pass unnoticed in spite of equipment alarms.
3. Constant attention by a qualified individual is required whenever a patient is measured with the canopy. Problems in delivery of fresh gas may pass unnoticed in spite of alarms. Use a pulse oximeter to ensure that the patient is sufficiently oxygenated.
4. Verify (before and during the test) that the $FeCO_2$ falls within the range 0.8%-1.3% and adjust the flow rate of the pump as necessary. If the $FeCO_2$ is too low you should decrease the flow rate and if the $FeCO_2$ is too high you
5. Do not place the canopy hood over a patient's head before connecting the tube and applying a continuous flow from the canopy blower.
6. In order to obtain reliable results, check the integrity of hood, veil and gasket before testing.

The ventilated bubble hood system allows a stream of air to pass across the face of the subject and mix with the air being collected by the transparent hood over the subject's head. The flow rate measurement system will calculate the oxygen consumption, CO_2 production and the Energy Expenditure.

Note: *Presence of a skilful staff member (adequately trained and educated on the testing) is necessary during REE testing procedure and device.*

C. Connecting the Canopy

1. Connect the Canopy unit to the mains by means of the medical grade AC/DC adapter provided.
2. Attach the vail to the bubblehood through the velcro strips.
3. Insert the adapter into the bubblehood from the outside and connect it by screwing the ring from the inside.
4. Connect the bubblehood to the wrinkled tube, interposing a bacterial filter.
5. Connect the wrinkled tube to the unit through the Flow in connector.
6. Connect the ID18 turbine to the output of the Canopy unit.

D. Preparing the patient/subject

1. Switch the Canopy unit on.
2. Switch the Canopy unit on (the switch is on the rear side of the Quark RMR).
3. The red LED on the front panel of the unit should flash for few seconds followed by an alarm beep. If the LED does not flash and/or the alarm does not beep the test cannot be performed because either the backup battery is exhausted or there is no backup battery.
4. When the green LED turns on you may begin the test. If the green LED does not turn on the test cannot be performed because the pump does not work or the mains is not powering the system.
5. After performing these checks, instruct the patient/subject to lie in a supine position.
6. Place the bubblehood with the vail on the patient's head. The tube should be placed near the patient's mouth.

Warning: *Do not place the canopy hood over a patient's/subject's head before the tube is properly connected and a continuous flow has been applied from the canopy blower.*

F. Performing a REE test

Press the Testing tile in the home panel, or **start** a new test from the subject database, then **Metabolic** and **Resting Energy Expenditure**, then **select** REE – Canopy.
The system is ready to read the subject's breaths. **Press** Start to start the data acquisition.

The system will start to display data according to the selected dashboard. This data are not saved until REC is pressed. Test starts automatically and begins to store data after the time set in Settings.
In order to start data storage, press **Rec**. The display will be cleared and data will now be saved in the database.

Note: *During test with Canopy, before starting data storage, please check the FeCO2 value in the Dilution widget.*
Data are displayed according to the selected dashboard. Please refer to the Utilities/Customise views section for more details about the dashboards and the presentation of data.

Important, during measurement/Testing, please consider the following:
The ***Variability AVG widget*** reports the time and the variability of $\dot{V}O_2$ and $\dot{V}CO_2$. The colors of the vertical bars of the Variability AVG widget show the acceptability of the main values:

- Green: acceptable value
- Yellow: border line value
- Red: not acceptable value

If a bar is red (and possibly if it is yellow also), please operate in order to bring the value in the normal range. In particular:
• The $\dot{V}O_2$ or $\dot{V}CO_2$ bar is red (or yellow) if the subject's breath is not enough regular. Please normalise the subject's breath.
• The time bar is red (or yellow) if the acquisition time is too short according to the international guidelines. It is green only if the acquisition time is more than 10 minutes.

The ***Dilution widget*** (test with Canopy only) shows the $FeCO_2$ value. As explained in the general warnings, this value must be kept within the acceptability range. The needle points in one of three different bands, whose color is described below:

- Green: acceptable value
- Red: $FeCO_2$ too high, please increment the flow
- Grey: $FeCO_2$ too low, please reduce the flow

This check should be performed before starting the data acquisition. In order to adjust the flow, please rotate the knob (On REE Measurement Screen) controlled via software.

Appendix B.21

University of Glasgow

Alcohol (Ethanol) Burning Indirect Calorimetry System Validation Test

(*Quark RMR* Indirect Calorimetry) SOP [*Hani v.1.0*]

The alcohol (Ethanol) burning kit module (COSMED, Italy) is designed to provide the user with a useful tool to validate the respiratory quotient and the CO_2 production measurements.
In order to validate the variability and accuracy of the equipment and the precision of the gas analysers, the ethanol combustion tests with different quantities can be performed. When a known quantity of ethanol is combusted, the volumes of O_2 consumed and CO_2 produced, involved in the oxidation process, can be calculated.

By combustion of 1 mol of ethanol, 3 mol of O_2 is consumed and 2 mol of CO_2 is produced:

$C_2H_5OH + 3\ O_2 = 2\ CO_2 + 3\ H_2O$
Molecule weight of Ethanol: 46.08

46.08 g Ethanol = 3 mol O_2 = 67.23 l O_2
1 g Ethanol= 67.23/46.08 = 1.459 l O_2

46.08 g Ethanol= 2 mol CO_2 = 44.82 l CO_2
1 g Ethanol = 44.82/46.08 = 0.973 l CO_2

Thus, RQ = 0.973/1.459 = 0.667

Alcohol Burning Test Steps for *Quark RMR* system:

Ethanol CO_2 quantitative test

1. Turn ON the Quark RMR and let it warm up for at least 30 minutes.
2. Turn on Hood blower during warm up period.
3. COSMED support team recommends removing the moisture absorbent round filter during this test
4. Quark RMR system is prepared and calibrated as for a normal experiment.
5. Make sure that alcohol burning Kit (COSMED, Italy) is cleaned and prepared according to the manufacturer's manual and safety instructions.
6. Start the *Omnia software* and perform a dummy test during at least 15 minutes, in order to properly warm up the Quark.
7. Connect the alcohol burning metallic base to the Quark RMR (use the plastic tube and its adapters avoiding leakages).
8. Turn on Hood blower.
9. Perform a flowmeter turbine calibration at constant flow of 600ml/s (about 5 seconds for each stroke).
10. Run a gas calibration.
11. Launch the RMR Canopy test (in the options/real time, remove the option related to automatic calibration and leave the other settings in the default mode).
12. Start the data acquisition.
13. Verify that the FeO_2 and $FeCO_2$ are those of the ambient air (respectively, 20.93% and 0.03%). If this requirement is not met, please abort the test and repeat from step #10.
14. Fill the ceramic vessel with a known quantity of ethanol (during this operation is extremely important to deliver the exact quantity of liquid; avoid air bubbles and be sure that all the amount of liquid is poured in the vessel). Use ≥ 99.99% Ethanol.
15. Light the ethanol and immediately cover the vessel base with the glass.
16. Stop the test when the flame is over and the VCO_2 on the screen is 0.
17. Press or select View/Information in order to modify the parameters of the test in the following way:
Temperature at flowmeter = Actual Temperature
Humidity at flowmeter = Actual Humidity

18. Take note of the STPD correction factor.
19. Export the test in the xls (Excel) format (Test/Send to Excel…) and open it.
20. Calculate* the predicted value as shown in the Background paragraph i.e. 1 g of Ethanol produces 0.973 liters of CO_2. For the calculations, use the quantity delivered in the vessel at step #12.
21. Sum the values in the CO_2exp column related to the steps in which the VCO_2 values are >0, subtract the CO_2exp value multiplied by the number of

steps used in the previous sum, and multiply the result for the STPD factor (CO_2exp is not STPD corrected) [Obtain data sheet example from the department].
22. The value obtained at the previous step must be equal to the predicted one (step #19).

* For *Omnia Software* use Excel macro sheet provided by COSMED v. 6.2016.

- Alcohol tests are performed approximately once a week, in the periods where projects are going on.
- The tests should always be done after reparations or if the gas cylinder has been changed.
- Consider that 5ml of ethanol with a Quark RMR ventilation set around 35~40 L/min burns in about 20 minutes. Usually tests are performed burning quantity in a range from 1ml to 5 ml.

Respiratory Quotient validation test

The test is the same of the above one, with the following differences:

1. It is not necessary to measure the exact amount of ethanol.
2. It is necessary to enable the automatic calibration.
3. The RQ value can be read in real time during the test (it is not necessary to export the test at the end).

Appendix C.1

Table C.1 STEMDRL laboratory age and gender-specific reference ranges for blood vitamins and trace elements

Vitamins /Trace Elements	Age	Normal Range
Vitamin A (µmol/L)	< 1 year	0.5 - 1.5
	1 - 6 years	0.7 - 1.5
	7 - 12 years	0.9 - 1.7
	13 - 18 years	0.9 - 2.5
Vitamin B12 (ng l^{-1})		193 – 982
Vitamin E (µmol/L)	< 1 year	5 – 19
	1 - 6 years	7 – 21
	7 - 12 years	10 – 21
	13 - 18 years	13 – 24
Serum Folate (µg l^{-1})		3 – 17
Red Cell Folate (µg l^{-1})		100 – 600
Vitamin D (nmol/L)	Deficient	< 25
	Insufficient	25 – 50
	Adequate	> 50
Copper (µmol/L)	0 - 3 months	1.5 - 7.0
	4 - 6 months	4.0 - 17.0
	7 - 12 months	8.0 - 20.5
	1 - 5 years	12.5 - 23.5
	6 - 9 years	13.0 - 21.5
	10 - 13 years	12.5 - 19.0
	Men	10 – 22
	Women	11 – 25
Selenium (µmol/L)	0 - 2 years	0.2 - 0.9
	2 - 4 years	0.5 - 1.3
	4 - 16 years	0.7 - 1.7
	Adults	0.8 - 2.0
Zinc (µmol/L)	0 - 9 years	10 - 18
	Male > 9 years	10.7 - 18
	Female > 9 years	10 - 18
Ferritin (µgl^{-1})	0 – 4 years	12 - 90
	4 – 12 years	17 - 90
	>12 years	20 - 300

STEMDRL, Scottish Trace Element and Micronutrient Diagnostic and Research Laboratory

Appendix C.2

Table C.2 UK's NDNS centile ranges (95% CI) results for blood vitamins and trace elements

Vitamins /Trace Elements	Centiles 2.5	Boys	Girls
		11-18 years	11-18 years
Vitamin A (mmol/L)	Lower	0.87	1.00
	Upper	2.58	2.69
Vitamin B12 (pmol/L)	Lower	140	130
	Upper	539	497
Vitamin E (mmol/L)	Lower	3.6	15.8
	Upper	36.5	87.8
Vitamin D (nmol/L)	Lower	12.0	12.5
	Upper	96.5	93.3
Folate [Serum] (nmol/L)	Lower	6	6.7
	Upper	51.2	39.1
Folate [RBC] (nmol/L)	Lower	280	156
	Upper	1079	915
Selenium (mmol/l)	Lower	0.60	0.66
	Upper	1.29	1.18
Zinc (mmol/l)2	Lower	11.3	10.2
	Upper	22.4	19.4
Ferritin (mg/L)	Lower	11	5
	Upper	137	101

NDNS, National Diet and Nutrition Survey (2008-2009)