UNIVERSITÀ DEGLI STUDI DI MILANO

PhD program in NUTRITIONAL SCIENCES

XXIX cycle

PHENYLKETONURIA DIET: EFFECT ON CARDIOVASCULAR RISK FACTORS AND FECAL SHORT CHAIN FATTY ACIDS

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Academic Year
2015-2016

«Ci sono battaglie che non abbiamo scelto.
Poi c'è la vita. E io quella non smetterò mai di sceglierla.»

(Carlotta Nobile,
Il Cancro E Poi)

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Riassunto

La fenilchetonuria (PKU) è una malattia genetica, dovuta a mutazioni del gene codificante l'enzima fenilalanina idrossilasi (PAH), che catalizza l'idrossilazione della fenilalanina (Phe) in tirosina. Una ridotta attività di PAH, porta ad elevate concentrazioni di fenilalanina nel sangue, nei fluidi corporei e a concentrazioni cerebrali tossiche (Blau 2016). Nonostante ci siano continui progressi nelle terapie, la restrizione della Phe dalla dieta rimane l'elemento cardine nella gestione della PKU (Giovannini et al. 2012). Le differenti combinazioni di mutazioni di PAH portano a diversi fenotipi metabolici che vanno dall' iperfenilalaninemia lieve (MHP), nella quale restrizione dietetica non è necessaria, alla PKU lieve, moderata e classica, che richiedono il trattamento dietetico (Regier, Greene 2000). La dieta per le persone affette da PKU è composta da alimenti a basso contenuto di proteine naturali (ortaggi, frutta), prodotti speciali a basso contenuto proteico (varianti a basso contenuto proteico di alcuni alimenti come pane, pasta e biscotti) e miscele amminoacidiche Phe-free, che forniscono gli amminoacidi in opportune proporzioni (Giovannini et al. 2012).

Poiché i bambini affetti da PKU devono iniziare il trattamento dietetico pochi giorni dopo la nascita e lo devono seguire per tutta la vita, risulta importante analizzare la sicurezza a lungo termine di questo trattamento dietetico e il suo potenziale impatto sul successivo rischio di *non-communicable diseases*.

L'obiettivo generale di questa tesi di dottorato è stato quello di studiare l'impatto della dieta "PKU", in modo particolare la qualità dei carboidrati, sui fattori di rischio cardiovascolare e sulla produzione di acidi grassi a corta catena (SCFA) fecali in bambini affetti da fenilchetonuria.

I risultati principali sono stati: 1) nei bambini PKU il trattamento dietetico potrebbe essere associato ad un più elevato indice e carico glicemico giornaliero, ad un'elevata assunzione di carboidrati e fibre e ad una bassa assunzione di proteine rispetto bambini sani. Inoltre, nei bambini PKU potrebbe esserci un'associazione tra la qualità dei carboidrati consumati e il *tryglicerid glucose index*, che sembra indicare insulino-resistenza periferica. 2) Per quanto riguarda i fattori di rischio cardiovascolare, è stato osservato: uno spessore dell'intima media carotidea più sottile, minori livelli di colesterolo totale, colesterolo LDL e di omocisteina e un basso in bambini PKU rispetto ai bambini sani. Al contrario, in bambini PKU sono stati osservati elevati i livelli ematici di trigliceridi, l'*atherogenic index of plasma* e il rapporto trigliceridi/HDL rispetto ai bambini sani. Nel gruppo PKU, non sono state osservate associazioni tra lo spessore dell'intima media carotidea e l'indice glicemico e il carico glicemico giornalieri. 3) Dal confronto tra la biodiversità del microbiota intestinale e la sua produzione di SCFA in bambini

PKU con quella di bambini MHP è emerso che i bambini PKU hanno una ridotta biodiversità del microbiota intestinale e una minore produzione fecale di SCFA, sia totale che di butirrato, rispetto ai bambini MHP.

In conclusione, dovrebbe essere posta una maggiore attenzione alla qualità dei carboidrati presenti nella dieta "PKU", in modo particolare nei prodotti speciali a basso contenuto proteico. Studi longitudinali, adeguatamente progettati, sono necessari per chiarire le relazioni tra la dieta con un limitato apporto di fenilalanina e i fattori di rischio cardiovascolare/variabili metaboliche in bambini affetti da PKU. Inoltre, ulteriori studi, che utilizzano tecniche di sequenziamento innovative, sono necessari per indagare meglio disbiosi del microbiota intestinale nei bambini affetti da PKU e per poter aprire la strada ad integrazioni di pre/probiotici in questa popolazione.

Abstract

Phenylketonuria (PKU) is a genetic disorder, caused by mutations in the gene encoding phenylalanine hydroxylase (PAH). PAH catalyzes the hydroxylation of phenylalanine (Phe) to tyrosine. Loss of PAH activity results in increased concentrations of phenylalanine in the blood and fluids throughout the body and toxic concentrations in the brain (Blau 2016). Despite continuing progress in the treatment, the restriction of dietary Phe remains the mainstay of PKU management (Giovannini et al. 2012). Various combinations of PAH mutations result in a full spectrum of metabolic phenotypes ranging from mild hyperphenylalaninemia (MHP), in which dietary restriction is not necessary to mild, moderate and classical phenylketonuria, which require dietary management (Regier, Greene 2000). PKU diet is mainly made up of low-protein natural foods (vegetables, fruits), special low protein products (low-protein variants of some foods such as bread, pasta and biscuits) and Phe-free protein substitutes, which provide amino acids in suitable proportions (Giovannini et al. 2012).

Because PKU children have to start a Phe restricted diet as soon as possible and to follow this throughout life, the long-term safety of this dietary treatment and its potential impact on later non-communicable diseases risk need to be better evaluated.

The general aim of this PhD thesis was to investigate the impact of phenylketonuric diet, principally carbohydrates quality, on cardiovascular risk factors and fecal short chain fatty acids (SCFA) production in children with phenylketonuria.

The key findings were: 1) in PKU children the dietary treatment could be associated with higher overall glycemic index and load, fiber and carbohydrate intakes and lower protein intakes than healthy children. Moreover, in PKU children a relationship between the quality of carbohydrates consumed and the *triglyceride glucose index,* that seems to reflect mainly peripheral insulin resistance, may exist. 2) Regarding cardiovascular risk factors, PKU children showed a thinner intima media thickness, lower total cholesterol, LDL cholesterol and homocysteine levels than healthy children. Conversely, PKU children had higher triglyceride levels, *atherogenic index of plasma* and higher triglycerides/HDL ratio than healthy children. In PKU group, no associations of intima media thickness with overall glycemic index and glycemic load were observed. 3) Comparing gut microbiota biodiversity and its production of SCFA in PKU children and MHP children, PKU children showed a lower degree of microbial diversity in gut microbiota and minor total fecal SCFA and butyrate production than MHP children.

In conclusion, a greater attention should be paid to the management of dietary carbohydrate quality in PKU diet, with focus on special low protein products. Longitudinal, adequately-

powered studies are needed to clarify the interrelationships between the diet and cardiovascular risk factors/metabolic profile of PKU children. Moreover, further studies using innovative sequencing techniques are needed to better investigate gut microbiota dysbiosis in PKU children and to eventually pave the way for pre/probiotic supplementations in this population.

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- Regier DS, Greene CL. Phenylalanine Hydroxylase Deficiency. 2000 Jan 10 [Updated 2017 Jan 5]. In: Pagon RA, Adam MP, Ardinger HH, et al., editors. GeneReviews® [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2017.

Chapter 1: INTRODUCTION

1.1 Phenylketonuria and dietary treatment

1.1.1 Phenylketonoria: overview

Phenylketonuria (PKU; OMIM 261 600) is an autosomal recessive disorder of Phenylalanine (Phe) metabolism [1], caused by mutations of phenylalanine hydroxylase (PAH) gene, which is located on the long arm of chromosome 12 in the band region q22-q24 (Figure 1). Until now, over 950 different mutations have been identified [2,3]. With an average incidence of 1:10.000, PAH deficiency is the most common inborn error of metabolism in Europeans [4].

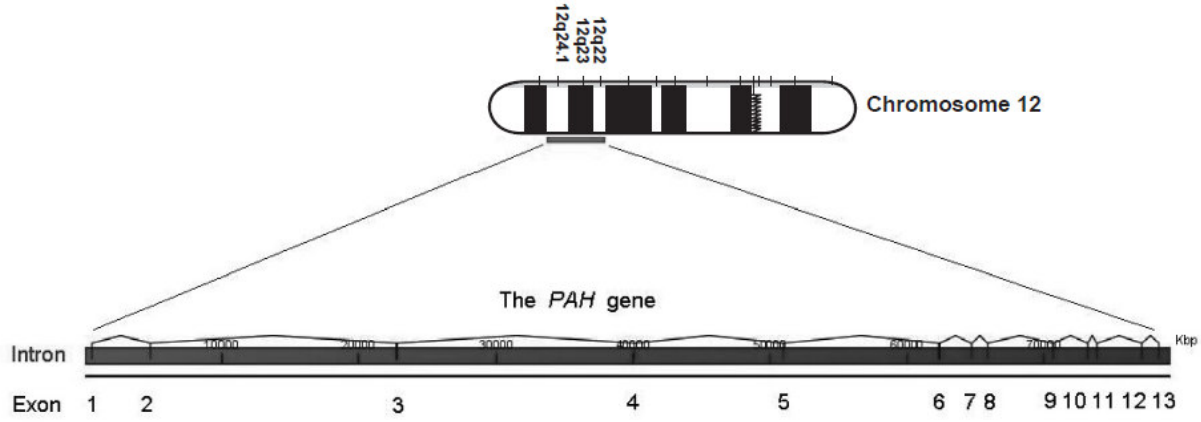

Figure 1 The basic structure of the human PAH gene. Found on the long arm of chromosome 12 (12q23.2), the human PAH gene contains 13 exons which encode a polypeptide of 452 amino acid [3].

The PAH is enzyme responsible for the majority of the catabolism of dietary phenylalanine and is located mainly in the liver. Phe is derived from dietary protein and turnover of endogenous pools. Disposal of Phe is by hydroxylation to Tyrosine (Tyr), incorporation into bound (polypeptide) pools, transamination and decarboxylation (Figure 2). At physiological levels, hydroxylation to Tyr accounts for 75%, and incorporation into protein for 25% of the total Phe disposal. PAH, with tetrahydrobiopterin (BH4), as a cofactor, plus molecular oxygen and iron, catalyzes the hydroxylation of Phe to Tyr. Loss of PAH activity, with consequently little or no conversion of phenylalanine to tyrosine, results in increased concentrations of phenylalanine in the blood and fluids throughout the body and toxic concentrations in the brain [1].

The position and nature of the mutation on PAH influence the activity of the PAH enzyme, which determines the hyperphenylalaninemia phenotype of the patient. Little or no enzyme activity results in the classic phenylketonuria phenotype. Other mutations only partly inhibit enzyme activity, giving rise to mild hyperphenylalaninemia or mild phenylketonuria [1].

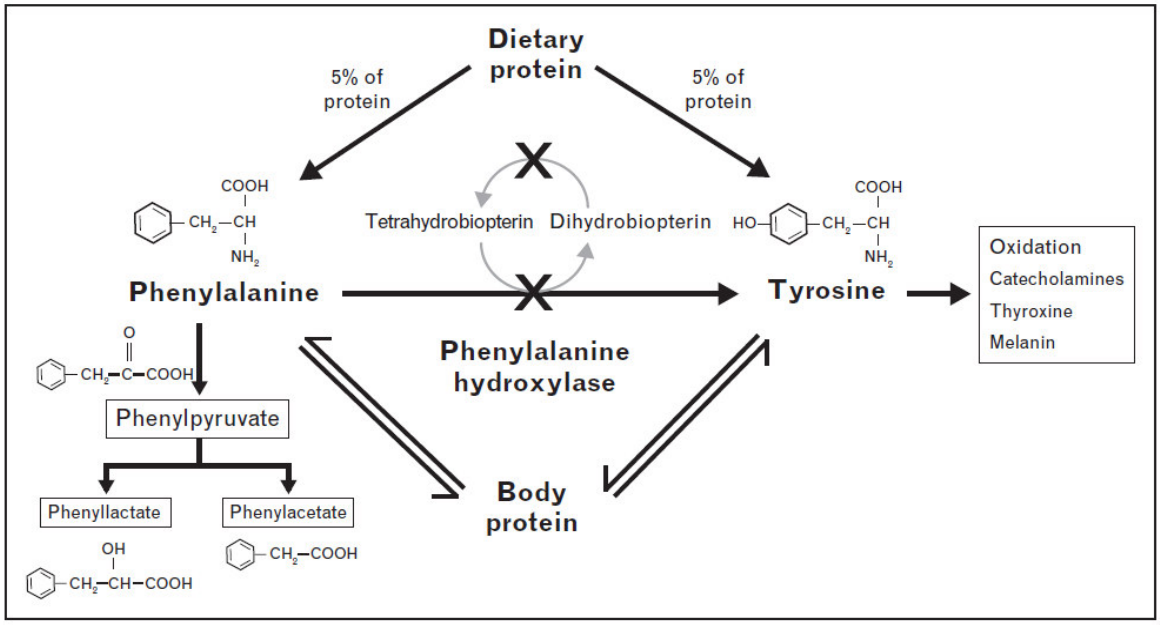

Figure 2. Phenylalanine metabolism in phenylketonuria. As indicated by the 'X', PKU results from mutations that affect the hepatic phenylalanine hydroxylase (PAH) enzyme needed for the hydroxylation of the indispensable amino acid phe to tyrosine. Due to these mutations which reduce the conversion of phe to tyrosine, phe accumulates in blood and is transaminated and decarboxylated into many compounds which appear in blood and urine; three of the compounds which are measured clinically are shown. Tyrosine, a precursor for multiple biological products, becomes an indispensable amino acid (AA) and must be provided by the diet for those with PKU. Under physiological conditions, PAH catalyzes about 75% of the phe input from the diet and protein catabolism [5].

Most severe are individuals with complete enzyme deficiency whose untreated blood Phe levels are typically >1200 µmol/L (mean normal level: 60 µmol/l); this phenotype is consistently termed "classical PKU." It should be noted that infants diagnosed and treated earlier in life might have a peak PHE level <1200 µmol/L and still have complete PAH deficiency [6].

A minority of HPA cases (1-2%) is due to mutation of one of the enzymes involved in the synthesis or recycling of PAH genes cofactor, tetrahydrobiopterin (BH4) [7,8].

Untreated outcomes and pathophysiology

Prognosis and outcome of HPA depend strongly both on the diagnosis and treatment time and on the type of mutation.

Older children with PKU, if untreated, can show microcephaly, epilepsy, a musty body odour, decreased skin and hair pigmentation, eczema, severe intellectual disability and behaviour problems as well as structural brain changes visible on magnetic resonance imaging (MRI) [9]. Therefore, the major effect of hyperphenylalaninemia in PKU patients is on the brain function and development. Although the exact pathophysiologic mechanism by which HPA causes the neurocognitive damage is still not completely dissolved, there are several hypotheses supported with evidence, which address the different possible causes of the neurotoxicity secondary to elevated blood Phe levels:

1) High concentrations of phenylalanine in the blood can inhibit large neutral amino acid carrier L-amino acid transporter 1 (LAT1), that mediates the entry of phenylalanine, tyrosine and tryptophan into the brain. Because tyrosine is a precursor of dopamine and norepinephrine, and tryptophan is a precursor of serotonin, the high concentrations of phe, obstructing their entry into the brain, could be the cause of neurotransmitter dysfunction and their availability for protein synthesis in PKU patients [8,10].
2) Imaging studies have described white matter lesions associated with reduced formation of myelin in brain white matter, although no definite causative link between dysmyelination and neuropsychological impairment has been established [11,12].
3) Other possible mechanisms for hyperphenylalaninemia induced damage to the brain include reduced activity of pyruvate kinase, disturbed glutamatergic neurotransmission, reduced activity of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase, and the function of monoamine oxidase B as a modifying gene [13].

Patients with non-phenylketonuric hyperphenylalaninemia have a lower risk of neuropsychological dysfunction than do those with phenylketonuria, although compared with healthy controls, some might have decreased executive functioning. Adequate control of blood phenylalanine is effective in prevention of most of the central nervous system (CNS) deficits associated with phenylketonuria [1].

Diagnosis

Affected newborns show no clinical signs of hyperphenylalaninemia, so until the introduction of the newborn screening, most children born with phenylketonuria became profoundly mentally disabled. Since the '60s, the newborn screening was gradually introduced in several countries, starting from the USA and Europe. In Italy, neonatal screening has been required by law since 5 February1992, but in the Lombardy region has been undertaken since 1 January 1977 [14].

Newborn screening is based on the detection of hyperphenylalaninemia in blood spots, as Phe is the marker for PAH deficiency. Phe levels in blood spots can be measured by several possible methods, but currently tandem mass spectrometry is the routine method of analysis [4].
In most cases, after the routine newborn screening test (typically performed 2-7 days after birth, using samples drawn by neonatal heel prick), a repeat test is done at approximately two weeks of age to verify the initial test and uncover any phenylketonuria that was initially missed.
Every infant identified in newborn screening with hyperphenylalaninemia should be assessed at a metabolic centre as early as possible. If the baby has hyperphenylalaninemia, blood amino acid analysis will reveal increased phenylalanine concentrations (>120 μmol/L), normal or reduced tyrosine concentrations (phenylalanine to tyrosine ratio >2), and normal concentrations of the remaining amino acids [1]. This is the pattern of all forms of hyperphenylalaninemia/ phenylketonuria. In these patients two differential diagnostic assessments should be done. First, whether or not the patient has a defect in BH4 synthesis or recycling should be clarified. If they do not have such defects, whether the patient with hyperphenylalaninemia/ phenylketonuria can be treated with diet restrictions only, or can at least be helped in part with BH4 alone or together with a restricted diet should be assessed [1].
Early diagnosis and prompt intervention have undoubtedly allowed most individuals with phenylketonuria show normal overall development, attain expected educational standards, lead independent lives as adults, form normal relationships, and obtain employment [15,16].

Phenotypes

Depending on the enzyme defect, the genotype and the severity of the disease, different forms of PKU with different clinical phenotypes have been described. Thus, different classifications for PKU phenotypes have been established. PKU may be classified as classic PKU and as variant PKU which includes all milder forms of PKU (moderate PKU and mild PKU), as mild HPA or non-PKU HPA, and additionally, as BH4-responsive PKU. Definition of PKU phenotypes may be essential in establishing treatment options, foe example (e.g.) new therapeutic strategies, in counseling and in prediction of the outcome, and in pregnancy. Pre-treatment blood Phe levels, the individual Phe tolerance may help to discriminate the different phenotypes of PKU [17]. Concerning “mild HPA-gray zone” there is disagreement about the need to treat and there are conflicting data on the effects on cognition and executive function, while there is general consensus regarding “mild HPA-NT” that no treatment is required [16].

Table 1. Phenotypes of PAH deficiency based on blood Phe levels and treatment indication (Adapted from [16]).

PAH deficiency	Pretreatment blood Phe level
Requiring treatment	
Classical PKU	>1200 μmol/L
Moderate PKU	900–1200 μmol/L
Mild PKU	600–900 μmol/L
Mild HPA-gray zone	360–600 μmol/L
Not requiring treatment	
Mild HPA- NT	120–360 μmol/L

The goal of the treatment is to maintain plasma concentrations of Phe within an acceptable range. The acceptable, or target, range for Phe levels varies throughout the world and across ages [1].

In Italy, the target blood Phe concentration recommended for children under twelve is 120-360 μmol/L, and for those over twelve it is 120-600 μmol/L. For individuals who are on a low Phe diet, it is important to monitor their Phe levels to make sure they are remaining within their target zones. The monitoring of blood Phe levels is typically done through blood spot analysis. During infancy adjustments to an individual's diet may need to be assessed weekly, whereas later, assessments will not have to occur as close together.

1.1.2 Phenylketonuria diet

There is general consensus that the current standard of care and primary treatment for PKU is dietary and that this treatment should be continued throughout an individual's lifetime to prevent adverse clinical outcomes and cerebral MRI changes, and to promote normal cognitive development [16].

Adherence to the diet is usually straightforward in the neonatal period and during early childhood, as the child's parents control the diet. However, dietary compliance becomes increasingly difficult as children approach adolescence due to palatability of the diet. This is clearly reflected by poor control of blood phenylalanine concentrations in a proportion of individuals in this age group [18]. Dietary restriction of phenylalanine was introduced in 1953

by Bickel and colleagues [18].

The restriction of dietary Phe usually begins immediately after confirmation of this condition in a newborn. During infancy breastfeeding or human milk meals, to provide natural protein according to the individual Phe tolerance, and/or a Phe-free infant formula are recommended. With the introduction of solid foods, PKU subjects have to avoid foods rich in protein (meat, fish, eggs, standard bread, dairy products, nuts and seeds); only in some cases, according to the individual Phe tolerance, minimal amounts of animal products (usually milk) are allowed. Therefore, PKU diet is mainly made up by low-protein natural foods (vegetables, fruits and some cereals), to reach the Phe supply necessary for growing processes. For these reasons, PKU diet tends to contain high carbohydrate, low saturated and polyunsaturated fat, cholesterol, iron, zinc, selenium and some vitamins (in particular B12) intake, because of the very low assumption of Phe-containing animal foods [15].

Phenylalanine exchange systems

There is no standardized method on dietary management of PKU patients.

A study showed that important differences among centres across Europe in the dietary management of PKU and in support systems designed to assist patients in managing their diets, exist [19].

In some centers patients are instructed with the Phe content of foods, whereas centres, such as in Milan, Italy, in Denmark and United Kingdom, lists of food-Phe equivalence in varying milligram exchange amounts (10–50 mg Phe exchanges) are released. The idea is that one exchange of Phe can easily be swapped for another exchange of equivalent value [19]. In some centers (e.g. Norway), patients choose between adherence to a total daily amount of Phe or use of an exchange system [19].

Some centers (e.g. in Milan, Italy) are opposed to the incorporation of most higher protein foods to make up Phe allowance because of the potential for patients to become accustomed to the taste, which could lead to a desire for more of it and the consumption of more than the required amount, which would thereby reduce compliance [19].

The dietary schedules of phenylalanine exchange system are different among PKU centres. These schedules are food lists in which is present an exact quantity (e.g. 10 or 20 mg) of Phe for a given weight of food. In the Table 2 are shown examples of the equivalents from vegetables and fruits (20 mg of Phe) and the equivalent from grains and milk (10 mg of Phe) used at San Paolo Hospital, Milan, Italy. In the list of equivalents from grains and milk are present all low-protein products commercialized. In figure 3 is shown an example of diet prescribed to PKU

children.

Table 2. Example of Phenylalanine exchange systems.

EQUIVALENT FROM VEGETABLES AND FRUIT (1 Equiv. = 20 mg of Phe)		**EQUIVALENT FROM GRAINS AND MILK (1 Equiv. = 10 mg of Phe)**	
Vegetables	**g**	**Grains**	**g**
Basil	12	Pasta (Aproten)	33
Chard	30	Pasta (Harifen)	100
Broccoli	15	Pasta (Taranis)	80
Artichokes	15	Lp-Pasta (Milupa)	100
Carrots	65	Bread “Pane biscottato” (Aproten)	20
Cauliflower	25	Bread “Ciabatta” (Aprotide)	65
Cucumber	140	Chocolate Wafer	70
Green bean	25	Cracker Loprofin	100
Fennel	25	Cake Mix (Loprofin)	60
Lettuce	35	Kidbar (Sineamin)	100
Potato	20	Melba Toast (Aproten)	15
Tomato	80	Lp-flakes with honey	200
Spinach	20	Lp-Ringlets	25
Fruit		Rice	2,5
Apple	220	**Milk**	
Kiwi	40	Milco	100
Grape	60	Whole milk	6
Pear	85	Whole yogurt	5
Strawberry	80	Fruit yogurt	4,5

DIETA PER: ….....

ETA' : 11 anni

PESO: 46.4 Kg

Alimenti	**quantità**	**Sostituzioni**
<u>Colazione</u>		
Latte Milco	250 g	2 e ½ equiv. di cereali
Biscotti Aproten	30 g	2 equiv. di cereali
Zucchero	20 g	oppure 20 gr di miele o
marmellata		
X-PHE Junior	35 g	
<u>Spuntino</u>		
Crostini Mevalia	3 pz (30g)	1 equiv. di cereali
Pranzo		
Pasta ipoproteica	80 g	1 equiv. di cereali
Pomodori pelati	60 g	1 equiv. di verdura
Zucchine	75 g	3 equiv di verdure
Carote	65 g	1 equiv. di verdure
Olio ex. v. di oliva	20 g	
Pesca	55 g	½ equiv. di frutta
X-PHE Junior	20 g	
<u>Merenda</u>		
Mela	220 g	1 equiv. di frutta
Pane a fette Mevalia	3 fette (60g)	1 equiv. di cereali
<u>Cena</u>		
Pasta ipoproteica	80 g	1 equiv. di cereali
Pomodori pelati	60 g	1 equiv. di verdura
Zucchine	75 g	3 equiv di verdure
Patate	20 g	1 equiv. di verdure
Olio di oliva	20 g	
X-PHE Junior	20 g	

Figure 3. Example of PKU diet (in Italian).

Phenylalanine requirements in PKU

In PKU, the individual dietary phenylalanine tolerance is pragmatically determined and is influenced by many factors, such as growth rate, age, gender, compliance, target blood phenylalanine concentration. It is defined as the amount of phenylalanine per kg/body weight that is tolerated to achieve plasma phenylalanine concentrations within a target range. In PKU, generally phenylalanine tolerance/requirements are highest in early infancy (ranging from 55 mg/kg/day at 0–3 months of age to 27 mg/kg/day at 12 months) [20]. After the age of 1 year, there is a slow and steady decline in tolerance per kg/body weight, and even from the early days of treating PKU with diet it has been recognized that children with classic PKU, on dietary treatment usually only tolerate between 200 and 500 mg/day (0.2–0.5 g daily). Patients with a milder form of PKU (untreated blood phenylalanine concentrations less than 1000 μmol/l), usually tolerate in excess of 500 mg/day dietary phenylalanine [21].
Moreover, it is important to highlight that in PKU also tyrosine is an indispensable amino acid, because it is not supplied endogenously via phenylalanine hydroxylation or only to a very limited degree [21].

Micronutrient status and long-chain polyunsaturated fatty acids in PKU

No tailored micronutrient dietary reference values have been established for PKU, so the optimal intake of micronutrients on a low phenylalanine diet is unknown, despite the fact that the majority of nutrient sources are chemically derived (European Commission/Scientific Committee on Food 1999) [22]. However, clinical studies indicate that any deficiency or nutrient imbalance is mainly (but not entirely) due to inadequate dietary intake, and there is little to suggest that dietary reference values should vary from the healthy population. Therefore, normal population dietary reference values for micronutrients are used as a guideline [23]. However Individual polymorphisms and genotype may be important in determining individual nutrient requirement [24,25].

- Vitamins

An absence of meat or fish in the diet without supplementation increases the risk of Vitamin B12 deficiency, because plant do not not synthesize this vitamin. Therefore, vitamin B12 deficiency is mainly reported in adolescents or adult patients who have stopped or relaxed their diets and who are less adherent with phe-free L-amino acid supplements [26]. Symptoms such as spastic paraparesis, tremor, and slurred speech are associated with deficiency but patients may be

unaware of the early manifestations of vitamin B12 deficiency as they appear insidiously.
In PKU, fractures and reduced bone mineral density have been commonly reported in all age groups, but it is unclear if this is due to inadequate mineral, vitamin D, or natural protein intake (either due to low intake or poor adherence with phenylalanine-free protein substitute), lack of non-essential amino acids such as proline which are abundant in collagen, limited exercise, poor blood phenylalanine control or is a consequence of the disorder itself [26].

- Minerals

The trace mineral status has been widely studied in PKU, but particularly zinc and selenium.
Concerning zinc status in PKU, there have been conflicting reports. Assessment and interpretation of marginal biological zinc deficiency is difficult. There are no reports of clinical symptoms of zinc deficiency in PKU but low plasma zinc is still described, despite generous zinc supplementation in to phe-free protein substitutes.
Regarding selenium status, in the early years of PKU treatment, biochemical selenium deficiency was common in PKU. The selenium is found predominantly as the amino acid selenomethionine (cereal products) and selenocysteine (animal products) and is rich in foods such as Brazil nuts, cereals, offal, fish and eggs but low in fruits and vegetables. For this reason, the selenium content of a non-supplemented low phenylalanine diet is likely to be low. The majority of selenium in a low phenylalanine diet is sourced from added selenium to phe-free protein substitutes [26].

- Long-chain polyunsaturated fatty acids

Long-chain polyunsaturated fatty acids (LCPUFA) with 20 and 22 carbon atoms are metabolically derived from a-linolenic acid (ALA) and linoleic acid (LA) by consecutive enzymatic desaturation and chain elongation. Although LCPUFA including DHA (22:6n-3) and AA (20:4n-6) can be synthesized from ALA and LA in mammals, respectively, the activity of conversion is low in humans [15].
PKU children who are compliant with the recommended low protein diet are devoid of natural dietary sources of n-3 LCPUFA, as LCPUFA rich foods also contain high amounts of protein. Accordingly, blood concentrations of n-3 LCPUFA and especially of DHA are reduced in plasma and red blood cell phospholipids of PKU children relative to omnivorous children with a more marked relative depletion of DHA than arachidonic acid, the major n-6 LCPUFA, throughout childhood [15,25,27]. Therefore, given the functional effects, DHA is conditionally essential substrates that should be supplied with PKU diet [15].

Phe free protein substitutes and special low protein foods

The required amount of daily proteins is obtained from phe-free protein substitutes providing essential amino acids and some micronutrients in suitable proportions [15]. Moreover, low-protein variants of some foods are commercially present, such as low-protein bread and low-protein pasta (special low protein foods, SLPF). It would be difficult to achieve acceptable blood Phe control without them because they provide an important source of low-phe kilocalories in the diet [19].

Phe free protein substitutes

In PKU, the majority of non-phenylalanine protein is supplied by phe-free amino acids. The number of protein substitutes available for PKU patients is increasing constantly with time. The absence of Phe is the one constant in these mixtures; but there is variability in the presence or absence of lipids, vitamins and minerals with differences also in the amount [15]. Amino acid patterns of phe-free amino acids designed for infants, children and adults are primarily based on that of human milk [21]. The phe-free protein substitutes for infants meets infants' requirements; most of the different protein substitutes contain DHA and AA and some formulations are supplemented with prebiotics. Many products are available with different composition and presentation for children, adolescents and adults; this is a great advantage for patients as they can choose the product most likely to meet their needs [15].
The metabolic and dietary handbooks advise to divide the daily intake of phe-free amino acids into at least three equal parts, and to combine the intake of natural protein with the amino acid supplement [21].

The major progresses in protein substitutes are:

- Palatability and caloric content: better taste of amino acid powder/lower calories (lipid and carbohydrate composition of preparations have been improved);
- Compliance (all ages): better compliance due to different formulations (powder, tablets, creams etc)
- Age-related problems with diet (childbearing age, adolescence, adult age): different age-related formulas and composition individualized dietary treatment. Improvements in the palatability, presentation, convenience and nutritional composition of substitutes have helped to improve long term compliance with PKU diet. Although it can be expected for further improvement in this area, PKU patients can choose the product most likely to

meet their needs [15,16]. Studies in healthy adults, investigating the bioavailability of amino acids mixtures compared to natural protein, demonstrate differences in the rate of absorption in the gut. Amino acids delivered as dietary protein (casein) may support whole-body protein metabolism better than ingestion of crystalline [21].

Recently, other than these progresses, a new low phenylalanine whey based protein substitute has been developed for PKU, called glycomacroprotein (GMP). It is derived from cheese whey (naturally low in phenylalanine) and it is supplemented with valine, isoleucine and threonine. Studies suggest that PKU patients find foods containing GMP more palatable than their usual amino acid formula, preferring a diet supplemented with GMP. The potential benefits of having GMP in the PKU diet have been explored and data showed that the GMP diet significantly reduced ureagenesis, improved protein retention and Phe utilization. Another study found that consuming the GMP diet for breakfast promoted satiety as reflected by decreased levels of the postprandial ghrelin concentration (associated with greater feelings of fullness) when compared to an amino acid diet [21]. However, further studies are needed to evaluate the safety and efficacy of GMP consumption for long term [18].

Studies in healthy adults, investigating the bioavailability of amino acids mixtures compared to natural protein, demonstrate differences in the rate of absorption in the gut. Amino acids delivered as dietary protein (casein) may support whole-body protein metabolism better than ingestion of crystalline L-amino acids, casein hydrolysates or soy protein [21].

Special low protein foods (SLPF)

The function of special low protein foods in the PKU diet is to provide energy and variety in the diet. It is estimated that the patient's SLPF intake will depend on disorder severity, providing around 50% of the total energy intake in the most severe forms of the disorder. Milder phenotypes, with a higher natural protein tolerance are likely to be less dependent on their use [29].

SLPF have a low protein and Phe content but their micronutrient composition is unclear. In a normal diet, healthy energy food sources commonly provide other macro and micronutrients. In direct contrast, SLPF micronutrient fortification is uncommon and it is assumed that the majority of vitamins and minerals are provided from L-amino acids supplemented with micronutrients [30,31].

SLPF are regulated by the European legislation 'Foods for Special Medical Purposes' (Commission Directive 1999/21/EC of 25 March 1999; amended in Directive 2006/141/EC).

This Directive sets out rules for the composition and labeling of foods that are specifically formulated, processed and intended for the dietary management of diseases, disorders or medical conditions of individuals who are being treated under medical supervision. The nutritional substances that may be used in the manufacture of foods for special medical purposes are also outlined in legislation: Commission Regulation (EC) No 953/2009 [32]. In addition, all Foods for Special Medical Purposes have to follow the European Food Information to Consumers Regulation No 1169/2011 and Regulation No 609/2013 which is only just being enforced in many countries. Manufacturers must provide information concerning the energy value and principal nutrients contained in such foods but only have to declare the vitamins and/or minerals if they are present in "significant amounts". Seems that in Italy there is the major number of SPLF (n = 256) compared with other countries [33].

A recent study [33] compared Portuguese special low protein foods with corresponding regular Portuguese foods: in 75 % of the SLPF subgroups the energy content was higher than in regular foods. Moreover, Portuguese SPLF were found higher in lipid (in 58% of SLPF subgroups) and carbohydrates (in 92% of SLPF subgroups) content. Another common feature of SLPF is lack of label micronutrient information when compared with regular matched-foods. Although micronutrients are mainly consumed through L-amino acid supplements [23,26]. SLPF should contain a warning indicating that their nutritional profile does not replicate regular foods because patients, caregivers and health professionals may assume they provide other nutrients other than energy. At present, there are no detailed studies outlining their full nutritional contribution to a low Phe diet [33].

In table 3 is shown a comparison between special low protein foods available in Italy with the corresponding regular Italian foods. The energy content of low protein foods seemed higher in 2 ("cookies" and "breakfast cereals") of the 5 food groups analyzed. Low protein breakfast cereals seemed to have a higher soluble sugar intake than regular ones (32.5g vs. 10.4g), and low protein breads seemed to have higher fat contents than "regular bread". Fiber content of low protein foods was higher in 2 ("bread" and "melba toast") of the 5 food groups analyzed.

Table 3. Energy and nutrient content of low protein foods compared with protein containing equivalent foods available in Italy. Values express for 100 g of product.

	ENERGY (kcal)		PROTEIN (g)		CARBOHYDRATES (g)		SOLUBLE SUGAR (g)		FAT (g)		FIBER (g)	
	Regular foods	Low-protein	Regular foods	Low-protein	Regular foods	Low-protein	Regular foods	Low-protein	Regular foods	Low-protein	Regular foods	Low-protein
Pasta	353	356.5	10.9	0.6	79.1	85.2	4.2	1.0	1.4	1.0	2.7	3.4
Bread	289	248.3	8.6	0.7	66.9	45.4	1.9	1.9	0.4	5.2	3.2	8.0
Cookies	429	473.5	7.2	1.1	73.7	77.7	22	20.8	13.8	17.5	1.9	1.2
Melba Toast	408	393.7	11.3	1.2	82.3	78.0	2.2	3.8	6	7.1	3.5	8.8
Breakfast cereals	361	380.7	6.6	0.5	87.4	91.8	10.4	32.5	0.8	1.3	3.8	2.3

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1.2 Carbohydrate quality

As previously written, PKU diet is characterized by a high carbohydrate intake. For this reason, this subchapter deals with this macronutrient and its qualities.

1.2.1 Definition and category of carbohydrates

Chemically, carbohydrates include a range of components such as polyhydroxy aldehydes, ketones, alcohols and acids, as well as their derivatives and polymers, e.g. starch and other polysaccharides. The chemical classification of carbohydrates is usually based on molecular dimensions and monomeric composition, three principal groups being sugars (1–2 monomers), oligosaccharides (3–9 monomers) and polysaccharides (10 or more monomers) [1].

From nutritional point of view, it is important to differentiate between two broad categories of carbohydrates: those digested and absorbed in the human small intestine, providing carbohydrates to body cells and those passing to the large intestine, forming substrate for the colonic microflora [2]. A FAO/WHO Expert Consultation on Carbohydrates in Human Nutrition [1] recommended the introduction of the concept ″glycemic carbohydrate″, meaning providing carbohydrate for metabolism, which corresponds to the previously used term ″available carbohydrates″ and to ″carbohydrates″ according to the European legislation. The nondigestible (unavailable) carbohydrates are commonly referred to as ″dietary fiber″ [1,3].

Table 4. The main types of dietary carbohydrates [3].

Class (DP[a])	***Subgroup***	***Principal components***
Sugars (1-2)	Monosaccharides Disaccharides Polyols (sugar alcohols)	Glucose, fructose, galactose Sucrose, lactose, maltose, trehalose Sorbitol, mannitol, lactitol, xylitol, erythritol, isomalt, maltitol
Oligosaccharides (3-9) / short-chain carbohydrates	Malto-oligosaccharides (α-glucans) Non- α-glucan oligosaccharides	Maltodextrins Raffinose, stachyose, fructo and galacto oligosaccharides, polydextrose, inulin
Polysaccharides (≥10)	Starch (α-glucans) Non-starch polysaccharides (NSPs)	Amylose, amylopectin, modified starches Cellulose, hemicellulose, pectin, arabinoxylans, β-glucan, glucomannans, plant gums and mucilages, hydrocolloids

[a]Degree of polymerization or number of monomeric (single sugar) units.

1.2.2 Glycemic carbohydrates: metabolism

The glycemic carbohydrates provide carbohydrate to body cells, mainly in the form of glucose. The main glycemic carbohydrates are: glucose and fructose (monosaccharides), sucrose and lactose (disaccharides), malto-oligosaccharides, starch (polysaccharide). In general, only monosaccharides are absorbed in the small intestine. The enzymatic degradation of starch begins by the action of salivary amylase and is continued in the small intestine by pancreatic amylase. The degradation products – mainly maltose and oligosaccharides – are hydrolysed further to glucose by a set of enzymes "disaccharidases", bound to the brush border membrane of the enterocytes. The same enzymes hydrolyse the dietary disaccharides. Glucose and galactose are absorbed efficiently by a secondary active carrier coupled with sodium (sodium glucose transporter 1, SGLT1), whereas fructose is absorbed by facilitated diffusion that does not involve sodium co-transport (GLUT5). The absorption of monosaccharides is regarded as the rate-limiting step. Absorbed monosaccharides are transported to the liver and then to the systemic circulation. The cellular uptake is mediated by a number of glucose transporters (GLUT1–4), variously expressed in different tissues. Insulin is a key hormone for the uptake and metabolism of glucose. The plasma insulin concentration increases immediately after ingestion of glycemic carbohydrates. Unlike glucose, fructose enters body cells without the need for insulin. The metabolism of fructose, therefore, favours lipogenesis more than glucose. In liver cells, fructose is phosphorylated to fructose-1-phosphate that can be converted to fatty acids, providing a route for lipogenesis in addition to that shared with glucose (via glucose/fructose-6-phosphate). Both fructose and galactose, the latter arising from hydrolysis of lactose, are also transformed to glucose mainly in the liver [1].

1.2.3 Glycemic index (GI) and glycemic load (GL)

The concept of glycemic index (GI) was introduced by Jenkins and co-workers in 1981, in order to rank foods in a standardized way according to their effects on blood glucose levels after a meal [4]. The FAO/WHO Expert Consultation defined GI as the incremental area under the blood glucose response curve during 1.5–3 hours after intake of a 50 g carbohydrate portion of a test food (usually glucose or white bread), and expressed as a percentage of the response to the same amount of carbohydrate from a standard food taken by the same subject [5].

Foods having carbohydrate that is digested, absorbed and metabolized quickly are considered high GI foods (GI ≥ 70 on the glucose scale) whereas those that are digested, absorbed and

metabolized slowly are considered low GI foods (GI ≤ 55 on the glucose scale) [6].

Glucose or white bread is used as standard. GI values obtained with the white bread standard are typically about 40% higher than those obtained with the glucose standard which is the generally preferred standard. GI values for about 750 foods have been published [7] and updated with additional data to contain 2480 individual food items [8]. Recently, in Italy, Scazzina et al. published GI values of 141 Italian commercial foods [9]. Whereas it was previously assumed that sugars are rapidly absorbed and therefore have a higher GI than polysaccharides (e.g. starch), which are slowly absorbed, a number of food-related factors have been identified to determine the GI: fructose has a low GI (30 with the white bread reference as 100) and sucrose an intermediate GI (lower than white bread) [10]. Starchy foods, on the other hand, can have low, intermediate or high GI, depending on their composition (amylose/amylopectin ratio) and physical/chemical state. The swelling and dissolution of starch at wet heat treatment, known as gelatinization, is particularly important in making starch more readily accessible to digestive enzymes. Physical barriers such as in intact cereal grains, cellular structures in leguminous seeds, parboiled rice and whole fruits, and the protein network in pasta products, are food-related factors lowering the GI. Organic acids (acetic, propionic and lactic acid) decrease the glycemic response to foods or meals, mainly due to inhibition of gastric emptying [11]. Viscous, soluble types of dietary fiber may also delay gastric emptying, in addition to their inhibitory effect on diffusion and transport in the small intestine [12].

In practice, the blood glucose response after a meal is influenced by both the GI and the amount of carbohydrate in a portion of a food. Consequently, the glycemic load (GL) concept was introduced in 1997 to quantify the glycemic effect of a portion of food [13]. GL is defined as the mathematical product of the grams of available carbohydrate in the food portion and the food's GI, divided by 100, and the sum of singular GL values for foods and meals has been used to estimate the glycemic load of the whole diet.

Studies have shown that the glycemic response to a meal can be predicted from properly determined GI of the constituent foods. However, the glycemic response can also be influenced by the protein and fat content, and by the type and amount of beverage taken with the food [14].

1.2.4 Factors influencing the blood glucose responses of foods

Factors affecting the rate of glucose absorption from starchy food and therefore the GI value include: 1) the nature of the food and 2) the type and extent of food processing (Table 5). The former includes the ratio of amylose to amylopectin present in the raw food and the type of

monosaccharide components, the amount and type of dietary fiber, the presence of large amounts of fat or protein, antinutrients such as phytic acid, lectins and tannins and nutrient – starch interactions in carbohydrate-containing foods, such as in wheat products. Extrusion, flaking, grinding, canning, storing and cooking of the carbohydrate-containing foods can affect the particle size and the integrity of the starch granules and plant cell walls, making the carbohydrate portion more accessible to digestive enzymes. Fat and protein may modify the glycemic response to a carbohydrate food by slowing gastric emptying and increasing insulin secretion, respectively [15].

Table 5. Factors that influence the glycemic response and the glycemic index.

Factors that affect GI	***Factors that decrease GI***	***Factors that increase GI***
Nature of starch	↑ Amylose/amylopectin	↓ Amylose/ amylopectin
Nature of monosaccharide components	Fructose Galactose	Glucose
Viscus fiber	↑ Guar ↑ β-glucan	↓ Guar ↓ β-glucan
Cooking/food processing	Parboiling Cold extrusion	Extruding Flaking Popping
Particle size	Large particles	Grinding (small particles)
Ripeness and food storage	Unripeness Cooling	Ripeness
α-Amylase inhibitors	↑ Lectins ↑ Phytates	↓ Lectins ↓ Phytates
Nutrient starch interactions	↑ Protein ↑ Fat	↓ Protein ↓ Fat

↑=high levels.
↓=low levels.

1.2.5 GI/GL during childhood and its relevance for metabolic outcomes

Growing evidence underscores the important role of glycemic control in health and recovery from illness [16]. Carbohydrate quality and digestibility can influence post-prandial plasma glucose concentration and the inflammatory response, which is now known to underlie the development of insulin resistance, metabolic syndrome, and type 2 diabetes mellitus (T2DM). Foods with high GI and GL are associated with increased risk of such diseases [16]. Conversely, lowering dietary GI and GL can improve metabolic control [16].

Recent use of meta-analytical methods has been illuminating in understanding the role of GI/GL in the development and management of some diseases, such as diabetes mellitus type 2 and cardiovascular diseases [6]. To date meta-analyses indicate, as for T2DM, a stronger beneficial

relation between CHD and lower GI/GL in non-diabetic women more than in men among prospective cohort studies [17-19]. Reasons for difference between men and women in respect of both incident T2DM and incident CHD remain to be elucidated [6].

Evidence on the relevance of GI/GL for risk markers of Diabetes Mellitus type 2 and CVD in children and adolescents is still emerging. Smaller intervention studies suggest some benefits of low GI/GL diets specifically for insulin resistance [20-22]. However, in a recent 2-year intervention on 113 obese hispanic children the examined diets did not differ in their effect on changes in insulin resistance or markers of the metabolic syndrome [23]. Similarly, in a sub-sample of 253 children and adolescents participating in the DiOGenes study, dietary GI did not affect cardiovascular risk markers [24]. Conversely, prospective cohort studies suggest long-term adverse health consequences of a habitually higher GI or GL during adolescence. In an Australian adolescent cohort, increases in dietary GI and GL between age 12 and 17 years were related to substantial concurrent increases in systolic blood pressure among 278 girls [25]. Consideration of the GI in the diet of children and adolescents is of long-term relevance, since nutritional behaviors are shaped during childhood and adolescence. Of note, analysis of dietary GI in a representative sample of Australian children and adolescents revealed that a preferred selection of carbohydrates from low GI sources may indeed confer benefits for overall nutrient adequacy [26]. By contrast, adherence to the current recommendations to increase whole grain consumption and/or reduce intake of sugary foods cannot be expected to translate into a lower dietary GI/GL. Associations of dietary GI to dietary fiber are neither strong nor uniform across pediatric populations [27,28].

To confirm this, in the DONALD cohort, 76% of the whole grains consumed by healthy adolescents came from sources with higher dietary GI (GI ≥55) [29] which reflect the fact that many whole grain products have a relatively high GI [8].

Also, contrary to the popular belief a higher dietary sugar intake is not related to a higher dietary GI because all common sugars, except glucose, are of moderate (sucrose) or low GI (fructose and lactose) [8,30].

Since dietary GI is not closely related to dietary fiber or dietary sugar intake it needs to be addressed as a separate entity in nutritional recommendations given to children and adolescents. Efforts to reduce the dietary GI and GL in children and adolescents should best be targeted to energy-dense starchy food, since these make a considerable contribution to total dietary GL in children and adolescents [28].

In conclusion, a recent International Scientific Consensus Summit from the International Carbohydrate Quality Consortium (ICQC) [6], showed that there is convincing evidence that

diets low in GI reduce the risk of type 2 diabetes, improve glycemic control in people with type 2 and type 1 diabetes and reduce the risk of coronary heart disease, meanwhile probable evidence has emerged for low GI/GL diets in reducing total body fat mass and in weight management. Therefore, the consensus recommended that foods low in GI and GL should be considered in the context of a healthy diet [6].

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Nutrition, Metabolism & Cardiovascular Diseases (2016) **26**, 171–177

Available online at www.sciencedirect.com

Nutrition, Metabolism & Cardiovascular Diseases

journal homepage: www.elsevier.com/locate/nmcd

REVIEW

Diet in children with phenylketonuria and risk of cardiovascular disease: A narrative overview

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Received 19 May 2015; received in revised form 18 September 2015; accepted 11 October 2015
Available online 3 November 2015

KEYWORDS
Phenylketonuria;
PKU diet;
Cardiovascular risk;
Nutrition;
Obesity;
Lipid profile

Abstract *Aims:* The aim of this paper is to review the possible relationship of restricted phenylalanine (Phe) diet, a diet primarily comprising low-protein foods and Phe-free protein substitutes, with major cardiovascular risk factors (overweight/obesity, blood lipid profile, plasma levels of homocysteine, adiponectin and free asymmetric dimethylarginine (ADMA), oxidative stress and blood pressure) in PKU children.
Data synthesis: In PKU children compliant with diet, blood total cholesterol, low-density lipoprotein cholesterol (LDL-C), plasma ADMA levels and diastolic pressure were reported to be lower and plasma adiponectin levels to be higher compared to healthy controls. No difference was observed in overweight prevalence and in high-density lipoprotein cholesterol (HDL-C) levels. Inconsistent results were found for plasma homocysteine levels and antioxidant status.
Conclusions: PKU children compliant with diet seem to display non-different cardiovascular risks compared with the healthy population. Well-designed longitudinal studies are required to clarify the potential underlying mechanisms associated with PKU and cardiovascular risk factors.

Introduction

Phenylketonuria (PKU; OMIM 261 600) is an autosomal recessive disorder of phenylalanine (Phe) metabolism [1], primarily due to mutations in Phe hydroxylase (PAH) gene, which facilitates conversion of the essential amino acid Phe to tyrosine. Loss of PAH activity results in increased Phe concentrations in the blood (hyperphenylalaninaemia, HPA) and therefore in toxic concentrations in the brain. Various combinations of mutations [2] result in a full spectrum of metabolic phenotypes ranging from severe, moderate and mild PKU (blood Phe concentration >360 μmol/L), which require dietary management, to mild HPA (blood Phe concentration: 120–360 μmol/L), wherein dietary restriction is not necessary [3]. A minority of HPA

Abbreviations: ADMA, asymmetric dimethylarginine; BH4, tetrahydrobiopterin; BMI, body mass index; CVD, cardiovascular disease; HDL-C, high-density lipoprotein cholesterol; HPA, hyperphenylalaninaemia; LDL-C, low-density lipoprotein cholesterol; NO, nitric oxide; PAH, phenylalanine hydroxylase; Phe, phenylalanine; PKU, phenylketonuria.

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http://dx.doi.org/10.1016/j.numecd.2015.10.005
0939-4753/

cases (1–2%) are due to mutation of one of the enzymes involved in the synthesis or recycling of PAH gene cofactor, tetrahydrobiopterin (BH4) [4,5].

In developed countries, PKU subjects are identified at birth through newborn screening programmes and classified by their clinical phenotype. The minimum pretreatment Phe levels, requiring a Phe-restricted diet, vary among different countries [6]. Differential diagnosis between the two major forms of HPA (PAH or BH4 deficiency) is performed by BH4 loading test, analysis of urinary pterins, determination of dihydropteridine reductase activity in blood, analysis of pterins, folates and neurotransmitter metabolites in cerebrospinal fluid and enzyme activity measurement.

High blood Phe levels are neurotoxic mainly because of their inhibitory effect on the blood–brain barrier transport of free L-amino acids (leucine, isoleucine, valine, tyrosine, tryptophan and lysine) that are necessary for the synthesis of proteins and neurotransmitters (dopamine and serotonin) [3,5]. PKU children, if untreated, can exhibit microcephaly, epilepsy, a musty body odour, decreased skin and hair pigmentation, eczema, severe intellectual disability and behaviour problems as well as structural brain changes visible on magnetic resonance imaging [7]. Prognosis and outcome depend on both time of diagnosis and type of mutation [8].

Despite the new advancements and treatment strategies (e.g. large neutral amino acids, BH4, gene therapy, Phe ammonia lyase), dietary intervention remains the mainstay of PKU therapy [8].

PKU: dietary treatment

The main treatment for PKU is an early Phe-restricted diet, which aims at reducing blood Phe to non-toxic levels [8]. During infancy, breastfeeding is recommended to provide natural proteins according to the individual Phe tolerance, and/or whether necessary a Phe-free infant formula is used. With the introduction of solid foods, PKU infants have to avoid foods rich in protein (meat, fish, eggs, dairy products, standard bread, nuts and seeds). Therefore, PKU diet mainly comprises low-protein natural foods (vegetables, fruits and some cereals), to reach the ideal Phe levels necessary for growth processes. The allowed intake of low-protein natural foods is commonly calculated on the basis of their Phe content. Nowadays, low-protein variants of some foods are commercially present, such as low-protein bread and low-protein pasta [9]. The overall required amount of daily protein intake is obtained consuming additional Phe-free protein substitutes that supply essential amino acids in suitable proportions [3,8].

This type of dietary regimen usually includes high carbohydrate content, low saturated and long-chain polyunsaturated fat, cholesterol, carnitine, taurine, iron, zinc, selenium, calcium, folates, A, C, D, E and B2, B6, B12 vitamins, because of the very low consumption of Phe-containing animal foods [8,10,11]. Indeed, a study observed that PKU children compliant with diet consumed $<$ 7% of saturated fats including $<$50 mg cholesterol per day [12]. Moreover, PKU subjects must rely on the endogenous synthesis of long-chain polyunsaturated fatty acids from their precursors; the content of these acids is often suboptimal in dietary products for patients with PKU, particularly of α-linolenic acid, precursor of docosahexaenoic acid [8,10,13]. These characteristics of the diet for subjects with PKU may resemble those of a vegan diet with respect to the composition of permitted natural foods. Vegan diet has been recently considered within healthy populations for the presumed health benefits, including potential effects on cardiovascular health [14]. However, consumption of some foods of the usual vegan diet, rich in micronutrients (cereals, nuts, etc.), is restricted in PKU subjects because of their high protein content. PKU subjects need therefore nutritional supplements (by Phe-free protein substitutes or given separately) to meet the dietary reference intakes for micronutrients. Besides, it is worth noting that patients with severe PKU are provided with a significantly high percentage of dietary supplements such as folic acid and vitamin B12 [15–17].

Overall, while there is a general agreement that PKU patients need long-term dietary counselling and daily nutritional supplementation [8], particular attention should be paid concerning the micronutrient intake [17].

Cardiovascular disease

Cardiovascular disease (CVD) remains to be the major cause of deaths worldwide. More than 3 million of these deaths occurred before the age of 60, which could have largely been prevented. The percentage of premature deaths from CVD ranges from 4% in high-income countries to 42% in low-income countries [18].

The burden of disease will increase with an ageing population and increasing levels of obesity and sedentary lifestyles [18]. Diet has long been implicated in managing and reducing the risk of CVD [19]. The assessment of CVD risk factors relies mainly on the evaluation of dietary habits, anthropometric measurements, blood lipid profile, homocysteine and inflammatory biomarker levels, blood pressure as well as genetic and psychosocial factors [20].

Topic of review

This paper reviews the literature published over the past 15 years and discusses the potential relationship of restricted Phe diet in PKU children with cardiovascular risk factors (overweight/obesity; blood lipid profile; plasma levels of homocysteine, adiponectin and free asymmetric dimethylarginine (ADMA); oxidative stress; and blood pressure).

Methodology

Publications were identified from a PubMed search using the terms ‘obesity’, ‘overweight’, ‘lipid profile’, ‘cholesterol’, ‘long-chain polyunsaturated fatty acids’, ‘homocysteine’, ‘micronutrients’, ‘adiponectin’, ‘asymmetric dimethylarginine’, ‘nitric oxide’, ‘oxidative stress’, ‘blood

pressure' and 'phenylketonuria' and 'PKU diet'. Other publications came from the reference list of other papers, hand searches and from the personal reference databases of the authors.

CVD risk factors in PKU children on dietary treatment

Overweight/obesity

Obesity is strongly related to major cardiovascular risk factors, and its impact on CVD is well recognized [18,21].

A recent review [22] regarding overweight issue in PKU summarized that the earliest studies described a tendency for overweight in PKU, but the results are inconsistent, although an increasing number of studies described higher obesity prevalence in female PKU patients than healthy controls. A study, included in the review, demonstrated that in PKU children compliant with diet, the prevalence of overweight at 8 years of age (25%) was comparable to the values estimated in normal populations [23]. Similarly, another study found no statistically significant differences regarding the prevalence of overweight and obesity between PKU children (<10 years) and healthy controls (25% vs. 15.4%, respectively) [24]. In the same study, a higher prevalence of overweight and obesity was observed in PKU subjects (3–30 years) with poor metabolic control than in those with good metabolic control (42.9% vs. 27.9%, respectively) [24]. Moreover, Burrage et al. observed an overall overweight rate of 40% in PKU subjects (2–20 years) with sex difference (males 27% vs. females 55%) [25].

Following the review by Rocha et al. [22], other two studies were published investigating the risk of obesity in PKU [26,27]. The first study performed on 54 PKU patients, followed up from birth up to 18 years, found weight and body mass index (BMI) slightly higher in PKU patients compared to the reference population, but without reaching statistical significance [26]. The second is a retrospective longitudinal study evaluating the anthropometric characteristics of the PKU subjects collected every 6 months from birth to 18 years of age [27]. In this study, the prevalence of obesity was higher in PKU children than in general population, from 8 years of age, without sex difference [27].

In healthy population, an early age at adiposity rebound (age at the nadir of BMI) has been additionally suggested as a predictor of both higher BMI during adolescence and adult age and late onset of overweight [28]. Similarly, PKU children overweight at 8 and 18 years of age showed earlier adiposity rebound than non-overweight PKU children [23,27].

Although not significantly accurate, BMI is the most common surrogate measure of adiposity in children and adults [29]. A study did not find differences between paediatric PKU subjects (<19 years of age) and healthy control regarding fat and fat-free mass, using a single-frequency bioelectrical impedance analyzer [30]. Another study did not find differences in body composition (measured from bioelectrical impedance and skinfold thickness) between the PKU children (8 months to 7 years) and French reference population [31]. Conversely, a study evaluating the body composition using the BOD POD whole-body air displacement plethysmography method found that PKU children had a higher body fat percentage than healthy controls although BMI values in the two groups were similar [32]. In this study, no association was observed between the mean blood Phe levels and body fat percentage in PKU children [32].

Some factors could influence the tendency towards obesity, as reported in some studies, in PKU population. Probably the greater weight gain of PKU patients may be related to the intake of protein substitute having a high energy content [33]. In particular, from 10 years of age onwards, PKU patients may have less supervision from parents regarding consumption of Phe-free protein substitutes and meal preparation and consequently a higher energy intake [25]. Furthermore, it has been suggested that increased BMI observed in PKU subjects could be related to the avoidance of sporting activities due to the reported social isolation and anxiety of these patients [33]. Physical inactivity is an important modifiable risk factor in the development of CVD [34]. However, nowadays, no published paper evaluated the physical activity in PKU children. In conclusion, the majority of evaluated studies found that the prevalence of overweight/obesity in PKU population with good dietary compliance was not different from that of the general healthy population. Further studies are needed to confirm these data and to clarify the possible nutritional and lifestyle habit factors associated with overweight/obesity in the PKU population compliant with diet.

Blood lipid profile

The alteration of blood lipid profile is a metabolic risk factor that plays a key role in the aetiology of atherosclerosis, one of the major underlying pathological processes that lead to heart attacks and strokes [18]. Indeed, low-density lipoprotein cholesterol (LDL-C) is deposited in the walls of arteries and causes atherosclerosis. In addition, triglycerides increase the risk of atherosclerotic CVD. By contrast, high-density lipoprotein cholesterol (HDL-C) protects against vascular disease by removing the LDL-C from the walls of arteries and thus reducing the risk of atherosclerosis [18]. A healthy diet can contribute to a desirable blood lipid profile [18].

The reduction in the intake of animal lipids and cholesterol, a major feature of PKU diet, could be defined as a non-atherogenic diet [35]. The blood lipid profile of PKU children with good metabolic control has been analyzed in few studies [12,35–38]. In some studies, PKU children on diet exhibited lower cholesterol levels compared to the healthy controls [35,37,38]. However, a study showed that the cholesterol levels did not differ between PKU and healthy children [36].

Lower LDL-C levels were observed in PKU children on dietary treatment than in the healthy controls [35,37,38]. However, this difference was not found in another study [36].

The majority of the evaluated studies did not show differences between PKU children and controls for plasma HDL-C [35–38] and triglyceride levels [37,38]. However, a study demonstrated that the triglyceride levels were higher in PKU children with a good metabolic control compared to the healthy group [36].

LDL-C particle size is another relevant factor involved in the pathogenesis of CVD. High LDL-C/apolipoprotein B (Apo B, the almost exclusive apolipoprotein of LDL cholesterol) ratio is associated with the presence of larger and less atherogenic particles that are less susceptible to oxidative damage than small LDL particles [35]. The LDL particles size could be related to PKU diet. Indeed, a study observed that LDL-C/Apo B ratio was higher in the group of PKU children compliant with diet than in non-compliant PKU and healthy controls [35]. Larger and less atherogenic LDL particles were associated with a high Zn/Cu ratio in PKU children compliant with diet [35]. These data could suggest that some minerals may play an important role in the lipoprotein metabolism in patients with PKU on diet; they are involved in the metabolic activities and also are associated with the size of LDL particles. The PKU patients, strictly adhering to diet, may be not at risk of developing atherosclerosis as their blood lipid profile may be less atherogenic as compared to PKU not adhering to dietary treatment and healthy controls [35].

Finally, it is important to highlight that genetics may influence the response to diet. Indeed, genetic variations in Apo B may play a major role in modifying the response to diet and its fat composition [12]. A study performed on PKU children compliant with diet found that the Apo B XbaI polymorphism (X-) may be associated with a favourable response to long-lasting diets low in total fats, saturated fats and cholesterol [12].

In conclusion, results from literature suggest similar or better lipid profile in PKU children than healthy controls.

Plasma homocysteine level

Homocysteine, a sulphur-containing amino acid, is recycled into methionine by a transmethylation reaction requiring folate and vitamin B12. Folic acid and vitamin B12 and B6 deficiencies and reduced enzyme activities inhibit the breakdown of homocysteine, thus increasing the intracellular homocysteine concentration [39]. Plasma homocysteine levels are associated with the risk of CVD [39]. Some of the supposed mechanisms include an increase in proliferation of vascular smooth muscle cells, endothelial dysfunction, oxidative damage, an increased synthesis of collagen and deterioration of arterial wall elastic material [39].

Six studies evaluated the homocysteine levels in PKU children on dietary treatment [20,37,38,40–42]. Stolen et al. [42] showed that 68% of 34 PKU children on dietary treatment had plasma homocysteine concentrations below the reference range. In this study, 91% and 53% of the studied children had plasma folate and vitamin B12 levels above the upper reference level, respectively [42]. Similarly, Huemer et al. reported lower plasma homocysteine concentrations in 16 treated PKU children and adolescents than the age-matched controls [40]. No difference was found for folate levels [40]. However, in another study, no difference was observed in the homocysteine concentrations in PKU subjects (age: 4–20 years) on dietary treatment and in controls, although the folate and vitamin B12 levels were higher in PKU subjects [41]. Furthermore, Karam et al., [20] recently, showed no difference in the homocysteine levels between nine patients with PKU (eight children and one adult) and control group (30 healthy subjects, mean age: 12.1 years). On the contrary, Schulpis et al. found homocysteine concentrations twice higher in PKU children compliant with diet than in non-compliant PKU and in healthy controls [37,38]. Moreover, the PKU subjects compliant with diet showed a lower dietary intake of folate and vitamin B12 than the healthy controls [37,38].

In conclusion, the available studies on homocysteine concentrations in PKU children on dietary treatment found inconsistent results. Further studies are needed to understand the relationship between PKU diet and homocysteine levels taking into account that PKU children use Phe-free protein substitutes enriched with folate and vitamin B12 [15–17]. However, it should be noted that not all studies specified dietary compliance of PKU subjects, the folate and vitamin B12 levels in the Phe-free protein substitutes and the impact of genetic polymorphisms on plasma homocysteine levels.

Plasma adiponectin level

Adiponectin, an adipose tissue-derived hormone, prevents endothelial inflammation and early atherogenesis [43]. It seems to exhibit protective functions in an experimental model of vascular injury suppressing the attachment of monocytes/macrophages to endothelial cells, a fundamental early step in the atherosclerotic process [43]. Moreover, expression of this protein decreases with an increase in adiposity, and it also has an insulin-sensitizing effect mediated through binding to its receptors AdipoR1 and AdipoR2, thereby leading to the activation of different signalling pathways [44]. The study conducted by Schulpis et al. [37] showed that PKU children compliant with diet had higher plasma adiponectin levels compared to the healthy children. Interestingly PKU children not compliant with diet showed elevated adiponectin concentrations compared to those compliant with diet and the healthy controls [37]. The authors suggested that the increased adiponectin levels in non-compliant PKU patients might be due to the decreased inhibition by low concentrations of catecholamines, directly acting on adiponectin production and/or release in blood stream [37].

To our knowledge, this is the only study evaluating adiponectin concentrations in a population of PKU subjects. Further studies must be conducted to evaluate the existence of a possible relationship between PKU diet, dietary compliance and adiponectin levels for more scientific evidence.

Plasma-free asymmetric dimethylarginine level

There is growing interest in the nutritional factors modulating endothelial functions [45]. Free asymmetric dimethylarginine is a competitive inhibitor of nitric oxide (NO) synthases. NO is a potent vasodilatator, protects functional integrity of the endothelium and inhibits platelet aggregation. Therefore, suppression of NO synthesis is a risk factor for atherosclerosis [40]. In adults, an increase in plasma ADMA concentrations by 0.1 μmol/L results in an odds ratio of 2.61 for coronary heart disease [40]. However, in oxidative stress conditions, NO synthase inhibition by ADMA may exert protective effects [40,46]. The assessment of ADMA concentrations in PKU subjects could be justified considering that ADMA, synthesized by dimethylation of protein-bound L-arginine residues and consecutive degradation of these proteins, might be higher in PKU subjects than controls due to altered protein metabolism induced by PKU diet, containing large amounts of synthetic amino acids [40].

A cross-sectional study [40] investigated plasma-free ADMA concentrations in 16 PKU children (mean age: 10 years) on dietary treatment and 91 healthy controls. This study found that in contrast to the hypothesis, PKU children had lower ADMA concentrations compared to controls [40]. This result was confirmed also by another study [45].

Although the literature is scarce, a lower ADMA plasma concentration was found in PKU subjects on strict diet than in the control group. However, further investigations are needed to elucidate the role of PKU diet in ADMA concentrations.

Oxidative stress

Oxidative stress is involved in the development of many diseases, such as atherosclerosis, heart failure and myocardial infarction [47]. Oxidative stress is an imbalance between the systemic manifestation of reactive oxygen species and the ability of the biological system to readily detoxify the reactive intermediates or repair the resulting damage [47]. Modifications in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals, thus damaging all cellular components [47]. Altered antioxidant capacity is observed in PKU, both in human and animal models, and markers of lipid, protein and DNA damage have been shown to suggest an increased production of reactive species in this disease, probably due to the depletion of antioxidant micronutrients [16].

The PKU diet includes good sources of dietary antioxidants (phytochemicals, some vitamins and minerals), but it could be a risk factor for some micronutrient deficiencies (selenium, zinc, ubiquinone-10 and L-carnitine) unless supplemented [48,49]. PKU children compliant with diet showed a significant elevation of β-carotene and α-tocopherol intake as well as in blood concentrations compared to those not compliant with diet and healthy children [50]. These high levels of antioxidants probably may contribute to increased antioxidant status [50]. In a more recent study, total antioxidant status was higher in PKU children on strict diet than in those on loose diet, with no difference between PKU on strict diet and controls [51].

In conclusion, evaluated studies found that PKU children compliant with diet may have a “better antioxidant status” than not accurately compliant children. Results evaluating the antioxidant status of PKU children than healthy controls were inconsistent.

Blood pressure

Some long-term cohort studies have shown that hypertension in adolescence or young adulthood is strongly related to later risk of stroke or coronary heart disease, independent of blood pressure in mid-life [52]. Two studies evaluated the blood pressure of PKU subjects (both adults and children) on dietary treatment compared to age-matched controls. Rocha et al. [24] observed a lower diastolic and systolic blood pressure in PKU subjects than controls (48 vs. 52 mmHg and 104 vs. 110 mmHg, respectively). Huemer et al. [40] showed that children with PKU on dietary treatment had a lower diastolic pressure than controls (64 mmHg vs. 66 mmHg; $p < 0.0001$) without any difference in systolic pressure (108 mmHg vs. 117 mmHg) [40].

In conclusion, few studies have been conducted in PKU subjects about this cardiovascular risk factor. These data do not allow us to draw a conclusion about a possible protective effect of PKU diet on blood pressure.

Results and discussion

A summary of the major conclusions from this review is presented in Table 1. These are based on the possible association of restricted Phe diet in PKU children with main cardiovascular risk factors.

Out of the evaluated literature [23,24,26], no difference between PKU compliant with diet and healthy population was observed for overweight/obesity prevalence while only a study found higher prevalence of obesity in PKU subjects aged 8–18 years [27]. PKU children showed lower diastolic pressure than healthy controls [24,40], but the observed values were within the reference range both in PKU and healthy children. Concerning blood lipid profile, PKU children showed lower total cholesterol and LDL-C [35,37,38] levels than healthy controls, but no difference was observed for HDL-C levels [35–38]. Although the observed lower levels of LDL-C suggest a better lipid profile, a recent review highlighted that non-HDL-C is a better predictive indicator of CVD in children [53]. Unfortunately, there are no studies currently evaluating non-HDL-C levels in PKU subjects. Only a case–control study assessed adiponectin levels in PKU children and found higher levels in PKU than healthy controls. The protective effects of higher adiponectin levels against CVD may be worth noting [37]. Although plasma-free ADMA levels were found to be lower in PKU children than healthy controls [40,45], inconsistency about their antioxidant status [50,51] does not allow

Table 1 Possible effects of the diet on cardiovascular risk factors in PKU children compliant with diet.

Cardiovascular risk factor	PKU versus healthy children	References (study design)
Overweight/obesity	No difference in prevalence of overweight/obesity [23,24,26]. Higher prevalence of obesity only in Ref. [27]	23, 26, 27 (retrospective longitudinal) 24[a] (case–control, cross sectional)
Blood lipid profile	Lower total cholesterol and LDL-C [35,37,38] except in Ref. [36]. No difference in HDL-C [35–38]. No difference in triglycerides [37,38], higher triglycerides in Ref. [36].	35[b]–38 (case–control, cross-sectional)
Plasma homocysteine level	Inconsistent results No difference [20,41]; higher [37,38]; lower [40]	20, 37, 38, 40, 41 (case–control, cross-sectional)
Plasma adiponectin level	Higher	37 (case–control)
Plasma-free asymmetric dimethylarginine level	Lower	40, 45 (case–control, cross-sectional)
Oxidative stress	Inconsistent results Higher β-carotene and α-tocopherol blood concentrations [50]; no difference [51]	50, 51 (case–control)
Blood pressure	Lower systolic pressure [24] Lower diastolic pressure [24,40]	24,[a] 40 (case–control)

[a] This study also includes adults.
[b] Triglycerides were not evaluated in this study.

any resounding inference on the protective effects of ADMA against PKU. Inconsistent results have been found for plasma homocysteine levels [20,37,38,40,41], an emerging cardiometabolic risk factor [39].

Finally, the study of the relationship between PKU diet and cardiovascular risk would be of great interest if this aspect could vary depending on dietary compliance and tolerance to Phe. It was not possible to discuss this issue because of the lack of data in most of the assessed studies.

Conclusions

Dietary treatment remains essential for PKU, despite the appearance of new advances and strategies [8]. Long-term dietary guidance and monitoring of the nutritional status of subjects with PKU should be part of a life-long follow-up programme. Doctors and dieticians should prescribe and carefully monitor macronutrient and micronutrient intake, growth and physical activity in PKU subjects.

The existing literature about the possible relationship of PKU diet with cardiovascular risk in children is scanty. This narrative overview did not consider studies that included only PKU children not compliant with diet. Although PKU children compliant with diet may exhibit lower blood total cholesterol and LDL-C levels than the healthy population, they seem to display non-different cardiovascular risks. Nevertheless, well-designed longitudinal studies, with adequate statistical power, are still required to clarify the potential underlying mechanisms related to PKU diet and cardiovascular risk factors.

Author contributions

Dr Elvira Verduci is primarily responsible for manuscript management and contributed to the writing of the manuscript.

Dr Giuseppe Banderali, Dr Francesca Moretti and Dr Carlotta Lassandro critically performed the literature research about this issue and contributed to the writing of the manuscript.

Dr Graziella Cefalo, Dr Giovanni Radaelli and Dr Elisabetta Salvatici contributed to the writing of the manuscript.

Prof Marcello Giovannini supervised the editorial project and contributed to the writing of the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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1.4 Diet and gut micromiota: relationship and metabolic effect

1.4.1 Definition of gut microbiota

Over the last decade, the microbial composition of the gut (gut microbiota) has been widely investigated, and recognized as having an impact in various physiological and pathological conditions [1]. The gut microbiota includes more than 100 trillion cells of 400 species which is equivalent to ten times the total number of cells in the human body [2].

Currently, four major microbial phyla are known to represent over 90% of the bacterial component of gut microbiota: *Firmicutes, Bacteroides, Proteobacteria* and *Actinobacteria.* The majority of ″good″ bacteria harbouring the human gut microbiota are represented by *Firmicutes* and *Cytophaga-Flavobacterium-Bacteroides* (CFB). *Firmicutes* are sub-grouped in *Clostridium coccoides* (Clostridium cluster XIVa) and *Clostridium leptum* (Clostridium cluster IV); whereas CFB group is represented mainly by *Bacteroides phyla* with a great number of *Prevotella* and *Porphyromonas* [3]. Moreover, the human gut microbiota includes viruses, especially phages, Eukarya, as Fungi, Blastocystis, Amoebozoa, and Archaea [3].

Gut microbiota has been classified into three main enterotypes, each one owning specific metabolic features. Each enterotype is characterized by the relative abundance of one of the following genera: *Bacteroides* (more represented in enterotype 1), *Prevotella* (more abundant in enterotype 2), *Ruminococcus* (prevalent in enterotype 3). Prevalence of a specific enterotype can depend on long-term dietary habits; indeed high-fat and protein diet enhances the growth of enterotypes 1 and 3, while a diet rich in carbohydrates supports the raise of enterotype 2. Recent findings suggest that gut microbiota composition is influenced also by short-term dietary changes [3]. Both genetic and environmental factors are involved in influencing the inter-individual diversity of gut microbiota. Among environmental ones, the interest of the scientific community in dietary factors has progressively increased, and the role of diet on the composition of gut microbiota has been particularly emphasized over the last years [3].

1.4.2 The role of phenylketonuria diet on gut microbiota composition

Diet is known to have a strong influence on the composition of intestinal microbiota [4].

The nutritional composition of PKU diets might change gut microbial ecology and affect host physiology [5]. At the present time only a Brazilian study analyzed gut microbiota in PKU children (age [DS] 4.24 [1.74]) in comparison to healthy children (6.06 [1.78]) [5]. In this study, as previously observed in healthy humans [6,7] and in PKU mice [8], the *Bacteroidetes* and

Firmicutes were the most dominant phyla found within in PKU and healthy children stool samples. However, significative difference in gut microbiota between PKU and healthy children were observed. The most significant differences in gut microbiota between patients and controls were observed for members of the *Clostridiaceae*, *Erysipelotrichaceae*, and *Lachnospiraceae* family, *Clostridiales* class, *Coprococcus, Dorea, Lachnospira, Odoribacter, Ruminococcus*, and *Veillonella* genera, which were enriched in the control group. Furthermore, three bacterial members—belonging to genera *Prevotella and Akkermansia and to the Peptostreptococcaceae* family—were enriched in the PKU group. According to metagenome prediction, the microbiome of PKU patients presented fewer genes involved in starch and sucrose metabolism and in glycolysis/gluconeogenesis. Differences in microbial starch and glucose metabolism might also affect production of short-chain fatty acids (SCFAs), which might suggest lower SCFA production by the gut microbiota of PKU patients [5].

1.4.3 Short chain fatty acids: pathways

SCFAs are saturated aliphatic organic acids that consist of one to six carbons of which acetate (C2), propionate (C3), and butyrate (C4) are the most abundant (≥95%). Acetate, propionate, and butyrate are present in an approximate molar ratio of 60:20:20 in the colon and stool [9].

Acetate, which is the most abundant SCFA, is produced by most enteric bacteria as a fermentation product, but it is also formed by acetogenic bacteria, such as B. hydrogenotrophica, from H2 and CO2 or from formate via the Wood–Ljungdahl pathway (Figure 1). Acetogenic bacteria can produce three molecules of acetate from one molecule of glucose, but non-acetogenic anaerobes, which comprise most of the microbiota, must dispose of reducing equivalents by forming other products in addition to (or instead of) acetate, including succinate, propionate, butyrate, formate, d-lactate, l-lactate and ethanol (Figure 1). The relative synthesis of the different fermentation products varies according to the composition of the microbiota and environmental conditions, including pH, hydrogen partial pressure and available substrates [9].

Propionate is mostly formed via the succinate pathway by Bacteroidetes and by some Firmicutes that belong to the Negativicutes class (such as Phascolarctobacterium succinatutens, *Dialister spp*. and *Veillonella spp.*). Succinate is a metabolic end-product for some bacteria under some environmental conditions, but specialist succinate utilizers, such as P. succinatutens, convert most of the succinate that is produced into propionate. Two other pathways also contribute to the formation of propionate: the acrylate pathway and the propanediol pathway (Figure 1). The acrylate pathway, in which lactate is a substrate, shows a limited distribution, whereas the

propanediol pathway, in which deoxyhexose sugars (such as fucose and rhamnose) are substrates, is present in some Firmicutes (including Roseburia inulinivorans and Ruminococcus obeum) and in Proteobacteria. The proportion of propionate that is present in total faecal SCFA correlates with the relative abundance of Bacteroidetes, which confirms that the succinate pathway is the dominant source of propionate [9].

Butyrate is produced by some Firmicutes using either the butyryl-CoA:acetate CoA-transferase enzyme or, less commonly, phosphotransbutyrylase and butyrate kinase to catalyse the final steps of the pathway (Figure 1). Species that use the butyryl-CoA:acetate CoA-transferase route include several of the most abundant species in the healthy gut microbiota (including *Faecalibacterium prausnitzii*, *Roseburia spp.*, *Eubacterium rectale, Eubacterium hallii and Anaerostipes spp.*) which are generally net users of acetate, such that the concentration of acetate in the gut lumen is determined by the balance of production, use and mucosal uptake. A subset of Lachnospiraceae, including *E. hallii* and *Anaerostipes spp.*, can use lactate and acetate to produce butyrate. Thus, these organisms may have an important role in stabilizing the microbial ecosystem by preventing the accumulation of lactate. Only a few anaerobes are known to produce both propionate and butyrate, and they do so from different substrates: *R. inulinivorans* produces butyrate from glucose and produces propionate from fucose, whereas *Coprococcus catus* produces butyrate from fructose and produces propionate from lactate (via the acrylate pathway).

Acetate, propionate and butyrate are rapidly adsorbed from the gut lumen, but their subsequent distribution, fate and effects on host cell metabolism differ. Butyrate is preferentially used as an energy source by gut epithelial cells, and its concentration in the systemic circulation is low. Propionate is mostly metabolized in the liver, and only acetate achieves relatively high concentrations (0.10–0.15 mM) in peripheral blood [10,11].

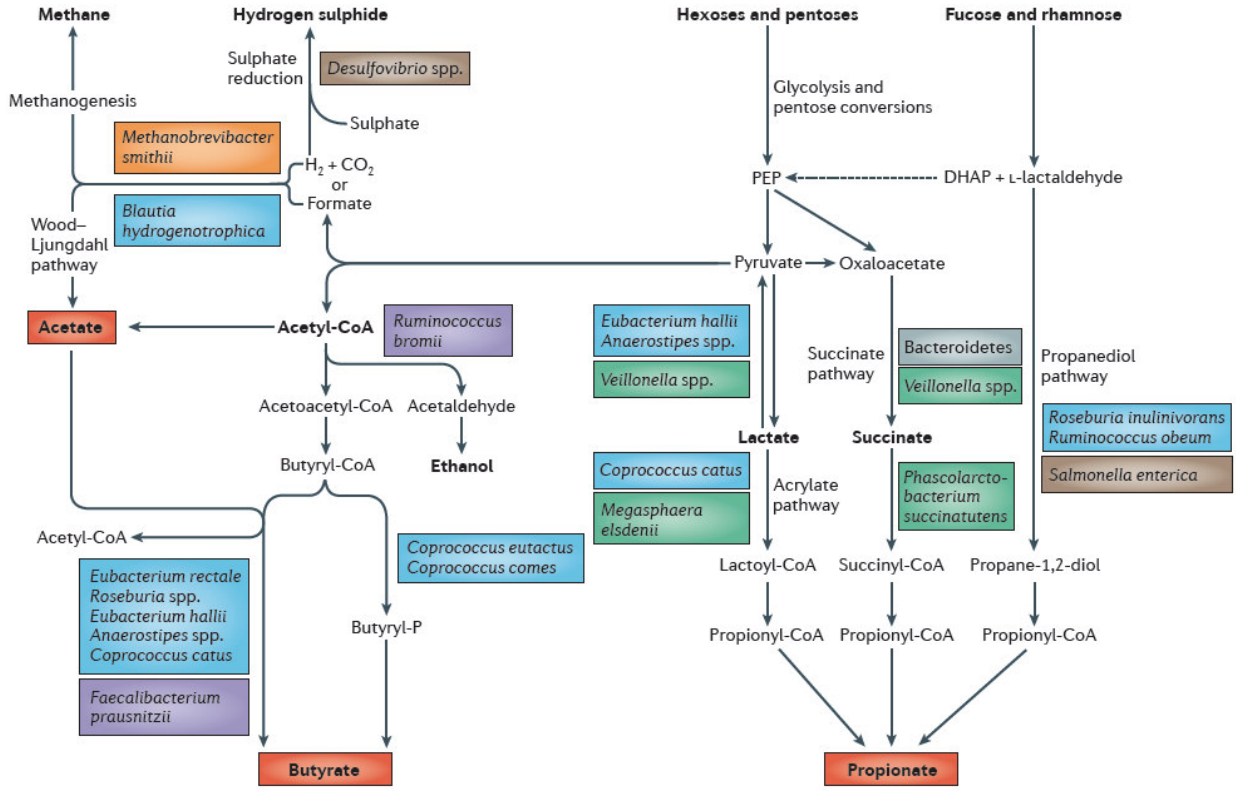

Figure 1. Pathways that are responsible for the biosynthesis of the major microbial metabolites that result from carbohydrate fermentation and bacterial cross-feeding.

1.4.4 Short chain fatty acids: functions

Short chain fatty acids have a wide range of actions. They are absorbed and metabolized rapidly by colonocytes, providing 60–70% of their energy requirements [12]. They also regulate fluid and electrolyte uptake via activation of apical NaC/HC exchange [12]. Their presence in the colon lowers the pH, thus preventing the overgrowth of pH-sensitive pathogenic bacteria. Human rectal SCFA infusions have also shown to increases planchnic blood flow and decrease gastrict one [13]

Butyrate, in particular, plays an important role in intestinal health. It has been shown to have atrophic effect on colorectal and ileal mucosal cells but despite this, is able to maintain normal colonic phenotype via growth arrest, differentiation, and apoptosis, thereby lowering the risk of malignancy. Importantly, butyrate enhances the gastrointestinal innate immunity by acting as a relay for transducing information from the luminal environment to the mucosal immune system via up-regulation of toll like receptors expression [14]. These toll like receptors enable the epithelium to differentiate commensal flora from pathogens, via recognition of bacterial molecular patterns called PAMPs and induce the transcription of a panel of genes mediating immune and inflammatory responses [14].

In the last few decades, it has been hypothesized that SCFAs might play a key role in the prevention and treatment of metabolic syndrome, bowel disorders, and cancer [15,16]. Butyrate and propionate were shown to protect against diet-induced obesity and regulated the gut hormones [17]. Clinical studies showed that the administration of SCFAs has a positive effect on the treatment of ulcerative colitis, Crohn's disease, and antibiotic-associated diarrhea and obesity [18,19]. In obese subjects, propionate significantly increased the release of postprandial plasma peptide YY (PYY) and glucagon-like peptide-1 (GLP-1) from colonic cells, and reduced the energy intake. Inulin-propionate ester administrated at 10 g per day over a period of 24 weeks significantly reduced weight gain, intra-abdominal adipose tissue distribution and intrahepatocellular lipid content, and improved insulin resistance in the inulin control group [19]. On the other hand, SCFAs also utilize as host energy source, therefore SCFAs are regarded as cause of increasing energy harvest from diet, linked to the obese phenotype by changes of gut microbiota composition [2].

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1.5 Aims of the study

The general aim of this PhD thesis was to investigate the impact of phenylketonuric diet, principally carbohydrates quality, on metabolic profile, cardiovascular risk factors and fecal short chain fatty acids (SCFA) production in children with phenylketonuria.

The primary aim was to evaluate nutritional intakes, especially dietary daily glycemic index and gycemic load, in PKU children compared with healthy children matched for age, sex and BMI z score and to evaluate whether an association may exist between the carbohydrate quality of the diet and the metabolic profile in PKU children. The results of this aim are presented in **chapter 2**.

The secondary aim, covered in **chapter 3**, was to determine cardiovascular risk factors in PKU children, on low-Phe diet, in MHP and in healthy children, on unrestricted diet, matched for age, sex and BMI z score and to evaluate whether an association may exist between the carbohydrate quality of the diet and the risk factors in PKU children.

The third aim, described in **chapter 4**, was to compare gut microbiota biodiversity and its production of short chain fatty acids in PKU children and MHP children matched for age, sex and BMI z score.

Chapter 2: DIETARY INTAKE AND CARBOHYDRATE QUALITY IN CHILDREN WITH PHENYLKETINURIA: EFFECT ON METABOLIC PROFILE

Nutrition, Metabolism & Cardiovascular Diseases (2017) 27, 176–182

Available online at www.sciencedirect.com

Nutrition, Metabolism & Cardiovascular Diseases

journal homepage: www.elsevier.com/locate/nmcd

Dietary glycemic index, glycemic load and metabolic profile in children with phenylketonuria

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Received 18 July 2016; received in revised form 28 October 2016; accepted 2 November 2016
Available online 21 November 2016

KEYWORDS
Phenylketonuria diet;
Glycemic index;
Glycemic load;
Metabolic profile

Abstract *Background and aims:* No data exist in the current literature on the glycemic index (GI) and glycemic load (GL) of the diet of phenylketonuric (PKU) children. The aims of this study were to examine the dietary GI and GL in PKU children on a low-phenylalanine (Phe)-diet and to evaluate whether an association may exist between the carbohydrate quality and the metabolic profile.
Methods: Twenty-one PKU children (age 5–11 years) and 21 healthy children, gender and age matched, were enrolled. Dietary (including GI and GL) and blood biochemical assessments were performed.
Results: No difference was observed for daily energy intake between PKU and healthy children. Compared to healthy controls, PKU children consumed less protein ($p = 0.001$) and fat ($p = 0.028$), and more carbohydrate (% of total energy, $p = 0.004$) and fiber ($p = 0.009$). PKU children had higher daily GI than healthy children (mean difference (95% confidence interval), 13.7 (9.3–18.3)) and higher GL (31.7 (10.1–53.2)). PKU children exhibited lower blood total and low density lipoprotein cholesterol (LDL) levels ($p < 0.01$) and higher triglyceride level ($p = 0.014$) than healthy children, while glucose and insulin concentrations did not differ. In PKU children the dietary GL was associated with triglyceride glucose index (Spearman's correlation coefficient = 0.515, $p = 0.034$).
Conclusion: In PKU children a relationship of the dietary treatment with GI and GL, blood triglycerides and triglyceride glucose index may exist. Improvement towards an optimal diet for PKU children could include additional attention to the management of dietary carbohydrate quality.

Abbreviation list: BMI, body mass index; GI, glycemic index; GL, glycemic load; HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment of insulin resistance; ICQC, International scientific consensus summit from the International Carbohydrate Quality Consortium; LDL, low-density lipoprotein; MHP, mild hyperphenylalaninemia; PAH, phenylalanine hydroxylase; Phe, phenylalanine; PKU, phenylketonuria; QUICK, quantitative insulin sensitivity check index; TyG, triglyceride glucose index; WHO, World Health Organization.

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Introduction

Phenylketonuria (PKU; OMIM 261 600) is an autosomal recessive disorder of phenylalanine (Phe) metabolism mainly due to mutations of the phenylalanine hydroxylase (PAH) gene, which is needed to convert the essential amino acid phenylalanine to tyrosine [1]. The loss of PAH activity results in accumulation of Phe to neurotoxic levels [1]. Various combinations of mutations result in a full

http://dx.doi.org/10.1016/j.numecd.2016.11.002

spectrum of metabolic phenotypes ranging from mild hyperphenylalaninaemia (MHP, blood Phe 120–360 μmol/L) – in which dietary restriction is not necessary, to mild, moderate and classical phenylketonuria (blood Phe levels >360 μmol/L) which require dietary management [1].

The main goal of treatment for PKU is to maintain the blood Phe within safe limits to prevent mental retardation and ensure normal growth and life with good health through adulthood [2–4]. The dietary treatment usually begins immediately after confirmation of PKU diagnosis in newborns and should be continued throughout their lifetime [3,4].

Patients with PKU have to avoid foods rich in protein (meat, fish, eggs, conventional bread, dairy products, nuts, and seeds) [3]. Accordingly, the PKU diet is mainly made up of low-protein natural foods (vegetables, fruits) and special low protein products, which are low-protein variants of some foods such as bread, pasta and biscuits with a protein content <1 g/100 g [3]. In Italy, the quantities of the permitted natural foods are calculated from a method of Phe equivalence (dependent upon how much Phe a food contains for a given weight) [5]. The required amount of daily protein is obtained from Phe-free protein substitutes providing essential amino acids in suitable proportions [3].

In PKU patients the issue of overweight and obesity has been highlighted [6], although the prevalence of obesity was observed similar to general populations both in PKU children [7] and adults [8,9]. The metabolic profile of PKU patients has been studied in the past (e.g. Refs. [8–10]). Only a study compared the metabolic syndrome between PKU and healthy subjects and no difference in the prevalence was reported [8].

The high energy content of Phe-free protein substitutes and the “avoidance” of sporting activities have been suggested to be factors influencing the tendency to overweight/obesity in the PKU population [6]. The uncontrolled consumption of special low protein products could be another factor predisposing to obesity [6]. In severe PKU phenotypes (with natural protein tolerance less than 10 g/day), the special low protein products may provide up to 50% of energy requirements [6]. Moreover, Rocha et al. [6] highlighted that in some low protein products it is common to add glucose, dextrose or sugar as ingredients, and this may increase the sugar intake in PKU [6].

The glycemic index (GI) was developed to systematically classify foods according to their ability to raise postprandial glycemia [11]. Since the overall impact of one food on postprandial response is due to the combination of GI and the amount of carbohydrates in that food, a derived index has been proposed, namely glycemic load (GL) [11]. Subsequently, GL was standardized to the energy of the food portion consumed (GL/1000 kJ) for a better representation of carbohydrate-based foods combined with fat and protein [11].

In 2015 the International Scientific Consensus Summit from the International Carbohydrate Quality Consortium (ICQC) [12] recommended that low GI and low GL should be considered in the context of a healthy diet [12]. The ICQC stated that low GI/GL diets reduce the risk of type 2 diabetes and could reduce total body fat mass [12]. No data exist in the current literature on GI and GL in PKU children.

The primary aim of the present study was to examine the dietary glycemic index and glycemic load in PKU children on low-Phe diet. Secondary aim was to evaluate whether an association may exist between the carbohydrate quality and their metabolic profile.

Methods

This observational case-control study examined 21 PKU primary school children (age 5–11 years) gender and age (±6 months) matched with 21 healthy controls, consecutively admitted to the Department of Pediatrics, San Paolo Hospital, Milan, from January 2014 to September 2014. Inclusion criteria were: gestational age 37–42 week inclusive, weight at birth ≥2500 g, single birth, no congenital malformation, white parents. Exclusion criteria were: having endocrine disorders, chronic liver diseases or overweight/obesity. PKU children non-compliant with the recommended diet were also excluded. Overweight/obesity was defined in accordance with the International Obesity Task Force [13]. PKU children were defined as compliant to the diet when the annual mean Phe levels, monitored monthly by the Guthrie test [14], was within the range 120–360 μmol/L. Phenylketonuric children were detected by a newborn screening test and periodically monitored in our department since diagnosis.

A medical history was collected at recruitment from parents by a standardized questionnaire during a personal interview conducted by the same pediatrician that also saw the children for a general examination, and evaluated the Tanner stage of puberty [15]. Moreover, the pediatrician took anthropometric measurements of children, assisted by an experienced operator. Body weight and height were measured using a mechanical column scale (seca 711; seca GmbH & KG, Hamburg, Germany) with an integrated measuring rod (seca 220; seca GmbH & KG). The body mass index (BMI) was calculated from the ratio of weight to height squared (kg/m^2). BMI z-scores were calculated and adjusted for age and gender by using Cole’s LMS method [16] and Italian reference data [17]. The parents of eligible children or their legal guardian received a detailed explanation of the study, and signed a consent form. The hospital Ethics Committee approved the study protocol and gave ethical clearance.

Dietary assessment and daily GI and GL

For each child, the dietary intake, including beverages, was recorded by means of a food diary filled out by parents for three consecutive days (two weekdays and one weekend day). Parents received instructions about the method for weighing and recording food. They were trained by a dietitian to weigh each food item offered to their child before consumption, to weigh leftovers and to record the weights each time [18]. Quantification and analysis of the energy intake and nutrient composition were performed

with an ad hoc PC software (MètaDieta®, Me.Te.Da S.r.l., San Benedetto del Tronto, Italy). Vegetable intake quantification excluded tuber and legumes in accordance with the World Health Organization (WHO) [19].

For each food, the GI value was derived from the Italian [11] or International [20–22] tables. If there was a discrepancy between the tables [20–22] then the mean value was used. For a food not included in the Italian or International tables, the GI value was determined at the Department of food science, University of Parma (Italy), using the Food and Agriculture Organization/World Health Organization's method [23] and in accordance with the International Standards Organization guideline [24]. Then, to find out the GI of these foods healthy volunteers were recruited, after an informed consent was signed. On the day before the test the volunteers consumed a standardized dinner. Foods with known content of fermentable dietary fiber were forbidden in order to avoid any second meal effect. After fasting overnight, volunteers were tested for assessed foods (with 500 mL of still mineral water) once each, and the standardized food (50 g of glucose) three times, in random order on separate days. Blood samples were taken by finger-prick. Whole capillary blood glucose was measured by an automatic analyzer (YSI Stat2300, YSI Inc., Yellow Springs, OH). Blood samples were collected at 15, 30, 45, 60, 90 and 120 min after starting to eat.

Mean GI of each meal, was calculated by the following formula [25]:

$$GI_{meal} = (\Sigma_{i=1,\ \ldots,\ n}\ GI_{food\ i} * \text{grams of carbohydrates}_{food\ i})/\text{total grams of carbohydrates}_{meal}$$

Daily mean GI, was calculated as:

$$GI_{daily} = (\Sigma_{i=1,\ \ldots,\ n}\ GI_{meal\ i} * \text{grams of carbohydrates}_{meal\ i})/\text{daily total grams of carbohydrates}$$

The glycemic load for each meal was calculated:

$$GL_{meal} = \Sigma_{i=1,\ \ldots,\ n}\ GL_{food\ i}$$

Biochemistry

Fasting blood samples of our study population were analyzed at the hospital laboratory of biochemistry for total cholesterol, high-density-lipoprotein (HDL) cholesterol, low-density-lipoprotein (LDL) cholesterol, triglycerides, insulin and glucose on the Cobas® 6000 analyzer series, c501 and e601 modules (Roche Diagnostics GmbH, Hoffmann-La Roche ltd, Mannheim, Germany).

The triglycerides/HDL ratio was calculated as a predictor of smaller dense low-density-lipoprotein, a cardiovascular risk factor [26]. The homeostatic model assessment of insulin resistance (HOMA-IR) mainly reflecting insulin resistance in the liver, was calculated as the product of fasting glucose (mmol/L) and fasting insulin (μU/mL) divided by 22.5 [27]. The quantitative insulin sensitivity check (QUICK) index was calculated as 1/(log10 fasting plasma insulin in μU/mL + log10 glucose in mg/dL) to evaluate insulin sensitivity [28]. Pancreatic β-cell function was evaluated by HOMA-β as (20 × fasting insulin in μU/mL)/(fasting glucose in mmol/L−3.5) [27]. The triglyceride glucose index (TyG index), that seems to reflect mainly peripheral insulin resistance, was calculated as Log [fasting triglycerides (mg/dL) × fasting glucose (mg/dL)/2] [29,30].

Statistical analysis

Descriptive data are reported as mean and standard deviation (SD), and median and the 25th–75th centile. Normality of the distribution of continuous variables was assessed by the Kolmogorov–Smirnov test. For continuous variables, comparison between groups was performed by the Student's t-test and the Mann–Whitney or Wilcoxon test, as appropriate. Triglycerides, insulin, and HOMA-IR were not normally distributed, and log 10-transformation was used for analysis. For clarity of interpretation, results are expressed as untransformed values. The association of dietary daily glycemic index, glycemic load and GL/1000 kJ with glucose-metabolism and lipid variables was assessed by Spearman's correlation coefficient. All values of $p < 0.05$ were considered to indicate statistical significance (two-tailed test). The statistical package for social sciences (SPSS) version 20.0 (SPSS Inc., Chicago, IL, USA) for Windows (Microsoft, Redmond, WA, USA) was used for the statistical analysis.

Results

The sample examined comprised 9 boys and 12 girls for each group. Mean (SD) age of children was 8.0 (2.2) years. No significant difference between PKU and healthy children was found for BMI z-score (−0.08 (0.74) vs. 0.03 (0.65), $p = 0.801$). In PKU children, mean (SD) blood Phe levels at the recruitment was 243.7 (72.3) μmol/L.

Dietary intake of energy, macronutrients, fiber, fruit and vegetables are reported in Table 1. PKU children consumed lower proteins and fats, and higher carbohydrates (% of total energy) and fibers than healthy children. The recommended carbohydrate intake was exceeded by 71.4% of PKU children and 19.0% of controls ($p = 0.002$). All PKU children had a lower protein intake than the recommended upper limit compared to 57.1% of healthy children ($p = 0.001$). No significant difference between the two groups was observed for the proportion of children who were within the recommended range for energy and fat (minimum $p = 0.454$). Ten (47.6%) PKU children and 5 (23.8%) controls were in line with the recommendation for fiber intake (≥8.4 g/1000 kcal), $p = 0.197$. PKU children had a greater fiber intake from vegetables than controls ($p = 0.002$) and consumed more vegetables ($p = 0.013$). Table 2 shows the overall daily GI and GL of diet and of each meal in PKU and healthy children. Overall dietary GI, as well as at breakfast, lunch and dinner, was higher in PKU than healthy children ($0.001 < p < 0.026$). Overall dietary GL, as well as at lunch and dinner, was higher in PKU than healthy children ($0.004 < p < 0.011$). The mean

Table 1 Daily dietary intake of energy, macronutrients, fiber, fruit and vegetables in phenylketonuric (PKU) and healthy children.

Variable	PKU children (n = 21)		Healthy children (n = 21)		*P*-value[a]	Reference values[b]
	Mean (SD)	Median (25th–75th centile)	Mean (SD)	Median (25th–75th centile)		
Energy						
kcals	1480.5 (298.4)	1443.0 (1276.6–1795.3)	1540.3 (245.8)	1517.5 (1313.3–1746.6)	0.570	Boys: 1400–2610 kcal (AR) Girls: 1290–2360 kcal (AR)
kcals/kg weight	59.2 (13.4)	59.3 (46.6–69.2)	58.3 (14.1)	59.9 (48.9–67.4)	0.760	
kJ	6194.5 (1248.5)	6037.5 (5341.2–7511.4)	6444.7 (1028.2)	6349.3 (5494.7–7307.8)	0.570	
Protein						
g	36.6 (11.4)	34.8 (28.4–43.5)	57.6 (10.3)	55.0 (49.5–63.4)	0.001[d]	16–39 g (AR)
% energy	9.8 (1.9)	9.6 (8.3–11.2)	15.0 (1.7)	14.8 (14.1–16.2)	0.001[d]	12–15% (RI)
g/kg weight	1.4 (0.3)	1.5 (1.3–1.6)	2.1 (0.4)	2.1 (1.9–2.3)	0.002[d]	boys: 0.76–0.79 g/kg (AR) girls: 0.76–0.77 g/kg (AR)
Carbohydrate						
g	235.4 (58.7)	230.3 (193.5–276.5)	213.2 (31.9)	213.0 (188.5–235.0)	0.065	
% energy	63.4 (6.8)	63.1 (58.8–66.8)	55.6 (4.9)	55.7 (53.0–59.5)	0.004[d]	45–60% (RI)
Fat						
g	45.3 (12.7)	43.9 (37.7–55.1)	54.1 (12.7)	52.7 (44.3–63.9)	0.025[d]	
% energy	27.9 (7.0)	28.8 (23.4–31.4)	31.5 (4.5)	30.7 (28.4–35.7)	0.028[d]	20–35% (RI)
Fiber						
Overall (g)	13.2 (4.1)	13.0 (10.8–15.6)	10.5 (2.8)	10.5 (8.4–12.0)	0.009[d]	
Overall (g)/1000 kcal	9.2 (3.6)	8.4 (7.4–9.3)	6.9 (2.2)	6.6 (7.4–9.3)	0.012[d]	8.40 g/1000 kcal (AI)
From fruit (g)	2.0 (1.8)	1.4 (0.6–3.7)	1.6 (1.4)	1.6 (0.3–2.8)	0.377	
From vegetables (g)	3.2 (1.4)	3.2 (1.9–4.0)	1.5 (1.0)	1.2 (0.7–2.3)	0.002[d]	
From other foods (g)[c]	8.0 (4.3)	7.4 (4.5–9.5)	7.3 (2.0)	7.0 (5.8–8.8)	0.338	
Fruit and vegetables						
Overall (g)	281.7 (120.5)	253.3 (179.9–360.8)	219.6 (110.6)	214.1 (155.0–273.0)	0.086	≥400 g
Fruit (g)	98.4 (84.6)	80.0 (34.0–174.5)	108.6 (74.2)	111.7 (58.3–143.3)	0.713	
Vegetables (g)	183.3 (87.0)	173.3 (120.0–213.7)	111.0 (70.6)	102.4 (63.4–144.2)	0.013[d]	

AR, average requirement; RI, reference intake; AI, adequate intake.
[a] Student's t-test or Mann–Whitney test.
[b] Energy, macronutrients and fiber [34]; amount of fruit and vegetables [19].
[c] Excluded fruit and vegetables.
[d] Statistically significant.

(SD) daily GL/1000 kJ was higher in PKU than in healthy children (23.9 (8.4) vs. 18.2 (5.0) kJ^{-1}, $p = 0.001$).

Table 3 reports daily dietary intake of energy, macronutrients and fiber consumed by Phe-free protein substitutes and special low protein products in PKU children. These products accounted for more than 60% of overall daily energy, protein and carbohydrate intake, and for about a third of fat and fiber intake. Except for protein, intake of energy, macronutrients and fiber was higher for special low protein products than for Phe-free protein substitutes (maximum $p < 0.01$).

Table 4 shows the glucose metabolism and blood lipid profile variables in PKU and healthy children. Glucose metabolism variables did not differ between PKU and healthy children (minimum $p = 0.052$), except for triglyceride glucose index that was higher in PKU children ($p = 0.020$). Compared to healthy controls, PKU children exhibited lower blood total ($p < 0.001$) and LDL ($p = 0.006$) cholesterol, higher triglyceride levels ($p = 0.014$) and higher triglycerides/HDL ratio ($p = 0.015$). In PKU children the dietary GL was associated with triglyceride glucose index (Spearman's correlation coefficient = 0.515, $p = 0.034$). Association of dietary GL with blood triglycerides was close to statistical significance (Spearman's correlation coefficient = 0.446; $p = 0.064$). Neither GI nor GL were associated with any other metabolic variables (minimum $p = 0.104$).

Discussion

This is the first study evaluating the dietary glycemic index and glycemic load in PKU children on a strict diet.

The results showed that while daily energy intake was similar between PKU and healthy children, as previously reported [31,32], PKU children had lower fat and protein intake, and higher carbohydrate and fiber intake, as also observed in English/French [33] and Greek (except protein) [31] PKU children. Anyhow, it should be pointed out that in PKU subjects protein intake should be higher than the recommended value for healthy subjects [34,35], due to the decreased protein utilization for amino acids that

Table 2 Glycemic index and glycemic load of overall daily diet and meals in phenylketonuric (PKU) and healthy children.

Variable	PKU children (n = 21)		Healthy children (n = 21)		*P*-value[a]
	Mean (SD)	Median (25th–75th centile)	Mean (SD)	Median (25th–75th centile)	
Glycemic index					
Overall diet	62.2 (5.8)	61.6 (58.9–66.9)	48.5 (8.4)	50.6 (45.0–53.9)	<0.001[c]
Breakfast	51.6 (9.5)	52.8 (46.2–58.2)	40.8 (15.6)	42.7 (26.8–50.1)	0.026[c]
Morning snack[b]	48.8 (18.2)	42.6 (34.4–64.6)	49.7 (12.0)	46.7 (39.1–61.5)	0.792
Lunch	70.9 (5.5)	70.9 (66.4–76.1)	52.5 (4.5)	53.5 (48.4–56.2)	<0.001[c]
Afternoon snack	51.5 (11.5)	50.5 (44.8–57.9)	48.1 (11.8)	50.9 (42.3–57.4)	0.319
Dinner	68.2 (6.8)	70.0 (65.3–72.8)	50.5 (6.6)	52.6 (46.6–54.2)	0.005[c]
Glycemic load					
Overall diet	148.2 (35.2)	151.5 (123.4–173.2)	116.5 (33.9)	110.6 (95.0–132.3)	0.007[c]
Breakfast	17.8 (9.6)	15.9 (11.2–25.7)	14.6 (11.2)	12.2 (6.4–20.9)	0.327
Morning snack[b]	6.2 (8.3)	3.9 (1.0–7.1)	9.0 (4.9)	9.3 (5.3–13.0)	0.957
Lunch	53.3 (14.6)	51.6 (44.9–62.9)	38.5 (12.1)	36.0 (30.2–45.3)	0.004[c]
Afternoon snack	19.4 (12.6)	16.3 (10.2–27.3)	14.4 (8.6)	12.1 (7.7–23.7)	0.173
Dinner	55.2 (20.3)	53.2 (43.0–60.8)	38.3 (13.9)	40.7 (29.8–49.8)	0.011[c]

[a] Student's t-test or Mann–Whitney test.
[b] Nine PKU children and 9 healthy children did not report any morning snack during the food diary recording.
[c] Statistically significant.

Table 3 Daily dietary intake of energy, macronutrients and fiber, consumed by phenylalanine (Phe)-free protein substitutes and special low protein products in phenylketonuric children (n = 21).

Variable	Phe-free protein substitutes and special low protein products		Phe-free protein substitutes		Special low protein products		*P*-value[a]
	Mean (SD)	Median (25th–75th centile)	Mean (SD)	Median (25th–75th centile)	Mean (SD)	Median (25th–75th centile)	
Dietary intake							
Energy (kcal)	891.3 (239.9)	873.5 (764.0–1062.6)	204.4 (73.9)	211.0 (155.2–249.2)	686.9 (238.7)	684.5 (577.6–830.5)	<0.001[b]
Protein (g)	25.9 (8.9)	22.9 (19.6–31.4)	24.4 (8.6)	21.1 (18.0–30.8)	1.5 (0.7)	1.5 (0.8–2.0)	<0.001[b]
Carbohydrate (g)	161.5 (49.6)	154.5 (126.5–196.7)	17.8 (10.7)	17.3 (11.0–25.2)	143.6 (51.3)	138.5 (110.7–176.6)	<0.001[b]
Fat (g)	14.8 (6.2)	16.1 (10.4–18.7)	4.1 (4.3)	3.8 (0.1–7.1)	10.8 (5.8)	9.5 (5.9–16.5)	0.003[b]
Fiber (g)	4.8 (4.3)	3.7 (1.8–6.6)	0.04 (0.10)	0 (0.0–0.0)	4.7 (4.3)	3.7 (1.8–6.6)	<0.001[b]
Dietary intake/overall dietary intake							
Energy (%)	61.2 (14.4)	64.5 (57.6–70.7)	14.2 (6.3)	13.7 (10.6–16.3)	46.5 (14.3)	47.4 (43.9–56.2)	<0.001[b]
Protein (%)	70.1 (10.1)	72.1 (64.3–76.8)	65.9 (9.9)	68.0 (60.1–73.4)	4.1 (2.1)	4.0 (2.5–6.1)	<0.001[b]
Carbohydrate (%)	69.7 (16.8)	76.7 (61.3–79.6)	8.3 (6.2)	6.6 (4.1–12.0)	61.5 (17.7)	66.9 (56.3–71.3)	<0.001[b]
Fat (%)	32.9 (10.8)	34.9 (26.9–39.8)	9.2 (10.3)	6.3 (0.2–16.2)	23.8 (10.1)	22.6 (19.0–28.9)	0.004[b]
Fiber (%)	33.7 (21.3)	27.2 (17.8–51.6)	0.2 (0.5)	0 (0.0–0.0)	33.5 (21.1)	27.1 (17.8–51.6)	<0.001[b]

[a] Student's t-test or Mann–Whitney test (Phe-free protein substitutes versus special low protein products).
[b] Statistically significant.

are the primary source of protein in PKU disease [4]. Indeed, in this study, all PKU children had a protein intake lower than 12.3% of energy. The 71.4% and 14.3% of PKU children had higher carbohydrate and fat intakes, respectively, than the recommended upper limit for healthy children and 52.4% of children had an inadequate fiber intake [34]. Within the healthy group 19.0%, 42.9% and 28.6% of children exceeded the recommended intake for carbohydrate, protein and fat, respectively, while 76.2% had inadequate intake of fiber [34]. Unlike healthy children, PKU children showed an adequate mean fiber intake.

No data have yet been published on GI and GL in the PKU diet. This study showed that overall dietary GI and GL were higher in PKU than in healthy children. Only 9.5% of healthy children and none of the PKU children had GI $\leq$ 45, which could be associated with reduced risk of chronic disease in adult cohort studies [36]. This cut-off value remains to be further investigated in children. Additionally, PKU children showed higher GI and GL values at lunch and dinner and a higher GI value at breakfast than did healthy children. This could be due to consumption of special low protein products mainly in these specific meals. The special low protein products accounted for about 50% of total energy intake and more than 60% of carbohydrate intake, suggesting the importance of management of these products for possibly improving the dietary intake. Some special low protein products are commonly added with glucose, dextrose or sugar as ingredients, which may increase their glycemic index [6]. A higher carbohydrate composition than conventional foods was also found in 92% of special low protein products in a recent Portuguese study [37].

PKU children did not show alteration in blood glucose and insulin metabolism, despite the higher overall diet GI

Table 4 Glucose metabolism and blood lipid profile of phenylketonuric (PKU) and healthy children.

Variable	PKU children (n = 21)		Healthy children (n = 21)		*P*-value[b]	Reference values [35]
	Mean[a] (SD)	Median (25th–75th centile)	Mean[a] (SD)	Median (25th–75th centile)		
Glucose metabolism						
Glucose (mmol/L)	4.3 (0.3)	4.4 (4.1–4.6)	4.5 (0.4)	4.5 (4.2–4.8)	0.335	<5.55 mmol/L
Insulin (pmol/L)[c]	32.6 (13.3)	29.9 (20.1–42.4)	44.5 (17.8)	38.9 (35.3–57.1)	0.073	≤104.18 pmol/L
HOMA-IR[c]	0.9 (0.4)	0.8 (0.6–1.3)	1.3 (0.6)	1.2 (1.0–1.6)	0.063	
HOMA β[c]	137.1 (80.4)	103.2 (80.0–206.2)	151.8 (82.4)	128.2 (86.0–200.6)	0.424	
QUICK Index[c]	0.4 (0.0)	0.4 (0.4–0.4)	0.4 (0.03)	0.4 (0.4–0.4)	0.052	
Triglyceride Glucose Index	3.4 (0.2)	3.4 (3.4–3.5)	3.3 (0.2)	3.3 (3.2–3.4)	0.020[d]	
Lipid profile						
Total Cholesterol (mmol/L)	3.4 (0.5)	3.3 (3.1–3.7)	4.4 (0.8)	4.4 (3.8–4.9)	<0.001[d]	<4.66 mmol/L
LDL Cholesterol (mmol/L)	1.9 (0.4)	1.9 (1.6–2.2)	2.6 (0.6)	2.4 (2.1–3.1)	0.006[d]	<3.37 mmol/L
HDL Cholesterol (mmol/L)	1.4 (0.3)	1.4 (1.1–1.6)	1.5 (0.4)	1.5 (1.2–1.8)	0.078	>1.04 mmol/L
Triglycerides (mmol/L)	0.9 (0.4)	0.8 (0.7–1.0)	0.6 (0.2)	0.6 (0.5–0.8)	0.014[d]	boys: 0.34–1.41 mmol/L girls: 0.36–1.48 mmol/L
Triglycerides/HDL	1.6 (0.9)	1.4 (1.1–2.2)	1.0 (0.5)	0.8 (0.7–1.2)	0.015[d]	

HDL, high-density-lipoprotein; HOMA-IR, homeostatic model assessment of insulin resistance; LDL, Low-density-lipoprotein; QUICK, quantitative insulin sensitivity check. SI conversion factors: to convert glucose, divide values by 0.0555; to convert insulin, divide values by 6.945; to convert cholesterol, divide values by 0.0259; to convert triglycerides, divide values by 0.0113.

[a] Adjusted for Tanner stage of puberty.

[b] Student's t-test or Mann–Whitney test.

[c] Insulin value was not available in one PKU child.

[d] Statistically significant.

and GL than healthy controls. A Brazilian study evaluated insulin and HOMA in normal weight PKU children (mean age 9 years) observing a median of 26.4 pmol/L and 0.7, respectively [38], that are similar to values found in this study.

A positive association was observed between the overall diet GL and the triglyceride glucose index, suggesting a possible relationship between carbohydrate quality diet and peripheral insulin resistance. It should be pointed out that the lack of association between GL and other insulin sensibility and resistance indices, might be at least in part due to the low protein intake in the PKU diet. A higher protein intake, mainly from animal foods, could stimulate secretion of an insulin-like growth factor I (IGF-I) and insulin [39,40]. Moreover it should be noted that in this study insulin resistance and insulin sensitivity have been evaluated by surrogate indices rather than by a hyperinsulinemic-euglycemic clamp (the gold-standard method).

Regarding blood lipid profile, PKU children exhibited lower total and LDL cholesterol and higher triglyceride levels than did healthy controls, in accordance with previous studies [10,31]. These results may be attributed, at least in part, to the PKU diet, which is characterized by the avoidance of animal foods [3,38], and to a higher than the recommended upper limit of carbohydrate intake [34] in PKU children. Lastly, it should be noted that a relationship might exist between dietary GL and triglycerides, as previously found both in healthy children [41] and in adults [42].

Within the limitations of this study, it is possible to conclude that in PKU children the dietary treatment could be associated with higher dietary GI, GL and blood triglyceride level and lower total and LDL cholesterol levels. Moreover a relationship between the quality of carbohydrates consumed and the triglyceride glucose index may exist. An improvement towards an optimal diet for PKU children could include additional attention to the management of dietary carbohydrate quality, with particular focus on special low protein products. Longitudinal, adequately-powered studies are needed to clarify the mutual interrelationships between the diet and metabolic profile of PKU children.

Conflict of interest

The authors declare no conflict of interest.

Funding sources

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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Chapter 3:
METABOLIC PROFILE AND RISK OF CARDIOVASCULAR DISEASE IN CHILDREN WITH HYPERPHENYLALANINEMIA

3.1 Introduction

Reduction of animal origin lipids and cholesterol intakes, which is one of the main characteristics of the PKU diet, could be considered a non-atherogenic diet [1].

Arteriosclerosis is related to the presence and intensity of the known cardiovascular risk factors like family history, age, gender, nutrition/diet, physical inactivity, lipid levels, overweight/obesity, diabetes mellitus, inflammatory markers [2]. It has a long, asymptomatic, preclinical period. Pathological changes in the arteries of children develop decades before clinical manifestation of the disease. Some authors suggest that specific types of lesions occurring in younger ages (fatty streaks) are transformed into another type of lesions (fibrous plaques) in young adults and middle-aged persons, while others demonstrate that the progression from fatty streaks to plaque might be arrested in childhood and does not begin to a significant extent until after puberty in males and after menopause in females [3].

Carotid artery intima–media thickness (cIMT) is considered a significant predictive marker of generalized atherosclerosis because of its correlation with coronary artery disease and it may predict future cardiovascular events in adults [4]. It is also recommended by the American Heart Association as a noninvasive imaging parameter for detecting atherosclerosis [5,6]. In 2007, Lorenz *et al*. published a systematic review and meta-analysis of eight relevant general population-based studies that had reported on the ability of cIMT to predict future CV end points, involving a total of 37,197 subjects followed for a mean of 5.5 years [7]. They reported that for an absolute cIMT difference of 0.1 mm, the future risk of myocardial infarction increases by 10–15%, and the stroke risk increases by 13–18%.

The primary aim of this study was to determine early atherosclerotic changes (evaluated by cIMT), and several cardiovascular-related risk factors (for example blood pressure (BP), plasma total homocysteine (tHcy) levels, Apolipoprotein B/Apolipoprotein A1, lipoprotein (a)) in children with PKU, on phe-restricted diet and in MHP and in healthy children, on unrestricted diet. The secondary aim was to evaluate whether an association may exist between cIMT and their dietary glycemic index and glycemic load.

3.2 Materials and methods

This observational case-control study examined 31 PKU children (age 4–13 years) gender and age (± 6 months) matched with 31 MHP children and 31 healthy controls, consecutively admitted to the Department of Pediatrics, San Paolo Hospital, Milan, from March 2014 to

September 2015. Inclusion criteria were: gestational age 37–42 week inclusive, weight at birth ≥ 2500 g, single birth, no congenital malformation, white parents. Children with endocrine disorders and chronic liver diseases were excluded. PKU children non-compliant with the recommended diet were also excluded. PKU children were defined as compliant to the diet when the annual mean Phe levels, monitored monthly by the Guthrie test [8], was within the range 120–360 μmol/L. Phenylketonuric children were detected by a newborn screening test and periodically monitored in our department since diagnosis.

A medical history was collected at recruitment from parents by a standardized questionnaire during a personal interview conducted by the same pediatrician that also saw the children for a general examination, and evaluated the Tanner stage of puberty [9]. Moreover, the pediatrician took anthropometric measurements of children, assisted by an experienced operator. Body weight and height were measured using a mechanical column scale (seca 711; seca GmbH & KG, Hamburg, Germany) with an integrated measuring rod (seca 220; seca GmbH & KG). The body mass index (BMI) was calculated from the ratio of weight to height squared (kg/m^2). BMI z-scores were calculated using WHO Anthro Software [10]. Waist circumference (WC) was measured using the measuring tape seca 203 (seca GmbH & KG) to the nearest 0.1 cm at the mid-point between the iliac crest and the lower edge of the ribs at the end of a normal expiration. WC was defined high when was above 95^{th} percentile [11]. Blood pressure (BP) was measured by an oscillometric technique with Dinamap Procare blood pressure monitor (GE Medical Systems, Freiburg, Germany). Measurement and definition of high blood pressure were in accordance with the US National High Blood Pressure Education Program [12]. High blood pressure was defined as systolic BP and/or diastolic BP above the 95^{th} percentile according to gender, age, and height; while prehypertension was defined as systolic BP and/or diastolic BP from the 90^{th} to 95^{th} percentile on repeated measurements Physical activity of children included in the present study was assessed by validated questionnaires [13,14].

The parents of eligible children or their legal guardian received a detailed explanation of the study, and signed a consent form. The hospital Ethics Committee approved the study protocol and gave ethical clearance.

Dietary assessment and daily GI and GL

For each child, the dietary intake, including beverages, was recorded by means of a food diary (showed in figure 1) filled out by parents for three consecutive days (two weekdays and one weekend day). Parents received instructions about the method for weighing and recording food. They were trained by a dietitian to weigh each food item offered to their child before

consumption, to weigh leftovers and to record the weights each time [15]. Quantification and analysis of the energy intake and nutrient composition were performed with an ad hoc PC software (MètaDieta®, Me.Te.Da S.r.l., San Benedetto del Tronto, Italy). Vegetable intake quantification excluded tuber and legumes in accordance with the World Health Organization (WHO) [16]. Dietary glycemic index and dietary glycemic load were performed using a previously described method [17] (Chapter 2).

Diario alimentare

Data/giorno: _____ (giorno 1)

Ora/pasto	Luogo (casa, scuola)	Alimenti + bevande consumate (preparazione, marca, tipo, caratteristiche…)	Peso a cotto al netto del piatto	Quantità avanzata al netto del piatto

Figure 1. Example of food diary (in Italian).

Biochemistry

Fasting blood samples of study population were analyzed at the hospital laboratory of biochemistry for total cholesterol, high-density-lipoprotein (HDL) cholesterol, low-density-lipoprotein (LDL) cholesterol, triglycerides (Tg), insulin, apolipoprotein A1 (ApoA1) and apolipoprotein B (ApoB), lipoprotein (a), glucose, vitamin B12 (B12), homocysteine (Hcy), folate and high sensitivity C-Reactive Protein (hsCRP) on the Cobas® 6000 analyzer series, c501 and e601 modules (Roche Diagnostics GmbH, Hoffmann-La Roche ltd, Mannheim, Germany).

The Apolipoprotein B/Apolipoprotein A1 ratio was calculated to represent the balance between proatherogenic and atherogenic lipoproteins, it was considered a better predictor of cardiovascular risk in adults than any of the cholesterol indexes [18,19].

The LDL cholesterol/Apolipoprotein B and triglycerides/HDL cholesterol ratios were calculated as a predictor of the low-density-lipoprotein size and have been used as marker of atherogenicity of LDL cholesterol [1,20-22]. Moreover, Tg/HDL cholesterol was calculated to identify individuals with atherogenic dyslipidemia, cardiometabolic risk [23] and ratio ≥ 2.2 has been recently suggested as predictor of impaired glucose tolerance in overweight/obese youth [24].

The homeostatic model assessment of insulin resistance (HOMA-IR), mainly reflecting insulin resistance in liver, was calculated as the product of fasting glucose (mmol/L) multiplied for fasting insulin (μU/mL) divided by 22.5 [25]. Insulin resistance was defined as HOMA-IR >3.16 according to the most recent cut-off for the pediatric population [26]. The quantitative insulin sensitivity check (QUICK) index was calculated as 1/(log10 fasting plasma insulin in μU/mL + log10 glucose in mg/dL) to evaluate insulin sensitivity [27]. Pancreatic β-cell function was evaluated by HOMA-β as (20 × fasting insulin in μU/mL)/(fasting glucose in mmol/L−3.5) [25].

The triglyceride glucose index (TyG index), that seems to reflect mainly peripheral insulin resistance, was calculated as Log [fasting triglycerides (mg/dL) × fasting glucose (mg/dL)/2] [28,29].

Degree of background inflammation was measured by level of hsCRP. Values 1 to < 3 mg/l were considered to be mildly elevated, and those ≥3 mg/l were considered to be elevated [30].

Regarding tHcy, only reference values for adults were found in the literature [31]. Elevated plasma homocysteine levels (>12 μmol/l; moderate hyperhomocysteinemia) were considered cytotoxic [31].

The atherogenic index of plasma (AIP), a strong predictor of atherosclerosis [33] was calculated as: Log [fasting triglycerides (mg/dL)/ fasting HDL (mg/dL)]. AIP value of under 0.11 is

associated with low risk of CVD; the values between 0.11 to 0.21 and upper than 0.21 are associated with intermediate and increased risks, respectively [33].

Ultrasound examination

All ultrasound examinations of the study were performed with the ultrasound device: GE Logiq 9, GE Healthcare Medical System, Milwaukee, Wisc., USA, 10–12 MHz. The combined thickness of the intimal and medial arterial wall components of the common carotid artery was determined with a linear transducer by an experienced pediatrician. Subjects were examined in a supine position with a slightly overextended neck in a two-dimensional presentation of the longitudinal view of the vessel. cIMT of the far wall was measured 1–2 cm below the bifurcation of the common carotid artery at the late diastolic phase.

Measurement of fecal calprotectin

FC concentration was measured in samples using the CALPROLAB Calprotectin ELISA (Calpro, Lysaker, Norway) kit. The lower detection limit of the assay is 15.6 mg/kg.

Statistical Analysis

Descriptive data are reported as mean and standard deviation (SD), and median and the 25^{th}–75^{th} centile. Normality of the distribution of continuous variables was assessed by the Kolmogorov-Smirnov test. Comparison among groups was performed by Kruskal–Wallis test or ANOVA, as appropriated.

The association of dietary daily glycemic index, glycemic load and cardiovascular risk factors was assessed by Pearson's or Spearman's correlation coefficient as appropriate. Finally, we adjusted the obtained *p*-values using the Bonferroni correction. The statistical package for social sciences (SPSS) version 20.0 (SPSS Inc., Chicago, IL, USA) for Windows (Microsoft, Redmond, WA, USA) was used for the statistical analysis.

3.3 Results

The examined sample was comprised of 14 males and 17 girls for each group. Mean (SD) age of children was 7.5 (2.9) years. No significant differences among PKU, MHP and healthy children were found for anthropometric measurements and systolic and diastolic blood pressure (Table 1). In PKU children, annual mean (SD) blood Phe levels was 294.8 (128.3) μmol/L.

Table 1. Anthropometric measurements and systolic and diastolic blood pressure in PKU, MHP and healthy children.

	PKU children (n=31)	**MHP children (n=31)**	**Healthy children (n=31)**	***P*-value**
Variable	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	
Weight, kg	27.73 (12.06)	29.60 (13.08)	31.18 (12.99)	0.572
Height, m	1.23 (0.16)	1.27 (0.20)	1.29 (0.21)	0.840
BMI, z-score	0.67 (0.98)	0.67 (1.21)	0.53 (0.82)	0.938
Waist circumference, cm	57.65 (7.26)	58.41 (8.98)	60.30 (9.39)	0.687
Systolic blood pressure, mm Hg	101.97 (10.00)	101.04 (11.39)	99.90 (9.85)	0.737
Diastolic blood pressure, mm Hg	57.71 (6.47)	57.04 (6.30)	57.55 (8.92)	0.970

Dietary intake of energy, macronutrients, fiber, fruit and vegetables are reported in Table 2. PKU children consumed lower proteins and higher carbohydrates (% of total energy) and fibers than MHP and healthy children. Moreover, PKU consumed a higher fruit and vegetables (overall and only vegetables) intake than MHP and healthy children. The recommended carbohydrate intake [34] was exceeded by 74.2% of PKU children and by 19.4% of MHP and controls. Moreover, it should be noted that 16% of healthy children consumed carbohydrate intake less than recommended. Only 1 (3.1%) PKU, 11 (35.5%) MHP and 13 (41.9%) healthy children exceeded the recommended upper level for protein intake. Six (19.4%) PKU and MHP and 7 (22.6%) healthy children exceeded recommended lipid intake. Regarding the fiber intake, sixteen (51.6%) PKU, 3 (9.7%) MHP children and 2 (6.5%) healthy controls were in line with recommendation (≥8.4 g/1000 kcals). The World Health Organization (WHO) recommendation for fruit and vegetables consumption (≥400g/day) was reached by 8 (25.8%) PKU children and 1(3.1%) healthy children, no one of MHP children.

Overall daily GI and GL of diet and of each meal in PKU, MHP and healthy children are shown in Table 3. Overall dietary GI, as well as at breakfast, lunch and dinner, was higher in PKU than MHP and healthy children ($0.001<p<0.006$). Overall dietary GL, as well as at lunch and dinner, was higher in PKU than MHP and healthy children ($p<0.001$).

Carotid intima media thickness was observed thinner in PKU children than healthy children (0.36[0.05] vs 0.41[0.10] mm, respectively, $p=0.004$).

Table 4 shows the glucose metabolism in PKU, MHP and healthy children. Glucose metabolism variables did not differ among groups (minimum $p=0.153$), except for blood glucose that was lower in PKU children than healthy controls ($p=0.003$) and HOMA β that was higher in PKU children than healthy controls ($p= 0.049$). The condition of inuslin resistance (HOMA-IR > 3.16) was observed in 4 overweight/obese subjects (2 healthy children, 1 PKU and 1 MHP children).

Regarding blood lipid profile variables, as shown in Table 5, PKU children exhibited lower

blood cholesterols, apolipoprotein A1, higher triglyceride levels, triglycerides/HDL ratio and and atherogenic index of plasma than MHP and healthy children (maximum p=0.036). Percentages of PKU, mild MHP and healthy children with acceptable, borderline, and high/low plasma lipid, and apolipoprotein concentrations are shown in table 6.

Blood vitamins B (folate and vitamin B12) levels were observed higher in PKU children than MHP and healthy children, conversely PKU children showed lower homocysteine levels than other two groups (p <0.001), as shown in Table 7. Concerning inflammatory variables, hsCRP was lower in PKU children than MHP and healthy controls (PKU:1.54[3.38] mg/L, MHP:3.93[8.37] mg/L, healthy: 2.13[2.70] mg/L, respectively, p=0.012), whereas no statistical difference was found in fecal calprotectin among groups (PKU: 20.52[15.91] mg/kg, MHP: 27.65[30.66] mg/kg, healthy: 15.01(12.98) mg/kg, p=0.359). Results of physical activity questionnaires are shown in table 8. No statistical differences were observed among the three groups.

Associations with risk factors

In PKU group inverse associations between dietary glycemic index and Apo B (R = -0.396; p = 0.024), ApoB/ ApoA1 (R = -0.466; p = 0.012) were observed. When correlation analyses between overall glycemic load and cardiovascular risk factors were assessed, positive association between overall glycemic load and HOMA (R = 0.411 p = 0.030) and negative association between overall glycemic load and QUICKI were found.

Moreover, correlation analysis between cIMT and potential arteriosclerotic risk factors were assessed in PKU group. No associations of cIMT with overall glycemic index and glycemic load were observed. Instead, associations of cIMT and BMI z score (R = 0.552; p = 0.001), total cholesterol (R = 0.329; p = 0.07), LDL cholesterol (R = 0.358; p = 0.048), insulin (R = 0.423; p = 0.022), HOMA-IR index (R = 0.396; p = 0.033), Lp(a) (R = 0.494; p = 0.006), tyg index (R = 0.374; p = 0.038) were found. The associations between insulin, HOMA-IR index and cIMT were significant also after adjustment for gender, age, BMI z score and Tanner stage.

No association was observed between cIMT and annual mean Phe.

Table 2. Daily dietary intake of energy, macronutrients, fiber, fruit and vegetables in phenylketonuric (PKU), mild hyperphenylalaninemic (MHP) and healthy children.

	PKU children (n=31)	**MHP children (n=31)**	**Healthy children (n=31)**	***P*-value**
Variable	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	**Pku-Mhp-Healhty**
Energy				
kcals	1443.56(268.11)	1528.11(261.51)	1506.89(410.92)	0.539
kcals/kg weight	57.25(15.24)	58.27(19.69)	56.47(21.31)	0.784
Protein				
g	35.96(10.50)[a,b]	54.30(11.09)[a]	54.53(15.23)[b]	<0.001*
% energy	10.06(2.45)[a,b]	14.28(2.37)[a]	14.65(2.81)[b]	<0.001*
g/kg weight	1.39(0.36)[a,b]	2.07(0.80)[a]	2.05(0.84)[b]	<0.001*
Carbohydrate				
g	229.03(46.61)	211.53(37.52)	201.30(62.17)	0.098
% energy	63.20(6.35)[a,b]	55.48(5.67)[a]	53.35(7.31)[b]	<0.001*
Fat				
g	44.46(15.54)[b]	52.83(15.76)	53.05(14.23)[b]	0.037*
% energy	27.39(6.84)[b]	30.68(5.10)	32.08(5.59)[b]	0.017*
Fiber				
overall (g)	14.02(5.65)[a,b]	9.78(3.49)[a]	9.27(3.17)[b]	0.001*
overall (g)/1000 kcal	10.02(4.46)[a,b]	6.41(1.82)[a]	6.10(1.59)[b]	<0.001*
Fruit and vegetables				
overall (g)	304.00(150.09)[a,b]	193.65(80.05)[a]	178.31(82.16)[b]	0.002*
fruit (g)	125.86(107.60)	112.29(74.33)	93.40(73.55)	0.683
vegetables (g)	178.15(73.04)[a,b]	81.36(41.59)[a]	84.91(60.10)[b]	<0.001*

MHP vs. Heathy children: no significant difference for any dietary varibles.

[a] $p<0.05$ for PKU vs. MHP children.

[b] $p<0.05$ for PKU vs. Healthy children.

* Statistically significant

Table 3. Glycemic index and glycemic load of overall daily diet and meals in phenylketonuric (PKU), mild hyperphenylalaninemic (MHP) and healthy children.

	PKU children	MHP children	Healthy children	*P*-value
Variable	**Mean (SD) Median[25th;75th]**	**Mean (SD) Median[25th;75th]**	**Mean (SD) Median[25th;75th]**	**Pku-Mhp-Healhty**
Glycemic index				
Overall diet	64.4(7.93)[a,b] 63.38 [59.03;66.51]	54.92(4.22)[a,c] 54.58[52.91;55.74]	51.01(4.25)[b,c] 50.86[48.62;53.27]	<0.001*
Breakfast	56.50(30.48)[b] 52.97[45.35;56.65]	54.35(30.76)[c] 48.38[40.57;55.76]	39.17(11.68)[b,c] 43.27[29.62;47.33]	0.001*
Morning snack§	47.36(17.43) 41.27[35.43;61.65]	53.96(11.98) 52.00[47.56;63.26]	43.38(18.93) 46.07[31.15;62.00]	0.161
Lunch	69.11(6.70)[a,b] 70.94[63.76;75.06]	55.53(8.69)[a] 54.38[49.97;59.50]	52.38(9.59)[b] 54.92[49.86;57.99]	<0.001*
Afternoon snack§§	49.61(10.93)[a] 48.31[41.09;56.35]	56.92(9.86)[a,c] 55.00[50.85;61.30]	49.65(9.24)[c] 49.68[44.88;55.48]	0.016*
Dinner	69.69(5.01)[a,b] 69.54[66.18;74.17]	53.45(8.47)[a] 51.72[46.82;61.73]	53.33(10.56)[b] 53.81[48.20;58.09]	<0.001*
Glycemic load				
Overall diet	147.55(27.18) [a,b] 149.63[125.36;171.55]	113.85(24.66)[a] 106.86[99.54;135.90]	107.68(33.15)[b] 108.02[83.85;123.21]	<0.001*
Breakfast	18.89(11.53) 17.26[10.22;25.45]	17.44 (8.67) 16.96[11.00;22.03]	13.79(9.62) 11.60[5.74;20.54]	0.125
Morning snack§	7.43(7.61) 4.69[1.98;12.36]	7.60(5.53) 5.38[2.82;12.74]	8.15(6.61) 5.64[2.76;15.33]	0.787
Lunch	53.17(14.33)[a,b] 50.63[41.51;62.14]	37.53(14.68)[a] 33.36[26.38;47.29]	40.06(17.47)[b] 37.33[30.94;51.17]	<0.001*
Afternoon snack§§	15.38(13.34) 10.31[5.14;18.24]	16.35(9.54) 13.28[8.79;25.13]	13.83(8.26) 15.26[6.92;19.50]	0.547
Dinner	53.80 (19.48) [a,b] 53.55[43.38;64.71]	38.64(16.60)[a] 38.74[24.48;48.36]	34.83(18.90)[b] 37.71[18.94;49.49]	<0.001*

§ Thirteen PKU children; 14 MHP and 14 healthy children did not report any morning snack during the food diary recording.
§§Four PKU children; 4 MHP children; 5 healthy children did not report any afternoon snack during the food diary recording
[a] $p<0.05$ for PKU vs. MHP children.
[b] $p<0.05$ for PKU vs. Healthy children.
[c] $p<0.05$ for MHP vs. Healthy children
*Statistically significant.

Table 4. Glucose metabolism of phenylketonuric (PKU), Mild HyperPhenylalaninemic (MHP) and healthy children.

	PKU children	**MHP children**	**Healthy children**	***P* – value**
Variable	**Mean (SD)** **Median [25th;75th]**	**Mean (SD)** **Median [25th;75th]**	**Mean (SD)** **Median [25th;75th]**	**Pku-Mhp-Healhty**
Glucose, mg/dL	78.81 (6.90)[b] 79.00[74.75; 83.00]	78.40(6.09)[c] 79.00[74.75; 82.00]	84.35(5.77)[b,c] 86.00[80.00; 89.00]	<0.001*
Insulin, µU/mL	5.98 (3.85) 5.20[3.40; 7.00]	5.84(2.96) 4.80[4.30;5.80]	6.36(3.48) 5.70[4.28; 8.20]	0.621
HOMA-IR	1.18(0.85) 1.02[0.66-1.29]	1.14(0.60) 0.93[0.80-1.18]	1.33(0.78) 1.21[0.84-1.66]	0.288
HOMA β	148.47(78.43)[b] 122.40[91.43;174.86]	161.70 (103.86)[c] 133.50[99.66;207.00]	112.10(71.86)[b,c] 92.40[71.55;128.35]	0.021*
Log HOMA-IR	-0.01(0.27) 0.01[-0.18; 0.11]	0.01(0.18) -0.03[-0.10; 0.07]	0.06(0.24) 0.08[-0.08; 0.22]	0.288
QUICK Index	0.39(0.04) 0.38[0.37; 0.41]	0.38(0.03) 0.39[0.37; 0.40]	0.38(0.04) 0.37[0.35; 0.39]	0.287
Triglycerides/HDL	1.75(1.11)[a,b] 1.40[1.06; 2.11]	1.23(0.62)[a] 1.14[0.76; 1.43]	1.11(0.60)[b] 0.88[0.78; 1.19]	0.004*
Triglyceride Glucose Index	3.46(0.18) 3.43[3.37; 3.53]	3.38(0.16) 3.39[3.29; 3.48]	3.39(0.15) 3.38[3.29; 3.47]	0.153

HOMA-IR, homeostatic model assessment of insulin resistance; QUICK, quantitative insulin sensitivity check.
[a] $p<0.05$ for PKU vs. MHP children.
[b] $p<0.05$ for PKU vs. Healthy children.
[c] $p<0.05$ for MHP vs. Healthy children
* Statistically significant.

Table 5. Blood lipid profile of phenylketonuric (PKU), Mild HyperPhenylalaninemic (MHP) and healthy children.

	PKU children	**MHP children**	**Healthy children**	***P* – value**
Variable	Mean (SD) Median [25th;75th]	Mean (SD) Median [25th;75th]	Mean (SD) Median [25th;75th]	**Pku-Mhp-Healhty**
Cholesterol				
Total, mg/dL	134.55(20.56)[a,b] 135.00[121.00; 148.00]	155.78(28.07)[a] 155.00[133.00; 170.00]	164.23(25.80)[b] 159.00[148.00; 184.00]	<0.001*
LDL, mg/dL	79.61(15.87)[b] 81.00[66.00; 88.00]	93.72(27.64) 91.00[72.50;116.50]	92.94(22.42)[b] 90.00[80.00; 106.00]	0.036*
HDL, mg/dL	50.03(11.38)[b] 48.00[38.00; 59.00]	59.42(24.76) 55.00[45.75; 64.75]	59.29(11.00)[b] 59.00[53.00; 65.00]	0.018*
LDL/HDL	1.67(0.51) 1.62[1.22; 2.03]	1.74(0.81) 1.63[1.19; 2.02]	1.62(0.48) 1.55[1.34; 2.05]	0.951
Total/HDL	2.79(0.59) 2.74[2.20; 3.24]	2.88(0.94) 2.78[2.33; 3.23]	2.83(0.55) 2.90[2.49; 3.23]	0.852
Triglycerides, mg/dL	80.10(37.32)[b] 68.00[60.00; 84.00]	64.70(21.35) 65.00[51.00;76.00]	61.32(22.32)[b] 59.00[48.00; 70.00]	0.037*
Triglycerides/HDL	1.75(1.11)[a,b] 1.40[1.06; 2.11]	1.23(0.62)[a] 1.14[0.76; 1.43]	1.11(0.60)[b] 0.88[0.78; 1.19]	0.004*
Atherogenic Index of Plasma	-0.18(0.23)[a,b] -0.22[-0.33; -0.04]	-0.33(0.23)[a] -0.31[-0.48; -0.21]	-0.36(0.19)[b] -0.42[-0.47; -0.29]	0.004*
Apolipoprotein A-1, mg/dL	128.17(16.04)[b] 127.00[114.75; 141.50]	133.48(18.45) 134.00[120.00; 148.00]	143.88(18.38)[b] 142.00[133.00; 156.00]	0.011*
Apolipoprotein B, mg/dL	65.60(10.76) 65.00[57.65; 72.25]	71.48(19.20) 67.00[59.00; 85.00]	72.64(20.27) 65.00[61.50; 84.00]	0.397
Apo B/Apo A1	0.52(0.09) 0.51[0.45; 0.58]	0.55(0.19) 0.54[0.40; 0.59]	0.52(0.19) 0.45[0.39; 0.58]	0.526
LDL/Apo B	1.21 (0.10) 1.20 [1.14;1.27]	1.04 (0.52) 1.26 [1.18;1.34]	1.32 (0.42) 1.27 [1.16;1.54]	0.084
Lp(a), mg/dL	17.54(21.89) 9.00[3.00; 21.50]	19.24(26.25) 7.50[4.00; 21.25]	17.29(16.78) 8.50[4.00;28.75]	0.815

HDL, High-density-lipoprotein; LDL, Low-density-lipoprotein; Apo, Apolipoprotein; Lp(a), Lipoprotein (a)
MHP vs. Heathy children: no significant difference for any variables.
[a] $p<0.05$ for PKU vs. MHP children.
[b] $p<0.05$ for PKU vs. Healthy children.
*Statistically significant.

Table 6. Number (%) of phenylketonuric (PKU), mild hyperphenilalaninemic (MHP) and healthy children with acceptable, borderline, and high/low plasma lipid, and apolipoprotein concentrations.

	PKU children	**MHP children**	**Healthy children**	
Reference values [2]	**n (%)**	**n (%)**	**n (%)**	***P* – value**
Total cholesterol				
Acceptable [< 170 mg/dl]	29 (93.5)[b]	22(7.,0)	20 (64.5)[b]	0.019*
Borderline-high [170-199 mg/dl]	2 (6.5)[b]	5 (16.1)	9 (29.0)[b]	
High [≥ 200 mg/dl]		1(3.2)	2(6.5)[b]	
LDL cholesterol				
Acceptable [< 110 mg/dl]	27 (87.1)[a]	16 (51.6)[a]	25(80.6)	0.040*
Borderline [110-129 mg/dl]	1 (3.2)[a]	6(19.4)[a]	4(12.9)	
High [≥ 130 mg/dl]		1(3.2)[a]	2(6.5)	
Apolipoprotein B				
Acceptable [< 90 mg/dl]	31(100)	25 (80.6)	21(67.7)	0.285
Borderline [90-109 mg/dl]		5 (16.1)	2(6.5)	
High [≥ 110 mg/dl]		1 (3.2)	2(6.5)	
Triglycerides				
Acceptable [0-9y: < 75mg/dl; 10-19 y: < 90 mg/dl]	19 (61.3)[b]	21(67.7)	27(87.1)[b]	0.048*
Borderline [0-9y: 75-99 mg/dl; 10-19 y: 90-129 mg/dl]	7 (22.6)[b]	4(12.9)	3(9.7)[b]	
High [0-9y: ≥ 100 mg/dl; 10-19 y: ≥ 130 mg/dl]	4 (12.9)[b]	2(6.5)		
HDL cholesterol				
Low [< 40 mg/dl]	8 (25.8)	2(6.5)		0.795
Borderline [40-45 mg/dl]	2 (6.5)	4(12.9)	3 (9.7)	
Acceptable [> 45 mg/dl]	21 (67.7)	20(64.5)	28 (90.3)	
Apolipoprotein A-1				
Low [< 115 mg/dl]	7(22.6)	6(19.4)	1 (3.2)	0.285
Borderline [115-120 mg/dl]	1(3.2)	1 (3.2)		
Acceptable [> 120 mg/dl]	22(71.0)	22(71.0)	24 (77.4)	

MHP vs. Heathy children: no significant difference for any variable.

[a] $p<0.05$ for PKU vs. MHP children.

[b] $p<0.05$ for PKU vs. Healthy children.

* Statistically significant

Table 7. Vascular variables and vitamins in phenylketonuric (PKU), Mild HyperPhenylalaninemic (MHP) and healthy children.

	PKU children	MHP children	Healthy children	*P* – value
Variable	**Mean (SD)** **Median [25th;75th]**	**Mean (SD)** **Median [25th;75th]**	**Mean (SD)** **Median [25th;75th]**	**Pku-mhp-healhty**
Folate, ng/ml	17.35(5.23)[a,b] 21.00[13.53; 21.00]	5.33(2.76)[a] 4.62[3.80; 7.85]	6.78(3.83)[b] 5.5[4.30;7.97]	<0.001*
Vitamin B 12, pg/mL	783.30(216.82)[a,b] 786.50[689.50; 967.50]	569.90(179.86)[a] 574.00[438.00; 702.00]	558.4(208.78)[b] 571.00[380.75;700.00]	<0.001*
Homocysteine, µmol/L	4.53(1.17) [a,b] 4.43[3.83; 5.09]	6.76(1.72) [a] 6.52[5.50; 7.49]	5.78(1.71) [b] 5.5[4.55;6.62]	<0.001*

MHP vs. Heathy children: no significant difference for any varible.
[a]$p<0.05$ for PKU vs. MHP children.
[b]$p<0.05$ for PKU vs. Healthy children.
*Statistically significant.

Table 8. Physical activity of phenylketonuric (PKU), mild hyperphenilalninemic (MHP) and healthy children.

	PKU children (n=31)	MHP children (n=31)	Healthy children (n=31)	
Variable	**Mean(SD)**	**Mean(SD)**	**Mean(SD)**	***P* – value†**
Minutes spent watching TV/playing video games	123.79 (56.97)	112.57(75.43)	133.18 (95.86)	0.645
Physical activity of children ≤ 6 years				
Hours spent playing outside	2.96 (1.55)	2.88 (1.21)	3.04 (1.54)	0.998
Physical activity of children > 6 years				
Total Score	7.33 (1.10)	6.88 (1.40)	6.48 (3.03)	
Sport Score	3.14 (7.44)	3.03 (0.76)	3.69 (1.28)	0.388
Leisure Score	2.63 (0.89)	2.82 (0.76)	3.21 (0.82)	0.089
Work Score	1.42 (0.58)	1.19 (0.82)	1.02 (0.93)	0.246

†Kruskal Wallis test or One way ANOVA.

3.4 Discussion

The aim of this work was to determine cardiovascular risk factors in PKU children, on low-Phe diet, in MHP and in healthy children, on unrestricted diet, matched for age, sex and BMI z score and to evaluate whether an association may exist between the carbohydrate quality of the diet and the risk factors in PKU children.

The nutritional results, regarding the differences between PKU and healthy children, were in line with [17] (Chapter 2). Although daily energy intake was similar between PKU and healthy children, PKU children consumed lower proteins and fats, and higher carbohydrates (% of total energy) and fibers intakes than healthy children. When PKU diet, phe-restricted diet, was compared with MHP unrestricted diet, similar differences (except for no statistical difference was observed between the two groups regarding fat intakes) were observed. Overall glycemic index and glycemic load of PKU diet were shown higher than MHP and healthy children.

To our knowledge this is the first study that analyzes cIMT in PKU children. A reduced carotid intima media thickness was found in PKU children than healthy children (0.36 [0.05] vs 0.41[0.10], p=0.004). These findings were in contrast with Htun et al. [35], who evaluated cIMT in adult with PKU, they showed no differences between PKU and adult healthy controls (0.43[0.02] vs 0.40 [0.01], respectively) [35]. Instead, in accordance with the results observed in this study, the carotid intima media thickness of subjects adhering to vegan diet, considered similar to PKU diet [36], was observed thinner than subjects adhering to Western diet [37].

Differences among the 3 groups regarding blood parameters and fecal calprotectin concentrations were analyzed below.

Regarding blood lipid profile, PKU children showed lower total, LDL, HDL cholesterol levels in accordance with previous studies [1,38,39]. Similar results were observed also when lipid profile of PKU children was compared with that of MHP children. PKU children recruited in the present study had, also, lower Apo A1 levels than healthy children, but this result did not find confirmation in literature [1,39]. However, PKU children showed higher triglyceride levels and triglycerides/HDL than healthy as observed in previous studies [38,39]. Compared with MHP children higher triglyceride levels were observed in PKU children. These findings may be attributed, at least in part, to the PKU diet, which is characterized by avoiding animal foods [40, 41], and to a higher than the recommended upper limit of carbohydrate intake [34]. Moreover, atherogenic index of plasma, a strong predictor of atherosclerosis [32] was observed higher in PKU children, although in all groups the mean AIP value was under 0.11, associated with low risk of CVD in adults [33].

Consistent with a previously published study [42], our data confirmed decreased plasma Hcy levels in PKU patients compared with healthy children and MHP children. In contrast, Schulpis et al. showed that PKU patients, on strict diet, had higher Hcy concentrations (moderate hyperhomocysteinemia) than healthy children. Certainly, these divergences are determined by vitamin B12 and folate levels, both essentials in Hcy metabolism. Indeed, Shulpis et al. [39] found low vitamin B12 and folate levels in PKU children, differently from results showed in this study.

No significant difference among groups was found regarding fecal calprotectin concentrations. This result was in accordance with the only available study in literature that evaluated the fecal calprotectin concentrations in young-adult PKU compared with healthy patients [43], that did not observe difference in fecal calprotectin between the two groups.

Associations between GI/GL and cardiovascular risk factors were assessed in PKU group.

Inverse associations between dietary glycemic index and Apo B (R = -0.396; $p = 0.024$), ApoB/ ApoA1 (R = -0.466; $p = 0.012$) were found. In the literature are present only few results on the relationship between Apo B and carbohydrate quality [44]. When correlation analyses between overall glycemic load and cardiovascular risk factors were assessed, positive association between overall glycemic load and HOMA (R = 0.411 $p = 0.030$) and negative association between overall glycemic load and QUICKI were found. No associations between glycemic index and glycemic load of the diet and intima media thickness were observed in PKU group. Few studies investigated the influence of glycemic index diet on carotid intima media thickness [45,46]. An Italian study testing the effect of two hypocaloric diets (high-glycemic vs low-glycemic index diet) on weight loss and markers of subclinical atherosclerosis in obese children, observed that low glycemic diet performed much better than high glycemic diet in reducing carotid stiffening [45]. Instead, a Spanish study that assessed the association between glycemic index and glycemic load of the diet and intima media thickness in a population (mean age: 67 years) at high cardiovascular risk with no clinical symptoms, did not find significant association between IG or CG and cIMT [46].

Moreover, associations between cIMT and cardiovascular risk factors in PKU group were evaluated. Positive associations were observed between insulin, HOMA-IR and cIMT also after adjustment for gender, age, BMI z score and Tanner stage. Several observational studies (in overweight/obese chidren and in children with diabetes mellitus type 1) demonstrated an association between insulin resistance and carotid intima media thickness [47,48]. The association between cIMT and insulin resistance was observed also by Fang et al. [49]. They explained this association affirming that insulin not only directly stimulates the expression of

vascular cell adhesion molecule but disrupts the balance between the production of nitric oxide and endothelin-1 leading to endothelial dysfunction [49].

Moreover, in PKU group a positive association between cIMT and total, LDL cholesterol was found, although it was not significant after confounding factors. Indeed, it is well known in literature that carotid IMT is affected by serum lipids [49-51].

Although a study conducted in 297 healthy children observed an inverse association between cIMT and ApoA1 (R = -0.16; p = 0.01) and a positive association with ApoB (R = 0.13; p=0.02) and ApoB/ApoA1 ratio (R = 0.15; p = 0.01) at 5 years, in PKU group no associations were observed between lipoprotein levels and cIMT [52]. Other studies found, also, associations of cIMT with homocysteine levels and physical activity, but in the present study these associations were not observed [53,54].

In conclusion, the nutritional data obtained in this study showed significant differences between phe-restricted diet and omnivore diet (followed by MHP and healthy children). Compared with MHP and healthy children, PKU children had a higher overall glycemic index and load, fiber and carbohydrate intakes and lower protein intakes. Fat intakes of PKU children were lower only than healthy children. Regarding cardiovascular risk factors, PKU children showed a thinner intima media thickness, lower total cholesterol, LDL cholesterol and homocysteine levels, suggesting a lower cardiovascular risk in PKU children than healthy children. Conversely, PKU children had higher atherogenic index of plasma, triglyceride levels and higher triglycerides/HDL ratio than healthy children. Because PKU children have to start a Phe restricted diet as soon as possible and to follow this throughout life, the long-term safety of this dietary treatment and its potential impact on later non-communicable diseases risk need to be better evaluated.

Well-designed longitudinal studies, with adequate statistical power, still are needed to hopefully clarify the potential underlying mechanisms relating PKU diet and cardiovascular risk factors.

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Chapter 4: FECAL SHORT-CHAIN FATTY ACIDS IN CHILDREN WITH HYPERPHENYLALANINEMIA

4.1 Introduction

The aim of this work was to compare gut microbiota biodiversity and its production of short chain fatty acids (SCFA) in PKU children, on low-Phe diet, and MHP children, on unrestricted diet. It was conducted in collaboration with the Microbiology Unit, Department of Health Sciences, University of Milan. They performed gut microbiota analysis.

4.2 Materials and methods

This observational case control study included 21 phenylketonuric (PKU) children (age 4-18 years) gender and age (±12 months) matched with 21 children with mild hyperphenylalaninemia (MHP), consecutively admitted to the Department of Pediatrics, San Paolo Hospital, Milan, from December 2014 to May 2016. Inclusion criteria were: gestational age 37–42 week inclusive, weight at birth ≥ 2500 g, single birth, white parents. Exclusion criteria were: congenital malformation, having endocrine disorders, chronic liver diseases, chronic or acute intestinal diseases and treatments with antibiotic and probiotic/prebiotic in the previous 3 months. PKU children non-compliant with the recommended diet were also excluded. PKU children were defined as compliant to the diet when the annual mean Phe levels, monitored monthly by the Guthrie test [1], was within the range 120–360 μmol/L for children below 13 years and was under 600 μmol/L for patients >13 years. Phenylketonuric children were detected by a newborn screening test and periodically monitored in our department since diagnosis. A medical history was collected at recruitment from parents by a standardized questionnaire during a personal interview conducted by the same pediatrician that also saw the children for a general examination. Moreover, the pediatrician took anthropometric measurements of children, assisted by an experienced operator. Body weight and height were measured using a mechanical column scale (seca 711; seca GmbH & KG, Hamburg, Germany) with an integrated measuring rod (seca 220; seca GmbH & KG). The body mass index (BMI) was calculated from the ratio of weight to height squared (kg/m^2). BMI z-scores were calculated and adjusted for age and gender by using WHO anthro [2].

A fresh fecal sample was self-collected at home by each enrolled subject and stored immediately at -20° C. The collection took place in the same week as the dietary record. Subsequently, the fecal samples were transported to the laboratory and stored at -20° C until further analysis. The parents of eligible children or their legal guardian received a detailed

explanation of the study, and signed a consent form. The hospital Ethics Committee approved the study protocol and gave ethical clearance.

Dietary assessment

For each child, the dietary intake, including beverages, was recorded by means of a food diary filled out by parents for three consecutive days (two weekdays and one weekend day). Parents received instructions about the method for weighing and recording food. They were trained by a dietitian to weigh each food item offered to their child before consumption, to weigh leftovers and to record the weights each time [3]. Dietary glycemic index and dietary glycemic load were performed using a previously described (Chapter 2).

Bacterial DNA extraction

Total microbial DNA extraction was performed with the Spin Stool DNA Plus Kit (Stratec Molecular, Berlin, Germany) according to manufacturer instruction, using 200mg aliquot of wet feces.

The extraction kit provides homogenization of the fecal sample in a lysis buffer and incubation at 95° C for 10 minutes. The lysed sample was mixed with the matrix in InviAdsorb to remove most of the components that inhibit PCR. Proteinase K was added to the supernatant to digest and degrade proteins after incubation at 70° C. The purification of bacterial DNA was obtained by the addition of suitable buffers to remove other impurities. The filtrate was added with an elution buffer to obtain bacterial DNA in 100 µL. The microbial DNA extract concentration and purity were evaluated by NanoDrops (Spectrophotometer ND-1000). One microliter of each bacterial DNA extract solution was read at a wavelength of 260 nm.

Short Chain Fatty Acid (SCFA) measurement

SCFA concentrations were assessed in accordance with the method proposed by Weaver et al. (1997) modified as follows. Stool (200 mg) were suspended in 1 ml of double distilled water, homogenized on vortex mixer and, after 30 min, centrifuged (15000 rpm) for 15 min at 10° C. Aliquots (500 µl) of supernatant were added with:

- 200 µl 85% orthophosphoric acid
- 200 µl 2% (v/v) sulphuric acid
- 100 µl 2-ethyl-butyric acid (iSTD, Aldrich cat n 245526) 10 mM in HCOOH 12% as

internal standard. SCFA were gently extracted for 1 min with 1 ml ethyl-ether/heptan (1:1 v/v) and centrifuged for 10 min at 3000 rpm. The aqueous phase was frozen and the organic layer was removed for analysis by a Varian 3400 CX gas liquid chromatograph equipped with a Varian 8200 CX autosampler and a HP-FFAP fused-silica capillary column (30 m, 0.53 mm i.d. with a 1-mm film). Specific chromatography conditions were: gas carrier He with flow 15 ml/min; splitting 1:10 after 20 seconds injection; injection volume of 1 µl. Injector and detector temperatures were 110 and 260° C, respectively. The initial oven temperature was 60° C and was increased by 10° C min-1 to 110° C and then by 5° C min-1 and held at 200 for 5 min. Quantification of the SCFA was obtained through calibration curves of acetic, propionic, iso-butyric, butyric and iso-valeric acid in concentrations between 0.25 mM and 10 mM (10 mM 2-ethyl-butyric acid as internal standard).

Absolute quantification analyses in real time PCR (qRT-PCR)

The quantitative analysis of fecal DNA was carried out on the main of short-chain fatty acids producing species using Real Time PCR technique (qRT-PCR). The amplification reaction was conducted using as detector system SYBR® Green I dye, a DNA intercalating agent that has a minimal fluorescence in the initial mixture and emits fluorescence at 520 nm only when it is bound to dsDNA. Therefore, fluorescence intensity depends on the initial amount of the sample. The fluorescence emission during the PCR amplification was analyzed by SDS, Sequence Detection System software, which constructed amplification curve. The measurements, from the 3rd to the 15th cycle, were considered background noise (basic level) and on these standard deviations were calculated. The threshold cycle values were calculated by determining the point at which the fluorescence exceeds 10 times the standard deviation of the basic level. To determine the initial concentration of the sample, software calculated the threshold cycle of each sample and places this value in a standard curve of precisely known concentration a standard (Figure 1).

After DNA extraction with Prepman Ultra kit (Applied Biosystems, USA), the standard curve was determined by the following control strains: *roseburia intestinalis* DSM 14610 and DSM 17677 *Faecalibacterium prausntzii* of international collection DSMZ (Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH). Absolute quantification by real-time PCR was performed using the following control strains: *uniformis Bacteroides, Lactobacillus reuteri and Bifidobacterium animali* (from Clinical Microbiology Laboratory of the Department of Health Sciences of the University of Milan).

Statistical analysis

Variables were expressed as mean (standard deviation, SD). Statistical analysis was performed using ANOVA (for the comparison of variables between groups) and Whitney U-test (for comparison between subjects), using the statistical software Graph Pad Prism (Graph Pad Software, Inc., La Jolla, CA, USA). All adjusted values of $p < 0.05$ were considered statistically significant (two-tailed test).

4.3 Results

21 PKU and 21 MHP children were enrolled in the study, 10 boys and 11 girls for each group. Mean (SD) age of children was 8.81 (3.59) years. No significant differences in anthropometric parameters (weight, age and BMI z score) and mean blood Phe level were observed between PKU and MHP children (Table 1).

Table 1. Anthropometric parameters and blood Phenylalanine (Phe) levels in phenylketonuric (PKU) and mild hyperphenylalaninemic (MHP) children.

	PKU children (n=21)	**MHP children (n=21)**	***P*-value**
Variable	**Mean (SD)**	**Mean (SD)**	
Blood Phe levels, µmol/L	263.8 (95.1)	222.1 (74.7)	0.120
Weight, kg	35.5 (10.9)	29.9 (13.4)	0.068
Height, m	1.38 (0.17)	1.29 (0.17)	0.182
BMI, z-score	0.45 (1.26)	0.30 (0.98)	0.534

Dietary intake of energy, macronutrients, fiber, fruit and vegetables are reported in Table 2. PKU children consumed lower proteins and higher carbohydrates (% of total energy) and fibers than MHP and healthy children ($0.001<p<0.022$). Moreover, PKU consumed a higher fruit and vegetables (overall and only vegetables) intakes than MHP.

Overall daily GI and GL of diet and of each meal in PKU and in MHP children are shown in Table 3. Overall dietary GI, as well as at lunch and dinner, was higher in PKU than MHP children ($0.001<p<0.002$). Overall dietary GL, as well as at lunch and dinner, was higher in PKU than MHP children ($p<0.001$).

Table 2. Daily dietary intake of energy, macronutrients, fiber, fruit and vegetables in children with phenylketonuria (PKU) and with mild hyperphenylalaninemia (MHP).

	PKU children (n=21)	**MHP children (n=21)**	
Variable	**Mean (SD)**	**Mean (SD)**	***P*-value**
Energy			
kcals	1673.49 (430.20)	1472.23 (312.04)	0.171
kcals/kg weight	49.16(9.96)	50.96(24.48)	0.531
Protein			
total, g	43.21 (14.77)	51.37(11.16)	0.022*
% energy	10.32 (1.86)	14.26 (2.27)	<0.001*
g/kg weight	1.26(0.31)	1.78(0.79)	0.070
from vegetables, g	7.49(2.23)	16.68(5.37)	<0.001*
from animal sources, g	4.76(3.28)	27.57(8.62)	<0.001*
from formula, g	28.70(12.67)	0(0)	<0.001*
Carbohydrate			
g	252.90 (71.19)	207.57(47.07)	0.060
% energy	60.98 (6.97)	56.45 (6.97)	0.042*
Fat			
g	54.60(15.67)	50.80 (15.46)	0.531
% energy	30.66 (8.10)	32.31 (4.75)	0.135
Fiber			
overall, g	15.79 (8.95)	8.87 (2.74)	0.002*
overall, g/1000 kcal	9.58(4.82)	6.15(1.84)	0.021*
Fruit and vegetables			
overall, g	373.03(194.30)	199.71(80.30)	0.004*
fruit, g	138.76 (127.46)	119.81 (55.85)	0.867
vegetables, g	234.27 (116.93)	79.93 (50.85)	<0.001*

* Statistically significant

Table 3. Glycemic index and glycemic load of overall daily diet and meals in children with phenylketonuria (PKU) and with mild hyperphenylalaninemia (MHP).

Variable	PKU children (n=22) Mean (SD)	MHP children (n=22) Mean (SD)	*P* - value
Glycemic index			
Overall diet	64.82 (5.29)	52.78 (3.78)	<0.001*
Breakfast	51.38 (19.34)	47.03 (9.63)	0.249
Morning snack[§]	44.64 (23.45)	47.16 (24.11)	0.491
Lunch	68.99 (9.12)	54.11 (7.48)	<0.001*
Afternoon snack	46.06 (14.45)	51.70 (9.24)	0.067
Dinner	68.53 (5.67)	64.66 (41.25)	0.002
After-dinner snack	18.91 (14.49)	43.44 (15.23)	0.117
Glycemic load			
Overall diet	165.11 (47.57)	104.09 (29.79)	<0.001*
Breakfast	14.80 (8.15)	15.72 (6.75)	0.819
Morning snack[§]	9.55 (8.95)	4.03 (4.25)	0.141
Lunch	61.11 (21.97)	33.47 (15.22)	<0.001*
Afternoon snack	18.01 (12.76)	14.91 (10.93)	0.526
Dinner	61.75 (23.85)	36.43 (17.53)	0.001
After-dinner snack	5.24 (4.96)	3.24 (2.63)	0.648

[§]Four PKU children and 10 MHP children did not report any morning snack during the food diary recording.

* Statistically significant.

Quantification of short chain fatty acids (SCFA)

In Table 4 are reported fecal short chain fatty acids (SCFA) concentrations in children with phenylketonuria (PKU) and with mild hyperphenylalaninemia (MHP).

Table 4 SCFA(mg/g) in children with PKU and with mild hyperphenulalaninemia MHP.

SCFA	**PKU children**	**MHP children**
mg/g of faeces	**Mean (SD)**	**Mean (SD)**
acetate	3.0 (0.8)	3.6 (1.3)
propionate	1.1(0.4)	1.1(0.4)
i-butyrate	0.2(0.1)	0.2(0.1)
butyrate	1.0(0.3)*	1.3(0.4)*
i-valerate	0.3(0.2)	0.3(0.2)
Total	5.5(1.1)*	6.5(1.4)*

SD:Standard Deviation
*Statistically significant

PKU children showed lower total fecal SCFA and butyrate production than MHP children (p=0.04 e p=0.02, respectively), Figure 1. No differences were observed between the two groups regarding acetate, propionate, butyrate, iso-butyrate and iso-valerate.

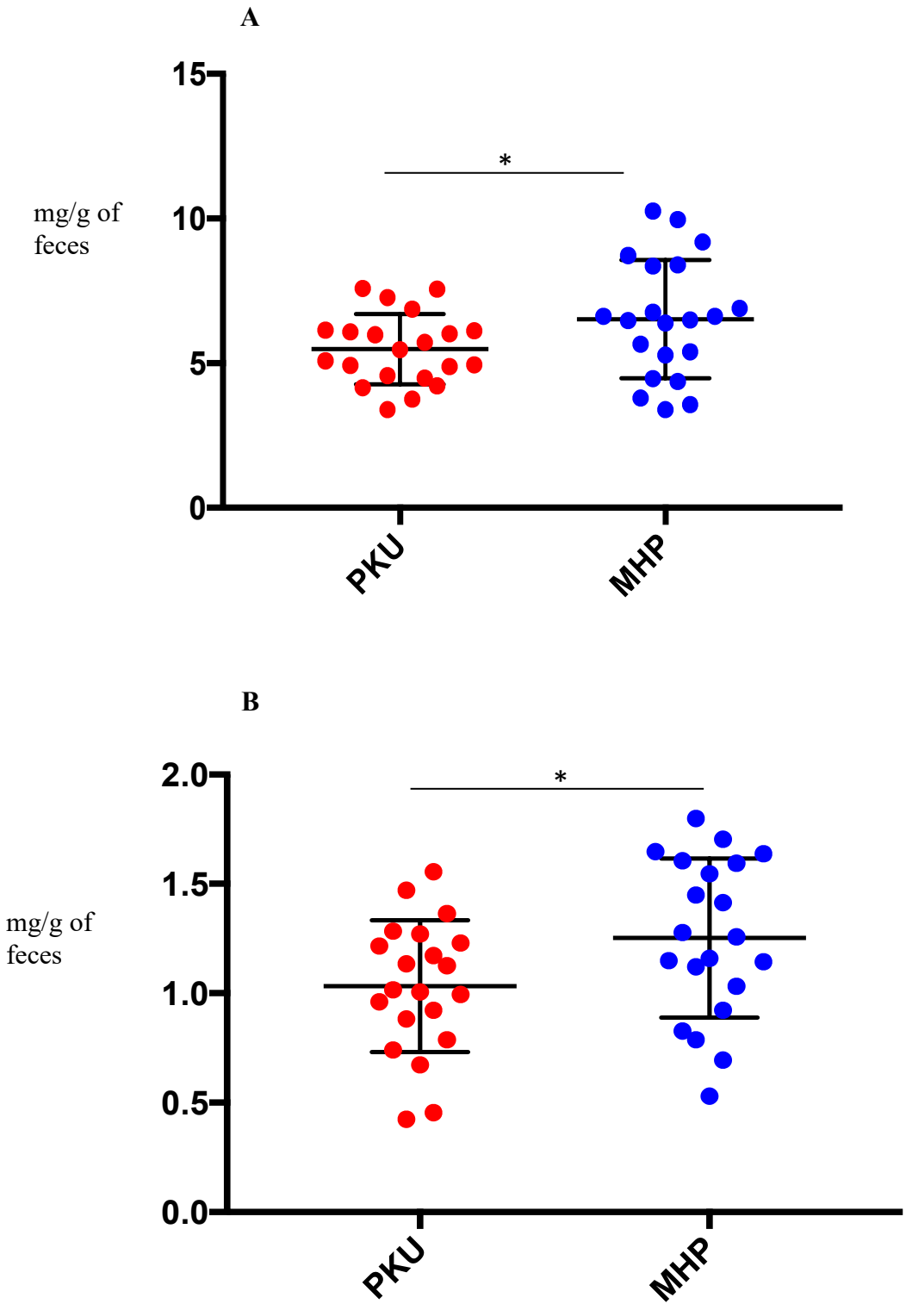

Figure 1 Total SCFA (**A**) and of butyrate (**B**) concentrations.

Absolute quantification analyses in real time PCR (qRT-PCR)

Real-Time PCR was performed to quantify the abundance of the two major groups of butyrate-producing bacteria. These analyses showed a significant reduction of *Faecalibacterium prausnitzii* species and *Roseburia spp.* (p = 0.02 and p = 0.03, respectively) in PKU compared with MHP children (Figure 2 A-B).

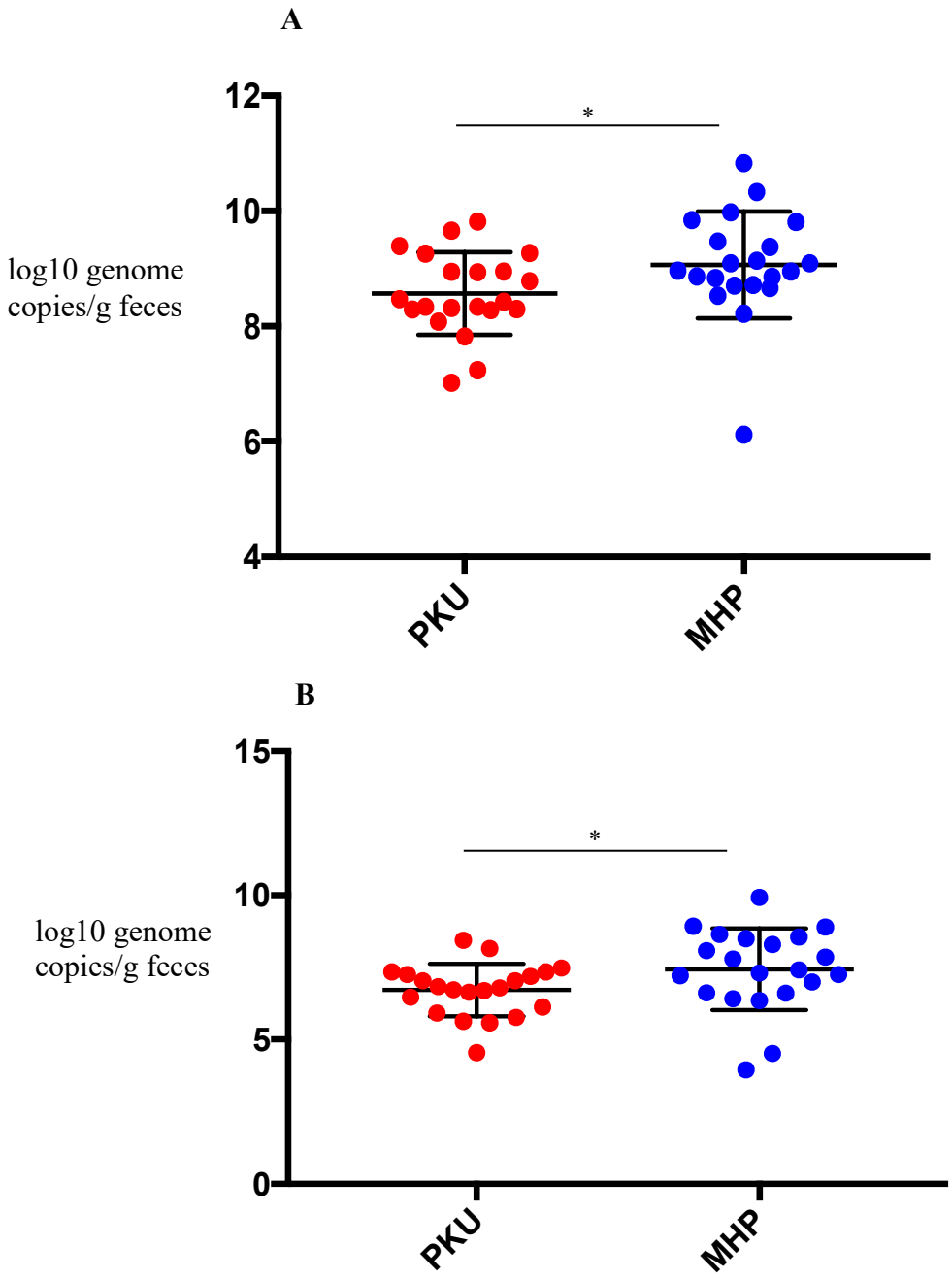

Figure 2. Microbial quantification, in phenylketonuric (PKU, red) and mild hyperphenylalaninemia (MHP, blue) children, of *Faecalibacterium prausnitzii* (A), and *Roseburia* spp (B). Abundances are expressed as log10 genome copies/g faeces.

qRT-PCR was also performed to evaluate possible differences in the abundance of two genera of lactate-producing bacteria (*Bifidobacterium spp.* e *Lactobacillus* spp).

PKU children showed a decrease in *Lactobacillus spp*. compared with MHP children (p=0.002) (Fig.3). No significant differences were observed for *Bifidobacterium spp*.

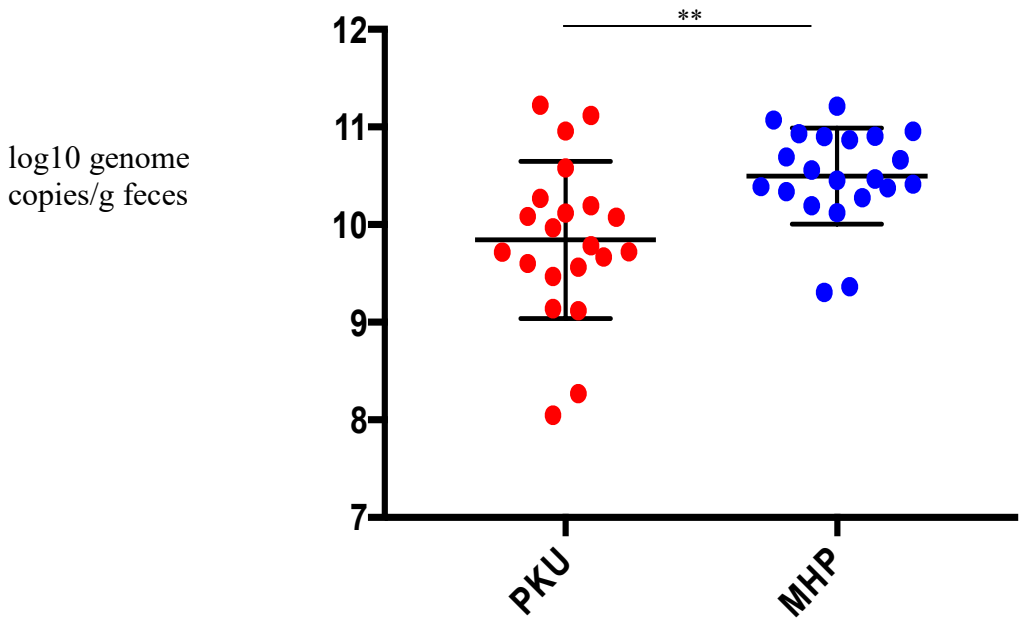

Figure 2. *Lactobacillus spp.* abundances (log10 genome copies/g faeces) in phenylketonuric (PKU, red) and mild hyperphenylalaninemia (MHP, blue) children.

4.4 Discussion

The aim of this work was to compare gut microbiota biodiversity and its production of short chain fatty acids (SCFA) in PKU children, on low-Phe diet, and MHP children, on unrestricted diet. Nutritional results showed differences in protein, carbohydrate and fiber intakes between PKU and MHP children, although similar energy intakes were observed in the two experimental groups. Specifically, PKU children showed a lower protein and higher carbohydrate and fiber intakes compared with MHP children. Moreover, vegetables intake, daily glycemic index and glycemic load were higher in PKU than MHP children.

Because changes in diet could result in different substrates for microbial fermentation, the main microbial metabolites, short chain fatty acids, were measured.

Acetate, butyrate, and propionate derive mainly from carbohydrates fermentation, whereas branched-chain fatty acids (BCFAs, 5% of total SCFAs), mainly iso-butyrate and iso-valerate, from proteins and amino acids fermentation by proteolytic bacteria [4].

Total fecal SCFA, as well as butyrate were decreased in PKU compared with MHP children. No differences were observed for acetate, propionate, and BCFAs.

Butyrate is the preferred energy source for colonocytes, and participates in maintaining gut homeostasis. Several families belonging to *Firmicutes* have a key role in butyrate production, especially *Faecalibacterium prausnitzii*, and *Eubacterium rectale/Roseburia* group [5]. Absolute quantification by qRT PCR showed decrease of both *F. prausnitzii* and *Roseburia spp.* as well as of *Lactobacillus spp.*. The latters produce lactate that can be further used as substrate for butyrate production.

These results seem to confirm what predicted by Pinheiro de Oliveira F and colleagues [6], which conducted the only available study in literature on PKU gut microbiota (a controlled study based on next-generation sequencing). The authors observed the presence of distinct bacterial taxonomic groups between PKU and healthy children and, according to metagenome prediction analysis, PKU microbiota presented fewer genes encoding for enzymes involved in starch and sucrose metabolism and in glycolysis/gluconeogenesis.

A reduction in SCFA production seems inconsistent with the higher fiber and vegetable (rich fiber foods) intakes that characterize PKU diets. Indeed, fibers are non-digestible carbohydrates that are fermented in the cecum and the large intestine by the anaerobic cecal and colonic microbiota allowing the production of short-chain fatty acids [7]. However, also the quality of fibers is a key factor in determining gut microbiota composition and its SCFA production. Studies highlighted that dietary fibers have a greater range of structures that could affect the gut

microbiota in different ways (bacteria use different metabolic methods to break down the sugar) [8]. Furthermore, Benus and colleagues, investigating the effect of dietary fiber exclusion and supplementation on the intestinal microbiota and SCFA concentrations in healthy subjects, observed large and statistically significant reductions in the abundance of *Faecalibacterium prausnitzii* and *Roseburia spp.* groups (butyrate producing bacteria analyzed also in the present study) during both fiber-free and fiber-supplemented diets. The authors demonstrated significant and strong positive correlations between *F. prausnitzii* abundance and fecal butyrate concentrations, and suggested that reduction of *Roseburia spp.* and *F. prausnitzii* during fiber-supplemented diet may be related to fibers that do not promote their proliferation [9].

DGGE analysis of fecal samples examined in this PhD thesis (data not shown) demonstrated a lower degree of microbial diversity in gut microbiota of PKU compared with MHP children. A reduced microbial richness is considered the first hallmark of gut dysbiosis. Changes in microbial population in PKU children results in altered SCFA production that could impact on, intestinal permeability.

In conclusion, the restricted PKU diet, characterized by a higher carbohydrate intake, including many simple sugars and non-digestible fiber, has been shown to increase glycemic index and glycemic load, resulting in a different quality of substrate for microbial fermentation.

Further studies using innovative sequencing techniques are needed to better investigate gut microbiota dysbiosis in PKU children and to eventually pave the way for pre/probiotic supplementations.

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ACKNOWLEDGEMENTS

I would like to thank:

- All patients and their families for kindly participating in the study.
- My tutor dr. Elvira Verduci.
- Carlotta, Benedetta, Fiammetta, Alice, Juri, Marta, Sara and all doctors who contributed to the realization of this sudy.
- Microbiology Units for the collaboration to realize microbiota analysis.

ANNEX: SCIENTIFIC PRODUCTIONS DURING PhD PERIOD

International Journal publications

- **Moretti F**; Pellegrini N; Salvatici E; Rovelli V; Banderali G; Radaelli G; Scazzina F; Giovannini M; Verduci E. Dietary glycemic index, glycemic load and metabolic profile in children with phenylketonuria. Nutr Metab Cardiovasc Dis. 2017;27:176-182.

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- **Moretti F,** Lassandro C, Pellegrini N, Radaelli G, Re Dionigi A, Verduci E. Dietary glycemic index and glycemic load in children with phenylketonuria: the effect on metabolic profile. Submitted for the "4th International Conference on Nutrition and Growth", March 2-4 2017, Amsterdam.

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