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Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfil the requirements of Master of Science degree in Biotechnology and Innovation

by
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Abstract

In Phenylketonuria (PKU), prior to BH_4 treatment, a loading test (BH_4-LT) is usually necessary to determine responsiveness. The Portuguese Society of Metabolic Disorders advocate a 72h BH_4-LT. Concurrently with a BH_4-LT, a strict dietary protocol should be followed, but adherence with this is rarely reported.

The aim of this study was to compare prescribed natural protein (NP, g/kg), Phenylalanine (Phe, mg/kg), L-amino acid supplements (AA, g/kg) and total protein (TP, g/kg) with reported dietary intake during 72h BH_4-LT's.

The sample included 78 PKU patients (20.9 ± 9.1 y; 4-48 y; 51% females; 41% classical PKU, 49% mild PKU, 4% hyperphenylalaninemia and 6% late diagnosed) who had a BH_4-LT between March 2015 and January 2017. Potential BH_4 responsiveness was considered with a blood [Phe] reduction $\geq$ 30%. Anthropometry, NP, Phe intake, AA and TP prescriptions were documented. A 3-day diet diary was used to calculate mean daily nutritional intake during BH_4-LT and to compare with diet prescription.

Prescribed NP and Phe were similar with reported nutritional intakes during BH_4-LT (0.80 ± 0.46 vs. 0.77 ± 0.44 g/kg, p=0.106; 38.13 ± 22.74 vs. 36.73 ± 21.37 mg/kg, p=0.116, respectively). In contrast, reported AA and TP intakes were significantly lower compared with dietary prescription (1.01 ± 0.37 vs. 1.05 ± 0.35 g/kg, p=0.006; 1.64 ± 0.49 vs. 1.71 ± 0.49 g/kg, p=0.003, respectively). Potential BH_4 responders (n=33) reported Phe and NP intakes in accordance with dietary prescription (43.10 ± 24.41 vs. 43.37 ± 24.26 mg/kg, p=0.922; 0.90 ± 0.50 vs. 0.91 ± 0.49 g/kg, p=0.721, respectively), while non-responders (n=45) reported lower Phe and TP intakes (32.05 ± 17.69 vs. 34.30 ± 21.00 mg/kg, p=0.048; 1.57 ± 0.50 vs. 1.66 ± 0.50, p=0.004, respectively). Children (n=29) had reported ingestions compliant with dietary prescription and adults (n=49) had a mean intake of AA and TP during the BH4-LT inferior to the amounts prescribed (0.94 ± 0.37 vs. 1.00 ± 0.35, p=0.007; 1.47 ± 0.39 vs. 1.56 ± 0.46, p=0.018, respectively). 57 of 78 patients (73.1%) reported ingestion of non-prescribed food items: e.g. soft drinks (47.4%), cakes and sweet desserts (26.3%) and potato chips (24.6%).

The results demonstrated incomplete dietary adherence with prescribed dietary protocols during BH_4-LT. It is important to fully monitor and support patients during BH_4-LT to ensure adequate consumption of prescribed NP, TP and AA in order to aid accuracy of outcome with BH_4-LT.

Keywords: *Phenylketonuria; BH4 loading test; diet; compliance*

Resumo

Antes de se iniciar tratamento com BH_4, os doentes com Fenilcetonúria (PKU) são submetidos a um teste de sobrecarga (BH_4-LT), para identificar os potenciais respondedores. A Sociedade Portuguesa de Doenças Metabólicas estipula um teste de 72h. Durante o BH_4-LT o plano alimentar deve ser estritamente cumprido e mantido constante. No entanto, existe pouca informação relativamente à adesão dos doentes à dieta.

Este estudo teve como objetivo comparar o aporte prescrito de proteína natural (NP, g/kg), fenilalanina (Phe, mg/kg), suplementação em aminoácidos (AA, g/kg) e proteína total (TP, g/kg) com a ingestão nutricional registada durante o BH_4-LT de 72h.

A amostra incluiu 78 doentes com PKU (20.9 ± 9.1 anos; 4-48 anos; 51% feminino; 41% PKU clássica, 49% PKU moderada, 4% HPA e 6% diagnóstico tardio) submetidos ao BH_4-LT entre março de 2015 e janeiro de 2017. Foi considerada como resposta positiva uma redução nos níveis sanguíneos de [Phe] $\geq$ 30%. Foram recolhidos dados antropométricos e os aportes de NP, Phe, AA e TP prescritos. Os diários alimentares de 3 dias foram utilizados para calcular a ingestão nutricional média diária durante o BH_4-LT e comparar com a dieta prescrita.

O aporte de NP e Phe prescrito foi semelhante à ingestão reportada durante o BH_4-LT (0.80 ± 0.46 vs. 0.77 ± 0.44 g/kg, p=0.106; 38.13 ± 22.74 vs. 36.73 ± 21.37 mg/kg, p=0.116, respetivamente). Pelo contrário, a ingestão reportada de AA e TP foi significativamente inferior quando comparada com a prescrição alimentar (1.01 ± 0.37 vs. 1.05 ± 0.35 g/kg, p=0.006; 1.64 ± 0.49 vs. 1.71 ± 0.49 g/kg, p=0.003, respetivamente). Os potenciais respondedores à BH_4 (n=33) reportaram ingestões de Phe e NP concordantes com a prescrição alimentar (43.10 ± 24.41 vs. 43.37 ± 24.26 mg/kg, p=0.922; 0.90 ± 0.50 vs. 0.91 ± 0.49 g/kg, p=0.721, respetivamente), enquanto que os não-respondedores (n=45) reportaram ingestões diminuídas de Phe e TP (32.05 ± 17.69 vs. 34.30 ± 21.00 mg/kg, p=0.048; 1.57 ± 0.50 vs. 1.66 ± 0.50, p=0.004, respetivamente). Os doentes pediátricos (n=29) reportaram ingestões nutricionais em conformidade com os aportes prescritos; os adultos (n=49) tiveram uma ingestão média de AA e TP durante o BH_4-LT inferior às quantidades prescritas (0.94 ± 0.37 vs. 1.00 ± 0.35, p=0.007; 1.47 ± 0.39 vs. 1.56 ± 0.46, p=0.018, respetivamente). 57 dos 78 pacientes (73.1%) reportaram a ingestão de alimentos que não estavam previstos na dieta - ex.: refrigerantes (47.4%), bolos e sobremesas doces (26.3%) e batatas fritas (24.6%).

Os resultados demonstraram uma adesão incompleta à dieta durante o BH_4-LT. É importante monitorizar e dar apoio aos pacientes durante o BH_4-LT para garantir a ingestão adequada de NP, TP e AA, e assim melhorar a precisão dos resultados do BH_4-LT.

Palavras-chave: *Fenilcetonúria; teste de sobrecarga à BH4; dieta; conformidade*

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Contents

List of abbreviations

AA – L-amino acid supplements
BH_4 – Tetrahydrobiopterin
BMI – Body Mass Index
CGM-CHP – Centro de Genética Médica do Centro Hospitalar do Porto
EMA – European Medicines Agency
FDA – US Food and Drug Administration
GMP – Glycomacropeptide
HPA – Hyperphenylalaninaemia
IQ – Intelligence Quotient
LAT1 – Large Neutral Amino Acid Transporter 1
LNAA – Large Neutral Amino Acids
LT – Loading Test
NP – Natural Protein
PAH – Phenylalanine Hydroxylase
PFCT – Portuguese Food Composition Table
Phe – Phenylalanine
PKU – Phenylketonuria
PS – Protein Substitutes
RCT – Randomized Controlled Trial
SLPF – Special Low Protein Foods
SPSS – Statistical Package for the Social Sciences
TP – Total Protein
Tyr – Tyrosine
WHO – World Health Organization

Phenylketonuria: definition, diagnose and pathophysiology

Phenylketonuria (PKU) is an inborn metabolic disease, characterised by an inability to break down the essential amino acid phenylalanine (Phe) into tyrosine (Tyr), which increases the plasmatic concentrations of Phe, leading to hyperphenylalaninaemia (HPA). This is caused by mutations in the phenylalanine hydroxylase (PAH) gene, resulting in a deficiency or inactivity of this hepatic enzyme (1). Different gene mutations (there are over 950 known (2)) have different impacts on the PHA activity, which can result in a complete inactivity of the enzyme (severe phenotypes) or in milder forms of the disease, where the activity of PAH is only partially affected (1). However, in 2% of the cases, HPA is due to defects in the synthesis of tetrahydrobiopterin (BH_4), the enzyme's cofactor (1).

In untreated PKU patients, the accumulation of Phe and the products of its metabolism has a toxic effect characterized by neurocognitive outcomes such as microcephaly and mental retardation, as well as other symptoms like growth impairment, eczema, seizures, decreased pigmentation and psychological disturbances (3,4). The diversity of genetic mutations identified in PKU patients can account, at least in part, for the great variety of phenotypes found.

In order to prevent neurological damage, early diagnose of the disease is essential to enable the initiation of dietary treatment soon after birth (3,5). PKU is diagnosed through newborn screening, with blood samples collected to filter paper cards (Guthrie Card). The Portuguese Commission of Early Diagnose classified disease severity according to blood Phe concentration at newborn screening (3): HPA is defined as blood [Phe] < 6 mg/dL, mild PKU as blood [Phe] ≥ 6 mg/dL and ≤ 20 mg/dL, and classical PKU as blood [Phe] > 20 mg/dL.

Although the pathophysiology of PKU is not yet clarified, altered protein metabolism and impaired neurotransmitters synthesis can possibly explain the neurological damage found in these patients (1). Phe is transported across the blood-brain barrier through the L-aminoacid transporter 1 (LAT1), that also carries other Large Neutral Amino Acids (LNAA). LAT1 has greater affinity for Phe, so high plasmatic concentrations of Phe compete with LNAA to enter the brain, limiting the transport of other amino acids for protein and neurotransmitter synthesis. Furthermore, raised levels of Phe were associated with poor myelination, decreased antioxidant defences, as well as with DNA, protein and lipid damage (1,6).

PKU treatment

It is important to initiate treatment as early as possible, in order to allow a better disease outcome. The treatment consists of a dietary therapy where protein and Phe are restricted. However, adherence and maintenance of this therapy are not always fully accomplished and other adjuvant therapeutics have emerged to improve treatment compliance and disease management.

Dietary therapy

A Phe restricted diet is the mainstay therapy for patients with PKU. The aim is to decrease the intake of Phe in order to lower its blood concentrations. Thus, food rich in Phe - protein sources, such as meat, fish, eggs, dairy, bread, rice, pasta etc., and products containing aspartame - are replaced by others with lower Phe content. The use of Phe-free protein substitutes (PS) - amino acid supplements - is essential to ensure protein requirements, since there is a restriction in natural foods (7). These PS can be responsible for the supply of 52-80% of protein and essential amino acids (8). Risk of nutritional deficiencies and consequent impaired development increases if Phe-restricted diet is not properly supplemented with PS, which allow an adequate intake of multiple nutrients, including protein, complex B vitamins, folate and magnesium (8). Also, a well distributed consumption of these products throughout the day promotes an optimized utilization of protein, improving metabolic control (8). In addition to Phe restriction and amino acid supplementation, Phe, protein and energy needs must be achieved to prevent protein catabolism which can increase blood Phe concentrations (7). To allow a better achievement of nutritional requirements, Special Low Protein Foods (SLPF) are included in the PKU diet to provide energy and variety. Depending on disease severity and natural protein tolerance, SLPF can account for up to 50% of the total energy intake of PKU patients (9).

Other auxiliary treatment options have emerged to improve PKU management.

Glycomacropeptide

Glycomacropeptide (GMP) is a Phe-free natural protein present in the cheese whey (10). Furthermore, as it is rich in essential amino acids, has convenient properties for the production of foods and beverages (heat stability, solubility, capacity to form gels and foams) and has acknowledged palatability, GMP emerges as an alternative approach to synthetic amino acids, improving taste, variety and convenience of food products, ultimately contributing for a better adherence to the Phe-restricted diet (10).

The high concentrations of LNAA present in GMP represents a possible explanation for the decrease of blood and brain Phe concentrations, as revealed in mice studies (10), since LNAA and Phe share the same carrier for transport across the blood-brain and intestinal barrier. Therefore, it is believed that increased levels of LNAA competitively inhibit Phe transport to the brain and intestinal absorption, lowering its concentrations.

GMP may also have a physiologic advantage compared to synthetic amino acids, as it provides intact protein instead of free amino acids (11). This property allows a slower absorption, simulating the ingestion of intact proteins, enhancing amino acid utilization for protein synthesis (increased protein retention with simultaneous decreased urea production and stimulated insulin release) and decreasing variations on blood Phe concentrations (11,12).

Some authors consider that GMP supplemented with some limiting amino acids (arginine, histidine, leucine, tryptophan and tyrosine) represents a complete source of natural protein, well accepted by PKU patients and with superior characteristics compared with synthetic amino acid products, facilitating the daily management of the disease (12).

Although current data seems to point out potential benefits with the use of GMP, human studies are limited, particularly in paediatric patients (13).

Large Neutral Amino Acids

Phenylalanine, tyrosine, tryptophan, threonine, methionine, valine, isoleucine, leucine and histidine are LNAA, and, with the exception of tyrosine, all of these are essential amino acids (14). As the ability to synthesize Tyr from Phe is compromised, in PKU patients, Tyr is also assumed to be an essential amino acid (conditionally essential).

With different aims and treatment protocols, LNAA supplementation has been suggested as an adjuvant treatment to improve neurocognitive outcome (14). There are several postulates proposed to support LNAA for PKU treatment (11,14):

(I) Since LNAAs share the same carrier in the blood-brain barrier, increased levels of LNAA compete with Phe for transport into the brain, reducing its concentrations;

(II) As LNAAs also share the same carrier in the intestine, treatment with LNAA may reduce the absorption of Phe, decreasing its blood levels;

(III) LNAA supplementation may also lower blood Phe concentrations due to higher net protein synthesis and consequent increased Phe utilization;

(IV) Supposing that reduced levels of essential amino acids in the brain (as seen in PKU patients) may impair protein metabolism and decrease neurotransmitters concentration, supplementation with LNAA potentially increases essential amino acid and neurotransmitter brain concentrations, improving disease outcome;

A study of 2015 (15) that investigated biochemical treatment objectives of LNAA supplementation in PKU mice, supported the mechanisms hypothesized. LNAA supplementation significantly reduced blood and brain Phe levels; attenuated brain deficiencies of some LNAA and increased brain serotonin and norepinephrine (but not dopamine) concentrations (15).

Depending on treatment aim and mechanism of action considered, distinct LNAA formulations (with different compositions) may be used – supplementation of all essential amino acids or of specific amino acids (14,16). Despite treatment protocol, LNAA has potential to enhance disease control, however more evidence is needed about the dose and composition of LNAA supplementation that results in long-term efficacy and improved tolerance (17).

Metabolic Control

The amount of Phe and natural protein ingested by PKU patients is determined based on individual Phe tolerance - the maximum amount of Phe that allows plasmatic concentrations within the recommended target ranges (7). Good metabolic control is defined as blood Phe concentrations below 6 mg/dL or below 8 mg/dL for patients under or above 12 years old, respectively (3). Patients with classical PKU usually do not tolerate more than 250 mg of Phe per day (5g of protein from natural foods), while individuals with HPA can tolerate more than 500 mg/day (1,8). For comparison, in the United States, the average dietary intake of Phe by non-PKU individuals is 3400 mg/day (8).

In order to assess Phe tolerance and adjust dietary prescription, patients need to monitor plasma Phe concentrations through blood sampling, regularly (5). It is important to point out that besides Phe and natural protein ingestion, there are other factors influencing Phe levels. Situations that induce protein catabolism, such as disease and/or an inability to achieve nutritional needs, increase protein breakdown and consequently the levels of Phe released to the blood stream (5).

Effectiveness of dietary treatment

An adequate lifelong Phe restricted diet is demonstrably effective in preventing neurological damage and improving intellectual performance (5). However, establishing a good metabolic control as early as possible (specially during the first decade of life) is crucial for the prevention of cognitive and neurologic impairments later in life (18). Some studies demonstrated that increases in blood Phe concentrations and/or delay on the onset of treatment are associated with decreases in Intelligence Quotient (IQ) score. Moreover, PKU patients appropriately treated still had lower IQ scores and mild executive function deficits, compared to non-PKU individuals (18). Additionally, the aim of the treatment should not only be to maintain blood Phe concentrations within the recommended limits, but also to control variability of those levels, as it seems to influence disease outcome (18).

Besides suboptimal clinical outcomes, the restrictive and unconventional dietary treatment often causes an important psychosocial impact to the patients and caregivers, which impairs therapy compliance. Limitations like low educational levels, poor social support, family dysfunction, difficulty in food preparation, cultural barriers, absence of symptoms and lack of motivation are some of the factors pointed as threats to dietary therapy adherence (19). Difficulties maintaining treatment are reported specially by teenagers and adults, compromising its effectiveness (20,21). For that reason, alternative therapies have been studied to increase compliance and improve disease control and quality of life of these patients.

Sapropterin dihydrochloride (Tetrahydrobiopterin)

Sapropterin dihydrochloride is a synthetic form of tetrahydrobiopterin (BH_4), cofactor of PAH (22).

As mentioned above, in PKU, the extent of deficient enzymatic activity varies among patients. Thereby, in some cases, PAH maintains some residual activity, which represents a potential opportunity for sapropterin to enhance the conversion of Phe into Tyr (22). BH_4 also catalyses the synthesis of several neurotransmitters, such as norepinephrine, dopamine and serotonine (22).

It would be expected that only patients with BH_4 deficiencies could respond to sapropterin treatment, where BH_4 supplementation replaces the absent cofactor. However, positive BH_4 responsiveness have been detected in patients without BH_4 defects for decades (23). Although the mechanism of action is not completely clarified, several postulates were proposed as possible explanations (23):

(I) Diminished binding affinity of the enzyme for the cofactor. In these cases, increases in cofactor concentrations (through BH_4 therapy) would activate the mutant enzyme;

(II) Protein stabilization by BH_4. It is believed that PKU can be caused by mutations that hamper the normal folding and oligomerization of PAH, which causes the enzyme to be more susceptible to degradation. BH_4 can act as a chaperone, stabilizing PAH, protecting the enzyme from proteolytic cleavage or degradation;

(III) Altered regulation of BH_4 biosynthesis. Oral therapy with BH_4 would possibly further increase the levels of BH_4, increasing PAH activity. It was also suggested that BH_4-responsive mutant PAH may not be dependent on Phe for activation, and so, the conversion of Phe to Tyr (with additional BH_4) is increased.

(IV) Induction of PAH expression by BH_4. It is suggested that regulation of PAH might be different in mutant PAH (in comparison to healthy situations) and that, in some cases, BH_4 plays a role in the regulation of PAH gene expression, increasing enzyme activity;

(V) Possible PAH mRNA stabilization by additional BH_4.

BH_4-responsiveness is most likely caused by several factors and its relationship with genotype is not yet completely understood.

Effects of sapropterin on metabolic control and dietary tolerance

Approved by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) in 2007 and 2008, respectively (22), sapropterin dihydrochloride (Kuvan®) emerges as the first adjuvant or alternative pharmacologic therapy able to increase Phe tolerance, enhancing metabolic control and/or decreasing dietary restrictions. In Europe, sapropterin is indicated for every sapropterin responder (regardless of age) with BH_4 deficiency, adults with HPA and PKU paediatric patients above 4 years old (24). In Portugal, dispensation of Kuvan was approved in 2014 (25).

Several studies (26–31) (Table 1) have revealed that BH_4 lowers blood Phe levels in PKU patients (mainly with mild to moderate forms of the disease, but also in patients with classical PKU), enhancing dietary Phe tolerance, which allows a more liberalized diet. However, most patients still need to do some dietary restrictions, but dietary Phe intake can increase 2 to 3 fold. In some particular cases, patients can have a normal diet, with a rapid introduction of natural Phe sources (32). An American survey conducted to understand the needs of PKU patients, concluded that patients treated with sapropterin considered easier to manage the disease than those without this pharmacologic treatment (20). Furthermore, BH_4 was found to be safe, without severe adverse effects. Headache and rhinorrhoea are the most frequent side effects reported with Kuvan (33). Approximately 25-50% of PKU patients seem to tolerate and respond to sapropterin (20).

Assessing BH_4 Responsiveness

BH_4 Loading Test

Treatment with BH_4 requires a previous loading test (LT) in order to determine responsiveness and identify patients that will potentially benefit from this therapy. Although a drop in blood Phe concentrations equal or superior to 30% during BH_4-LT is usually considered as a positive significant response, international consensus does not exist regarding the LT protocol and this threshold is arbitrary (22).

Fiege and Blau, 2007 (34) compared 2 LT modalities (8-hour and 24-hour protocols) and determined the prevalence of BH_4 responders depending on the criteria considered to define BH_4 responsiveness. Responsiveness rates (decreases in Phe levels of at least 30%) increased with the 24-hour LT (46%), compared to the 8-hour protocol (38%). Furthermore, responsiveness was lower when the cut-off assumed for Phe reduction was higher. Regardless of the LT protocol (duration and cut-off), prevalence of BH_4 responders was higher among patients with mild HPA, compared with mild and classical PKU patients. The authors alert that blood Phe concentrations influence BH_4 responsiveness and that higher levels might require additional BH_4 to enhance the enzyme's activity. Hence, the authors recommend the performance of a 24-hour LT (the most commonly used) with two administrations of 10-20 mg of BH_4 per kg of body weight and suggest that the cut-off considered should take into account the patient's phenotype (>20% reduction in Phe levels for classical PKU and >30% reduction in Phe levels for mild PKU). However, "slow" responders may require a longer LT (34).

Table 1: Effects of sapropterin treatment according to different studies

Reference (Year)	***Type of study***	***Patients***	***Location***	***Follow-UP***	***BH_4 Responsiveness assessement***	***BH_4 Treatment***	***BH_4 effects***
26 (2013)	Observational Retrospective	n = 147 PKU patients treated with BH_4 for at least 6m 68% Paediatric patients	6 European Countries (16 centres)	≥ 6 months	Test duration between 8h and 3 weeks 52.9% centres used 24-h LT 32.6% used 48-h LT 92.3% used 20 mg/kg BH_4	Duration: ≥ 6 m 63.9% treated with BH_4 alone 36.1% treated with BH_4 + low-Phe diet	Median ↓ in blood [Phe]: 62% 3.9x ↑ in Phe tolerance Blood [Phe] stable and within therapeutic range in all patients No severe adverse effects reported All side effects disappeared with reduced doses
27 (2015)	Observational, multicentre drug registry	n = 325 81.8% Paediatric patients 91.1% PAH deficiency 8.9% BH_4 deficiency	7 European Countries (55 sites)	1 year	Most frequent LT dose: 20mg/kg Most frequent LT duration: 24 to 48-hour	Median dose: 12.7 mg/kg/day (PAH deficiency) 5.0 mg/kg/day (BH_4 deficiency)	↑ in Phe intake No safety concerns identified
28 (2015)	Observational Retrospective	n = 8 Paediatric patients	Germany	4 years (1 prior to BH_4 and 3 under BH_4 treatment)	6 week test	Kuvan (Dose range: 10-19 mg/kg)	↑ in Phe tolerance (493.2mg/day under classical diet vs 2208.9 mg/day and 2021.93 mg/day, after 3 months and two years, respectively) ↑ intake of Phe containing food ↓ in consumption of special low protein products ↓ micronutrient intake (vitamin D, folic acid, iron, calcium, iodine), as the patients stopped taking amino acid mixtures
29 (2015)	Observational Longitudinal	n = 1189 97 patients < 4 y 42% of the patients were on continuous BH4 use for a median duration of 4 years 18% discontinued the drug within 3 months	United States (52 sites)	Up to 15 yeras			Average ↓ in blood [Phe] after 5 years: 43% ↑ in dietary Phe tolerance after 6 years: from 1000 to 1539 mg/day Non-serious adverse effects reported in 6% of the patients.
30 (2015)	Interventional trial	n = 17 12 Paediatric patients	Italy	60 to 84 m	48h LT 2 administrations of 20mg/kg BH_4-response: ↓ Phe >30% (Patients were hospitalized)	Initial dose: 10 mg/kg/day	↑ Phe tolerance by 2.3 – 11.6 fold 53% reached a Phe intake > 3000 mg/day with no need of aa and vitamin supplementation All patients maintained optimal metabolic control
31 (2015)	Systematic review of 2 RCT	Trial 1: n = 89; PKU patients > 8 years Trial 2: n = 46; PKU patients with 8-12 years	North America and Europe	Trial 1: 6 weeks Trial 2: 10 weeks	Trial 1: ↓ Phe ≥30% with 10mg/kg/d BH_4 dose Trial 2: ↓ Phe ≥30% with 20mg/kg/d BH_4 dose	Trial 1: 10mg/kg/d Trial 2: 20mg/kg/d	Trial 1: ↓ blood [Phe] in BH_4 group compared with placebo (without dietary restriction) Trail 2: ↓ blood [Phe] in BH_4 group compared with placebo (with dietary restriction) ↑ Phe tolerance in BH_4 group compared with placebo (from 0.5mg/kg/d to 20.3mg/kg/d) No serious adverse effects reported in both trials

The FDA protocol (24) for the implementation of treatment with sapropterin of patients with more than 4 years old, predicts an initial daily dose of 10 mg of sapropterin per kg of body weight for a week. When there is no significant reduction (no cut-off value specified) in Phe levels, the dose can be gradually increased up to 20 mg/kg/day for a maximum period of one month, during which blood Phe concentrations are monitored. For non-responders the treatment is ceased, and the daily dose of sapropterin is adjusted for those patients with a positive response.

Another protocol was proposed (24-hour LT) (24), indicating the administration of 20 mg of BH_4 per kg body weight and the monitoring of Phe levels during the following 24 hours. For this test protocol a positive response is considered for decreases in Phe levels equal or superior to 30%. The European working group (24), adjusted this protocol suggesting an 24-hour test to identify initial responders, and a second test for patients with responsiveness rates under 30%. Hence, the day before the start of the LT, patients collect 3 blood samples (at 8, 6 and 24h). On the first day, a dose of 20 mg of sapropterin per kg body weight is administered and 3 blood samples (at 8, 6 and 24h) are collected. Those with responsiveness rates above 30% proceed to the adjustment of the therapeutic dose. Patients with negatives responses, repeat the test (48-hour LT). If, even so, the decrease in Phe levels is inferior to 30%, the test is stopped (24).

In Portugal, a 72-hour test is applied, approved by the Portuguese Society of Metabolic Disorders in July 2014 (unpublished protocol). This LT protocol states that during the test patients collect blood samples every 8h (at 8, 16 and 24h). BH_4 is taken together with breakfast at the second and third day at a dose of 20mg/kg. All blood samples collected at 8 a.m. should be done in the fasted state. To assess responsiveness the mean value of blood Phe concentration (average of the 4 values obtained with sample collections) before the intake of the medicine is compared with the lowest value achieved within the following 48 hours. Patients with reductions in blood Phe concentrations equal or superior to 30% are considered potential BH_4 responders.

BH_4-LT accuracy

A Dutch study (35), evaluated the efficiency of the 48-hour BH_4 LT in identifying true responders. The test was based on the protocol described above, proposed by the European working group on PKU. A blood Phe concentration of at least 400 µmol/L (value considered high enough to allow detecting the effect of the drug, but close to the recommended range (32)) was required, and patients were asked to maintain the diet with which they reached those Phe levels. A 20 mg/kg dose of BH_4 was administered twice (at 0h and 24h), after blood sampling. Blood samples were collected every 8h (0, 8, 16, 24h) and at 48h. Potential responders were considered to be the ones, with a reduction of at least 30% in Phe levels at one or more points compared to 0h, and these patients proceeded to an extension phase. In this phase, blood Phe concentrations were monitored while Phe intake was increased, to assess improvements in Phe tolerance. A reduction of 30% in Phe levels with the same diet, and/or an increase in Phe tolerance of $\geq$ 50% or $\geq$ 4g of natural protein with blood Phe concentration within the recommended range, was assumed as true-positive BH_4 responsiveness. This study showed that 87% of the potential responders were true-responders, stating that the 48-hour BH_4 LT is a good method for the identification of patients who respond to BH_4. It is suggested that a 24-hour LT may prevent the identification of late responders, justifying the extension to 48 hours. However, late responders were less likely to be true-responders. The authors further affirm that longer tests are more susceptible to the influence of external factors (35).

McDonald *et al.* (32) suggest that fasting Phe levels must be monitored for at least 4 weeks previous to the BH_4 response LT, in order to attest each patient metabolic control with diet alone. It is also recommended a dietary registry during 3 days before the introduction of sapropterin, to establish actual Phe tolerance.

Concurrently with the BH_4-LT, a strict dietary protocol should be followed. An incapacity to maintain the diet throughout the test can jeopardize the interpretation of LT results, since dietary restrictions can lead to an apparent positive response. An accurate identification of BH_4-responsive patients is essential to avoid false positives and prevent misleading expectations and unnecessary costs. Hence, dietary compliance before and during loading test is essential to reduce false responsiveness. It is known that treatment compliance among PKU patients is not optimal and there is a trend to decrease with age. In addition, although it is advised that patients maintain their diet throughout the LT, unless the test is performed in a medical supervised environment, there is no guarantee that the diet remains constant. When it happens, that will be considered an issue regarding LT interpretation. To date, no studies have evaluated the level of dietary compliance during BH_4-LT and how it can impact responsiveness rates.

Treatment with sapropterin

After the identification of potential BH_4 responders through BH_4-LT, drug, dose and Phe tolerance should be assessed and adjusted to determine the dose and diet that best suits the patient, allowing the achievement and maintenance of good metabolic control, to initiate sapropterin treatment.

Kuvan® dose is usually adjusted between 5 and 20 mg/kg/day (32), and it is meant to be taken daily for long-term (33). It is advised that the pills are taken in the morning, dissolved in water, along with a meal (33).

Different protocols have been proposed for increases in Phe intake (consequent of enhanced Phe tolerance) during treatment with BH_4. Some authors suggest a 200 mg/day raise in dietary Phe per week, in mild or moderate PKU patients, while others advocate the increase of 10 mg per kg of body weight per day, or even the daily introduction of 100 mg along with regular Phe levels monitoring (32).

McDonald *et al*. (2011) (32) recommend that increases in dietary Phe should be systematic, by the same amount every 7 days, monitoring blood Phe concentrations to prevent them from raising above the target range and adjusting sapropterin dose when needed. Since there is great variability in BH_4 responsiveness, the protocol for Phe introduction (and its amount) should be determined based on initial individual tolerance.

During the introduction and adjustment phase of treatment with BH_4, besides deciding the dose and protocol that best suits each patient, it is important to take the type of protein source into account (32). It should be considered that sometimes, after the introduction of some protein foods (mainly high-protein sources), these need to be again removed from the diet if good metabolic control (after every adjustment) is not achieved, due to excess dietary Phe intake. This can represent a problem if the patient becomes accustomed to the taste. The first sources of protein being introduced to assess improvements in Phe tolerance should facilitate the patient's ability to return to their previous diet, if needed. On the contrary, there are also patients who are reluctant to the introduction of high-protein foods, since they always had to avoid them (32).

In addition to enabling a less restricted diet, with more natural dietary options, the reduction or removal of protein substitute products could be another advantage of BH_4 treatment for many patients. However, for decreases in protein substitutes to be made, an adequate supply of natural protein, vitamins and minerals should be achieved. Protein substitutes can account for the provision of 85% of protein requirements and represent a relevant source of essential amino acids, namely tyrosine, as well as some essential fatty acids, and are enriched with micronutrients (32). Since it is recognized the value of protein substitutes, they are not always completely removed from the patient's diet, in order to prevent the risk of nutritional deficiencies (32).

During sapropterin treatment, it is recommended the monitoring of several indicators, to assess treatment efficacy and to guarantee that nutritional requirements are achieved and that the patient is developing properly. Blood Phe and Tyr concentrations, dietary intake, growth and Body Mass Index (BMI) should be monitored regularly and, the assessment of biochemical nutritional markers is recommended once a year (32).

Study objectives

The aim of this study is to compare the natural protein, Phe, amino acid and total protein prescribed by the nutritionist before the BH_4 loading test with dietary intake during the loading test (recorded by the patients in a 3-day dietary registry). This analysis has the purpose to find out if potential responders restrict dietary ingestion during the loading test, to understand how the LT interpretation can be affected by dietary compliance.

Patients and Methods

This project was conducted at Centro de Genética Médica do Centro Hospital Porto (CGM-CHP), acknowledged as a Reference Center for Hereditary Metabolic Diseases, since 2016, integrating the European network.

The National Program of Early Diagnose started, in 1979, in this Centre, known for the follow-up and monitoring of patients with hereditary metabolic diseases. Besides, it also works as the central of acquisition, storage and distribution of SLPF, managed by the nutritional department since 1990.

Patients

The sample included 78 PKU patients (51% females; 37% pediatric patients) in follow-up at CGM-CHP, who underwent BH_4 Loading Test (LT) between March 2015 and January 2017. Out of these 78 studied patients (Table 2), 41% revealed classical PKU and 49% were mild PKU, 4% had hyperphenylalaninemia (HPA) and 6% were late diagnosed, according to the newborn screening blood [Phe] (3).

Table 2: Characterization of the patients studied (n = 78)

Gender		**Age at the time of LT**						**Disease Severity**			
F	M	min	max	mean	st dev	adults	children	HPA	Mild PKU	Classical PKU	Late Diagnose
40 (51%)	38 (49%)	4	48	20.91	9.08	49 (63%)	29 (37%)	3 (4%)	38 (49%)	32 (41%)	5 (6%)

All patients with hyperphenylalaninemia (HPA) and phenylketonuria (PKU), under treatment with a phenylalanine restricted diet and older than 4 years were invited to participate in the study. Exclusion criteria included pregnant woman, or woman with the desire to get pregnant in the near future, and late diagnosed patients with neurological impairment in whom the metabolic team considered that immediate clinical benefits would be less obvious, deciding that LT should be proposed later.

Study Design

This was a retrospective, observational study.

The LT protocol used was the one approved by the Portuguese Society of Metabolic Disorders in July 2014 (Figure 1) – a 72h test during which patients collect blood samples every 8h. BH_4 is taken together with breakfast at day 1 and 2 at a dose of 20mg/kg. Blood samples at 8 a.m. are always collected in the fasted state. To assess responsiveness the mean value of blood Phe concentration before the intake of the drug (average of the 4 values obtained with sample collections) is compared with the lowest value achieved within the following 48 hours. Potential BH_4 responsiveness is considered for blood [Phe] reductions ≥ 30%.

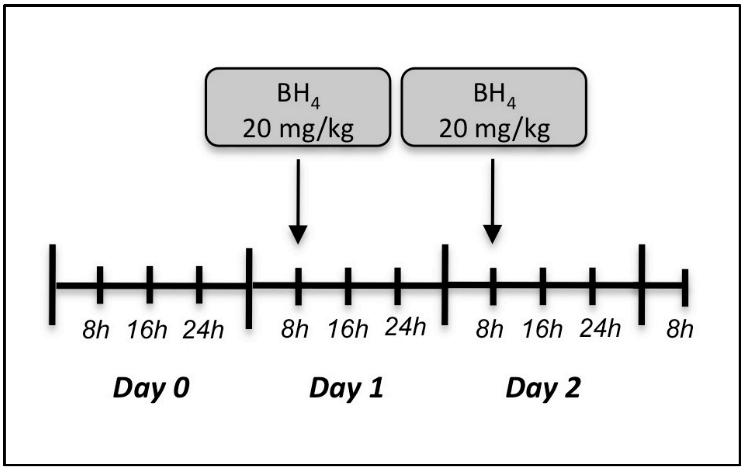

Figure 1: Portuguese BH_4 Loading Test Protocol
(Note: Each trace represents a blood sample collection)

Before the start of the BH_4-LT, all patients receive a nutritional prescription to follow throughout the test. Patients record their entire dietary intake during the 3-day test. This registry was used to evaluate the level of compliance with the diet previously prescribed (Figure 2). Association between the level of compliance with the nutritional prescription and the BH_4 responsiveness (obtained with the LT) was then tested.

Metabolic control was defined using median blood Phe concentrations in the two years preceding the LT.

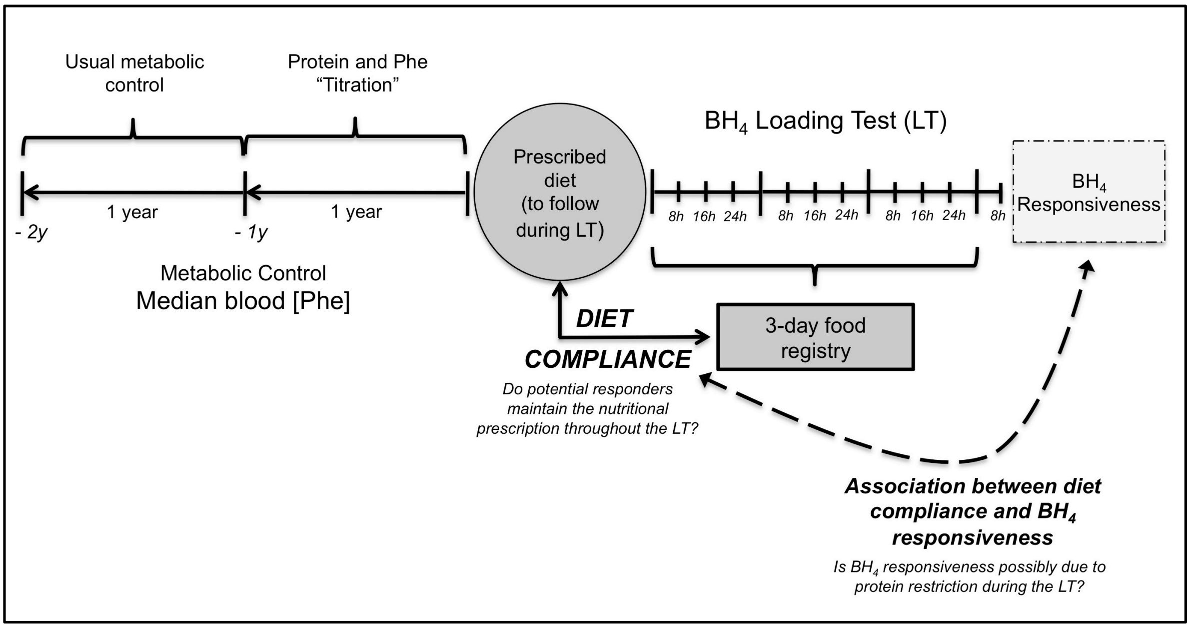

Figure 2: Study Design

The patients' data collected, were: gender; age at the time of LT; newborn screening blood Phe concentration; disease severity; anthropometric measures at the time of LT; metabolic control (median blood Phe concentrations in the two years preceding the LT); prescribed intake of natural protein, phenylalanine, amino acid and total protein, before the LT; dietary intake during the LT (natural protein, phenylalanine, amino acid and total protein), and responsiveness to the LT.

Data collection

All data was collected retrospectively from the clinical records available in the informatic software and databases of Reference Center for Hereditary Metabolic Diseases of Centro Hospital Porto (CHP).

Diagnose

Disease severity was classified according to blood Phe concentration at newborn screening, according to the Portuguese recommendations: hyperphenylalaninemia is defined as blood [Phe] < 6mg/dL, mild PKU as blood [Phe] ≥ 6 mg/dL and ≤20 mg/dL, and classical PKU as blood [Phe] > 20mg/dL (3).

Anthropometry

Weight, height and BMI were recorded in the last nutrition appointment scheduled before the LT. In children and adolescents, calculated z-scores were used to classify nutritional status, according to WHO BMI-for-age growth curves (36,37), whereas in adults usual WHO BMI classification was considered (38).

Metabolic control

To assess metabolic control before the LT, it was calculated the median blood Phe concentrations in the two years prior to the LT.

Median blood Phe concentrations in the year previous to the LT may not be an accurate indicator of usual metabolic control, since during this period it was necessary to titrate natural protein and Phe intakes, in order to adjust Phe tolerance and to maintain blood Phe concentrations above 8 mg/dl (32). For that reason, the median of blood Phe concentrations in the second year prior to the LT (year -2) was used to characterize the usual metabolic control of each patient (Figure 2).

In accordance with the Portuguese guidelines (3), good metabolic control was defined as blood Phe concentrations of ≤ 6 mg/dL, for patients under 12 years old, and ≤ 8 mg/dL for older patients.

Dietary assessment

Nutritional intake was assessed in two different moments: before and during the LT.

To assess the recommended nutritional intake for the LT, it was considered the diet prescribed in the last nutrition appointment scheduled before the LT. This was analyzed in terms of natural protein (NP, g/kg/d), phenylalanine (Phe, mg/kg/d), L-amino acid supplements (AA, g/kg/d), and total protein (TP, g/kg/d) content.

3-day food registries recorded by the patients were analyzed and corresponding NP, Phe, AA and TP intakes were registered, for assessment of dietary intake during the LT.

The tables used in clinical practice at CHP to calculate dietary plans and the Portuguese Food Composition Table (PFCT) were used as a reference to estimate the nutritional content of the diets. For food items not present in the PFCT, it was considered the information present on the nutritional label. To estimate the amount of Phe of non-prescribed food items it was assumed 5% of its protein value (8).

Compliance with the diet prescribed before the LT was assessed with the application of a difference test, as well as through the comparison between the two dietary assessments to evaluate the level of dietary compliance. Compliance was considered as variations between diet prescribed immediately before BH4-LT and mean nutritional intake reported during the test inferior to 10%. This was a criteria already used by other authors to define dietary compliance (39). Hence, patients with ingestions between 90-110% of the dietary prescription were considered compliant, while patients with ingestions below 90% or above 110% were considered non-compliant.

Food patterns

The analysis of the 3-day food diaries of the patients studied was used to verify if patients included foods that were not prescribed by the nutritionist in their dietary plan. Food patterns used in PKU treatment were also described based on the qualitative assessment of those registries, allowing the identification of natural protein sources present in PKU patients' diet.

Ethical statement

Data collected for this study was under the ethical approval consented by the Ethics Committee of Centro Hospitalar do Porto, EPE, on the 22nd of March 2017, to the investigation project "Dietary compliance during BH4 loading test in patients with phenylketonuria", with the reference 2016.268 (231-DEFI/220-CES).

Written informed consents were obtained from each patient or caregiver.

Statistical analysis

Statistical analysis was performed using the software SPSS® (Statistical Package for the Social Sciences) version 24.0 for Macintosh.

Normal distribution of variables was tested with Kolmogorov-Smirnov test.

Descriptive statistical analysis was made for continuous variables, while frequencies were used to report categorical variables.

Compliance with the diet prescribed before the LT was assessed with the application of Wilcoxon test, a difference test for non-parametric variables.

In all the tests, statistical significance was considered for p values inferior to 5%.

Results

Nutritional Status

Of the 78 patients, 61.5% were classified as normal weighted and 24.4% were overweight (Table 3). Assessing patients' nutritional status by age, show that 31% of paediatric patients and 26.5% of the adults were overweight or obese at the time of the LT. Only 2 of the 29 children submitted to the LT were underweight. For the adults, the percentage of underweight was 12.2%.

Table 3: Patients' nutritional status at the nutrition appointment before the BH_4-LT

Nutritional Status [a]	Adults (≥ 18 y)		Children (< 18 y)		TOTAL	
Underweight	6	12.2%	2	6.9%	8	10.3%
Normal weight	30	61.2%	18	62.1%	48	61.5%
Overweight	11	22.4%	8	27.6%	19	24.4%
Obesity I	1	2%	1	3.4%	2	2.6%
Obesity II	1	2%	0	0%	1	1.3%
TOTAL	49	62.8%	29	37.2%	78	100%

[a] Nutritional status in children and adolescents was classified considering z-scores, according to WHO BMI-for-age growth curves; for adults it was considered WHO BMI classification

Analysis of nutritional status by disease severity (Table 4) showed that the prevalence of overweight and obesity was 33.3%, 29%, 21.9% and 60.0% for patients with HPA, mild PKU, classical PKU and late diagnosed, respectively.

Table 4: Patients' nutritional status at the nutrition appointment before the BH_4-LT, according to disease severity [a]

Disease Severity	Nutritional Status [b]					TOTAL
	Underweight	*Normal Weight*	*Overweight*	*Obesity I*	*Obesity II*	
HPA	0	2 (66.7%)	1 (33.3%)	0	0	3
Mild PKU	3 (7.9%)	24 (63.2%)	9 (23.7%)	2 (5.3%)	0	38
Classical PKU	5 (15.6%)	20 (62.5%)	6 (18.7%)	0	1 (3.1%)	32
Late Diagnose	0	2 (40.0%)	3 (60.0%)	0	0	5
TOTAL	8 (10.3%)	48 (61.5%)	19 (24.4%)	2 (2.6%)	1 (1.3%)	78

[a] Absolute number of patients (and relative percentage) are presented

[b] Nutritional status in children and adolescents was classified considering z-scores, according to WHO BMI-for-age growth curves; for adults it was considered WHO BMI classification

Metabolic Control

Before the BH_4-LT, 60% and 45% of the patients had a bad metabolic control in the year before the LT and in the preceding year, respectively (Table 5). Three patients didn't have data concerning metabolic control in the year -2.

Analysing the metabolic control of the participants before the LT (Table 5), the minimum value of the median blood Phe concentrations in the year before the LT is superior to the minimum value of the preceding year, while the maximum value of the median blood Phe concentration decreased from year -2 to year -1. Results also suggest that the children's median blood Phe concentrations is lower than that of the adults, both in year -1 and -2.

Table 5: Metabolic control as median [Phe] mg/kg/d, in the years before the BH_4-LT

		Metabolic Control (Median [Phe])	
		Year -1	**Year -2**
Adults (n = 49)	*Mean*	9.95	9.68
	St Deviation	0.41	0.54
	Median	9.30	9.10
	Minimum	4	3.30
	Maximum	18	19.30
	Good Control [a, b]	14 (28.6%)	17 (34.7%)
	Bad Control [a, b]	35 (71.4%)	30 (61.2%)
	Missing	0	2
Children (n = 29)	*Mean*	6.70	5.42
	St Deviation	0.23	0.33
	Median	6.60	5.15
	Minimum	4.8	1.6
	Maximum	9	9.30
	Good Control [a, b]	17 (58.6%)	24 (82.8%)
	Bad Control [a, b]	12 (41.4%)	4 (24.1%)
	Missing	0	1
Total Sample (n = 78)	*Mean*	8.74	8.09
	St Deviation	2.86	3.71
	Median	8.14	6.9
	Minimum	4	1.6
	Maximum	18	19.30
	Good Control [a, b]	31 (39.7%)	41 (54.7%)
	Bad Control [a, b]	47 (60.3%)	34 (45.3%)
	Missing	0	3

[a] Good metabolic control was defined as blood [Phe] of ≤ 6 mg/dL, for patients under 12 years old, and ≤ 8 mg/dL for older patients

[b] Absolute number of patients (and relative percentage) are presented

Nutritional Ingestion and dietary compliance during BH_4-LT

Table 6 presents the mean values of prescribed natural protein, Phe, AA and total protein before the BH_4-LT, as well as their mean ingested values reported during the test.

Table 6: Prescribed and reported nutritional ingestion by patients (n = 78) during BH_4-LT

Nutritional Ingestion	**Prescribed Diet (before LT)**		**Day 0**		**Day 1**		**Day 2**		**3-day Average**	
	mean	std deviation	mean	std deviation	mean	std deviation	mean	std deviation	mean	std deviation
Natural Protein (g/kg)	0.80	0.46	0.77	0.45	0.78	0.46	0.77	0.44	0.77	0.44
Phe (mg/kg)	38.13	22.74	36.45	22.12	37.20	22.33	36.42	21.61	36.73	21.37
AA (mg/kg)	1.05	0.35	1.03	0.37	1.00	0.40	1.00	0.38	1.01	0.37
Total Protein (g/kg)	1.71	0.49	1.65	0.49	1.63	0.51	1.63	0.51	1.64	0.49

The average nutritional ingestion during the 3-day test is seemingly inferior to the amounts prescribed in the diet that patients were asked to follow throughout the test (Table 6). However, those differences are only statistically significant (Table 7) for the ingestion of amino acids and total protein (1.05 ± 0.35 vs. 1.01 ± 0.37 g/kg, p=0.006; 1.71 ± 0.49 vs. 1.64 ± 0.49 g/kg, p=0.003, respectively).

Table 7: Differences between nutritional ingestion prescribed and registered during BH$_4$-LT

	Nutritional Ingestion	**Prescribed Diet**		**Ingestion during LT (3-day Average)**		***p*** [a]
		mean	std deviation	mean	std deviation	
Total Sample (n = 78)	*Natural Protein (g/kg)*	0.80	0.46	0.77	0.44	0.106
	Phe (mg/kg)	38.13	22.74	36.73	21.37	0.116
	AA (mg/kg)	1.05	0.35	1.01	0.37	**0.006**
	Total Protein (g/kg)	1.71	0.49	1.64	0.49	**0.003**
Potential Responders (n = 33)	*Natural Protein (g/kg)*	0.91	0.49	0.90	0.50	0.721
	Phe (mg/kg)	43.37	24.26	43.10	24.41	0.922
	AA (mg/kg)	1.01	0.35	0.97	0.37	0.063
	Total Protein (g/kg)	1.79	0.48	1.74	0.46	0.208
Non-Responders (n = 45)	*Natural Protein (g/kg)*	0.72	0.43	0.68	0.36	0.069
	Phe (mg/kg)	34.30	21.00	32.05	17.69	**0.048**
	AA (mg/kg)	1.07	0.35	1.04	0.37	0.053
	Total Protein (g/kg)	1.66	0.50	1.57	0.50	**0.004**

[a] Non-parametric Wilcoxon test for paired samples

Potential BH$_4$ responders (Table 7) reported natural protein, Phe, AA and total protein intakes in accordance with dietary prescription (0.91 ± 0.49 vs. 0.90 ± 0.50 mg/kg, p=0.721; 43.37 ± 24.26 vs. 43.10 ± 24.41 g/kg, p=0.922; 1.01 ± 0.35 vs. 0.97 ± 0.37 g/kg, p=0.063; 1.79 ± 0.48 vs. 1.74 ± 4.61 g/kg, p=0.208, respectively), while non-responders reported lower Phe and TP intakes compared with diet prescriptions (32.05 ± 17.69 vs. 34.30 ± 21.00 mg/kg, p=0.048; 1.57 ± 0.50 vs. 1.66 ± 0.50, p=0.004, respectively).

Children had reported ingestions of NP, Phe, AA and TP compliant with dietary prescription (Table 8). In adults, mean intakes of AA and TP during the BH$_4$-LT was inferior to the amounts prescribed (0.94 ± 0.37 vs. 1.00 ± 0.35 mg/kg, p=0.007; 1.47 ± 0.39 vs. 1.56 ± 0.46, p=0.018, respectively).

Table 8: Differences between nutritional ingestion prescribed and registered during BH_4-LT by age group

	Nutritional Ingestion	Prescribed Diet		Ingestion during LT (3-day Average)		p^a
		mean	std deviation	mean	std deviation	
Children (n =29)	*Natural Protein (g/kg)*	0.98	0.39	0.96	0.46	0.194
	Phe (mg/kg)	46.94	19.21	45.57	22.44	0.107
	AA (mg/kg)	1.13	0.33	1.13	0.34	0.715
	Total Protein (g/kg)	1.97	0.45	1.93	0.46	0.060
Adults (n = 49)	*Natural Protein (g/kg)*	0.70	0.47	0.66	0.38	0.310
	Phe (mg/kg)	32.92	23.23	31.49	19.07	0.441
	AA (mg/kg)	1.00	0.35	0.94	0.37	0.007
	Total Protein (g/kg)	1.56	0.46	1.47	0.39	0.018

[a] Non-parametric Wilcoxon test for paired samples

According to the "level of dietary compliance" (Table 9), although the mean percentage of compliance for the ingestion of NP and Phe was 99.22 and 99.56, respectively, less than half of the patients were considered compliant (34.6% and 37.2%, respectively). Regarding the AA intake, albeit 84.6% of the patients were compliant, it was found that there were patients decreasing its ingestion to a minimum of 33.66% of the amount prescribed.

Table 9: Patients' dietary compliance during BH_4-LT

Compliance (%) [a]	**Nutritional Ingestion**			
	Natural Protein	***Phe***	***AA***	***Total Protein***
Mean	99.22	99.56	96.05	96.12
SD	25.05	25.74	11.75	12.48
Minimum	48.89	42.16	33.66	55.96
Maximum	176.36	179.26	113.60	128.06
Level of compliance [b, c]				
Compliance	27 (34.6%)	29 (37.2%)	66 (84.6%)	50 (64.1%)
Decreased Ingestion	33 (42.3%)	31 (39.7%)	11 (14.1%)	19 (24.4%)
Increased Ingestion	18 (23.1%)	18 (23.1%)	1 (1.3%)	9 (11.5%)

[a] Compliance (%) between diet prescribed immediately before BH4-LT and mean nutritional intake reported during BH4-LT

[b] Level of compliance was defined as: Compliance = 90-110%; Decreased ingestion = Compliance < 90%; Increased ingestion = Compliance > 110%

[c] Level of compliance: absolute number of patients (and relative percentage) is presented

In the group of patients with a positive response to the BH$_4$-LT (Table 10), 33.3% reported decreased ingestions of NP and Phe, compared to dietary prescriptions, while 48.9% and 44.4% of non-responders had mean ingestions of NP and Phe, respectively, inferior to the amounts prescribed by the nutritionist.

The percentage of patients compliant with dietary prescription tends to be lower for the group of non-responders, compared to responders, for all the nutritional intakes evaluated, except for the ingestion of AA (NP: 26.7% vs. 45.5%; Phe: 33.3% vs. 42.4%; AA: 86.7% vs. 81.8%; TP: 62.2% vs. 66.7%).

Table 10: Dietary compliance during BH$_4$-LT in responders and non-responders patients

	Compliance (%) [a]	**Nutritional Ingestion**			
		Natural Protein	***Phe***	***AA***	***Total Protein***
Potential Responders (n = 33)	*Mean*	100.93	101.30	95.94	98.15
	SD	23.56	23.48	11.69	12.68
	Minimum	55.11	54.35	55.34	57.88
	Maximum	169.57	175.84	113.60	128.06
	Level of compliance [b, c]				
	Compliance	15 (45.5%)	14 (42.4%)	27 (81.8%)	22 (66.7%)
	Decreased Ingestion	11 (33.3%)	11 (33.3%)	5 (15.2%)	6 (18.2%)
	Increased Ingestion	7 (21.2%)	8 (24.2%)	1 (3.0%)	5 (15.2%)
Non – Responders (n = 45)	**Compliance (%)** [a]	**Nutritional Ingestion**			
		Natural Protein	***Phe***	***AA***	***Total Protein***
	Mean	97.97	98.28	96.13	94.64
	SD	26.28	27.47	11.93	12.25
	Minimum	48.89	42.16	33.66	55.96
	Maximum	176.36	179.26	106.10	126.45
	Level of compliance [b, c]				
	Compliance	12 (26.7%)	15 (33.3%)	39 (86.7%)	28 (62.2%)
	Decreased Ingestion	22 (48.9 %)	20 (44.4%)	6 (13.3%)	13 (28.9%)
	Increased Ingestion	11 (24.4%)	10 (22.2%)	0	4 (8.9%)

[a] Compliance (%) between diet prescribed immediately before BH4-LT and mean nutritional intake reported during BH4-LT

[b] Level of compliance was defined as: Compliance = 90-110%; Decreased ingestion = Compliance < 90%; Increased ingestion = Compliance > 110%

[c] Level of compliance: absolute number of patients (and relative percentage) is presented

Out of the 29 paediatric patients, 41.4% reported decreased ingestions of NP and Phe, compared to dietary prescriptions. All the children had an AA intake compliant to the therapeutic indications.

In the adults, 42.8%, 38.8%, 22.4% and 26.5% had mean ingestions of NP, Phe, AA and TP, respectively, more than 10% inferior to the amounts prescribed by the nutritionist (Table 11). The percentage of children compliant with dietary prescription was higher, compared to the adults, for all the nutritional intakes evaluated (NP: 41.1% vs. 30.6%; Phe: 44.8% vs. 32.6%; AA: 100% vs. 75.5%; TP: 69% vs. 61.2%).

Table 11: Patients' dietary compliance during BH4 loading test by age group

	Compliance (%) [a]	**Nutritional Ingestion**			
		Natural Protein	***Phe***	***AA***	***Total Protein***
Children (n = 29)	*Mean*	95.49	94.59	99.54	97.36
	SD	18.00	17.17	2.45	9.59
	Minimum	59.62	57.82	90.91	84.08
	Maximum	136.00	136.74	106.10	128.06
	Level of compliance [b, c]				
	Compliance	12 (41.4%)	13 (44.8%)	29 (100%)	20 (69.0%)
	Decreased Ingestion	12 (41.4%)	12 (41.4%)	0	6 (20.7%)
	Increased Ingestion	5 (17.2%)	4 (13.8%)	0	3 (10.3%)
Adults (n = 49)	**Compliance (%)** [a]	**Nutritional Ingestion**			
		Natural Protein	***Phe***	***AA***	***Total Protein***
	Mean	101.42	102.50	93.99	95.39
	SD	28.36	29.44	14.36	13.95
	Minimum	48.89	42.16	33.66	55.96
	Maximum	176.36	179.26	113.60	126.45
	Level of compliance [b, c]				
	Compliance	15 (30.6%)	16 (32.6%)	37 (75.5%)	30 (61.2%)
	Decreased Ingestion	21 (42.8%)	19 (38.8%)	11 (22.4%)	13 (26.5%)
	Increased Ingestion	13 (26.5%)	14 (28.6%)	1 (2.0%)	6 (12.2%)

[a] Compliance (%) between diet prescribed immediately before BH4-LT and mean nutritional intake reported during BH4-LT

[b] Level of compliance was defined as: Compliance = 90-110%; Decreased ingestion = Compliance < 90%; Increased ingestion = Compliance > 110%

[c] Level of compliance: absolute number of patients (and relative percentage) is presented

Through a qualitative assessment of the 3-day food diaries it was possible to analyze the ingestion of foods that were not foreseen in the dietary plan prescribed by the nutritionist. Out of the 78 patients, 57 (73.1%) reported ingestion of non-prescribed food items. The three most frequent examples (Figure 3) were soft drinks (47.37%), cakes and sweet desserts (26.32%) and potato chips (24.56%).

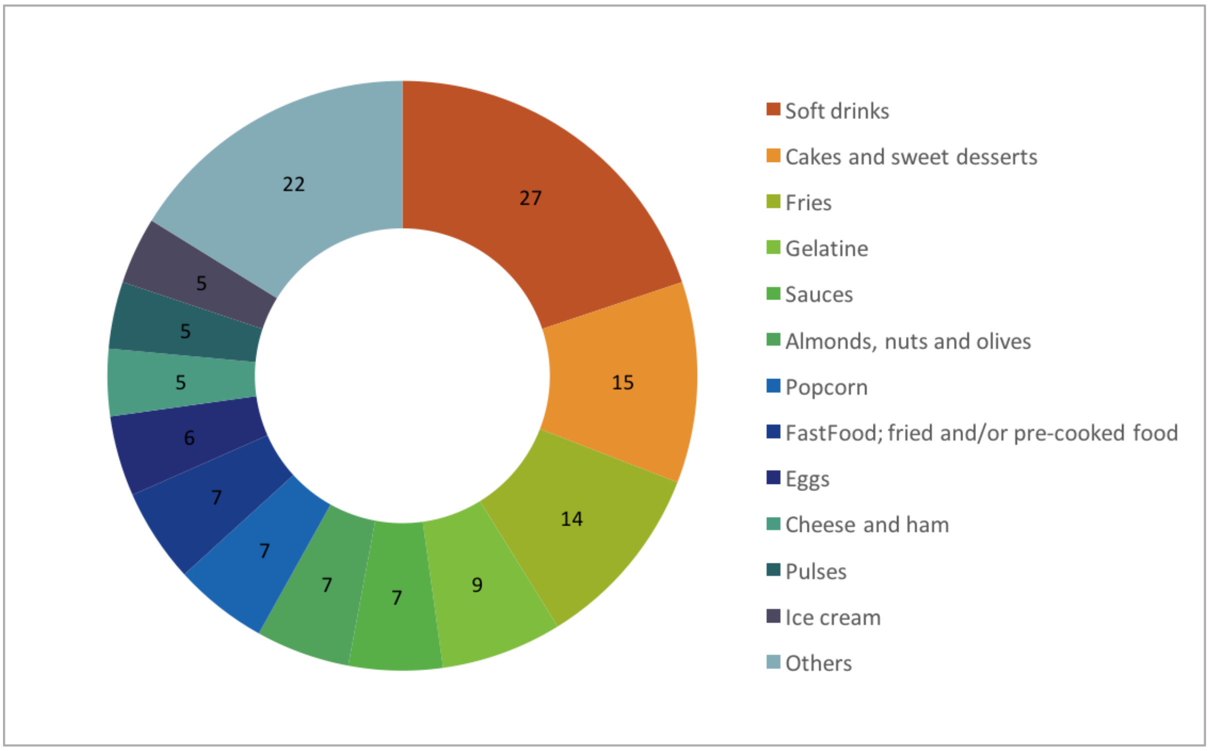

Figure 3: Patients' ingestion of non-prescribed foods during the BH_4-LT
(Note: For each food, the absolute number of patients that ingest it is referred)

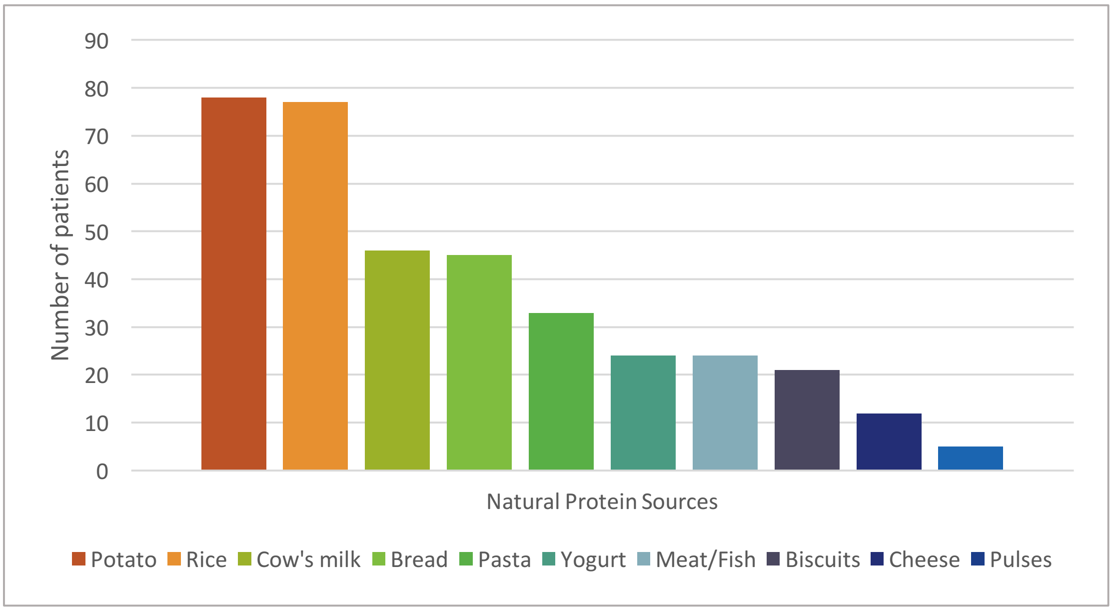

Figure 4: Natural protein sources present in PKU patients' diet

This analysis also allowed the identification of PKU patients' food patterns, namely concerning natural protein sources (Figure 4). All the patients participating in this study were consuming potato at the time of the BH_4-LT. Other natural protein sources often present in PKU patients' diet are rice (98.7%), milk (59%) and bread (57.7%).

Discussion

With this study, it was found that PKU patients did not achieve full dietary compliance with prescribed dietary protocols during BH_4-LT.

Although, at first sight, nutritional ingestion reported during the BH_4-LT seems to be lower than the one prescribed, statistically significant differences were only found for the ingestion of AA and TP, when considering the total sample (p=0.006; p=0.003, respectively). This can lead to the interpretation that sometimes PKU patients, intentionally or obliviously, do not take AA, essential to ensure their protein requirements. Some authors consider that, despite the improvements in formulation and packaging, the odor, taste and the fact of being different from conventional food products available for the healthy population, hinders the consumption of AA formulas (5).

When analysing the differences in nutritional ingestion by responsiveness, to understand if potential responders restrict dietary ingestion during the BH_4-LT, it was shown that potential BH_4 responders reported nutritional intakes in accordance with dietary prescription (p=0.721; p=0.922; p=0.063; p=0.208, respectively), while non-responders reported lower Phe and TP intakes compared with diet prescriptions (p=0.048; p=0.004, respectively). Contrary to what was suspected, potential responders do not seem to restrict (intentionally or not) their dietary ingestion, which leads to believe that interpretation of LT results is not being influenced by variations in the diet, minimizing the chance of identifying false responders. On the other hand, non-responders had ingestions of Phe and TP inferior to the amounts expected. With this observation, it can be speculated that the identification of these patients was accurate, since even with restrictions in Phe ingestion the decreases in blood Phe concentrations were not significant, meaning that probably the patients are true non-responders.

Concerning age group, it was shown that reported nutritional ingestion by children was compliant with dietary prescription, whilst adults decreased their intakes of AA and TP during the BH_4-LT (p=0.007; p=0.018, respectively). These results corroborate the evidence stating that treatment compliance (and particularly, dietary therapy) decreases with age, as it is no longer controlled by a caregiver and/or other external factors impairing patients' motivation to strictly follow the recommended therapy (19). During infancy, feeding is controlled by caregivers and peer pressure is reduced, therefore compliance with dietary treatment is usually favourable. Compliance begins to decline when children start school, become responsible for their meals and develop taste preferences, which aggravates during adolescence (5). This is also supported by the results obtained for metabolic control. In this study children's median blood Phe concentrations was lower than that of the adults, both in year -1 and -2 (Table 5), which also suggests that metabolic control tends to worsen with age. This is supported by the tendency showed by some studies that blood Phe concentrations increase with age, denotative of poor compliance (19,20).

Other variations were also found in median blood phenylalanine concentrations, used to characterize the metabolic control in the two years before the BH_4-LT. The maximum value of the median blood Phe concentrations from year -2 to year -1 decreased (Table 5). However, the mean value of the median blood [Phe] increases. This can be explained by the possible need of natural protein and Phe titration before the BH_4-LT, in order to maintain blood Phe concentrations above 8 mg/dl (5).

In addition to the application of Wilcoxon difference test, it was also defined "level of dietary compliance" to assess the percentage of patients that were compliant with the dietary prescription. Through this analysis, it was found that mean percentage of compliance for nutritional ingestion is high (Table 9), suggesting that these patients were compliant with the dietary prescription presented before the BH_4-LT. However, less than half of the patients were compliant with the diet prescribed by the nutritionist regarding the amounts of NP and Phe. Besides, the variations in dietary compliance are high, with patients decreasing the intake of AA to a minimum of 33.66% of the amount prescribed and others increasing the ingestion of NP to 176.36%, for example. These results demonstrate that, despite an apparent adherence to the nutritional recommendations, compliance with prescribed dietary protocols during BH_4-LT is incomplete.

In the group of patients with a positive response to the BH_4-LT (Table 10), 11 reported decreased ingestions of NP and Phe, compared to dietary prescriptions. It would be important to ascertain, long term, if these potential responders continue to respond to the BH_4 treatment, to verify if they are true responders and understand whether the dietary restriction made during the BH_4-LT jeopardized the accuracy of the test.

About half the non-responders had mean ingestions of NP and Phe (48.9% and 44.4%, respectively) inferior to the amounts prescribed by the nutritionist, which is in line with the results of the aforementioned differences test, suggesting that, although not compliant, these patients are probably true non-responders.

The analysis of the percentage of patients compliant with dietary prescription also allows the identification of possible patterns. In this study, the percentage of patients compliant with dietary prescription tends to be lower in the group of non-responders, compared to responders, for all the nutritional intakes evaluated, except for AA ingestion. These results, and the mean percentages of dietary compliance, suggest that responders tend to be more adherent to the prescribed therapeutic.

Once again, a tendency of decreased compliance with age was revealed with higher percentages of children compliant with dietary prescription, compared to adults. Besides, while all children took the AA accordingly, 22.4% of the adults decreased its ingestion, implying, once more, that the consumption of AA formulas is hampered by the fact of being unconventional, especially in adolescence and adulthood, when external control is also minor (5).

In addition to the comparison of nutritional intakes, the qualitative assessment of the food diaries also provides information about the presence of foods items that were not foreseen in the dietary plan prescribed by the nutritionist (information that could be missed if only the former analysis was performed). These data indicate that the majority (73.1%) of PKU patients included in this study reported the ingestion of non-prescribed food items. The most frequent examples chosen by patients were mainly carbohydrates sources [soft drinks (47.37%), cakes and sweet desserts (26.32%) and potato chips (24.56%)]. This suggests that patients are aware of the food items with the greater potential to increase blood Phe levels, impairing metabolic control. It could be speculated that, because of the restrictive and unconventional diet, these patients try to find some “normality” and pleasure in conventional foods that are simultaneously energy sources and popular among the general population. In fact, in PKU, since dietary treatment consists in a restriction of natural protein foods, other energy sources are included, namely carbohydrate rich foods. For that reason, it has been suggested that these patients would have a higher risk of overweight and obesity. However, study results are inconsistent and PKU patients do not appear to have higher overweight prevalence compared to the general population (40).

In this study, the prevalence of overweight and obesity was 28,2%, which is similar to the results found by Rocha *et al.* (2012) (41) for patients with a good metabolic control. Data from a national survey (42) shows that 34,8% and 22,3% of the Portuguese population are overweight and obese, respectively. The prevalence of overweight and obesity, respectively, by age, is 17,3% and 7,7%, in children, up to 10 years old; 23,6% and 8,7% in adolescents (10-17 years); and 36,5% and 21,6%, in adults (18-64 years) (42). From the 78 PKU patients evaluated in this study, 31% of paediatric patients and 26.5% of the adults were overweight or obese at the time of the LT. Despite the small sample size, these results corroborate that, apparently, the risk of overweight and obesity does not increase in PKU patients, when compared to the general population.

Like any study, this research also faced some limitations that can potentially impact the quality of the results obtained.

Daily food record is a validated and recognized dietary assessment method, nevertheless, it is not free of disadvantages that can affect its accuracy. Even though patients and caregivers received detailed orientation about food diaries fulfillment and were advised to be rigorous in the description of their food consumption, appropriate recording is variable. Daily food record is dependent on the individuals' literacy and motivation, as well as their ability to measure or estimate portion. If the food is not recorded at the time of consumption, accuracy of the diary is also dependent on memory (43). Besides, as the BH_4-LT is performed in outpatient setting, we cannot guarantee that patients consume exactly what they report, undermining the reliability of the food diaries.

The interpretation of the food diaries and estimation of nutritional intakes is also subject to error. The PFCT and nutritional labels used to estimate nutritional content only includes protein content, making it necessary to estimate the amount of Phe, assuming 5% of its protein value.

In this study, dietary compliance was assessed in terms of natural protein, Phe, AA and total protein intake. Other nutritional parameters, such as total energy intake, was not considered. Blood Phe concentrations fluctuate in situations of protein catabolism, which must be prevented by the achievement of protein and energy needs, as well as a proper distribution of feeding throughout the day (7,8). However, the medical metabolic team assured that the BH_4-LT was only performed if the patients were not sick, to minimize catabolism. Assessment of energy requirements and meal's schedules compliance could have contributed to a better understanding of the results.

Conclusions

This was to date, the first known study to evaluate dietary compliance in patients with PKU during BH_4-LT. The results obtained allowed the following main conclusions:

- Reported ingestion of AA and TP during BH_4-LT was inferior to nutritional prescription;
- Potential BH_4 responders reported nutritional intakes in accordance with dietary prescription;
- Non-responders reported lower Phe and TP intakes compared with diet prescriptions;
- Reported nutritional ingestion by children was compliant with dietary prescription, whilst adults decreased their intakes of Phe and TP during the BH_4-LT;
- Most PKU patients reported the ingestion of non-prescribed food items.

Despite an apparent adherence to the nutritional recommendations, compliance with prescribed dietary protocols during BH_4-LT was not fully achieved.

Dietary compliance before and during loading test is essential to reduce false responsiveness and allow an accurate identification of BH_4-responsive patients, to prevent misleading expectations and unnecessary costs. For future researches, it would be important to evaluate potential BH_4 responders long-term, after the onset of treatment, to ascertain if they maintained the treatment, in order to verify if they are true responders and understand whether dietary restrictions possibly made during the BH_4-LT could jeopardize the accuracy of the test.

This study highlights the role of the nutritionist and the importance of monitoring and supporting patients during BH_4-LT to ensure rigorous dietary compliance to improve accuracy of the test. Since international consensus does not exist regarding the BH_4-LT protocol, it would be important to revise the protocol, to minimize the influence of confounding variables that can influence the results of the test. It could be considered, for example, the performance of the BH_4-LT in a medically supervised environment, to ensure that the diet remains constant and a proper nutritional ingestion.

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