Phenylketonuria Treatment’s Impact on Physical Growth: A Spanish Retrospective Longitudinal Study

Abstract

Background: Phenylketonuria treatment based on phenylalanine-restricted diet has proven to be effective in preventing the development of long-term neurological damage. However, such diets have occasionally been reported to hinder normal development. Furthermore, to improve outcomes in these patients therapy based on administration of the cofactor BH\textsubscript{4} has emerged. However, little is known about how BH\textsubscript{4} treatment affects physical development.

Methods and Findings: Firstly, in order to evaluate the impact of the phenylketonuria diet on anthropometric characteristics we conducted a retrospective longitudinal study. Anthropometric characteristics and nutrition were evaluated from birth to adulthood in a cohort of phenylketonuria and mild-hyperphenylalaninemia patients, who were exclusively on protein-restricted diets, and were compared to the Spanish reference population. Patients with phenylketonuria showed growth impairment in early stages, with higher phenylalanine intakes being associated with improved developmental outcomes over this period. Secondly, we conducted a retrospective longitudinal study in a cohort of patients with phenylketonuria on BH\textsubscript{4} treatment and compared their developmental outcomes with those of a group of patients on a phenylalanine-restricted diet, in order to determine whether BH\textsubscript{4} treatment was associated with an improvement in growth development. No improvement was observed in the anthropometric variables in the BH\textsubscript{4}-treated group, from prior to initiating treatment to when they had taken the drug for 2 or 5 years. In addition, growth impairment was also observed in patients on low-phenylalanine diets. In fact, individuals on long-term BH\textsubscript{4} treatment seemed to achieve similar developmental outcomes to those on more restricted diets.

Conclusions: Our results suggest that prescribing very stringent diets in early stages might predispose these patients to later growth retardation, with growth outcomes in adulthood being well below the 50th percentile for healthy subjects. In conclusion, our study identified growth impairment in patients with phenylketonuria on BH\textsubscript{4} treatment, despite the fact that their natural protein intake increased.

Keywords: Phenylketonuria; Phe-restricted diet; tetrahydrobiopterin (BH\textsubscript{4}); physical outcomes; growth

Abbreviations: BH\textsubscript{4}; (6R)-L-erythro-5,6,7,8-tetrahydrobiopterin; BMI: Body mass index; GR: Growth rate; HPA: Hyperphenylalaninemia; PAH: Phenylalanine 4-hydroxylase; Phe: Phenylalanine; PKU: Phenylketonuria; RDA: Recommended dietary allowance; WHO: World Health Organisation

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Introduction

Phenylketonuria (PKU; OMIM 261600) is an autosomal recessive inborn error of metabolism affecting the phenylalanine (Phe) metabolic pathway, which converts Phe to tyrosine via phenylalanine 4-hydroxylase (PAH; EC 1.14.16.1) and its cofactor (6R)-L-erythro-5,6,7,8-tetrahydrobiopterin (BH$_4$). Depending on the plasma Phe levels at the time of diagnosis and the Phe tolerance, patients are classified into four phenotypic categories: mild-hyperphenylalaninemia (HPA), which does not require further treatment; mild-PKU; moderate-PKU and classic-PKU. If untreated, PKU can result in impaired cognitive development as a consequence of the neurotoxic effect of Phe accumulation [1].

PKU treatment consists of strict vegetarian diets, with very low Phe intake and supplemented with Phe-free protein substitutes and specially-manufactured low-protein foods. This Phe-restricted diet aims to maintain blood Phe levels below pre-established “safe” thresholds; and has proven to be effective in preventing the development of long-term neurological damage caused by Phe accumulation [2,3]. However, such diets have occasionally been reported to hinder normal development, since some individuals presented growth retardation and malnutrition [4-6]. Several studies have attempted to establish a connection between a reduction in natural protein intake and growth retardation, but their findings have been inconsistent. Therefore, we firstly conducted a retrospective longitudinal study, in order to evaluate the impact of PKU protein-restricted diet on anthropometric characteristics. The aim was to further explore the hypothesis of higher natural protein intake being associated with attaining improved physical outcomes in patients with PKU.

In this study, anthropometric characteristics and nutrition were evaluated from birth to adulthood in a cohort of patients with PKU. We conducted a retrospective, longitudinal, multicentre study on BH$_4$ treatment and compared their developmental outcomes between a reduction in natural protein intake and growth retardation [4-6]. Several studies have attempted to establish a connection between reduced Phe intake below recommended dietary allowances (RDAs) [9], as well as increasing patients’ dietary adherence. PAH-deficient patients positively responding to BH$_4$ treatment were observed for the first time in 1999 by Kure et al. [10]. Therapy based on this cofactor has opened up new treatment possibilities for a significant proportion of these patients, namely those responding to a BH$_4$ loading test. So far, clinical data on BH$_4$ treatment’s benefits have highlighted a remarkable increase in Phe tolerance, a reduction in the fluctuation of blood Phe levels and an improvement in nutritional status and quality of life [9,11-13]. In addition, BH$_4$ treatment allows patients with PKU to partially or totally liberalise their Phe-restricted diet, reducing their need to be supplemented with protein substitutes and enabling them to increase their intake of natural proteins. However, so far there is little clinical evidence regarding the impact of BH$_4$ treatment on PKU patient physical growth and the results from the few published studies have been inconclusive [14,15]. Therefore, we secondly conducted a retrospective longitudinal study of anthropometric characteristics in a cohort of patients with PKU on BH$_4$ treatment and compared their developmental outcomes with those of a group of patients on a Phe-restricted diet, in order to determine whether BH$_4$ treatment was associated with an improvement in growth development. We also assessed nutritional status, in order to further investigate the association of higher natural protein intake with improved developmental outcomes [16].

Study Design

Study population

We conducted a retrospective, longitudinal, multicentre study of PAH-deficient patients, who were regularly treated and monitored at several Spanish hospitals. Patients were classified into one of the four phenotypic categories according to their blood Phe levels, which were measured at diagnosis: classic PKU (Phe > 1200 mol/L), moderate PKU (Phe: 600-1200 mol/L), mild PKU (Phe: 360-600 mol/L) and mild-HPA (Phe < 360 mol/L). The inclusion criteria for the study were: (1) early diagnosis of PKU or mild-HPA; (2) in the case of patients on a PKU diet, exclusive and continuous treatment with Phe-restricted diet, supplemented with Phe-free substitutes and specially manufactured low-protein foods, the diet being initiated within days or weeks of birth; (3) absence of any other diseases known to affect physical development; (4) Caucasian race; and (5) regular attendance to their scheduled clinical check-ups. A total of 505 PAH-deficient patients were included in this study: 279 were diagnosed with PKU (130 males, 149 females), and 226 with mild-HPA (106 males, 120 females). The PKU group consisted of 43 patients with mild PKU, 78 with moderate PKU and 158 with classic PKU. Their ages ranged from 1 to 36 years.

Patients with PKU below 17 years of age were included in a second retrospective longitudinal study from thirteen hospitals in Spain. The study included patients with classic, moderate and mild PKU. They were divided into two groups according to their treatment: (1) BH$_4$ treatment (until 2009 as [6R]-L-erythro-5,6,7,8-tetrahydrobiopterin [Schircks Laboratories, Jona, Switzerland], and thereafter sapropterin dihydrochloride [KUVAN®, Merck, Madrid, Spain]), and (2) exclusively Phe-restricted diet. The inclusion criteria for this study were: (1) early diagnosis, confirmed by mutation analysis of PAH gene; (2) in the case of patients on a PKU diet, early and continuous treatment with Phe-restricted diet, supplemented with Phe-free substitutes and specially manufactured low-protein foods; (3) in the case of BH$_4$ patients, responsiveness to BH$_4$ treatment (a defect in the BH$_4$ pathways being ruled out by analysing urinary pterin levels); (4) absence of any other diseases known to affect physical development; (5) Caucasian race and (6) regular attendance to their scheduled clinical check-ups. Weight and height were collected over a period of two or five years, every 6 months, from the date when BH$_4$ treatment was initiated. The same timetable was used in the diet-only group, in order to compare the evolution of anthropometrics and nutrition between both populations. Height, weight, BMI and GR measurements were converted into Z-scores by subtracting...
the expected mean measurement for the corresponding age and gender and dividing by its standard deviation. We used the 2008 Spanish Growth Studies as the source of data for the reference population [17,18].

Biochemical measurements

Blood Phe concentrations were measured at diagnosis and every 6 months thereafter, in order to evaluate the effectiveness of the prescribed diet for each patient and monitor their adherence to the treatment. According to the Spanish protocol for treating and monitoring patients with PKU [19,20], blood Phe levels should remain below cut-offs established for each age group: < 360 µmol/L for individuals < 6 years of age; < 480 µmol/L for those from 6 to ≤ 10 years of age, and < 600 µmol/L for those > 10 years of age.

Nutrition

With regard to nutrition, dietary intervention was started at diagnosis. Patients were on Phe-restricted diets, supplemented with Phe-free amino acid mixtures and specially-manufactured low-protein foods. Specifically, they had a restricted intake of protein-rich foods (such as meat, fish, eggs, milk and dairy products, and legumes) as well as foods with moderate protein content (such as cereals). The daily amount of natural protein prescribed for each individual was modified according to their age and the most recent blood Phe values. The recommended daily intake of protein substitutes was also based on patients’ age and they were advised to divide it into at least four daily doses. Dietary parameters (such as intakes of natural protein, protein contained in amino acid mixtures, total protein and Phe) were calculated by an experienced dietician from the 3-day food records completed by parents/legal guardians. In the present study, nutritional data were analysed every 6 months from birth to 18 years of age for 98 out of the 279 patients with PKU; but not in the case of patients with mild-HPA due to incomplete records.

BH₄ loading test

Generally, BH₄ responsiveness is evaluated using a BH₄ loading test. The BH₄ loading test used in this study was based on recommendations in the Spanish protocol for treating and monitoring patients with PKU [7,16,19,20]. Briefly, participants were loaded with Phe before BH₄ administration. In general, an individual was considered to be a primary responder to BH₄ treatment when blood Phe levels fell by at least 30 % within 8 h after BH₄ administration. Patients who showed a reduction in blood Phe levels of at least 30 % within 12 h after BH₄ administration were classified as late responders. From 2005 onwards, BH₄ responsiveness was evaluated based upon the 50 % criterion at Virgen del Rocio University Hospital (for a full description of the protocol, see reference [21]). This BH₄ loading test was designed as a two-stage protocol. In the first stage (the 24-h test), participants were loaded with Phe before BH₄ administration. With this initial stage of the protocol, an individual was considered BH₄ responsive when blood Phe levels fell by at least 50 % within 24 h after BH₄ administration. PKU patients who did not meet the aforementioned criterion for responsiveness underwent the therapeutic test. In this second stage of the protocol, patients were administered a BH₄ dose of 20 mg/kg per day for one week together with a daily intake of protein, set on a case-by-case basis in line with age- and gender-specific RDAs. The results of the therapeutic test were considered to be positive when Phe levels remained below an established threshold (< 360 µmol/L, for individuals < 6 years of age; < 480 µmol/L for those from 6 to ≤ 10 years of age, and < 600 µmol/L for those > 10 years of age). Patients who met this criterion were classed as late responders.

Statistical Analysis

Statistical analysis was performed with the Statistical Package for the Social Sciences, SPSS® 20.0 for Windows (IBM, Chicago, IL, USA). The threshold for statistically significant differences was set at p < 0.05. Initially, all data were analysed using the Kolmogorov-Smirnov test, to assess whether the data were normally distributed. Descriptive statistics are presented as mean ± standard deviation (SD) for those variables which did satisfy the normality assumption. For non-parametric variables, descriptive statistics are presented as median and interquartile range. Differences between groups were assessed by using Student’s t-test for paired data. Pearson correlation coefficients (r) were calculated to assess bivariate correlations for parametric variables.

Results

Anthropometric characteristics and nutrition in a Spanish cohort of PAH-deficit patients exclusively treated with protein-restricted diets.

Clinical, biochemical and nutritional data were collected for the first 18 years of life in patients with PKU and for the first 12 years of life in patients with mild-HPA. The anthropometric characteristics which were assessed in our study were: height, weight, body mass index (BMI) and growth rate (GR), which were converted into Z-scores by subtracting the expected mean measurement for the corresponding age and gender and dividing by its standard deviation. BMI was calculated using the formula BMI = weight (kg)/ height² (m²). GR was calculated from two height measurements, which were taken approximately 1 year apart. We used the 2008 Spanish Growth Studies as the source of data for the reference population [17,18]. The statistical analysis indicated that mean Z-score for weight gradually decreased during the first 2 years of life to a nadir of -0.59 ± 0.95 and -0.51 ± 0.99 in male and female patients with PKU, respectively. From 2 to 3.5 years of age, it gradually increased and remained almost steady until 16.5 years of age (mean Z-scores for weight close to 0 and -0.2, in the male and female group, respectively). In adulthood, mean Z-score for weight in the male group fell well below the 50th percentile for healthy subjects (-0.51 ± 0.82), and it rose above this threshold in the female group (0.34 ± 1.13). In contrast, mean Z-score for weight in patients with mild-HPA remained close to 0 over the observation period. With regard to height Z-scores in the PKU group, they behaved similarly to weight Z-scores. They fell well below 0 during the first 2 years of life (mean Z-scores for height:
-0.87 ± 1.14 and -0.68 ± 1.16 in the males and female group, respectively, and then recovered slightly towards 0. From 16.5 to 18 years, mean Z-score for height in the male group dropped to a final value of -0.89 ± 1.03. In the female group, mean Z-scores for height decreased steadily from 12 years of age to adulthood (final value -0.88 ± 0.81). On the other hand, mean Z-scores for height in male patients with mild-HPA were above the 50th percentile for the healthy population, but fell to -0.49 ± 1.06 at 12 years of age; while in the female group, it fell to a nadir of -0.59 ± 1.27 at 7.5 years of age, and was close to 0 at 12 years of age. Our results are consistent with the findings of several previous European studies on physical development in patients with PKU [22-24]. In marked contrast to our findings, a Spanish study has observed normal developmental outcomes in PAH-deficient patients over an observation period from 0 to 18 years of age; even in the most severe phenotypes, who usually are on a strict diet [25].

No significant variation was observed in the evolution of BMI Z-scores in the male PKU group, its mean value was close to the 50th percentile for healthy subjects over the observation period. However, the female PKU group showed an increase in the mean Z-score for BMI at 8 years of age (0.98 ± 0.93); and, in contrast to the male group, BMI Z-score was well above 0 at 18 years of age. Mean Z-scores for BMI in the mild-HPA group were approximately 0 from 6 months to 12 years of age, in both the male and female groups. Finally, mean Z-scores for GR in the PKU group showed a gradual decrease between 1.5 and 4.5 years of age, and a sharp fall around puberty in both sub-groups. In the mild-HPA group, mean Z-scores for GR fell steadily in early stages of growth, and remained above the 50th percentile for healthy subjects until 11 years of age, dropping below this threshold from this age onwards.

We also performed paired-sample t-tests, in order to analyse intra-group (male vs. female) and inter-group (male/female-PKU vs. male/female-mild-HPA) differences in anthropometric characteristics. Statistically significant differences in mean Z-scores for height and GR (PKU group: male vs. female) were observed using the Student’s t-test (p < 0.05). In the mild-HPA group, we found significant differences in BMI Z-scores between male and female patients (p < 0.05).

As we expected, mean blood Phe levels were significantly higher in the PKU group than in the mild-HPA group. However, no significant differences were observed in blood Phe levels between the male and female groups.

The intake of nutrients was analysed by age (0-2; 2-9; 9-12 and 12-18 years of age) [Table 1]. The statistical analysis indicated that median intakes of natural protein, protein contained in amino acid mixtures, total protein and Phe were above the guidelines for normal growth [26]. With regard to Phe intake, its median met the recommendations in the Spanish protocol for treating PKU in each group, but was lower than the guidelines of the WHO.

### Table 1: Dietary parameters (intakes of natural protein, protein contained in amino acid mixtures, total protein and Phe) in the PKU group. Values are expressed as median (interquartile range).

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Natural protein (g/kg/day)</th>
<th>Protein in amino acid mixtures (g/kg/day)</th>
<th>Total protein (g/kg/day)</th>
<th>Phe (mg/kg/day)</th>
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<tbody>
<tr>
<td>0-2</td>
<td>0.8 [0.5-1.0]</td>
<td>1.4 [1.0-1.7]</td>
<td>2.2 [1.8-2.8]</td>
<td>28.2 [20.8-36.2]</td>
</tr>
<tr>
<td>2-9</td>
<td>0.5 [0.3-0.9]</td>
<td>1.3 [1.0-1.7]</td>
<td>1.8 [1.4-2.3]</td>
<td>15.5 [10.9-20.4]</td>
</tr>
<tr>
<td>9-12</td>
<td>0.3 [0.2-0.5]</td>
<td>1.2 [0.9-1.4]</td>
<td>1.2 [1.0-1.7]</td>
<td>8.6 [7.0-11.3]</td>
</tr>
<tr>
<td>12-18</td>
<td>0.3 [0.2-0.5]</td>
<td>1.0 [0.7-1.3]</td>
<td>1.1 [1.0-1.4]</td>
<td>7.6 [5.4-10.3]</td>
</tr>
</tbody>
</table>

Phe: Phenylalanine; PKU: Phenylketonuria
Total protein: combination of both natural protein and protein contained in amino acid mixtures.

In addition, we further evaluated the influence of the Phe-restricted diet on physical growth in patients with PKU using bivariate correlation analysis. There was no association between higher protein intakes and attaining improved developmental outcomes in the PKU group. On the other hand, we observed a significant positive correlation between higher Phe intakes and achieving better height Z-scores in the PKU female group (Pearson’s correlation coefficients (r) = 0.76, 0.56 and 0.69, p < 0.05, at 1.5, 2.5 and 4.5 years of age, respectively) as well as in the PKU male group (r = 0.61, 0.73, 0.78, 0.74, 0.86, 0.72 and 0.83, p < 0.05, at 2.5, 3.5, 4.0, 4.5, 5.0, 5.5 and 11 years of age respectively). In addition, weight Z-scores positively correlated with Phe intake in the PKU female group (r = 0.52 and 0.46, p < 0.05, at 1.5 and 2.5 years of age, respectively) and in the PKU male group (r = 0.68, 0.61, 0.74 and 0.78, p < 0.05, at 3.5, 4.0, 4.5 and 5.0 years of age, respectively).

In summary, our findings showed growth impairment in the group of patients with PKU. In particular, there were two well-differentiated periods of age (from birth to 2 years of age, and from end of growth to adulthood) when height Z-score fell well below 0. We also found a significant positive correlation between height and Phe intake, as well as between weight and Phe intake. On the contrary, in patients with mild-HPA, no growth retardation was observed.

### Impact of phenylketonuria on growth in BH₄-treated and Phe-restricted diet-only patients

Diet-only patients were on Phe-restricted diets, supplemented with Phe-free amino acid mixtures and specially manufactured low-protein foods (further details are described in the subsection Study 1). With regard to BH₄ patients, in an attempt to enable them to consume a normal diet, they gradually increased their intake of certain protein-rich foods, while maintaining BH₄ treatment, and consequently they reduced their intake of protein in amino acid mixtures. Although patients with a Phe reduction below 50% in the BH₄ loading test could not adopt a normal diet, they obtained at least 70% of total protein from natural sources.
Dietary parameters (such as intakes of natural protein, protein contained in amino acid mixtures, total protein and Phe) for both groups were calculated as described above.

**Two-year follow-up**

Follow-up data from patients with PKU were collected every 6 months over a period of 2 years. The group on BH₄ treatment included 36 patients (18 males and 18 females): 5 with classic PKU, 24 with moderate PKU and 7 with mild PKU. This BH₄ group represented the 33% of total patients with PKU included in this study. The rest of the cohort (72 patients (36 males and 36 females)) was on exclusively Phe-restricted diet supplemented with amino acids. There were 10 patients with classic PKU, 48 with moderate PKU and 14 with mild PKU. Thus, there were two diet-only patients for each BH₄ patient matched for age and type of PKU. Overall, there were 54 females and 54 males with an initial age range from 6 months to 15 years.

The comparison of anthropometric characteristics between the BH₄ and the diet-only group is shown in Table 2. The negative values of weight and height Z-scores in both groups, which were below the Spanish healthy population, may reflect the impact of a Phe-restricted diet until BH₄ treatment was initiated in one of the groups. When we compared both groups, there were no significant differences in the mean Z-scores for height over the observation period. With regard to weight, we observed significant differences (p < 0.05); while Z-scores were higher in the BH₄ group comparing with the diet-only group at 6 and 12 months; the mean Z-score for weight was higher in the diet-only group than the BH₄ group at 18 and 24 months. BMI Z-scores were significantly higher in the BH₄ group than the diet-only group within the two-year follow-up period (p < 0.05). However, this seems not to be related with treatment, since such difference was already significant before treatment was initiated. Consistent with the lack of significant difference in height Z-scores between groups, there was no significant difference in mean Z-scores for GR between them. However, we observed a trend towards decreased GR in the diet-only group comparing with the BH₄ group. Moreover, we analysed individually each treatment; there was no significant difference (p > 0.05) between initial and final values in the mean Z-scores for weight, height, BMI or GR, which indicated that the treatment had no substantial effect on those parameters.

With regard to blood Phe levels, mean values were lower in BH₄-treated patients than in the low-Phe diet group. There were significant differences between both groups at initial levels (255.2 ± 146.8 mol Phe/L and 418.4 ± 339.6 mol Phe/L, BH₄ and diet-only group, respectively; p = 0.003) and between initial and final mean values in the BH₄ group (initial: 255.2 ± 146.8 mol Phe/L and final: 365.5 ± 226.5 mol Phe/L; p = 0.005). Furthermore, BH₄ administration led to a decrease in the variability of mean blood Phe levels. It should be highlighted that stabilising blood Phe levels in patients with PKU is crucial to prevent neurocognitive impairment, especially during the first years of life. In this regard, it was described that blood Phe values above pre-established “safe” thresholds and its high variability correlated with neurocognitive impairment and psychosocial dysfunction [2,3].

With regard to Phe tolerance over the two-year follow-up, we observed that it increased in 28 of the BH₄-treated patients and remained steady in the other 8. Within the group in which Phe tolerance increased, 11 patients gradually managed to adopt a non-restricted diet; while Phe tolerance increased by 329.2 ± 230.2 mg Phe/day in the other 17 patients, who remained on the restricted diet. Daily intake of natural protein slightly increased from prior to initiating BH₄ treatment to when patients had been taken the drug for 2 years (Table 3).

**Five-year follow-up**

Follow-up data from patients with PKU were collected every 6 months over a period of 5 years. The BH₄ group included 10 patients (9 with moderate PKU and 1 with mild PKU). The rest of the cohort (20 patients: 12 males and 8 females) was on Phe-restricted diet: 18 with moderate PKU and 2 with mild PKU. Thus, there were two diet-only patients for each BH₄ patient, matched for age and type of PKU. The final mean age for the BH₄-treated group was 10.2 ± 3.1 years, and for the diet-only group was 10.2 ± 3.0 years.

The comparison of anthropometric characteristics between the BH₄-treated and the diet-only group is shown in Table 4. As in the two-year follow-up period, mean Z-scores for height in both groups were below the average for healthy population. Although mean Z-scores for weight in the BH₄ group were also higher than in the diet-only group, the difference did not reach statistical significance (p > 0.05). Furthermore, there was no statistically significant difference between initial and final mean values within each group for mean Z-scores for weight, height, BMI and GR.

With regard to blood Phe levels, mean values were slightly higher in the diet-only group comparing with the BH₄-treated group, but this difference did not reach statistical significance at any time point (p > 0.05). In addition, there was no statistically significant

| Table 2. Anthropometric characteristics (weight, height, BMI and GR Z-scores) in the BH₄-treated group comparing with the diet-only group over the two-year follow-up period. Values are expressed as mean ± standard deviation (SD). |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | Weight Z-score | Height Z-score | BMI Z-score    | GR Z-score     |                |                |                |
| BH₄             | diet-only       | BH₄             | diet-only       | BH₄            | diet-only       | BH₄            | diet-only       |
| Initial         | -0.16 ± 1.03   | -0.55 ± 0.83   | -0.71 ± 1.25   | -0.76 ± 1.03   | 0.13 ± 1.03*   | -0.17 ± 0.88*  | 0.15 ± 2.18    | 0.02 ± 2.37    |
| 6 months        | -0.08 ± 0.99*  | -0.56 ± 0.89*  | -0.62 ± 1.13   | -0.74 ± 0.99   | 0.28 ± 0.82*   | -0.19 ± 0.96*  | 0.61 ± 2.15    | -0.01 ± 1.23   |
| 12 months       | -0.13 ± 1.13*  | -0.71 ± 0.7*   | -0.69 ± 1.1    | -0.91 ± 0.88   | 0.33 ± 1.21*   | -0.27 ± 0.77*  | 0.08 ± 1.67    | 0.35 ± 1.59    |
| 18 months       | -0.79 ± 1.17   | -0.46 ± 0.9    | -0.72 ± 1.12   | -0.81 ± 0.98   | 0.51 ± 1.22*   | -0.14 ± 0.87*  | -0.01 ± 1.52   | 0.09 ± 1.73    |
| Final           | -0.75 ± 1.04*  | -0.52 ± 0.85*  | -0.73 ± 0.96   | -0.90 ± 1.00   | 0.37 ± 1.09*   | -0.12 ± 0.89*  | 0.05 ± 2.71    | 0.10 ± 2.26    |

Statistically significant differences between the groups: BH₄ vs. diet-only group: *p < 0.05
Initial: Data were collected immediately before initiation of BH₄ treatment. Data were collected every 6 months after initiation of treatment and over the two-year observation period.

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difference between initial and final mean blood Phe levels in either group (BH4 group, initial: 204.2 ± 143.9 and final: 289.6 ± 30.6 mol Phe/L; and diet-only group, initial: 391.8 ± 233.5 and final: 440.6 ± 298.4 mol Phe/L). In the five-year follow-up study, we observed that Phe tolerance increased in 6 patients, whereas it remained steady in 2 patients. This level of tolerance did not fall below baseline value in any patient. Within the group in which Phe tolerance increased, 2 patients gradually managed to adopt a non-restricted diet; while Phe tolerance increased by 684.7 ± 348.0 mg Phe/day in the other patients, who required a restricted diet. Daily intake of natural protein slightly increased in the BH4 group at the end of these periods, although the differences did not reach statistical significance. In addition, growth impairment was noted in patients on low-Phe diets. Furthermore, we found no statistically significant differences between both groups at any time point. It should be noted that individuals on BH4 treatment increased their natural protein intake and, in some cases, this allowed them to consume normal diets with protein intake meeting the RDAs. However, no association was observed between higher protein intake and growth.

**Discussion**

Dietary intervention is one of the currently available treatments options for PKU. This approach, which consists of a marked reduction in Phe intake, has shown to prevent severe neurological impairment which was observed in untreated patients. However, while several reports indicated that an excessive reduction in Phe intake resulted in poor developmental outcomes in patients with PKU; others have not found evidence of the PKU diet negatively affecting physical development. Considering the importance of attaining optimal growth in patients with PKU, we conducted a retrospective longitudinal study on the developmental outcomes in a Spanish cohort of PAH-deficient patients (PKU and mild-HPA) who were exclusively treated with protein-restricted diets. The main conclusion from this study was that patients with PKU showed growth impairment in early stages; whereas physical growth was not impaired in patients with mild-HPA. Since BMI remained relatively steady and close to Z-score=0 from birth to 18 years of age; malnutrition can be ruled out as the leading cause of growth impairment in our patients with PKU. Higher protein intakes have been suggested to be related to improved developmental outcomes, although it has not yet been defined which protein fraction (natural protein, protein substitutes or the combination of both [total protein]) exerts the most influence on physical development. In our cohort, we observed that higher Phe intake in the PKU group was associated with attaining a combination of both [total protein]) exerts the most influence on physical development. In our cohort, we observed that higher Phe intake in the PKU group was associated with attaining improved developmental outcomes in early stages. Therefore, we suggest that prescribing very stringent diets during this period (first 4 years of life) might predispose patients with PKU to later growth retardation, with growth outcomes in adulthood being well below the 50th percentile for healthy subjects. Moreover, it may be possible that insufficient intake of other nutrients, such as sources of energy or trace elements (selenium or zinc), underlie growth impairment in the PKU population, as it has been previously proposed [27]. Unfortunately, no data on these nutrients were available in our study. However, since patients with PKU on glycomacropeptide-diet and patients with mild-HPA seem to have better growth outcomes than patients on a protein-restricted diet, we hypothesize that the most likely cause of growth impairment is the low protein intake. We consider that amino acids in the supplements may be less efficient than natural

### Table 3. Dietary parameters (intakes of natural protein, total protein, protein contained in amino acid mixtures and Phe) in PKU patients on BH4 treatment and on a Phe-restricted diet. Data from the two-year and five-year follow-up studies are included. Values are expressed as median (interquartile range).

<table>
<thead>
<tr>
<th></th>
<th>two-year follow-up</th>
<th>five-year follow-up</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>BH4 group</td>
<td>Diet-only group</td>
</tr>
<tr>
<td>Phe intake (initial)</td>
<td>29.9 [18.3-52.3]</td>
<td>19.2 [9.3-31.6]</td>
</tr>
<tr>
<td>(mg/kg/day)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phe intake (final)</td>
<td>41.2 [22.9-48.9]</td>
<td>12.9 [7.9-20.3]</td>
</tr>
<tr>
<td>(mg/kg/day)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Natural protein</td>
<td>0.7 [0.4-1.1]</td>
<td>0.3 [0.3-0.6]</td>
</tr>
<tr>
<td>intake (initial)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(g/kg/day)</td>
<td>0.8 [0.5-1.0]</td>
<td>0.4 [0.3-0.5]</td>
</tr>
<tr>
<td>Natural protein</td>
<td>0.9 [0.7-1.2]</td>
<td>1.3 [1.0-1.8]</td>
</tr>
<tr>
<td>intake (final)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(g/kg/day)</td>
<td>0.5 [0.2-1.3]</td>
<td>1.2 [0.9-1.8]</td>
</tr>
<tr>
<td>Protein in amino</td>
<td>1.8 [1.0-3.6]</td>
<td>2.0 [1.3-2.4]</td>
</tr>
<tr>
<td>acid mixtures (initial)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(g/kg/day)</td>
<td></td>
<td>1.4 [1.0-2.4]</td>
</tr>
<tr>
<td>Protein in amino</td>
<td>1.5 [0.7-2.2]</td>
<td>1.4 [1.0-2.4]</td>
</tr>
<tr>
<td>acid mixtures (final)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(g/kg/day)</td>
<td></td>
<td>1.4 [1.0-2.4]</td>
</tr>
</tbody>
</table>
amino acids, because of faster oxidation and differences in their metabolism. Further research would be required to elucidate the mechanisms that underlie growth impairment in the PKU population, to confirm the association between reduced protein intakes and poorer growth outcomes. In addition, it would be interesting to evaluate epigenetics and creative deficit and their relationship with growth outcomes in PKU.

Over the last decade, BH₄ therapy has been extensively used to treat PAH-deficient patients who respond to a BH₄ loading test. This treatment has improved the management of these patients [28,29]. However, very few studies have explored growth outcomes in patients on BH₄ treatment. Among them, Singh et al. reported that height Z-score increased significantly after 2 years on BH₄ treatment [14]. In addition, they observed that natural protein became the main protein source in the diet, although amino acid supplementation was not always discontinued. Hence, our study aimed to assess to what extent growth in patients with PKU was influenced by BH₄ administration, since it allows them to consume larger amounts of natural proteins. The main conclusion was that BH₄-treated patients displayed growth improvement, despite the fact that their natural protein intake increased. Moreover, individuals on long-term BH₄ treatment seemed to achieve similar developmental outcomes to those on more restricted diets. In fact, mean Z-score for almost all anthropometric variables in both groups fell to the same extent, reaching similar final values. It should be noted that we also observed growth impairment in patients on protein-restricted diets, which is consistent with previous reports [22-23,27,30-31]. However, in contrast to previous studies, increasing natural protein intake did not seem to have a positive effect on developmental outcomes. In fact, both types of diets seemed to lead patients with PKU to physical development impairment, attaining similar growth outcomes at the end of the observation period. As abovementioned, we concluded that prescribing very stringent diets during the first years of life might lead to growth retardation in PKU. Moreover, our findings suggested that BH₄ therapy, along with the subsequent increase in natural protein intake in these patients, might not counteract the negative impact of the deprived diet during the period prior to initiating BH₄ treatment.

One of BH₄ therapy’s aims is to enable patients with PKU to consume normal diets and free them from the need to take Phe-free substitutes. It should be mentioned that many patients on BH₄ treatment in our cohort did not manage to liberalise their diet at the end of the observation period. Moreover, no increase in Phe intake was observed in some BH₄ patients in either two-year or five-year follow-ups. Taking into account these findings, the question arises as to whether the current protocols to assess BH₄ responsiveness, in particular the 30 % criterion, lead to the detection of true responders to the cofactor-based treatment. In our opinion, a shift towards a stricter criterion for responsiveness might ensure that responders to BH₄ treatment would not withdraw from this treatment at later stages. In fact, a new protocol for assessing BH₄ responsiveness based on a 50 % criterion has been recently published [26]. Analysis of the protocol showed that, with this criterion, all responders to the BH₄ loading test were true responders to long-term BH₄ treatment, and all these patients consumed normal diets with no need to take amino acid supplements.

To the best of our knowledge, this is the first time that anthropometric characteristics have been assessed in such a large cohort of patients with PKU on BH₄ treatment and over longer observation period (five- vs. two-year follow-up) comparing with previously published studies. Nevertheless, we consider that further research would be necessary in order to explore in more detail how BH₄ treatment affects the health status of patients with PKU.

Acknowledgments

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Compliance with Ethical Standards

The study protocol was approved by Clinical Research Ethics Committees at each hospital involved in this study. Written informed consent was obtained from patients and parents or legal guardians of all the children included.
References


