Methods

New evidence for assessing tetrahydrobiopterin (BH₄) responsiveness

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ABSTRACT

Objective. To evaluate the protocol we propose for detecting BH₄-responsive patients and the possibility of delimiting more precisely the population to be tested.

Methods. We recruited 102 phenylketonuric patients on a phenylalanine (Phe)-restricted diet. The initial stage of the protocol was a 24-h BH₄ loading test involving Phe loading and subsequent ingestion of the cofactor, a 50% fall in blood Phe levels being considered a positive response. The non-responders at this stage then completed a one-week therapeutic test combining BH₄ administration and daily protein intake meeting recommended dietary allowances, to assess whether the 24-h test had detected all responders.

Results. The 24-h test detected almost all BH₄ responders (30.3% of the 99 patients included in the analysis), with just two patients (2.0%) subsequently responding positively to the therapeutic test. The 24-h test did not give any false positive results.

Conclusions. The 24-h BH₄ loading test is clinically useful for screening phenylketonuric patients. Specifically, 95% of patients with Phe levels <700 μmol/L, and none with Phe levels >1500 μmol/L were BH₄-responsive. Given these results, we conclude that patients with Phe levels <700 μmol/L or >1500 μmol/L probably do not need to be tested, prioritising the identification of BH₄-responsiveness among individuals with intermediate Phe concentrations, between the aforementioned values. Additionally, our results suggest that the therapeutic test only needs to be performed in cases where the reduction in blood Phe levels after cofactor administration is within the range 40%–50%.

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Abbreviations: BH₄, (6R)-L-erythro-5,6,7,8-tetrahydrobiopterin; DGGE, denaturing gradient gel electrophoresis; FDA, Food and Drug Administration; HPLC, high performance liquid chromatography; NO, nitric oxide; PAH, phenylalanine hydroxylase; Phe, phenylalanine; PKU, phenylketonuria; RDA, recommended dietary allowance; SD, standard deviation; Tyr, tyrosine; WHO, World Health Organisation.

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1. Introduction

Phenylketonuria (PKU; OMIM 261600) is an inborn error of metabolism resulting from the impairment of the phenylalanine (Phe) metabolic pathway, which involves the conversion of Phe to tyrosine (Tyr), via phenylalanine 4-hydroxylase (PAH, EC 1.14.16.1) and its cofactor (6R)-L-erythro-5,6,7,8-tetrahydrobiopterin (BH₄). In order to prevent the accumulation of Phe and its metabolites, which would cause severe damage to the developing brain, the treatment must be implemented as early as the diagnosis is made. Traditionally, PKU treatment is comprised of a strict vegetarian diet with very low Phe intake, supplemented by Phe-free formula to compensate for the eliminated foods. Since proposed by Bickel [1], the PKU diet has proven to be effective in preventing the appearance of long-term neurological sequelae in these patients. Nevertheless, there may be secondary complications associated with the restricted diet (growth retardation, early development of osteopaenia/osteoarthritis, suboptimal neurocognitive or psychosocial outcomes) owing to the daily nutrient intake being insufficient to meet recommended dietary allowances (RDAs) and therefore, the delicate health status of PKU patients may worsen [2]. In addition, adhering to the strict diet is onerous and rates of dietary compliance usually decrease with patient age, especially around puberty. In recent decades, in an attempt to improve outcomes in these patients, several new treatments have been developed, including BH₄ therapy, large neutral amino acid supplementation, enzyme replacement therapy and gene therapy [3].

The first BH₄-based therapy (using an unregistered formulation) for the treatment of PKU, owing to defective or deficient PAH, was not described until 1999 [4], notwithstanding preceding evidence in several PAH-deficient patients of a reduction in Phe concentration levels after BH₄ loading [5,6]. This cofactor-based therapy opened up new treatment possibilities for a significant proportion of patients with a diagnosis of mild or moderate PKU who followed vegan-like diets. In many cases, the BH₄ therapy allows PKU patients to eat a near-normal diet, which in turn should increase dietary compliance, thereby improving patients’ and their families’ quality of life [7–9]. Moreover, benefits of administering BH₄ to PKU patients have also been noted with regard to fluctuation in Phe levels, growth and nutritional status [10].

Previous research on the BH₄ therapy has highlighted that the mechanisms underlying BH₄ responsiveness are complex and multifactorial [3,11], and thus, individuals with the same genotype could have different responses to BH₄ loading while patients classified as non-responders on the basis of their mutations could be BH₄-responsive. The response to BH₄ therapy is generally evaluated using a BH₄ loading test. However, there is no consensus on the method that should be used to perform the loading test, or on whether PKU patients should maintain their usual dietary regimen during the test, nor is there an internationally-accepted standard protocol to interpret the results, as highlighted by a recent European survey [12].

We herein present the results of a pilot study evaluating and validating a BH₄ loading test protocol over the period 2005–2009, comparing it with previously published protocols. We also address the possibility of delimiting more precisely the population to be tested, in order to reduce the overall expenditure on screening.

2. Methods

2.1. Patients

A total of 169 PAH deficient patients were diagnosed with PAH deficiency in Andalusia from 1979 to 2009, either in the neonatal period, through the routine Newborn Screening Programme for PAH or at a later stage, by analysing the blood Phe concentration of individuals with psychomotor retardation or a family history of PAH deficiency. We initially selected 102 patients monitored at Virgen del Rocío Hospital who fulfilled the criteria for inclusion in the evaluation of the BH₄ loading test: diagnosed with PKU and on a Phe-restricted diet. Exclusion criteria were pregnancy or breastfeeding in women. The diagnoses were confirmed by genetic analysis. Cofactor deficiency was dismissed in view of normal urine pterin profile measured by HPLC and normal dihydropteridine reductase activity in erythrocytes (The Centre for Molecular Diagnostics (CEDEM), Madrid, Spain). All patients or their parents or legal guardians, in the case of children, gave written informed consent to participate in the study. The study protocol conformed to the tenets of the Declaration of Helsinki (Version Seoul 2008) and was approved by the Ethics Committee at Virgen del Rocio Hospital.

2.2. BH₄ loading test

The BH₄ loading tests considered in this study were performed between 2005 and 2009. Patients were instructed to maintain their usual dietary habits before and during the test (note that, here and elsewhere, in the case of paediatric patients, instructions were given to parents or guardians for them to follow with respect to the patient in their care). After a fasting period of at least 4h, the first blood sample was drawn to determine the baseline Phe concentration (T=0). Subsequently, a load of L-Phe (100mg/kg; Nutricia S.R.L., Madrid, Spain) was administered to each patient, while the fast was maintained, in order to ensure that Phe concentration would rise sufficiently that significant decreases in Phe levels could be observed during the period of the test. Three hours after the Phe overload, (6R)-BH₄ was administered (in a single dose of 20mg/kg) by asking patients to swallow the tablets (Schicks Laboratories, Jona, Switzerland). In instances where patients could not swallow them, the tablets were dissolved in water (20mL), under low light conditions, and the solution was administered within 30min. At this stage, Phe concentration was measured by dried blood spots analysis (T=1). Patients were instructed to end the fasting period 30min after the administration of (6R)-BH₄. The remaining Phe measurements, also by dried blood spot analysis, correspond to blood samples drawn 7 (T=2), 12 (T=3) and 24h (T=4) after the (6R)-BH₄ was administered. Patients were not admitted to hospital in order to collect these blood samples, rather they were taken at patients’ own homes.
This initial stage of the protocol considered a reduction in blood Phe of at least 50% 24 h after BH₄ ingestion as the criterion for considering and individual to be BH₄ responsive. Responders were prescribed BH₄ treatment combined with a natural protein intake meeting RDAs. The BH₄ dose ranged from 5 to 20mg/kg/day. These values represent the minimum and maximum amount of BH₄ that can be administered to patients in an attempt to enable them to resume a normal diet, while maintaining blood Phe levels within a safe range [13].

Phenylketonuric patients who did not meet the aforementioned criterion for responsiveness subsequently underwent the therapeutic test, in order to confirm whether non-responders in the 24-h test were indeed non-responders to BH₄ treatment. This second stage of the protocol entailed administering a BH₄ dose of 20 mg/kg per day for one week and a daily protein intake meeting patients’ age- and sex-specific RDAs. At the end of this period, blood Phe was measured, again by dried blood spot analysis. A Phe level remaining below a defined threshold (<360 μmol/L, for individuals<6 years of age; <480 μmol/L for those 6 to<10 years of age, and<600 μmol/L for those>10 years of age) [14] was considered a positive result in the therapeutic test. Patients meeting this criterion were classed as late responders and, like the faster responders detected in the 24-h test, were prescribed BH₄ treatment and a natural protein intake meeting RDAs. Finally, patients whose Phe concentration rose above the therapeutic target range were considered to have a true negative response to the BH₄ loading test.

2.3 Genotyping

DNA extracted from whole blood samples from PKU patients and their parents were analysed by DGGE (ABI Prism 3700, Applied Biosystems®, Madrid, Spain) and subsequent sequencing (BigDye Terminator v 3.1, Applied Biosystems®). In instances where only one allele carrying a mutation was detected, the exonic regions of which PAH gene is composed were also sequenced.

2.4 Statistical analysis

Statistical analyses were performed using the statistical software program SPSS® 18.0 for Windows (Statistical Package for the Social Sciences, Chicago, IL, USA). Statistically significant difference was set at p<0.05. Initially all data were analysed using the Kolmogorov–Smirnov test, to assess the goodness-of-fit to a normal distribution. As they did satisfy the normality assumption, descriptive statistics are presented as mean±SD and range and differences between groups were tested using the Student’s t test for paired data. Differences between two time-related measurements of a variable were assessed using the t test for related samples. For comparing categorical data the chi square test was used.

3. Results

3.1. BH₄ loading test

During the performance of the BH₄ loading test, vomiting was observed in 3 patients, who were therefore excluded from the analysis. Thus, 99 phenylketonuric patients were finally included in the evaluation of the BH₄ loading test.

A positive response to the 24-h test (Phe reduction>50% after 24h) was observed in 30 of the 99 patients assessed (30.3%). Demographic and clinical characteristics of these BH₄-responsive patients are summarised in Table 1. The baseline blood Phe level in this group was significantly lower than among non-responders (Table 1). In general, among these responders Phe loading induced a marked increase in blood Phe levels (T=1, Table 1), while the subsequent administration of BH₄ caused a drastic decrease in these levels (T=4, Table 1). The response varied, however, with falls in Phe levels of 51.1% to 96.1%. These patients were prescribed BH₄ treatment, which involved taking a BH₄ dose of 5–20mg/kg/day. Four of the 30 patients stopped the long-term BH₄ treatment at some stage and sex-specific RDAs, for one week. Interestingly, in two

<table>
<thead>
<tr>
<th>Table 1 – Characteristics of BH₄ responsive and non-responsive patients.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Responsive patients (24-h test) (n=30)</strong></td>
</tr>
<tr>
<td>Sex (M/F)</td>
</tr>
<tr>
<td>Age at BH₄ loading (years)</td>
</tr>
<tr>
<td>Phe at diagnosis (μmol/L)</td>
</tr>
<tr>
<td>Phe at T=0 (μmol/L)</td>
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<tr>
<td>Phe at T=1 (μmol/L)</td>
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<td>Phe at T=2 (μmol/L)</td>
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<td>Phe at T=3 (μmol/L)</td>
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<tr>
<td>Phe at T=4 (μmol/L)</td>
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<tr>
<td>% response</td>
</tr>
</tbody>
</table>

Data represent mean, standard deviation and range for each variable. Statistical differences between groups were also assessed and p values are presented in the last column.
cases blood Phe concentrations decreased below the recommended levels for PKU patients (<360 μmol/L, <6 years of age; <480 μmol/L, 6 to 10 years; and <600 μmol/L, >10 years) [14] and, therefore, these individuals were classed as late responders to BH4 treatment. During the first stage of the test, the Phe levels of these two patients had decreased by 45.3% and 41.7%, with blood Phe levels at diagnosis of 950.4 and 1029.1 μmol/L. The remaining 67 patients in this group were considered true non-responders to the BH4 loading test.

PKU patients who were non-responders to the 24-h BH4 loading test had a significantly higher Phe concentration at diagnosis (Table 1) (p<0.001) than those with a positive response in this test.

3.2. Genotyping

Genetic analysis revealed that the profile of BH4-responsive patients encompassed 26 different mutant genotypes for the PAH gene (plus one patient in whom the genotype could not be ascertained since the second allele was not identified). Only one of the patients was homozygous for a given mutation although three other patients had two different mutations affecting the same codon. All other patients were compound heterozygotes of mutations affecting different codons. In total, 29 mutations including four novel mutations (IVS4nt+5G>A, E66K, R155C and R281L) were identified in these patients (Table 2). Twenty seven patients presented at least one mutation that had been reported associated to BH4-responsiveness [15]. However, three previously reported sponders to BH4 treatment. During the first stage of the test, and, therefore, these individuals were classed as late responders [15].

Concerning the R281L and IVS4nt+5G>A mutations, in all cases these were harboured in combination with mutations known to be related to BH4-responsiveness (Table 2; note that the IVS4nt+5G>A mutation was found in four patients, one of them with no mutation identified in the other allele). The R281L and IVS4nt+5G>A mutations are new and thus, their functionality has not yet been examined by in vitro expression analysis. Therefore, we cannot a priori state whether these mutations are involved in the BH4 response. Further research is needed to provide more information on this subject. Indeed, the R155C/G289R genotype (patient 26, Table 2) was also observed, that is, two mutations with unknown biological significance. Consequently, their role in the response to BH4 could not be established, although it is clear that either one or both of them confer BH4-sensitivity.

Genotypes of BH4-unresponsive patients are included as supplementary material.

3.3. Long-term BH4 treatment and follow-up

After the new diet was prescribed, patients were instructed to collect weekly blood samples on a Guthrie card at home and post it to our hospital for monitoring of their blood Phe levels. Clinical appointments were scheduled every three months in order to collect anthropometric and clinical data, monitor potential side effects of BH4 treatment and adjust the BH4 doses if needed.

<table>
<thead>
<tr>
<th>Number</th>
<th>Allele 1</th>
<th>Allele 2</th>
<th>Number</th>
<th>Allele 1</th>
<th>Allele 2</th>
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<tbody>
<tr>
<td>1</td>
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<td>F55L</td>
<td>17</td>
<td>Y414C</td>
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</tr>
<tr>
<td>2</td>
<td>I65T</td>
<td>V388M</td>
<td>18</td>
<td>Y414C</td>
<td>G46S</td>
</tr>
<tr>
<td>3</td>
<td>IVS10nt-11G&gt;A</td>
<td>N61K</td>
<td>19</td>
<td>R261Q</td>
<td>A403V</td>
</tr>
<tr>
<td>4</td>
<td>L348V</td>
<td>R261P</td>
<td>20</td>
<td>Y414C</td>
<td>G46S</td>
</tr>
<tr>
<td>5</td>
<td>IVS1nt+5G&gt;T</td>
<td>E390G</td>
<td>21</td>
<td>IVS4nt+5G&gt;A</td>
<td>E390G</td>
</tr>
<tr>
<td>6</td>
<td>C217G</td>
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<td>22</td>
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<td>IVS4nt+5G&gt;A</td>
</tr>
<tr>
<td>7*</td>
<td>R261Q</td>
<td>R261P</td>
<td>23</td>
<td>V388M</td>
<td>R261P</td>
</tr>
<tr>
<td>8</td>
<td>R68S</td>
<td>IVS4nt+5G&gt;A</td>
<td>24</td>
<td>E66K</td>
<td>S349P</td>
</tr>
<tr>
<td>9</td>
<td>I65T</td>
<td>R261Q</td>
<td>25</td>
<td>R68S</td>
<td>P362T</td>
</tr>
<tr>
<td>10</td>
<td>R261Q</td>
<td>R261P</td>
<td>26</td>
<td>A104D</td>
<td>R158Q</td>
</tr>
<tr>
<td>11</td>
<td>IVS4nt+5G&gt;A</td>
<td>Y414C</td>
<td>27</td>
<td>R155C</td>
<td>G289R</td>
</tr>
<tr>
<td>12*</td>
<td>IVS12nt+1G&gt;A</td>
<td>R68S</td>
<td>28</td>
<td>I65T</td>
<td>F55L</td>
</tr>
<tr>
<td>13</td>
<td>R261Q</td>
<td>R261P</td>
<td>29</td>
<td>R158Q</td>
<td>I65T</td>
</tr>
<tr>
<td>14</td>
<td>E390G</td>
<td>V388M</td>
<td>30</td>
<td>R261Q</td>
<td>V388M</td>
</tr>
<tr>
<td>15</td>
<td>R281L</td>
<td>F55L</td>
<td>31</td>
<td>IVS4nt+5G&gt;A</td>
<td>n.f.</td>
</tr>
<tr>
<td>16</td>
<td>R241H</td>
<td>S349P</td>
<td>32</td>
<td>R261Q</td>
<td>R261Q</td>
</tr>
</tbody>
</table>

n.f., a second mutant allele was not found in this patient.

Mutations reported previously in BH4-sensitive and BH4-insensitive patients are represented, respectively, in bold-type or in normal type without underlining. Underlined mutations are those associated here for the first time with BH4-sensitivity.

* These patients did not respond to BH4 in the 24-h test, but they did show BH4-responsiveness in the therapeutic test.

b Novel mutations.
Before starting BH₄ treatment, all patients adhered to a strict vegan-like diet in order to maintain their blood Phe concentration below the aforementioned threshold levels [14]. It can, however, be hard to comply with dietary restrictions and, as noted earlier, compliance diminishes as phenylketonuric patients grow up, especially around puberty. Accordingly, responders to the BH₄ loading were prescribed long-term BH₄ treatment, at doses ranging from 5 to 20 mg/kg/day according to patient requirements, that is, the dose was adjusted in order to enable them to eat a normal diet, while maintaining blood Phe within the safe range. The mean BH₄ dose used was 11.9 mg/kg/day (5.0–20.0 mg/kg/day), a dose of 5–15 mg/kg/day being administered to 75% of these patients, while the other 25% needed a higher dose (>15 mg/kg/day, up to a maximum of 20 mg/kg/day).

The mean blood Phe concentration measured one year after commencing the BH₄ therapy was 318.0±92.0 μmol/L (132.0–480.0 μmol/L). Statistical analysis revealed that this level (after one year on BH₄) was significantly lower than the mean blood Phe concentration at diagnosis (629.6±339.0 μmol/L; 181.6–1453.0 μmol/L) (p<0.001). That is, administering BH₄ to patients with PKU proved to be effective in controlling blood Phe levels, achieving an almost 2 fold reduction, while allowing patients to resume a normal diet with a daily protein intake in line with the RDAs established by the World Health Organization (WHO) [16]. In fact, responders to the test increased their protein intake more than 3 fold, from prior to starting the BH₄ treatment (0.6±0.3 g/kg/day) to when they had been taking the cofactor for a year (1.9±0.7 g/kg/day).

The impact of the BH₄ therapy on the nutritional status of PKU patients was explored by measuring the plasma levels of transferrin, albumin, prealbumin and retinol-binding protein one year after treatment was initiated (Table 3), as these are considered good biochemical markers of dietary protein intake. Moreover, prealbumin has been proposed as a reliable analytical marker of growth status in this population [17]. Plasma levels of the aforementioned biochemical markers slightly increased after one-year of treatment except for albumin, which slightly decreased, although none of these changes were statistically significant (Table 3). Transferrin, albumin and retinol-binding protein plasma levels fell within the reference interval, both before initiating BH₄ treatment and at the end of the follow-up. On the other hand, the mean plasma prealbumin concentration was slightly below the lower limit of the reference interval at both time points.

As mentioned above, four patients who were BH₄-responsive finally withdrew from the long-term BH₄ treatment at some stage during the study. One patient stopped taking BH₄ as blood Phe levels remained above recommended levels after starting the treatment. The other three individuals in this group did not keep their appointments at our hospital and, therefore, could not be monitored and BH₄ treatment was discontinued.

In order to monitor the appearance of side effects that could be related to taking pharmacological doses of BH₄, especially in the subset of patients given BH₄ treatment in the neonatal period, patients or their parents/guardians were asked to report any changes in their health status while on the treatment. During the period of our study, we did not detect any effects secondary to the long-term administration of BH₄ and assessing the minimum effective dose for patients to be on a normal diet, BH₄ response appeared to remain stable throughout the follow-up.

<table>
<thead>
<tr>
<th>Table 3 – Long-term follow-up biochemical measurement.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Biochemical variable</strong></td>
</tr>
<tr>
<td>Blood Phe (μmol/L)</td>
</tr>
<tr>
<td>Phe tolerance (mg/day)</td>
</tr>
<tr>
<td>Protein intake (g/kg/day)</td>
</tr>
<tr>
<td>Transferrin (mg/dL)</td>
</tr>
<tr>
<td>Prealbumin (mg/dL)</td>
</tr>
<tr>
<td>Albumin (mg/dL)</td>
</tr>
<tr>
<td>Retinol-binding protein (mg/dL)</td>
</tr>
</tbody>
</table>

Data represent mean, standard deviation and range for each variable. In the case of reference intervals, only range for each variable was considered. Statistical differences between two time-related measurements of a variable were also assessed and p values are presented in the last column.

a These values are taken by the Biochemistry Laboratory at Virgen del Rocio Hospital as reference intervals for routine analyses and were obtained based upon measurements in a cohort of healthy subjects.

4. Discussion

The first study on treating PAH-deficient patients with BH₄ was reported in 1999 by Kure et al. [4]. Since then, researchers have not succeeded in reaching an international consensus either on evaluating the response to BH₄ or on defining long-term BH₄ treatment. The findings of a European multicentre research project on the diagnosis, treatment and monitoring of PKU were published in 2010 [12]. The authors highlighted that approximately 50% of the participating research centres systematically performed a BH₄ loading test for all PKU patients. The researchers did not, however, apply a common methodology either for performing the test, or for interpreting its results. In most instances, the BH₄ loading test entailed administering a single dose of BH₄, with measurements over a 24h period, but it was not mentioned whether PKU patients ingested BH₄ after Phe was administered or after a period consuming a unrestricted diet. Further, the criterion for considering phenylketonuric patients to be BH₄-responsive differed considerably between centres. In some instances, a
decrease in blood Phe levels of more than 30%, either 8 or 24h after BH4 administration, represented a positive response to the test, while other researchers applied the 50% criterion (blood Phe reduction > 50% after 24h) for the response to the loading test to be considered positive.

In general, there is no consensus between hospitals performing BH4 loading tests as to whether BH4 intake should be preceded by Phe load [4,14,18–23]. Additionally, it has been reported that some patients with PKU are not detected by short BH4 tests but might respond positively to the test if BH4 treatment were to be prolonged for 3–7 days [24].

In addition to the lack of consensus, previous research describing protocols to select PAH-deficient patients that could be treated with BH4 has been based on small cohorts [20,21,25,26]. In other cases, in which large populations were studied, authors have not described key details, such as whether the results of the test had accurately predicted the response to long-term BH4 treatment [19] or whether patients were instructed to discontinue the PKU diet for the BH4 loading test [27]. A reason why the latter aspect is important is that dietary compliance sometimes diminishes among patients attempting to adhere to their usual dietary habits after being able to eat an unrestricted diet, so it is a factor that could affect compliance in the period after the test.

In our study, the BH4 loading test proved to be effective in identifying BH4-responsive individuals among a relatively large group of patients with PKU. The initial stage of the protocol (24-h test) based upon the 50% criterion would be suitable to screen the entire PKU population and would detect those who respond relatively fast. In addition, allowing PKU patients to maintain their usual dietary habits would avoid the risk of individuals failing to adhere to their PKU diet following this test. The second stage would then be focused on detecting individuals who respond more slowly to BH4. We did not measure biopterin levels to assess BH4 absorption, since previous research found that the response to BH4 loading (percent change in blood Phe levels) and postload total urinary biopterin concentrations were not significantly correlated [18]. On the other hand, BH4 was administered to patients during medical appointments, thereby ensuring that the full dose was ingested. All patients who responded positively to BH4 in either of the tests in the protocol received the cofactor therapy, freeing them from the restrictive diet and the need to take amino acid supplements.

Some other interesting results were also observed. One patient who showed BH4-responsiveness (blood Phe reduction of 77.8% after 24h) did not respond to the BH4 long-term treatment, despite carrying a mutation related to BH4-responsiveness (V388M). This patient had been previously diagnosed with moderate PKU, based on Phe concentration at diagnosis (780 μmol/L), and this was consistent with his blood Phe level 3h after Phe loading (684 μmol/L). We attribute this phenomenon to the multiple intercurrent illnesses this patient developed in the period during which BH4 was administered (6 months), this period coinciding with him starting school.

Concerning blood Phe levels at diagnosis, in cases in which blood Phe was above 1440 μmol/L, the response to the BH4 test was always negative, while 95% of the patients presenting blood Phe levels below 720 μmol/L responded positively to the test. This suggests that blood Phe concentration at diagnosis could be a valid predictor of BH4-responsiveness. Accordingly, consistent with the proposal of Baldeleou et al. [26], patients with Phe values below 700 μmol/L or above 1500 μmol/L at diagnosis may not need to be tested; this would enable priority to be given to identifying BH4-responsive individuals among patients with Phe levels between the aforementioned values.

Regarding the therapeutic test, 2 of the 69 patients who completed this longer test responded to the treatment. Reductions in blood Phe levels in these two individuals in the earlier 24-h test had been in the range 40%–50%. The results therefore suggest that performance of the therapeutic test could be restricted to PKU patients with Phe levels in this range. Indeed, we demonstrated that patients among whom Phe levels decreased by less than 40% were non-responders to the BH4 therapy and, thus, this percentage could be used as a threshold for omitting the therapeutic test in some patients.

The BH4 loading protocol we present herein was applied to our PKU patients for the period 2005–2009. During the period of our study and, more recently, during the process of preparing the manuscript for publication, two new BH4 loading protocols have emerged; both attempt to establish a standardised procedure to discriminate between responders and non-responders to the BH4 therapy but they differ considerably. The FDA-approved Prescribing Information for Kuvan® (sapropterin dihydrochloride), a relatively new synthetic form of BH4 available in the USA and Europe, systematically proposes screening all PAH-deficient patients by performing a BH4 loading that entails administering the drug for a period that could extend to several weeks [13]. In marked contrast to this proposal, Anjema et al. [23] have recently published a new approach to selecting BH4-responsive PKU patients based upon the recommendations of the European Working Group for Phenylketonuria [21]. This protocol involves administering two doses of BH4 (20 mg/kg/day), at baseline and 24h with measurements up to 48h, to identify PKU patients who could benefit from treatment with the cofactor. Despite their differences, both protocols are able to detect both early and late responders to BH4. Our results are more in line with those of the study of Anjema et al. [22], in that they indicate that the screening and selection of early responders to BH4 treatment only require a short test, avoiding the need for some patients to be involved in prolonged testing. As far as late responders are concerned, although the 48-h test seemed to effectively detect these patients, the authors recognised that the design might not be sufficiently long for this purpose in patients with very slow responses, that is, some responders to BH4 might not be identified. Based on our experience, the therapeutic test combining BH4 and protein intake meeting patients’ specific sex- and age-RDAs seems to be more appropriate to avoid missing any BH4-responsive patients, and would also ensure that responders to the therapeutic test are real responders to the long-term BH4 treatment.

BH4 therapy arose as an alternative to the usual protein-restricted diet for treating PKU, with the aims of enabling patients to be prescribed a diet that is easier to follow and ameliorating the secondary effects on growth often seen in these patients due to the limited diet [17], by allowing them to...
increase their dietary natural protein intake. To evaluate the impact of the BH₄ therapy on our patients’ nutritional status, we compared plasma concentrations of various biochemical markers directly linked to protein intake, including prealbumin, since it has been reported to be related to growth restriction in the PKU population [28]. Surprisingly, no significant alterations were observed in these biomarkers one year after treatment was commenced, despite natural protein intake meeting RDAs. Accordingly, it seems that PKU patients can achieve a similar nutritional status by consuming either an adequate amount of synthetic supplements or a natural protein intake in accordance with recommended allowances. Analogous results have been documented by other researchers analysing the nutritional status of their PKU patients undergoing BH₄ therapy [7,29].

Concerning long-term secondary effects of administering pharmacological doses of BH₄, there is so far little experience worldwide. BH₄ acts as the cofactor of the hydroxylases related to Phe, Tyr and tryptophan and thus, could prove beneficial to regulating the synthesis and function of neurotransmitters [30]. On the other hand, long-term BH₄ treatment might also trigger central nervous system disorders, since high doses of BH₄ could affect the synthesis of catecholamines and biogenic amines and, in turn, their effect on neurons [31]. This cofactor is also closely related to NO synthesis and vascular endothelial function [32–34], areas in which consequences of the BH₄ long-term therapy have not been thoroughly investigated.

All BH₄-responsive patients took 5mg/kg/day (6R)-BH₄ (Shircks Laboratories, Switzerland) at the beginning of the treatment and the dose was gradually augmented according to patient requirements, to ascertain the minimum effective dose for each individual and thereby minimise, as far as possible, the potential adverse effects of long-term BH₄ treatment. Patients were carefully monitored, especially focusing on those individuals who had begun treatment before 4 years of age (9 patients were aged 1 to 4 years and 1 patient was a neonate). We did not detect any effects secondary to long-term BH₄ treatment. So far, there are little data on the use of BH₄ at such young ages. In cases reported, consistent with our experience, the administration of the drug was found to be safe and no significant side effects were noted [35]. Nevertheless, none of our PKU patients have undergone BH₄ treatment for a very long period and, therefore, we cannot be sure that they will not suffer from any secondary effects at a later stage. Consequently, long-term follow-up is required to monitor the appearance of any clinical symptoms that might be related to the cofactor intake affecting any of the aforementioned metabolic pathways.

In conclusion, the protocol for performing the BH₄ loading test we describe proved to be effective in selecting patients with PKU who are BH₄-responsive. In our opinion, the main strengths of our study are that we tested a large cohort of PKU patients and applied a clearly defined protocol. Further, the findings of our two-stage design allowed us to suggest the possibility of restricting the scope of the BH₄ loading test. Bearing in mind the high cost of the drug, it is debatable whether the entire PKU population should be screened, and new thresholds based upon blood Phe concentration at diagnosis could be set to decide which PKU patients do not need to be tested. In addition, our experience with the long-term administration of the BH₄ treatment indicates that the test is able to effectively detect BH₄-responsive patients, since none of the patients withdrew from the treatment due to a misdiagnosis in the loading test. Nevertheless, we must acknowledge that further research is needed to collect data in different settings to confirm our findings, especially concerning the possibility of restricting the scope of the BH₄ loading test and also the nutritional status of responders to BH₄ therapy. Extending the follow-up period could be helpful to gather more information on how BH₄ impacts PKU patients’ health status and growth, especially in patients who start cofactor treatment early in life. Supplementary materials related to this article can be found online at http://dx.doi.org/10.1016/j.metabol.2012.07.015.

Authors contributions

Luis Aldámiz-Echevarría and María L Couce reviewed the literature and conceived the study. Manuel Pérez and María Bueno were involved in protocol development and patient recruitment and monitoring at Virgen del Rocío Hospital. María Bueno and Fernando Andrade interpreted the results. Carmen Delgado was involved in blood sample handling and performing biochemical analyses at Virgen del Rocío Hospital. Domingo González-Lamuño analysed genetic profiles of phenylketonuric patients enrolled in the study. Sergio Lage was involved in statistical analysis and drafted the manuscript, which was reviewed and approved by all authors.

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Conflict of interest

None of the authors have conflicts of interest to declare.

REFERENCES


