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The first European guidelines on phenylketonuria: Usefulness and implications for BH$_4$ responsiveness testing

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Abstract

Objective: This study aimed to investigate and improve the usefulness of the 48-hour BH$_4$ loading test and to assess genotype for BH$_4$ responsiveness prediction, using the new definition of BH$_4$ responsiveness from the European guidelines, as well as an amended definition.

Method: Applying the definition of the European guidelines ($\geq$100% increase in natural protein tolerance) and an amended definition ($\geq$100% increase in natural protein tolerance or tolerating a safe natural protein intake) to a previous dataset, we first assessed the positive predictive value (PPV) of the 48-hour BH$_4$ loading test using a cutoff value of 30%. Then, we tried to improve this PPV by using different cutoff values and separate time points. Last, using the BIOPKU database, we compared predicted BH$_4$ responsiveness (according to genotype) and genotypic phenotype values (GPVs) in BH$_4$-responsive and BH$_4$-unresponsive patients.

Results: The PPV of the 48-hour loading test was 50.0% using the definition of the European guidelines, and 69.4% when applying the amended definition of BH$_4$ responsiveness. Higher cutoff values led to a higher PPV, but resulted in an increase in false-negative tests. Parameters for genotype overlapped between BH$_4$-responsive and BH$_4$-unresponsive patients, although BH$_4$ responsiveness was not observed in patients with a GPV below 2.4.

Conclusion: The 48-hour BH$_4$ loading test is not as useful as previously considered and cannot be improved easily, whereas genotype seems mainly helpful in excluding BH$_4$ responsiveness. Overall, the definition of BH$_4$ responsiveness and BH$_4$ responsiveness testing require further attention.

KEYWORDS
definition, European guidelines, loading test, phenylketonuria, responsiveness, tetrahydrobiopterin

1 | INTRODUCTION

The cornerstone of treatment in phenylketonuria (PKU; MIM 261600) is restricting phenylalanine (Phe) intake by a natural protein-restricted diet combined with intake of Phe-free amino acid supplements. Additionally, some patients benefit from pharmacological treatment with tetrahydrobiopterin (BH$_4$), which can increase residual phenylalanine hydroxylase

activity leading to better metabolic control and/or an increase in natural protein tolerance. However, different definitions of BH_4 responsiveness exist.[1-3]

Recently, the first European guidelines on PKU were published.[4,5] In these guidelines, BH_4 responsiveness is defined as "establishing an increase in natural protein tolerance of ≥100% with blood Phe concentrations remaining consistently within the target range" or by improved metabolic control, which is defined as ">75% of blood Phe levels remaining within target range without any decrease in natural protein intake associated with BH_4 treatment." Since these criteria are stricter than previously used in the Netherlands,[1] some patients in our population might no longer be considered BH_4 responsive when applying this definition. We noticed that this would even be the case for some patients who could actually tolerate a safe natural protein intake as a result of BH_4, meaning these patients could meet their protein requirements (according to WHO guidelines) using only natural protein sources, therefore not requiring additional amino acid supplements. Since these patients clearly benefit from BH_4 treatment, we felt that the definition of BH_4 responsiveness from the European guidelines may need to be amended to include patients who can tolerate a safe natural protein intake due to BH_4.

The European PKU guidelines also give recommendations on the method of BH_4 responsiveness testing. With the exception of patients with a genotype consisting of two null mutations, in whom BH_4 responsiveness does not need to be further considered, it is recommended that BH_4 responsiveness testing is performed by a 48-hour BH_4 loading test. If Phe concentrations decrease with at least 30% during this test, a treatment trial should be performed to evaluate whether the patient is indeed BH_4 responsive. Although the 48-hour BH_4 loading test with a cutoff value of 30% is often cited as a reliable way to predict BH_4 responsiveness,[1,6] its predictive value has not been assessed using the expert-based definition of BH_4 responsiveness that is stated by the European guidelines. Specifically, the study by Anjema et al. is cited as conformation of the utility of the 48-hour BH_4 loading test, but this study defined BH_4 responsiveness as an increase in natural protein intake of ≥50% or ≥4 g/day, which is a much lower threshold.[1] Therefore, the present study aimed to investigate and improve the usefulness of the 48-hour BH_4 loading test, and to assess the predictive value of genotype, first using the new definition of BH_4 responsiveness from the European guidelines, and second using an amended definition that also includes patients who can tolerate a safe natural protein intake due to BH_4.

2 | METHODS

2.1 | Patients and protocol

We used data that were collected for a previous study on BH_4 responsiveness testing.[1] Detailed information on the data collection, subjects and protocol were described by Anjema et al.[1] Here, the most relevant methodological aspects are summarized.

Data were collected retrospectively from 183 pediatric and adult patients who performed the 48-hour BH_4 loading test between November 2009 and December 2010. None of these tests took place in the neonatal period. For the 48-hour BH_4 loading test, baseline Phe concentrations were required to be over 400 μmol/L. Patients who had Phe concentrations below 400 μmol/L were, therefore, supplemented with Phe. Patients received 20 mg/kg BH_4 for 2 days (at $t = 0$ and 24 hours), while blood samples were collected at $t = 0$, 8, 16, 24, and 48 hours. Patients who showed a reduction in blood Phe concentrations ≥30% compared to $t = 0$ at any moment during the loading test were invited for a BH_4 treatment trial. Three-day dietary records were taken before and after the treatment trial to assess natural protein intake. During this treatment trial, BH_4 was introduced at 20 mg/kg/day (with a maximum of 1400 mg/day), dietary Phe was increased to reach the maximal Phe tolerance, and BH_4 dose was finally decreased if possible. In the original protocol, true BH_4 responsiveness was defined according to previously used guidelines in the Netherlands as "a reduction in blood Phe concentrations of 30% or more compared to mean blood Phe concentrations prior to the 48-hour BH_4 loading test with the same diet, and/or an increase in dietary Phe tolerance of ≥ 50% or ≥ 4 grams of natural protein without increasing the Phe concentrations above the upper target." Data on genotype was collected if available. In total, 175 PKU patients correctly performed the 48-hour BH_4 loading test, and 65 patients performed the treatment trial (Table 1). Two patients from the original cohort were excluded, since it was found out that these patients had a DNAJC12 deficiency.[7]

2.2 | Genotype

Using the BIOPKU database (http://www.biopku.org, accessed on 29 January 2019), we assessed two ways for using genotype to predict BH_4 responsiveness. First, we used the BIOPKU database to collect the percentage of BH_4-responsive patients (including "slow" responders) with a corresponding genotype, when information on BH_4 responsiveness was available for ≥5 cases. Second, we used the BIOPKU database to assign genotypic phenotype values (GPVs) to the genotypes of the patients in this cohort. GPVs are as a numerical representation of predicted PAH activity

TABLE 1 Demographic and clinical details of the study cohort

48-hour BH$_4$ loading test result Treatment trial result	No potential BH$_4$ responsiveness[a] (n = 97)	Potential BH$_4$ responsiveness[a] (n = 78)		
		BH$_4$ responsive[b] (n = 31)	BH$_4$ unresponsive[b] (n = 34)	Other[c] (n = 13)
Gender (% female)	51.5	64.5	47.1	46.2
Age (years)	14.7 (10.3-23.0)	12.0 (6.6-17.1)	12.5 (7.9-22.2)	14.1 (10.4-19.4)
Baseline Phe (μmol/L)[d]	663 (530-915)	458 (362-549)***	509 (408-623)**	485 (386-590)*
Phe supplementation (%)	29.9	71.0***	58.8*	92.3***
Phe supplementation (mg/day)[e]	200 (125-300)	335 (202-500)	350 (150-1000)	400 (210-1000)*
Natural protein intake prior to treatment trial (g/kg bw)		0.34 (0.23-0.43)[f]	0.52 (0.29-0.70)	

Note: Data are presented as median (IQR) or as percentage of patients.

[a]Based on a decrease in Phe levels of 30% as cutoff value.

[b]Based on an increase in natural protein tolerance of 100% as cutoff value.

[c]No treatment trial despite a positive 48-hour BH$_4$ loading test.

[d]At $t = 0$ during the 48-hour BH$_4$ loading test.

[e]Amount of Phe supplementation in patients who were supplemented with Phe.

[f]$P < .05$ compared to BH$_4$-unresponsive patients (Mann-Whitney U test).

*$P < .05$; **$P < .01$; ***$P < .001$ compared to patients with no potential BH$_4$ responsiveness (Mann-Whitney U test, corrected for multiple comparisons according to Bonferroni).

in PKU patients, ranging from 0 (lowest PAH activity) to 10 (highest PAH activity).[8] Since BH$_4$ responsiveness is associated with higher levels of residual PAH activity, it was hypothesized that GPVs could be helpful in predicting BH$_4$ responsiveness. Patients who had a 48-hour BH$_4$ loading test that was considered positive (eg, ≥30% reduction in Phe levels) but did not perform a treatment trial were not included in these analyses.

2.3 | Analyses

No analyses were copied from the original study. BH$_4$-responsiveness was assessed based on natural protein intake. The positive predictive value (PPV) of the 48-hour BH$_4$ loading test was calculated as the number of BH$_4$-responsive patients (based on the results of the treatment trial, using different definitions) divided by the number of potentially BH$_4$-responsive patients (based on the results of the 48-hour BH$_4$ loading test, using different cutoff values). Similarly, the negative predictive value (NPV) was calculated as the number of BH$_4$-unresponsive patients divided by the number of not potentially BH$_4$-responsive patients. Descriptive statistics were used to present most of the data. Normality of data was checked visually using histograms and QQ-plots, and tested using the Shapiro-Wilk test. Other statistical tests are mentioned where used. A two-tailed P-value $<.05$ was considered statistically significant. Statistical analyses were performed using IBM SPSS Statistics version 23 and GraphPad Prism version 7 for Windows.

3 | RESULTS

3.1 | BH$_4$ responsiveness as defined by the European guidelines: The 48-hour BH$_4$ loading test and genotype

Of the 65 patients who performed a treatment trial, three had two putative null mutations (here defined as a GPV of 0 in the BIOPKU database) and were therefore excluded from the analyses in this part, as they would not have been involved in any BH$_4$ responsiveness testing following the recommendations of the European guidelines. All three patients were not considered to be BH$_4$ responsive in the original protocol. The PPV of the 48-hour BH$_4$ loading test was 50.0% when using the recommended cutoff value of 30% and the definition of BH$_4$ responsiveness recommended in the European guidelines.

Increasing the cutoff value to 35% led to a PPV of 57.4%, but beyond that, higher cutoff values were associated with a reduction in NPVs (Table 2). The PPV of separate time points varied between 51.7% (at $t = 8$) and 59.1% (at $t = 24$) using 30% as a cutoff value, with higher cutoff values again resulting in lower NPVs (Supplemental material 1). Furthermore, again looking at separate time points, 51.6%, 29.0%, 16.1%, and 6.5% of BH$_4$-responsive patients showed no Phe decrease ≥30% at $t = 8$, $t = 16$, $t = 24$, and $t = 48$, respectively.

In total, 58 patients had a genotype for which the percentage of BH$_4$-responsive patients in the BIOPKU database with a corresponding genotype (≥5 cases) was available

(Figure 1A). This BIOPKU responsiveness percentage was compared between patients considered BH_4 responsive and BH_4 unresponsive in this study. Significant differences were found between BH_4-responsive patients vs a total group of BH_4-unresponsive patients (including patients with no potential responsiveness after the loading test), as well as between patients considered BH_4 unresponsive after the loading test vs patients considered BH_4 unresponsive after the treatment trial. However, within the group of patients who performed the treatment trial, there was no difference between BH_4-responsive and BH_4-unresponsive patients. In 106 patients, it was possible to assign GPVs to their genotype (Figure 1B), showing a similar pattern. The lowest GPV in a BH_4-responsive patient was 2.4.

3.2 | BH_4 responsiveness using an amended definition: the 48-hour BH_4 loading test and genotype

For a second set of analyses, we defined BH_4 responsiveness as "an increase in natural protein tolerance $\geq 100\%$ or tolerating a safe natural protein intake," in which a safe natural protein intake was determined based on gender and age according to recommendations.[9] Using this definition, the number of BH_4-responsive patients in our cohort increased from 31 to 43, which is a significant increase of 38.7% ($P < .001$, McNemar test). Equally, again excluding the three patients with two null mutations, the PPV of the 48-hour BH_4 loading test (using the 30% cutoff value) increased from 50.0% to 69.4%.

Higher cutoff values resulted in higher PPVs, but were also associated with a decrease in the NPV (Supplemental material 2). The PPVs of having at Phe decrease $\geq 30\%$ at separate time points were between 71.7% (at $t = 48$) and 82.8% (at $t = 8$) (Supplemental material 3), with higher cutoff values again generally resulting in lower NPVs. Of BH_4-responsive patients, 44.2%, 23.8%, 19.0%, and 11.6%

TABLE 2 Predictive values using different cutoff values for a decrease in Phe levels during the 48-hour BH_4 loading test

Phe decrease[a]	PPV[b]	Phe decrease[a]	NPV[c]
$\geq 30\%$	50.0%		
$\geq 35\%$	57.4%	30-35%	100.0%
$\geq 40\%$	58.7%	30-40%	75.0%
$\geq 45\%$	57.5%	30-45%	63.6%
$\geq 50\%$	56.8%	30-50%	60.0%

[a]Maximum Phe decrease compared to the baseline value.
[b]Positive predictive value (PPV) using the corresponding cutoff value.
[c]Negative predictive value (NPV) for a decrease in Phe in the corresponding range.

showed no decrease in Phe $\geq 30\%$ at $t = 8$, $t = 16$, $t = 24$, and $t = 48$, respectively.

Parameters of genotype showed similar patterns as with the definition in European guidelines, although significant differences were now found between BH_4-responsive and BH_4-unresponsive patients who performed the treatment trial (Supplemental material 4). With the amended definition, the lowest GPV in a BH_4-responsive patient again was 2.4.

4 | DISCUSSION

Following the publication of the first European guidelines for PKU, this study assessed the usefulness of the 48-hour BH_4 loading test and genotype for BH_4 responsiveness prediction. Our results indicate that the 48-hour BH_4 loading test is not useful for predicting BH_4 responsiveness as defined by the European PKU guidelines, whereas genotype seems mainly helpful in excluding BH_4 responsiveness. Additionally, we introduced an amended definition of BH_4 responsiveness, which in our opinion better defines which patients benefit from BH_4 treatment. This definition also leads to an increase in BH_4-responsive patients and results in a somewhat more effective 48-hour BH_4 loading test.

Before discussing our findings in more detail, some limitations of this study need to be addressed. Since this study used retrospectively collected patient care data, patients with a negative 48-hour BH_4 loading test did not perform a treatment trial, and the number of false negative tests is therefore unknown. Furthermore, we determined BH_4 responsiveness based on natural protein intake, and therefore did not assess using ">75% of blood Phe levels remaining within target range" as a definition. We anticipate however that this definition will be less regularly used, mostly since increasing the natural protein tolerance is the main goal of BH_4 treatment, as shown by longer-term follow-up studies of BH_4-treated patients.[10-12] Besides these points, it is important to note that this protocol of the 48-hour BH_4 loading test involved Phe supplementation in case of too low Phe concentrations, and did not take measurements of Phe levels at $t = 32$ and $t = 40$.

Since the introduction of BH_4 as a new treatment option for PKU, different definitions of BH_4 responsiveness have been proposed ([1-4]). The definition of BH_4 responsiveness recently proposed in the European guidelines is stricter than the definition previously used in the Netherlands, as shown by a decrease in the number of BH_4-responsive patients in this study compared with the original publication (33 vs 58 BH_4-responsive patients). As mentioned in the introduction, we noticed that some patients who did not meet the criteria of BH_4 responsiveness set by the European guidelines could tolerate a safe natural protein intake due to BH_4.

(A)

(B)
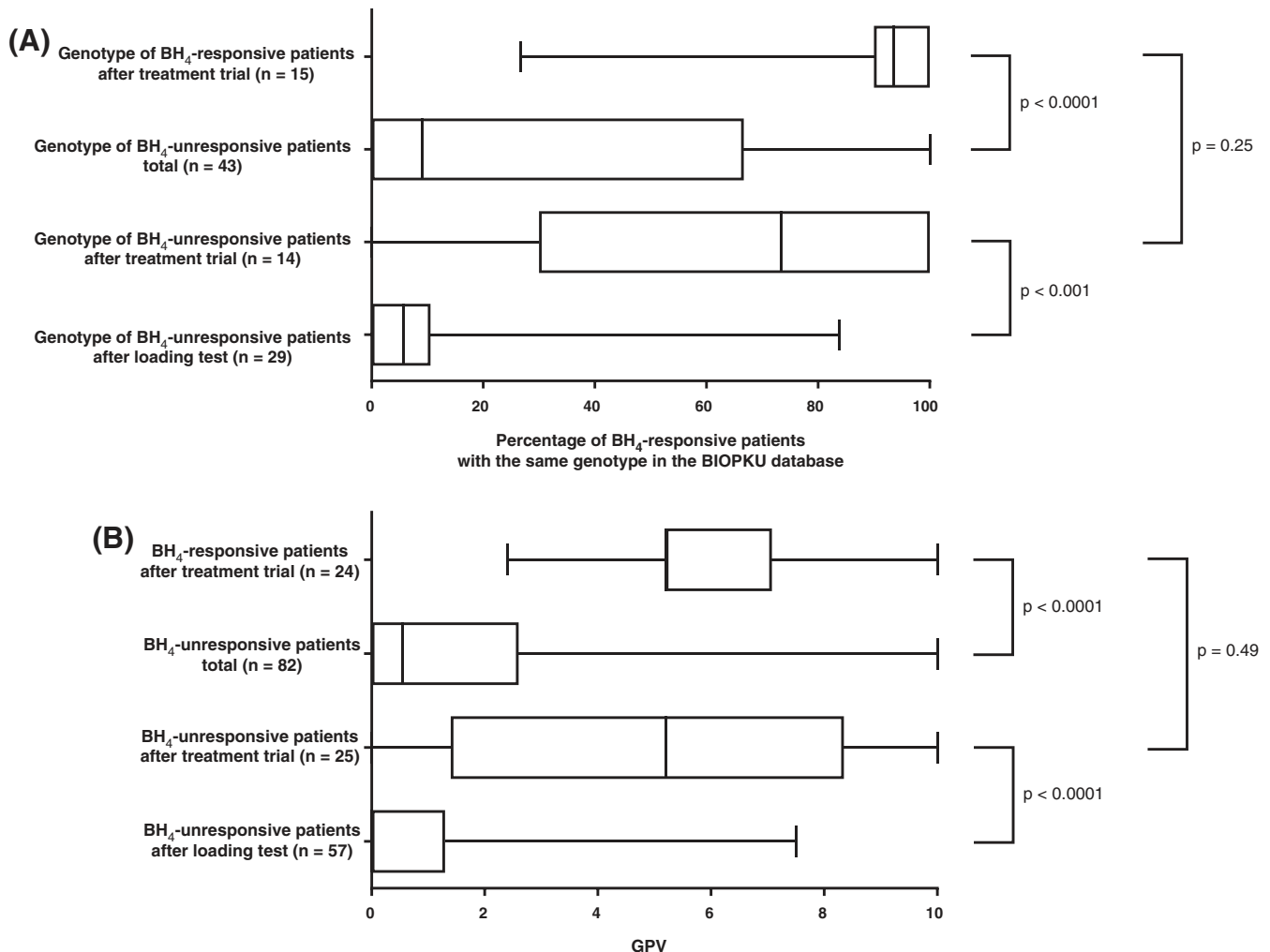
FIGURE 1 Association between genotype and BH$_4$ responsiveness. A, Boxplots (median, 25th-75th percentile, min, max) of the percentage of BH$_4$-responsive patients with a similar genotype in the BIOPKU database in BH$_4$-unresponsive and BH$_4$-responsive patients according to the definitions of the European PKU guidelines. Differences are tested with the Mann-Whitney U test. B, Boxplots (median, 25th-75th percentile, min, max) of the genotypic phenotype value (GPV) in BH$_4$-unresponsive and BH$_4$-responsive patients according to the definitions of the European PKU guidelines. Differences are tested with the Mann-Whitney U test

In our cohort, this was the case for 12 patients. These patients could increase their tolerance with a mean of 22 g of natural protein (range: 13 to 35 g) during the BH$_4$ treatment trial, enabling them to adopt a more normal diet. Moreover, these patients typically did not need amino acid supplementation anymore. Considering these major advantages of dietary liberalization to this extent, the consensus-based definition in the European guidelines could, in our opinion, be improved by defining BH$_4$ responsiveness as "an increase in natural protein tolerance ≥ 100% *or tolerating a safe natural protein intake*."

Clearly, this amended definition is arbitrary as well. The criterion of increasing the natural protein tolerance by at least 100% may still be too strict, and seems especially difficult for patients with a relatively high baseline natural protein tolerance. This is also indicated by a significant difference in baseline natural protein intake between BH$_4$-responsive and BH$_4$-unresponsive patients (according to the definition of the European PKU guidelines) who performed a treatment trial (Table 1). Ultimately, to be able to give a more definitive definition of BH$_4$ responsiveness that is evidence based rather than consensus-based, long-term follow up studies comparing outcomes in BH$_4$ treated to only dietary-treated PKU patients are necessary. Such studies should identify the exact advantages of better metabolic control and/or increased natural protein intake as a result of BH$_4$ treatment in PKU. Since these data are not yet available, developing a (better) consensus-based definition of BH$_4$ responsiveness may be the best alternative. However, different definitions of BH$_4$ responsiveness may require different BH$_4$ responsiveness testing methods, as shown by the results in this study.

Our results show a low PPV of the 48-hour BH_4 loading test when applying the definition of BH_4 responsiveness from the European guidelines. Using the amended definition resulted in a higher PPV, albeit still much lower than the PPV of 87% that was reported in the original publication. Given the widespread use of the loading test, this is a worrying result, since it impairs the cost-effectiveness of BH_4 testing and may also lead to an increase in disappointed patients with a false-positive BH_4 loading test. Using the amended definition, our data show that a larger reduction in Phe during the loading test is associated with a higher chance of being BH_4 responsive. However, considering the increase in false negatives with larger cutoff values, using a cutoff value higher than 30% would not be recommended according to the results in this cohort. Equally, the PPV of a decrease in Phe at separate time points may be somewhat higher, but is also associated with an increase in false-negative tests. Overall, the 48-hour BH_4 loading test cannot be improved easily, and its shortcoming should be kept in mind, especially when using the definition for BH_4 responsiveness stated in the European PKU guidelines.

To improve testing for potential BH_4 responsiveness, the simple 48-hour BH_4 loading test may need to be developed further. In this study, we unsuccessfully tried to improve the test by selecting higher cutoff values and by using separate measurements, but we were unable to investigate the predictive value of Phe measurements at $t = 32$ and $t = 40$, and Phe-to-tyrosine ratios. These latter approaches may thus deserve further attention. Additionally, extending the BH_4 loading test to 7 days[13] or even longer may be helpful in differentiating between daily Phe variation and a BH_4-induced decrease in Phe. On this topic, a comparison between the outcomes of the BH_4 testing regime in Europe (using a 48-hour BH_4 loading test) and the United States (using a 28-days BH_4 loading test) would be interesting. Recently, a group of experts proposed a BH_4 testing algorithm which combines the 48-hour BH_4 loading tests with testing periods up to 4 weeks.[14] Apart from the fact that Muntau et al. did not recommend a specific definition of long-term BH_4 responsiveness, the value of the recommended testing protocol remains to be established. Alternatively, it could be argued that completely different testing methods are simply superior. Potential examples of this include comparing simple Phe loading with Phe + BH_4 loading,[15] using a [^{13}C]Phe breath test,[16] or assessing genotype.[8]

With regard to genotype, results of this study showed that all patients with a GPV below 2.4 showed no (potential) BH_4 responsiveness, which is in line with the report of Gerbade et al. that mentions a cutoff value of 2 as a minimum for (potential) BH_4 responsiveness.[8] Nevertheless, in line with that study, we found considerable overlap between BH_4-responsive and BH_4-unresponsive patients, with some unresponsive patients having a GPV as high as 10 (using the definition of the European guidelines) or 8.9 (using the amended definition). It should be noted that many BH_4-unresponsive patients only performed a loading test and no treatment trial, and some of them might therefore be false negatives. Nevertheless, this is an important finding that requires further examination. Overall, GPVs seem to be primarily helpful to exclude BH_4 responsiveness in patients in case of low-residual PAH activity. Predicting BH_4 responsiveness using data on BH_4 testing in the BIOPKU database from patients with the same genotype shows similar results. Importantly, the BIOPKU database defines BH_4 responsiveness as a decrease in Phe levels of at least 30% during a short-term BH_4 loading test only, regardless of the specific protocol used. While genotype may be strongly related to the results of 48-hour BH_4 loading test,[8] our results show that the 48-hour BH_4 loading test is not a good predictor for BH_4 responsiveness. Therefore, although genotype is helpful in excluding BH_4 responsiveness, it is not an overall good predictor of BH_4 responsiveness. These results show the shortcomings of both genotype and the 48-hour BH_4 loading to predict BH_4 responsiveness, thereby underlining the importance of a treatment trial.

To conclude, the 48-hour BH_4 loading test with a cutoff value of 30% does not effectively predict long-term BH_4 responsiveness as defined by the European PKU guidelines, whereas genotype seems mainly helpful to exclude BH_4 responsiveness. We recommend amending the definition of BH_4 responsiveness from the European PKU guidelines to include patients who can tolerate a safe natural protein intake as a result of treatment with BH_4. Methods to predict BH_4 responsiveness require further attention, although a sound definition of BH_4 responsiveness may be even more important to optimize BH_4 responsiveness testing as well as treatment.

CONFLICTS OF INTEREST

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AUTHOR CONTRIBUTIONS

R.A.F.E. designed the study, analyzed the data, interpreted the results, and was the lead writer of the manuscript. A.M.J.v.W. designed the study, interpreted the results, and was the second lead writer of the manuscript. K.A. collected the data, interpreted the results, and cowrote the manuscript. C.M.A.L., E.v.D., D.v.V., and N.B. interpreted the results and cowrote the manuscript. F.J.v.S. interpreted the results, cowrote the manuscript, and supervised the project. All authors read and approved the final version of the manuscript.

DATA ACCESSIBILITY

Original data will be made available on reasonable request.

ETHICAL APPROVAL

For the original data collection, the BH_4 testing protocol was considered standard patient care by the Medical Ethical Committee of the University Medical Center Groningen. Informed consent was not required for this study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.