University of Groningen

Tetrahydrobiopterin in phenylketonuria

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DOI:
[10.33612/diss.135584531](https://doi.org/10.33612/diss.135584531)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2020

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):
Anjema, K. (2020). *Tetrahydrobiopterin in phenylketonuria: Who can benefit?* [Thesis fully internal (DIV), University of Groningen]. University of Groningen. <https://doi.org/10.33612/diss.135584531>

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Tetrahydrobiopterin in Phenylketonuria

Who can benefit?

Karen Anjema

Tetrahydrobiopterin in Phenylketonuria. Who can benefit?

The author gratefully acknowledges the financial support for printing his thesis by:

BioMarin

Nutricia Nederland

Rijksuniversiteit Groningen

Universitair Medisch Centrum Groningen

Groningen University Institute from Drug Exploration (GUIDE)

ISBN:

978-94-034-2557-3 (printed)

978-94-034-2556-6 (digital)

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Cover-design: Peter van Hout

Layout: Karen Anjema & Ridderprint | www.ridderprint.nl

Printed by: Ridderprint | www.ridderprint.nl

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Tetrahydrobiopterin in Phenylketonuria

Who can benefit?

Proefschrift

ter verkrijging van de graad van doctor aan de
Rijksuniversiteit Groningen
op gezag van de
rector magnificus prof. dr. C. Wijmenga
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op

dinsdag 20 oktober 2020 om 11.00 uur

door

Karen Anjema

geboren op 22 mei 1985
te Tietsjerksteradeel

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List of abbreviations

5-HIAA	5-hydroxyindoleacetic acid
5-HT	Serotonin
AF	Allele frequency
BBB	Blood-brain barrier
BH4	Tetrahydrobiopterin
BLT	BH4 loading test
CSF	Cerebrospinal fluid
DA	Dopamine
HPA	Hyperphenylalaninemia
IQR	Interquartile range
L-DOPA	L-3,4-dihydroxyphenylalanine
NE	Norepinephrine
PAH	Phenylalanine hydroxylase
Phe	Phenylalanine
PKU	Phenylketonuria
PPV	Positive predictive value
QoL	Quality of life
TH	Tyrosine hydroxylase
TPH-2	Tryptophan hydroxylase
Trp	Tryptophan
Tyr	Tyrosine
WT	Wild type

GENERAL INTRODUCTION AND SCOPE OF THE THESIS

1

General introduction and scope of the thesis

PKU and its history

Phenylketonuria (PKU, OMIM 261600) is an autosomal recessive disease that is caused by a mutation in the gene encoding for the enzyme phenylalanine-hydroxylase (PAH). As a result of this mutation the enzyme is not functioning properly or not functioning at all. PAH is a mainly hepatic working enzyme, which under normal circumstances converts the essential amino acid phenylalanine (Phe) into tyrosine. As Phe cannot be converted it accumulates in blood and tissue.

The disease was first described in 1934 by dr Asbjørn Følling after investigating two mentally disabled siblings.¹ He discovered that their urine contained high amounts of phenylketone bodies, hence the name phenylketonuria, and turns dark green after adding ferric chloride. After this discovery many patients from institutions were tested for PKU. All positively tested patient had many symptoms in common; progressive mental deterioration, neurological symptoms (epilepsy, motor deficits), behavioral and psychiatric disorders, microcephaly, a mousy odour and decreased pigmentation resulting in blond hair and blue eyes.² Its autosomal inheritance was described by Penrose.³ Jervis also demonstrated the deficiency in the enzyme PAH.⁴ In the 1950's the dietary treatment of PKU was developed by Horst Bickel as it was possible to remove Phe from a protein hydrolysate. It soon became clear that diet greatly improved the condition of PKU patients, but that early initiation was necessary to have a better outcome.^{5,6} With the development of the bacterial inhibition assay by Robert Guthrie in 1963, neonatal screening for PKU at a large scale was made possible.⁷ In the Netherlands, PKU screening was started by the University of Groningen in 1969 as a pilot study in the Northern provinces. Subsequently a national newborn screening program was started in 1974. Since then, the national newborn screening program has been expanded to include 22 diseases, of which 16 are inherited metabolic diseases. Although it is the most prevalent disorder in amino acid metabolism and is one of the most prevalent inherited metabolic diseases with a prevalence of 1:18,000 newborns in The Netherlands,⁸ PKU is a rare disease both in the Netherlands, Europe and in the world.

Genetics and biochemistry

The PAH gene discovered by Woo et al⁹ is located on the long arm of chromosome 12 (q22-q24), spans about 90 kb and contains 13 exons. Until now over 1100 different mutations have been documented.¹⁰ Deficiency of PAH can be caused by homozygous mutations as well as compound heterozygosity, which means two different mutations on the two different alleles. The mutations in the PAH gene can result in a wide spread of biochemical phenotypes, spanning from –if untreated- very mild hyperphenylalaninemia to very severe elevations of Phe concentration. In

individuals without PKU, normal fasting blood Phe concentrations range from approximately 23 - 83 $\mu\text{mol/l}$ (depending on age, gender and method of measurement). In PKU this can be increased to over 40 times. Higher concentrations of blood Phe are associated with worse intellectual outcome and the strictness of treatment required to prevent damage differs immensely among patients.¹¹ However, it is still hard to predict clinical severity right after birth. Multiple attempts have been made to categorize clinical severity mainly based on blood Phe concentrations and the amount of Phe tolerated to achieve acceptable Phe concentrations.^{12,13} Although hyperphenylalaninemia has been shown to be largely due to PAH deficiency, some patients have a deficiency in one of the enzymes related to the metabolism of its cofactor tetrahydrobiopterin,¹⁴ while very recently a defect in the DNAJC12 gene, encoding a PAH chaperone protein, has been discovered to cause mild elevation of phenylalanine as well.^{15,16}

Pathogenesis

Although in the last 80 years a lot of knowledge was gained regarding PKU and its treatment, the pathogenesis of the disease is still not completely known. Several theories have been posed. First of all, hyperphenylalaninemia has been shown to affect myelination in brain white matter.¹⁷ However, the significance of white matter pathology on functional impairments is uncertain, with some evidence that abnormalities extending into subcortical and frontal regions might be associated with functional outcome.¹⁸ Secondly, alterations in neurotransmitters may play a role as dopamine and serotonin levels in brains of deceased patients have been found to be clearly decreased.¹⁹ Both neurotransmitters have an amino acid precursor, respectively tyrosine and tryptophan. As Phe hydroxylation in PKU is diminished, some patients have lower blood tyrosine levels in general. Another explanation could be that Phe in the brain inhibits the enzymes tyrosine hydroxylase and tryptophan hydroxylase that are the rate limiting step of cerebral synthesis of dopamine and serotonin.²⁰⁻²² Yet, another option could be that Phe competes with other large neutral amino acids (LNAA's) for the transport over the blood-brain-barrier, which may further reduce the availability of tyrosine and tryptophan. The latter mechanism could have more consequences. Transport of Phe and eight other LNAA's (i.e. tyrosine, tryptophan, valine, isoleucine, leucine, threonine, methionine, and histidine) across the blood-brain-barrier occurs most importantly by the LAT1 transporter. As blood concentrations of Phe are high and the LAT1's affinity for Phe is also high, transport of Phe into the brain will be increased in expense of the other LNAA's.^{23,24} High Phe concentrations may also affect HMG-CoA reductase,²⁵ disturbed glutamatergic neurotransmission,^{26,27} glycolysis via reduced activity of pyruvate kinase^{28,29} and may assemble into cytotoxic fibrils³⁰. It also seems important that low cerebral concentrations of other LNAA's can result in decreased protein synthesis^{31,32}. Cerebral protein synthesis is essential for brain development and function.³³ For further review I refer to Surtees and Blau, and van Spronsen.^{23,34}

Treatment of PKU

Dietary treatment:

Amino acids are molecules that combine into peptide chains to form the building-blocks of proteins. After water, proteins comprise the largest component of human tissue. A lot of proteins function as enzymes that catalyze reactions in metabolism and can be used as energy source as well. To build those proteins, adequate amounts of the necessary amino acids must be available. Amino acids are derived by the diet or by protein breakdown (catabolism). Some amino acids can be formed from other amino acids (non-essential or dispensable amino acids), whereas others cannot be synthesized by the body itself (essential or indispensable amino acids). Phe is an essential amino acid, which means that the concentration solely depends on intake and the balance between protein anabolism and catabolism, while tyrosine is a non-dispensable amino acid but in case of PAH deficiency has become an essential amino acid. The fact that Phe is an essential amino acid is the crux to the PKU diet. Phe restriction in the diet realized by a natural protein restriction will result in a decrease in blood Phe concentrations. A relatively small amount of Phe is still needed to allow for basal metabolism and a variable amount for growth, depending on growth velocity. All other amino acids are supplemented by an artificially produced amino acid mixture lacking Phe, enriched with tyrosine and containing vitamins and trace elements. Although the theory of the diet is simple, in practice it is hard to follow as nearly all daily used food products contain protein and therefore Phe (also non-animal food products, e.g. bread and potatoes). Rigorous planning is needed, which can lead to social restrictions.³⁵

Guidelines

Among countries there has been a debate on what target Phe concentrations lead to the most optimal outcome. Research has shown that treatment during childhood and adolescence is essential for a normal development,³⁶ and even during adulthood more and more evidence is showing that the diet is beneficial as well.³⁷ The very recent European guidelines proclaim target blood Phe concentrations of 120-360 $\mu\text{mol/l}$ in children under 12 years of age, whilst 120-600 $\mu\text{mol/l}$ for those above 12 years. A special group of patients are the pregnant PKU women and those who are aiming to become pregnant (maternal PKU) for whom the guideline requires blood Phe concentrations between 120-360 $\mu\text{mol/l}$.³⁸ It is also advised to measure blood Phe concentrations by home blood sampling on filter paper weekly to monthly. Outpatient visits range from weekly to yearly according to the patients' age and, clinical and metabolic control.³⁸

Outcome

Most PKU patients treated at an early age show overall development within the normal range. Despite this tremendous improvement still some subtle differences exist when compared to non-PKU individuals. The optimal outcome seems highly dependent on metabolic control. Meta-

analysis showed that for early-treated patients every 100 $\mu\text{mol/l}$ increase in lifetime Phe results in a 1.9-4.1 reduction in IQ.³⁹ Furthermore, some degree of impairment in executive functioning is found, which are higher level cerebral processes such as planning and organizing.⁴⁰ Moreover, school problems, decreased social competence and low-self-esteem are seen.⁴¹ Psychological disturbances can also occur, for example depression, attention deficit disorder and agoraphobia.⁴²⁻⁴⁴ Last but not least, quality of life has been studied in PKU as well, these studies however have shown some contradictions. In one study decreased and delayed autonomy (exceptionally high percentage of patients still living with their parents), a lower number of relationships and having fewer children was reported.⁴⁵ Whereas in the other group no clear differences were seen, although it could be that the used questionnaire is not sensitive to some disease specific problems.⁴⁶ Recently, a PKU specific quality of life questionnaire has been developed.⁴⁷ A study using this questionnaire also showed an overall good quality of life in PKU patients, with a higher report rate of practical and emotional impacts of the diets and Phe-free amino acid supplement intake by patients with a more severe PKU phenotype.⁴⁸

BH4

History of BH4

The PAH enzyme activity depends on a number of cofactors including tetrahydrobiopterin (BH4), iron and oxygen. BH4 is synthesized from guanosine triphosphate (GTP) by multiple steps. BH4 donates hydrogen atoms to PAH to hydroxylate Phe, after which it becomes BH2, which can be recycled to BH4 again. In the 1970's, the first patients with high blood Phe concentrations not responding to the diet were described. In 1975 Kaufman et al published the first cases of BH4 deficiency.⁴⁹ In later years multiple enzyme deficiencies leading to BH4 deficiency were described.⁵⁰⁻⁵⁴ Furthermore, it was discovered that BH4 not only is a cofactor for PAH, but also for tyrosine hydroxylase and tryptophan hydroxylase. Both enzymes are the rate limiting step in the production of important neurotransmitters, dopamine and serotonin, respectively. Therefore, patients with a BH4 deficiency usually present with more severe neurological symptoms and need other or additional treatment to the Phe restricted diet. The differentiation between BH4 deficiency and PKU was traditionally made by conducting a BH4 loading test.^{55,56} In this test, BH4 is given to the patient and blood Phe concentrations are monitored. Patients with a BH4 deficiency usually show a fast and markedly decrease of Phe concentration (for most deficiencies within eight hours). Nowadays, most centers use other diagnostic tests to detect BH4 deficiencies (measurement of pterins and dihydropteridine reductase (DHPR) enzyme activity). BH4 deficiencies are beyond the scope of this thesis, but BH4 itself has shown to be a very important additional treatment option in PKU and is the main topic in this thesis.

BH4 responsiveness in PKU

In 1999, the first report of a Phe concentration reduction in PKU patients due to BH4 was published.⁵⁷ Since then, a lot more reports came out, showing that especially PKU patients with a milder biochemical phenotype respond to BH4.^{58,59} It soon became clear that the so-called BH4 responsive PKU patients usually take longer to show a Phe concentration reduction to BH4 when compared to BH4 deficient patients.⁶⁰⁻⁶² A randomized placebo controlled trial showed a clear effect of BH4 on Phe concentrations in a significant number of patients, but not in all patients.⁶³ The pharmaceutical formulation of BH4 used in this last study (sapropterin dihydrochloride; Kuvan®) became registered for PKU patients (USA 2007: all patients, except for pregnant women; Europe 2008: patients of four years or older, except for pregnant women; Europe 2015 extension of approval for children under four years of age). It is believed that BH4 acts as a pharmaceutical chaperone and thereby stabilizes the PAH protein.⁶⁴ Determining which patient is responsive to BH4 is not straightforward. Around the world many different methods are used. It was found that selection based on Phe concentrations or Phe tolerance is not reliable.⁶⁵ Most European centers use a BH4 loading test. Some aspects of the BH4 loading test are a matter of debate and will be discussed in this dissertation.

Scope of the thesis

Although BH4 seems a promising therapy for PKU patients, it is not clear-cut how to identify the patients who benefit from the drug. In small cohorts it has been shown that not all patients respond. However, it seems that determining responders is not as straightforward as initially thought. The following questions need to be answered: What should be the duration of the test, which dose of BH4 should be used, what should be the baseline blood Phe concentration starting the test and what are good predictors for a positive test. Furthermore, a lot of the tests used to predict BH4 responsiveness have not been compared to long-term treatment results and therefore have not been 'validated'. The introduction of BH4 for PKU in the Netherlands in 2009 posed an opportunity to study a large group of patients in a standardized manner.

The first part of this thesis aims at answering the following questions:

- Does the 48 hour BH4 loading test reveal new long-term responders (and therefore 'true' responders) compared to the 'traditional' 24 hour test? [Chapter 2](#)
- Does the course of blood Phe concentrations in the BH4 loading test predict the amount of Phe tolerance gained by BH4? [Chapter 3](#)
- Can BH4 responsiveness be predicted from genotype? [Chapter 3](#)
- How do we interpret 48 hour BH4 loading test results using different definitions of BH4 responsiveness? [Chapter 4](#)
- What can we learn from previous neonatal BH4 loading tests? [Chapter 5](#)
- What are the long-term effects of BH4 on metabolic control en dietary tolerance? [Chapter 6](#)

In PKU, the primary treatment aims at reducing Phe concentrations. However, as explained previously in this introduction, the pathophysiology of PKU is yet unraveled and one of the factors that could indirectly contribute to the brain dysfunction in PKU is a deficiency in the neurotransmitters dopamine and serotonin. These neurotransmitters are not only very important for neurocognition,³⁴ but also very important for mood and behavior. As addressed above, BH4 is a cofactor in the rate limiting step of cerebral dopamine and serotonin synthesis. Therefore, in theory, BH4 could have an effect on the neurotransmitter levels and thereby possibly affect mood and behavior. That led to the following questions, addressed in the second part of this thesis:

- What are typical behavioral disturbances in PKU and what is the effect of Phe? [Chapter 7](#)
- What is the effect of BH4 on prolactin (as dopamine marker) in PKU patients? [Chapter 8](#)
- What is the effect of BH4 on brain neurotransmitters in (PKU) mice? [Chapter 9](#)

Finally, an overview and discussion of this thesis is written in [Chapter 10](#)

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BH4 TREATMENT IN PKU

PART

1

THE 48-HOUR TETRAHYDROBIOPTERIN LOADING TEST IN PATIENTS WITH PHENYLKETONURIA: EVALUATION OF PROTOCOL AND INFLUENCE OF BASELINE PHENYLALANINE CONCENTRATION

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Abstract

Background: The 24- and 48-hour tetrahydrobiopterin (BH4) loading test (BLT) performed at a minimum baseline phenylalanine concentration of 400 $\mu\text{mol/l}$ is commonly used to test phenylketonuria patients for BH4 responsiveness. This study aimed to analyze differences between the 24- and 48-hour BLT and the necessity of the 400 $\mu\text{mol/l}$ minimum baseline phenylalanine concentration.

Methods: Data on 186 phenylketonuria patients were collected. Patients were supplemented with phenylalanine if phenylalanine was $<400 \mu\text{mol/l}$. BH4 20 mg/kg was administered at T=0 and T=24. Blood samples were taken at T=0, 8, 16, 24 and 48 h. Responsiveness was defined as $\geq 30\%$ reduction in phenylalanine concentration at ≥ 1 time point.

Results: Eighty-six (46.2%) patients were responsive. Among responders 84% showed a $\geq 30\%$ response at T=48. Fifty-three percent had their maximal decrease at T=48. Fourteen patients had $\geq 30\%$ phenylalanine decrease not before T=48. A $\geq 30\%$ decrease was also seen in patients with phenylalanine concentrations $<400 \mu\text{mol/l}$.

Conclusion: In the 48-hour BLT, T=48 seems more informative than T=24. Sampling at T=32, and T=40 may have additional value. BH4 responsiveness can also be predicted with baseline blood phenylalanine $<400 \mu\text{mol/l}$, when the BLT is positive. Therefore, if these results are confirmed by data on long-term BH4 responsiveness, we advise to first perform a BLT without phenylalanine loading and re-test at higher phenylalanine concentrations when no response is seen. Most likely, the 48-hour BLT is a good indicator for BH4 responsiveness, but comparison with long term responsiveness is necessary.

Introduction

The cornerstone of treatment of Phenylketonuria (PKU; phenylalanine hydroxylase (PAH) deficiency MIM 261600) is the life-long dietary restriction of phenylalanine (Phe).¹ Dietary treatment lowers the blood Phe concentration. Although the treatment is highly successful in preventing neurological damage, it is a major burden for patients and caregivers.

Recently, tetrahydrobiopterin (BH4) was introduced as a new treatment option, but only for BH4-responsive patients. Usually, these are patients with a milder PAH deficiency,² although BH4 responsiveness has also been reported in a few patients with more severe PAH deficiencies.³ Unfortunately, determining BH4 responsiveness is not explicit. Genotyping can be useful,⁴ but it tends to overestimate BH4 responsiveness.⁵ Selection based on either Phe concentrations and/or tolerance in day to day practice is also unreliable.⁶ Therefore, BH4 loading tests are used to discriminate between responders and non-responders. Different protocols of the BH4 loading test exist around the world. In Europe, the 48-hour test, developed by the “European working group for Phenylketonuria”, is commonly used. In this protocol a minimum Phe level of 400 $\mu\text{mol/l}$ is essential. A $\geq 30\%$ decrease of the Phe concentration compared to the baseline Phe concentration suggests long-term BH4 responsiveness,⁷ but data on long-term response need to prove the results.

BH4 (Sapropterin) was approved by the EMEA for PKU patients from four years of age, at the end of 2008.⁸ The Netherlands was one of the first countries in Europe where BH4 (Sapropterin) became available. We took the opportunity to test for BH4 responsiveness using a standardized national protocol based largely on the recommendations of the European working group.⁷ We would like to address two important questions to optimize the BH4 loading test 1. What are the differences in the results between the 24- and 48-hour BH4 loading test? 2. Does the BH4 loading test require a minimum Phe level of 400 $\mu\text{mol/l}$ at baseline?

Methods

Patients

Subjects were all PAH-deficient patients who were treated with a protein restricted diet and supplementation of amino acids, and also underwent the 48-hour BH4 loading test as standard patient care. Except for ten patients all of the others were above the age of 4 years. Data included are from patients from six of the eight University Medical Centers in the Netherlands that started to use the national 48-hour BH4 loading test protocol. The medical ethical committee of the University Medical Center of Groningen concluded that their approval was not required, since the 48-hour BH4 loading test is performed as standard patient care.

Procedure

All participating patients with plasma Phe concentrations below 400 $\mu\text{mol/l}$ were supplemented with Phe (L-Phe in powder, a protein rich supplement such as milk powder or an increase in natural protein intake) until the end of the test. The test consisted of two doses of 20 mg/kg body weight BH4 at T=0 and T=24 hours just after blood samples were taken. Blood samples on filter paper were taken at T=0, 8, 16, 24 and 48 h. Patients who (later) appeared to have developed a fever during the test or during the preceding day as well as patients who missed a dose of BH4 were excluded. Responsiveness was defined as a 30% or more reduction in Phe concentration at 1 or more moment(s) compared to baseline (T=0).

Blood Phe concentrations from dried blood specimens were measured according to current quantitative methods used in the various centers. These included methods such as high-performance liquid chromatography with fluorescence and tandem mass spectrometric detection.

Statistical analysis

All descriptive statistics were shown as medians with ranges. For comparing categorical data the chi square test was used. For continuous data the Mann-Whitney U test was used. A p-value ≤ 0.05 was considered to be statistically significant. Statistics were performed with PASW statistics 18.0; SPSS, Inc., Chicago, IL, USA.

Table 1

Demographic and clinical details of the $<30\%$ and $\geq 30\%$ response group

	$< 30\%$ response N=100	$\geq 30\%$ response N=86	P
Gender M/F	47/53	39/47	0.822
Age at BH4 loading	14.7 (0.5 – 46.0)	12.1 (3.6-35.2)	0.013
Baseline Phe ($\mu\text{mol/l}$)*	659 (203-1544)	479 (203-1181)	0.000
Mean Phe ($\mu\text{mol/l}$)**			
< 12 years	397 (137-920)	302 (142-566)	0.045
≥ 12 years	564 (159-1466)	336 (224-759)	0.000
Phe at diagnosis ($\mu\text{mol/l}$)†			0.000
< 600	3	34	
600-1200	14	33	
> 1200	66	18	

*Phe concentration at T=0 of the BH4 loading test. **Mean Phe concentration in one year prior to the BH4 loading test (min. 4 samples), value missing in N=15 and N=13 respectively. †First Phe concentration in hospital, missing in N=17 and N=1, respectively. P-values in bold are ≤ 0.05 .

Results

BH4 loading test data of 186 patients were collected over a period of 17 months following the introduction of Sapropterin in the Netherlands. The median age of the patients was 13.5 years (range 0.5–46.0 years). Eighty-six out of 186 patients (46.2%) had a $\geq 30\%$ Phe decrease

compared to T=0. Demographic and clinical details of the patients with <30% and $\geq 30\%$ response are shown in Table 1. The range of responses on BH4 included an increase of 27.3% to a decrease of 94.5%. In Figure 1 the prevalence of response rates is shown in patients with a $\geq 30\%$ response.

Figure 1 Distribution of maximal blood Phe reductions, in 86 patients responding to BH4 loading (20 mg/kg, twice) during the 48-hour BH4 loading test.

Duration of the test

In patients responding to BH4, Figure 2A shows the percentages of patients with a $\geq 30\%$ Phe decrease at the particular time points. When only one time point (either T=8, T=16, T=24 or T=48) was analyzed versus baseline, 48 (56%), 31 (36%), 28 (33%) and 14 (16%) patients would have been missed, respectively. Most patients (53.5%) had their maximal individual decrease at T=48 (Figure 2B). Fourteen patients (16%) had a $\geq 30\%$ response at T=48 and none before, they were regarded to as slow-responders. Table 2 presents the demographic and clinical details of normal responders versus slow responders. Additionally, three patients showed a response at T=8 and T=16, but not subsequently.

Baseline Phe concentration and Phe response

Before starting the BH4 loading test, 50% of all patients had Phe concentrations <400 $\mu\text{mol/l}$. All 92 patients were loaded with Phe (median 275 mg Phe, range 40–2000 mg). Despite Phe loading, 32% of these patients had a Phe concentration <400 $\mu\text{mol/l}$ at the time of the test. Out

of the patients who were not loaded with Phe, 11% of 94 patients had a Phe concentration <400 $\mu\text{mol/l}$ at baseline.

Figure 2 A. percentages of responders with $\geq 30\%$ Phe concentration reduction at specific time points. B. Moment of maximal reduction of blood Phe concentration (%) in patients responding to BH4 loading (of 20 mg/kg, twice) during the 48-hour BH4 loading test.

Table 2

Demographic and clinical details of patients responding to BH4 during the first 24 hours versus patients responding at T=48 (slow-responders)

	24h-responders N=72	Slow-responders N=14	P
Gender M/F	32/40	7/7	0.702
Age at BH4 loading	11.8 (3.6 – 35.2)	15.3 (5.5 – 28.6)	0.094
Baseline Phe (μmol/l)*	461 (203 – 969)	540 (352 – 1181)	0.017
Mean Phe (μmol/l)**			
< 12 years	298 (142 – 539)	462 (276 – 566)†	0.048
≥ 12 years§	323 (224 – 759)	414 (233 – 617)†	0.725
Phe at diagnosis (μmol/l)§§			-
<600	33	1	
600-1200	26	7	
>1200	12	6	

*Phe concentration at T=0 of the BH4 loading test. **Mean Phe concentration in one year prior to the BH4 loading test (min. 4 samples). §Value missing in N=8 and N=5 respectively. §§First Phe concentration in hospital, missing in N=1 and N=0, respectively. †N=4 patients. *N=5 patients. P-values in bold are ≤0.05.

Figure 3 shows that a decrease of ≥30% is not restricted to patients with plasma Phe concentrations above 400 μmol/l. Seventy-four percent of patients with a baseline Phe concentration <400 μmol/l (both Phe-loaded and not Phe-loaded patients) showed a ≥30% response. Baseline Phe concentrations of patients with a positive response ranged from 203 to 1181 μmol/l. The 203 μmol/l baseline Phe level in one patient was due to a combination of mild PKU and strict treatment as can be learned from the pre-treatment Phe level (740 μmol/l at day 10) and the present Phe intake of 450 mg (+500 mg extra Phe-supplemented during the test) at the age of 14 years. Figure 3 also shows that the higher the Phe concentration, the lower the chance of BH4 responsiveness.

Discussion

This is one of the first papers addressing issues that are important to establish the predictive value of the BH4 loading test.^{3,6,9-11} Many aspects deserve further attention to optimize the 24 and 48-hour BH4 loading test. The most important findings of the present study were that the second half of the 48-hour BH4 loading test seems more informative than the first 24 h, and that 400 μmol/l is at least not a necessary prerequisite in all patients to adequately test BH4 responsiveness.

As expected, sampling moments later than 24 h seem to be important. We found a considerable number of patients who responded to BH4 at 48 hours rather than before (Figure 2A), and therefore the 24-hour test is likely to miss patients that could benefit from BH4. This is in line with the report of Fiege et al., showing that 48 h seem to be useful to detect BH4-responsiveness in more severe phenotypes and “slow responders”.⁹ Furthermore, in most patients the largest response is seen at T=48. It has to be taken into account that 48 h might not be long enough to

detect even slower responders.¹² Nevertheless, it has to be considered that an early response might be a predicting factor for the long-term response. However, it would be fair to expect that in most of these patients, later samples would also show a decrease of $\geq 30\%$. It would be interesting to see whether samples during the second half of the 48-hour loading test (e.g. T=32 and T=40) would be more informative than earlier blood samples. This would also select fast responders/metabolizers and therefore possibly the samples at T=8 and T=16 could be left out as the test would be easier for patients and parents if the number of sampling moments could be reduced, especially during nighttime. However, it is not advisable to leave out the T=24-hour sample.

Figure 3 Maximal reduction in blood Phe concentration (%) in response to BH4 in the 48-hour BH4 loading test compared to the blood Phe concentration at the start of the loading test (T=0).

In the neonatal BH4 loading test, it has been considered that tests performed with baseline Phe concentrations $<400 \mu\text{mol/l}$ were inadequate to interpret BH4 responsiveness.¹³ Hence, Phe supplementation in case of Phe concentrations $<400 \mu\text{mol/l}$ was shown mandatory. However, data of the present study show that BH4 responsiveness can be observed in patients >4 years of age, when baseline Phe is $<400 \mu\text{mol/l}$, as already reported elsewhere.^{6,9,10} This is in line with the study by Staudigl et al.,¹⁴ showing that some mutations appear BH4 responsive, especially at lower Phe concentrations. In contrast, Staudigl's paper also shows that in some mutations BH4 responsiveness is observed in case of higher Phe concentrations.

Our data may change practice to load every patient with Phe before performing a BH4 loading test. Knowing the impact of this change, future studies have to confirm our data before such a change can become practice.

Some aspects need to be addressed when the method and results of this study are considered. First of all, each population has its own genetic background. Therefore, when results from different papers are discussed, the PKU genotype of each population should be taken into account. Second, quite unexpectedly, a large number of patients had Phe concentrations <400 $\mu\text{mol/l}$ at baseline, despite the Phe supplementation and the monitoring of Phe levels during the period of Phe loading. Due to the procedure, patients completed the BH4 loading test before baseline Phe concentrations were known. Third, for convenience of the patients and their families and financial reasons, the T=32 and T=40 blood samples were not included in our protocol. As discussed above, it probably would have been better to incorporate additional blood sampling at T=32 and T=40 as advised by the European working group on PKU.⁷ Additionally, the ultimate test of BH4 responsiveness is the long-term experience in patients, showing adequate increase in Phe tolerance and/or adequate decrease in plasma Phe concentration. Due to the limited time for follow-up we are currently collecting data of the patients who continued BH4 treatment after a positive BH4 loading test.

Conclusion

A follow-up of 48 h in the BH4 loading test seems to predict the BH4 responsiveness more reliably when compared to 24 h. To further improve the 48-hour BH4 loading test we suggest to include additional blood samples between T=24 and T=48 h (e.g. T=32 and T=40). This will improve the knowledge on BH4 response in the second 24 h of the BH4 loading test.

Furthermore, it seems that BH4 responsiveness can be predicted by BH4 loading test even when the baseline blood Phe concentration is <400 $\mu\text{mol/l}$. If these results are confirmed by other data and data on long-term BH4 responsiveness, it could be advised to first perform a BH4 loading test without Phe loading and re-test at higher Phe concentrations when no response is seen.

Probably the BH4 loading test is a good indicator for BH4 responsiveness, but a comparison with long term treatment results is necessary to really answer this question. Other issues still need to be solved to optimize this testing procedure.

Acknowledgments

We are very thankful to all the patients and families who participated in this study. Also we would like to thank the analysts of the metabolic laboratories for analyzing the phenylalanine samples.

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TETRAHYDROBIOPTERIN RESPONSIVENESS IN PHENYLKETONURIA: PREDICTION WITH THE 48-HOUR LOADING TEST AND GENOTYPE

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Abstract

Background: How to efficiently diagnose tetrahydrobiopterin (BH4) responsiveness in patients with phenylketonuria remains unclear. This study investigated the positive predictive value (PPV) of the 48-hour BH4 loading test and the additional value of genotype.

Methods: Data of the 48-hour BH4 loading test (20 mg BH4/kg/day) were collected at six Dutch university hospitals. Patients with $\geq 30\%$ phenylalanine reduction at ≥ 1 time points during the 48 hours (potential responders) were invited for the BH4 extension phase, designed to establish true-positive BH4 responsiveness. This is defined as long-term $\geq 30\%$ reduction in mean phenylalanine concentration and/or ≥ 4 g/day and/or $\geq 50\%$ increase of natural protein intake. Genotype was collected if available.

Results: 177/183 patients successfully completed the 48-hour BH4 loading test. 80/177 were potential responders and 67/80 completed the BH4 extension phase. In 58/67 true-positive BH4 responsiveness was confirmed (PPV 87%). The genotype was available for 120/177 patients. 41/44 patients with ≥ 1 mutation associated with long-term BH4 responsiveness showed potential BH4 responsiveness in the 48-hour test and 34/41 completed the BH4 extension phase. In 33/34 true-positive BH4 responsiveness was confirmed. 4/40 patients with two known putative null mutations were potential responders; 2/4 performed the BH4 extension phase but showed no true-positive BH4 responsiveness.

Conclusions: The 48-hour BH4 loading test in combination with a classified genotype is a good parameter in predicting true-positive BH4 responsiveness. We propose assessing genotype first, particularly in the neonatal period. Patients with two known putative null mutations can be excluded from BH4 testing.

Introduction

In phenylketonuria (PKU, OMIM 261600), deficiency of the enzyme phenylalanine hydroxylase (PAH) leads to increased phenylalanine (Phe) concentrations. Untreated, this results in progressive and irreversible cerebral damage.^{1,2} For almost 60 years, a life-long low-Phe diet has been the only possible treatment for PKU. Dietary treatment has proved to be very effective in preventing the devastating consequences of PAH deficiency when started early in life (e.g. after neonatal screening). The cognitive outcome strongly depends on optimal metabolic control.^{3,4} Unfortunately, adherence to such a diet proves difficult.⁵ A pharmaceutical formulation of Tetrahydrobiopterin (BH4), the natural co-factor and co-substrate of PAH, is now an FDA and EMA-registered drug (Sapropterin dihydrochloride; Kuvan®) and provides a new treatment option in a significant number of patients. It acts as a pharmacological chaperone by stabilizing PAH.⁶ In BH4-responsive patients, BH4 decreases the blood Phe concentration and/or increases the dietary Phe tolerance.^{7,8} Correct and efficient identification of BH4-responsive patients is important, both to improve the fast assessment, as well as to avoid false expectations and unnecessary costs. Unfortunately, there is still no golden standard on how to assess BH4 responsiveness most efficiently.

Three methods have been proposed for the prediction of BH4 responsiveness: the 7–28 days BH4 challenge,^{9,10} the 48-hour BH4 loading test¹¹ and, the START (sapropterin therapy actual response test) BH4 challenge and genotyping protocol.¹² Genotype was frequently reported to be useful in predicting or excluding BH4 responsiveness.^{8,13–15} However, only small studies have correlated genotype data with in vivo BH4 challenge tests and long-term BH4 responsiveness.^{16–29} To improve the assessment of BH4 responsiveness, we investigated the positive predictive value (PPV) of the 48-hour BH4 loading test and additional value of genotype for BH4 responsiveness in a large cohort of Dutch PAH-deficient patients.

Methods

Subjects and protocol

In a national collaborative study, data were collected from 183 patients who participated in the 48-hour BH4 loading test between November 2009 and December 2010 in six Dutch university medical centres. Following the European Medicines Agency (EMA) and the Dutch regulations on BH4 prescriptions, only patients above four years of age, requiring a Phe restricted diet, and not pregnant or planning a pregnancy were considered for treatment with BH4. The 48-hour BH4 loading test was also performed in younger children. The protocol consisted of two parts: the 48-hour BH4 loading test to assess 'potential BH4 responsiveness' and the BH4 extension phase to establish 'true-positive BH4 responsiveness'. The BH4 protocol (the 48-hour BH4 loading test

and the BH4 extension phase) was considered standard patient care by the Medical Ethical Committee of the University Medical Center Groningen.

The 48-hour BH4 loading test was largely based on recommendations made by the European working group on PKU,¹¹ requiring baseline Phe concentrations over 400 $\mu\text{mol/L}$. Dutch treatment guidelines recommend blood Phe concentrations of 120–360 $\mu\text{mol/L}$ in patients under twelve years of age and 120–600 $\mu\text{mol/L}$ in patients over twelve years of age.³⁰ Therefore, well-controlled patients with Phe concentrations within the recommended range had to be supplemented with dietary Phe (L-Phe in powder, a protein rich supplement based on milk protein, or an increase in natural protein intake) to reach a stable Phe concentration 'just' over 400 $\mu\text{mol/L}$. The extra Phe intake was used until the test was finished. Typically, multiple blood samples were taken to assure stable Phe concentrations. Patients with Phe concentrations above 400 $\mu\text{mol/L}$ were instructed to continue their usual diet. All patients received 20 mg/kg BH4 (sapropterin dihydrochloride; Kuvan®) twice, directly after blood sampling at baseline ($T = 0$) and after 24 hours ($T = 24$). BH4 dosages were rounded up or down to the nearest 100 mg. Blood samples were collected after $T = 0, 8, 16, 24$ and 48 hours. Patients with a 30% or more reduction in blood Phe concentration at one or more points compared to the baseline ($T = 0$) were regarded as 'potential BH4-responsive' and were invited for the BH4 extension phase.

The BH4 extension phase consisted of three steps in which the blood Phe concentrations, which were measured one to three times a week, had to remain within the ranges of the guideline. Beforehand, a three-day dietary record was used to determine baseline Phe tolerance. BH4 was then introduced at 20 mg/kg/day, followed by: an increase of dietary Phe (to reach the maximal Phe tolerance), BH4 dose adjustment (decrease if possible) and finally adjustment to the Phe-free amino acid supplement according to the patients' total protein needs (age and sex dependent). Following this last step, if blood Phe concentrations exceeded the upper target limit, both the BH4 dose and the amount of Phe intake were reevaluated. Different methods were used to increase dietary Phe (natural protein or L-Phe powder/protein rich supplement first), but in all cases this was done gradually and on an individual basis. The method to determine the maximum Phe tolerance was not protocolized, although it was advised to keep or bring the Phe concentrations within treatment range. Typically, when Phe levels were above the target concentration, natural protein intake was decreased by 10-20% depending on the amount of increase in Phe concentration and the absolute concentration. The reduction of the Phe-free amino acid supplement was performed in multiple steps in case of large quantities. When the patients had Phe concentrations within target range or were stable, a three-day dietary record was used to determine the final Phe tolerance and dietary sufficiency for all nutrients. Dietary records were analysed by nutritionists qualified in metabolic diseases at the various centres. True-positive BH4 responsiveness was defined as a 30% or more reduction in blood Phe concentration compared to mean blood Phe concentrations prior to the 48-hour BH4 loading test

with the same diet, and/or an increase in dietary Phe tolerance of $\geq 50\%$ or ≥ 4 grams of natural protein without increasing the Phe concentrations above the upper target.

Assessments

Blood Phe concentrations in dried blood spots were measured by the patients' respective centre laboratory according to standard quantitative methods, with the same method per patient. To calculate the individual mean blood Phe concentration prior to the BH4 introduction, all known values from the year preceding the BH4 loading test were used, with a minimum of four measurements; alternatively, the period was extended to two years or this value was considered missing. The individual mean blood Phe concentration after the BH4 extension phase was calculated from all the Phe concentrations in the first three months after finishing the BH4 extension phase, with a minimum of four measurements. The period was extended until four samples were available in patients that did not send that many samples (maximum one year). Weight was usually measured at the first day of the BH4 loading test or at a recent outpatient visit, to determine the total daily dose of BH4. Otherwise, the most recent value was used. The medical history was obtained to ascertain the blood Phe concentration at diagnosis. Data on genotype were collected if available. A Medline database literature search was performed to compose a list of mutations associated with long-term BH4 responsiveness. This list consisted of thirty mutations. Mutations with a known residual in vitro activity of $\leq 1\%$, all nonsense mutations, variants altering the reading frame and splice-site mutations leading to exon skipping and disruption of the reading frame were regarded putative null mutations [13,14,20]. All genotypes are tabulated in the BIOPKU database (www.biopku.org) and compared with existing information.

Statistical analysis

Normality was defined according to the Shapiro Wilk test. As almost all data showed skewed distributions, data are presented as medians with inter-quartile ranges (IQR). The Mann-Whitney U test was used to compare independent continuous data. To compare related continuous data the Wilcoxon Signed Rank test was used. The Chi-square test was used for categorical data unless the minimum expected count was smaller than five, in which case Fisher's exact test was used. Statistical analyses were performed using PASW statistics version 18.0.3, SPSS, Inc., Chicago, IL, USA. A two-tailed P-value < 0.05 was assumed as statistically significant.

Results

Cohort

A total of 183 patients started the 48-hour BH4 loading test. Six patients were excluded because of illness, missing T = 48 blood sample or irregular BH4 administration (Figure 1). The median (IQR) age of the 177 patients that successfully completed the 48-hour loading test was 13.8

(8.6-21.1) years. Seven children were younger than four years old during the 48-hour BH4 loading test, but none during the BH4 extension phase. A female gender accounted for 52.5% of the study population. Phe was supplemented in 51 out of 66 patients younger than 12 years old and in 36 out of 111 patients of 12 years or older.

Figure 1 Study profile

48-hour BH4 loading test and BH4 extension phase

In the 48-hour BH4 loading test, 80 out of 177 (45.2%) patients were potentially BH4-responsive. Out of 80 patients with potential BH4 responsiveness, 67 (85%) started the BH4 extension phase. False-positive BH4 responsiveness was shown in nine of them. Therefore, the PPV of the 48-hour BH4 loading test was 87%. Demographic and clinical details of all patients with no potential BH4 responsiveness, false-positive and true-positive BH4 responsiveness as well as potentially responsive patients who did not participate in the BH4 extension phase are shown in Table 1.

Table 1

Demographic and clinical details of patients with non-potential BH4 responsiveness and False/True positives

BH4 loading test:	Non-potential BH4-responsive (n=97)	Potential responder (n=80)		
BH4 extension phase:		False-positive (n=9)	True-positive (n=58)	Other ¹ (n=13)
Sex				
Male	47 (48.5)	6 (66.7)	24 (41.4)	7 (53.8)
Female	50 (51.5)	3 (33.3)	34 (58.6)	6 (46.2)
Age (years) A*	14.7	8.0	12.3	14.1
	(10.3 – 23.0)	(6.4 – 11.9)	(7.7 – 17.9)	(10.4 – 19.4)
<12 years A**	30 (30.9)	7 (77.8)	25 (43.1)	4 (30.8)
≥12 years	67 (69.1)	2 (22.2)	33 (56.9)	9 (69.2)
Baseline Phe	663	620	465	485
(μmol/L) ² B*** C*	(530 – 915)	(528 – 831)	(358 – 555)	(386 – 590)
Mean Phe ^{3,4} B***	494	455	304	267
C**	(364 – 700)	(406 – 553)	(244 – 401)	(239 – 407)
<12 years B* C**	397	455	282	274
	(290 – 441)	(372 – 539)	(227 – 400)	(207 – 318)
≥12 years ⁴ B***	564	440, 759	332	264
	(427 – 773)	(n=2)	(255 – 414)	(239 – 456)
Phe at diagnosis ⁵				
B***, C***				
<600 μmol/L	3 (3.5)	0 (0.0)	26 (44.8)	4 (30.8)
600-1199 μmol/L	13 (15.3)	2 (22.2)	26 (44.8)	3 (23.1)
>1200 μmol/L	69 (81.2)	7 (77.8)	6 (10.3)	6 (46.2)

Data are n (%) or median (interquartile range). ¹ no BH4 extension phase. ² Phe concentration at T = 0 (with Phe loading when necessary). ³ Mean Phe concentration prior to the BH4 loading test. ⁴ Missing in n= 14 (<30%), n= 10 (True-positives) and n=1 (Other). ⁵ First Phe concentration in hospital, missing in n= 12 (<30%). **A)** comparing non-potential BH4-responsive and false-positive, **B)** comparing non-potential BH4-responsive and true-positive, **C)** comparing false-positive and true-positive. *P= <0.05, **P= <0.01, ***P= <0.001.

In patients with a 30-40% decrease in Phe concentrations (n = 18), the PPV was 67%, while this was 94% in patients with ≥40% reduction (n = 49), P = 0.009. The PPV of patients responding only at T = 48 (n = 11) was lower than patients who respond within 24 hours (n = 56) (PPV 64% and 91% respectively, P = 0.03). Patients who responded within 24 hours showed a median (IQR) Phe concentration decrease of 56 (42 – 57) percent at the 24 hour time point, whereas this was 24 (16 – 26) percent in patient who responded only at T = 48. At T = 48 this was 50 (35 – 68) and 36 (34 – 45) percent, respectively. Patients with at least 30% decrease in Phe concentration at two or more moments showed true BH4 responsiveness in 47 out of 50, whilst

this was ten out of 16 in patients with only one moment. Five patients had this one moment at another time point than T = 48, of them 3 showed true BH4 responsiveness.

Figure 2 Treatment results in patients with true-positive BH4 responsiveness **(A)** Histogram of the increase in natural protein intake (g/dag). **(B)** Histogram of the percentage change in phenylalanine concentration. **(C)** Combined overview of the increase in natural protein intake (g/dag) and the absolute change in phenylalanine concentration.

The duration of the BH4 extension phase ranged from six to 76 weeks in patients with true-positive BH4 responsiveness with a median of 24 weeks. During this period a median (IQR) amount of 40 (24 – 55) blood samples were taken per patient. For patients with a false-positive response the duration of the BH4 extension phase was 6 to 43 weeks, with a median of 13 weeks. In patients with true-positive BH4 responsiveness the median (IQR) natural protein intake increased significantly from 17 (12–28) g to 49 (34 – 57) g ($P = 0.000$). The distribution of increase in natural protein is shown in Figure 2A. In 41 patients four or more blood samples were taken within three months after stabilization (with a median of seven samples). Eleven patients collected four blood samples within one year, with a mean period of 20 ± 6.2 weeks. In six (adult)

patients too little samples were collected to calculate the mean Phe concentration after stabilization of BH4 treatment. These six patients were excluded from Phe concentration analysis. The median (IQR) of the mean Phe concentrations in patients with enough samples did not significantly change (350 (250–460) $\mu\text{mol/L}$ prior to BH4 treatment and 329 (272–415) $\mu\text{mol/L}$ after stabilization, $P = 0.716$). The distribution of the decrease in Phe concentration is shown in Figure 2B. An overview of changes in natural protein as well as Phe concentration can be seen in Figure 2C. Patients with a late response during the 48-hour BH4 loading test and who were true BH4 responders showed no difference in baseline natural protein intake compared to patients that responded within 24 hours (Median (IQR) 13 (7 – 21) g versus 17 (12 – 29) g, respectively, $P = 0.211$). Also the median amount of increase was not different (24 g and 25 g of natural protein respectively, $P = 0.674$).

Genotype

Genotype was determined in 127 patients and revealed heterozygosity in 102 patients and homozygosity in 18 patients, while only one mutation was identified in four patients and no PAH mutations were identified in two patients (siblings). Furthermore, one patient was found to have a silent mutation (p.V245V) and a p.G272* mutation, with no evidence of a third mutation. This patient had a severe phenotype (diagnostic Phe concentration of 1963 $\mu\text{mol/L}$ and a dietary Phe tolerance of 8.1 mg/kg/ day) and was potentially BH4-responsive (47% Phe reduction), but did not want to participate in the BH4 extension phase. An overview of all 120 complete genotypes including 48-hour BH4 loading test and BH4 extension phase results is available in the Additional file 1: Table S1. The table also shows four novel mutations of the PAH gene. An overview of the mutations associated with long-term BH4 responsiveness is shown in Table 2.

Table 2

Mutations associated with true BH4 responsiveness and mutations newly associated with true BH4 responsiveness

			New
p.F39L	p.V190A	p.K320N	
p.L48S	p.V230I	p.L348V	p.A104D
p.F55L	p.R241Q	p.P366H	p.T193I
p.I65T	p.R241H	p.A373T	p.P211T
p.R68S	p.R241C	p.E390G	p.T238A
p.D129G	p.V245A	p.A403V	p.P314H
p.P147L	p.P275S	p.F410S	p.R408Q
p.V177L	p.A300S	p.Y414C	
p.V177M	p.A309V	p.D415N	
p.E178G	p.A313T	p.P416Q	

Thirty-five patients had a genotype consisting of two putative null mutations, 28 a functionally hemizygous genotype (a combination of a putative null mutation on one allele and a mutation associated with true BH4 responsiveness on the other allele) and, three patients a combination

of two mutations associated with true BH4 responsiveness. For these patients the outcome is shown in Figure 3.

Figure 3 The outcome of the 48-hour BH4 loading test and the BH4 extension phase in patients with (A) 2 Putative Null mutations (B) Compound heterozygosity (C) 2 mutations associated with true BH4 responsiveness.

Seven patients with true-positive BH4 responsiveness were identified as having a genotype consisting of a putative null mutation and a mutation not yet associated with BH4

responsiveness or a homozygous genotype with mutations not yet associated with BH4 responsiveness (p.A104D, p.T193I, p.P211T, p.P314H, p.R408Q and the novel mutation p.T238A). One or more unclassified mutations including the p.R261Q and p.R158Q mutation were detected in 47 patients. Results regarding the 48-hour BH4 loading test and the BH4 extension phase are shown in Table 3.

Of the 44 patients with at least one mutation known to be associated with BH4 responsiveness, 41 showed potential BH4 responsiveness (three non-potential responders: c.1066-11G>A/p.L48S 27%, p.L48S/p.R261Q 28%, p.C203Y/p.L348V 25%). Of 41 potential BH4 responders 34 patients participated in the BH4 extension phase. Of those 34 patients with potential responsiveness and a favourable genotype, 33 were true responsive (97%, no true BH4 responsiveness was seen in p.I65T/p.L311P).

Table 3
Genotypes with Unclassified Mutations

No potential BH4 responsiveness (n)	Potential BH4 responsiveness (n)
c.168+5G>C ¹ /p.R261Q (1)	False Positive
c.842+1G>A ¹ /p.R243Q (1)	c.1315+1G>A ¹ /p.I174N (1)
c.842+5G>A ¹ /p.R158Q (1)	p.R158Q/p.R158Q (1)
c.1066-11G>A ¹ /p.D222G (1)	p.R158Q/p.Y277D (1)
c.1315+1G>A ¹ /p.I174N (1)	p.R243* ¹ /p.R261Q (1)
c.1315+1G>A ¹ /p.R261Q (4)	
c.1315+1G>A ¹ /p.R261P (1)	True Positive
c.1315+1G>A ¹ /p.Y277D (1)	p.L48S ² /p.R408W (1)
c.1315+1G>A ¹ /p.R408W (1)	p.A104D ³ /p.R158Q (1)
p.L48S ² /p.R261Q (1)	p.A104D ³ /p.R408W (1)
p.R111X ¹ /p.R408W (1)	p.R158Q/p.V190A ² (2)
p.R158Q/p.R158Q (1)	p.R158Q/p.Y414C ² (2)
p.R158Q/p.R243* ¹ (1)	p.I174N/p.P211T ³ (1)
p.R158Q/p.R261P (1)	p.E178G ² /p.Y343C (1)
p.R158Q/p.R408W ¹ (1)	p.R261Q/p.P281L ^{1,4} (1)
p.I174T/p.F299C ¹ (1)	p.R261P/p.Y414C ² (1)
p.C203Y/ p.L348V ² (1)	p.Y277D/p.P314H ³ (1)
p.R261Q/p.R261Q (2)	p.E280K/p.Y414C ² (1)
p.R261Q/p.E280K ¹ (2)	p.A300S ² /p.R408W (1)
p.R261Q/p.P281L ¹ (1)	
p.P281L ¹ /p.R413P (2)	No Extension Phase
	p.L48S ² /p.R158Q (1)
	p.Y277D/p.P314H ³ (1)

¹ Putative Null Mutation. ² Mutation associated with true BH4 responsiveness.

³ New Responsive Mutation. ⁴ Natural protein increase: 6.5 g/day (before treatment 10.5g/day) with no statistically significant Phe concentration increase [6%, P= 0.379].

Discussion

This study, in a large cohort of PKU patients, shows that the 48-hour BH4 loading test has a high PPV for true BH4 responsiveness. Furthermore, it shows that patients with genotypes including at least one mutation known to be associated with long-term BH4 responsiveness have a high rate

of true response. This is the first study to investigate the PPV for BH4 responsiveness of the 48-hour BH4 loading test and the additional value of genotype. Establishing a reliable pre-selection method is of high importance, since it could save time and money, and will prevent false expectations. The treatment of BH4 responsive PKU patients has been markedly changed since the therapy was introduced. Expansion of the Phe-restricted diet or even complete liberation can reduce a major burden. Although quality of life results obtained by standardized questionnaires (not suitable for PKU) do not show a significant improvement, patients do report an increased quality of life.^{27,28}

Recently, we amongst others suggested that 24 hours is not long enough to detect patients with a delayed response to BH4.^{16,31-33} Indeed in this study we confirm that patients with true BH4 responsiveness can be missed in a 24 hour test. Other studies suggest extending the BH4 loading test to beyond 48 hours.^{9,28,34} We detected some patients who tend to show a response at T = 48. However, we did not compare our results to a longer lasting test, such as the 7–28 day BH4 challenge. As our data indicate that patients who show a first response at T = 48 are less likely to show true BH4 responsiveness than patients who respond within 24 hours, prolongation of the BH4 loading test will possibly increase the number of patients with true BH4 responsiveness, but will also decrease the PPV and specificity. Also, influence of external factors increases with the length of the test. Moreover, patients with only one moment of response during the 48-hour BH4 loading test can show true BH4 responsiveness. Another strategy is published by Singh and Quirk. They chose to see whether it is possible to liberalize the diet in patients with just 15% decrease of Phe after one month of BH4 treatment.³⁵ Unfortunately however, this study does not show whether such low a cut off results in the detection of extra BH4 responsive patients.

As expected blood Phe concentrations at the diagnosis, baseline Phe concentrations and Phe concentrations prior to the BH4 loading test were significantly lower in patients with true-positive BH4 responsiveness than patients without potential BH4 responsiveness and false-positives. Nevertheless, these biochemical parameters can give an indication but show too much overlap for practical use in predicting BH4 responsiveness. Another notable finding was that a majority of false-positive patients are found in the younger age range. At present, we do not have a clear explanation for this finding. Possibly, younger patients have more variability in their blood Phe concentrations.

Since a few years it is recognized that a genotype can be helpful in predicting BH4 responsiveness and to do so a full genotype is required.^{14,15,36} Our results underline this assumption. Patients who have a functionally hemizygous genotype are frequently true BH4 responders. The three patients who were not potential BH4 responders all reached a 20-30% Phe concentration decrease in the 48-hour BH4 loading test and can potentially also benefit from the therapy. The patients with two mutations associated with true BH4 responsiveness were

all true BH4 responders. This concerns only three patients as patients with this genotype combination probably have mild hyperphenylalaninemia very often and do not always require treatment, which was an inclusion criterion. Furthermore, the results in this study support the suggestion in the literature that patients with two putative null mutations are not candidates for BH4 treatment.^{36,37} In a recent study by Sterl et al. the proportions of predicted BH4 responsiveness are fairly similar to our results.³⁸

An important remark is that a classified genotype is needed to predict BH4 responsiveness. As more than 400 missense mutations are described and 75 to 90 percent of the patients are compound heterozygous,^{8,13,27} this results in a large number of as yet unclassified genotypes. Additionally, some mutations are associated with an inconsistent BH4 response, for instance p.R158Q,^{8,13} p.I65T^{21,26,39,40} and p.R261Q.^{8,13,14,27} In our patients, where the p.R158Q mutation was homozygous or combined with a putative null mutation, it showed no BH4 responsiveness. The p.I65T mutation combined with a putative null mutation showed potential response in two out of two patients. However, unfortunately, one patient did not perform the BH4 extension phase (60% Phe concentration decrease) and the other was a false-positive. The p.R261Q mutation again showed variable results, with a response just under the threshold of 30% remarkably often. Patients who are functionally hemizygous for p.R261Q seldom show true BH4 responsiveness, but one of our patients and one patient described by Hennermann et al.,²⁷ both p.R261Q/L. P281L, show (modest) long-term BH4 responsiveness.

Since this study reports patient care results, patients without potential BH4 responsiveness did not participate in the BH4 extension phase. This limits the conclusion regarding its diagnostic value. Other results, however (number of false-positives in patients with borderline response), support the presumption that the number of false-negatives will be limited. A possible bias in the 48- hour BH4 loading test could be the supplementation of Phe to increase the baseline Phe concentration and the different methods to reach this increase. The influence of the different metabolic kinetics is unclear.^{41,42} With respect to the true BH4 responsiveness criteria, the extra 4 g of natural protein (180 mg Phe) tolerance increase criterion can be considered relatively modest. This age independent criterion was agreed within the Dutch group of metabolic physicians. If the criterion were doubled to 8 g of natural protein, four patients would not have met this tolerance requirement. Two out of these four patients also showed a 30% or more decrease in blood Phe concentration. Of the other two patients (both children), genotype showed heterozygosity for p.R261Q/p.P281L in one and c.1315+1G>A/p.L348V in the other. Furthermore, we anticipated that the BH4 extension phase would take less than six months. However, the actual duration exceeded this six months' time period in 41 percent of the patients, with three patients taking even longer than one year. There are several explanations for this longer duration of which the exploration of the possible speed of Phe tolerance increase is an

important one, as is the motivation of the patients during the last stages (especially blood sampling frequency).

Conclusions

In conclusion, notwithstanding the small number of patients, a classified genotype seems a good predictor of BH4 responsiveness. Therefore, assessing genotype first maybe an option, particularly in the neonatal period when in Europe BH4 is not yet approved for use. In case the protocol would be altered to assessing genotype first, the 48-hour BH4 loading test is an adequate and patient-friendly method for detecting BH4 responsiveness, especially for patients with an unclassified or unknown genotype. The duration of the test is adequate for many patients and whereas multiple time points with $\geq 30\%$ Phe concentration reduction are beneficial, one time point can indicate true BH4 responsiveness.

Acknowledgements

This study was sponsored by the Junior Scientific Masterclass, University of Groningen and an unrestricted grant from Merck B.V., The Netherlands. The funding source had no role in the writing of the manuscript, but was informed about the decision to submit the manuscript for publication. No authors were paid by a pharmaceutical company or other agency to write this article. We thank all participating institutions and their staff for their contributions to this study, particularly the dieticians Ninja ter Borg, Ems C. Carbasius Weber, Nienke M. ter Horst, Cora F. Jonkers, Elisabeth M.C. van der Ploeg, Renske G. Janssen-Regelink, Annemiek van Wegberg Heidi Zweers-van Essen, N. Ada P. van der Herberg-van de Wetering and the laboratory technicians for their excellent work.

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Supplementary Table: Overview of genotypes, results of the 48-hour BH4 loading test and the BH4 extension phase

Allele 1		Allele 2		Phe load†	T=0	Max Phe ↓ (%)	Extension phase
IVS2+5G>C‡§	c.168+5G>C	p.W187*‡	c.561G>A	No	741	4	No
IVS2+5G>C‡§	c.168+5G>C	p.W187*‡	c.561G>A	No	326	17	No
IVS2+5G>C‡	c.168+5G>C	p.R243*‡	c.727C>T	No	1049	25	No
IVS2+5G>C‡	c.168+5G>C	p.R261Q¶	c.782G>A	No	491	15	No
IVS5-21del177‡	c.510-21_665del	p.G272*‡	c.814G>T	No	855	5	No
IVS7+1G>A‡	c.842+1G>A	p.R243Q‡	c.728G>A	Yes	626	5	No
IVS7+5G>A‡	c.842+5G>A	p.R158Q¶	c.473G>A	Yes	896	-1	No
IVS7+5G>A‡	c.842+5G>A	p.Y414C	c.1241A>G	No	503	36	Yes positive
IVS7-5T>C‡	c.843-5T>C	p.P281L‡	c.842C>T	No	949	22	No
IVS10-11G>A‡	c.1066-11G>A	IVS10-11G>A‡	c.1066-11G>A	No	1224	22	No
IVS10-11G>A‡	c.1066-11G>A	IVS10-11G>A‡	c.1066-11G>A	Yes	550	14	No
IVS10-11G>A‡	c.1066-11G>A	IVS10-11G>A‡	c.1066-11G>A	No	705	3	No
IVS10-11G>A‡§	c.1066-11G>A	p.L48S	c.143T>C	Yes	478	56	No
IVS10-11G>A‡§	c.1066-11G>A	p.L48S	c.143T>C	No	845	27	No
IVS10-11G>A‡§	c.1066-11G>A	p.L48S	c.143T>C	Yes	459	48	No
IVS10-11G>A‡	c.1066-11G>A	p.D222G¶	c.665A>G	Yes	936	23	No
IVS12+1G>A‡	c.1315+1G>A	IVS12+1G>A‡	c.1315+1G>A	No	769	9	No
IVS12+1G>A‡	c.1315+1G>A	p.S16* ‡	c.47_48delCT	Yes	715	-4	No
IVS12+1G>A‡	c.1315+1G>A	p.S16* ‡	c.47_48delCT	No	451	12	No
IVS12+1G>A‡	c.1315+1G>A	p.S16* ‡	c.47_48delCT	No	599	0	No
IVS12+1G>A‡	c.1315+1G>A	p.L48S	c.143T>C	Yes	304	69	Yes positive
IVS12+1G>A‡	c.1315+1G>A	p.I65T	c.194T>C	Yes	587	60	No
IVS12+1G>A‡§	c.1315+1G>A	p.I174N¶	c.521T>A	Yes	692	35	Yes negative
IVS12+1G>A‡§	c.1315+1G>A	p.I174N¶	c.521T>A	Yes	663	17	No
IVS12+1G>A‡	c.1315+1G>A	p.W187*‡	c.561G>A	No	514	7	No
IVS12+1G>A‡§	c.1315+1G>A	p.P211T‡‡	c.631C>A	Yes	367	81	Yes positive

Allele 1	Allele 2		Phe load†	T=0	Max Phe ↓ (%)	Extension phase	
(Continued from previous page)							
IVS12+1G>A‡	c.1315+1G>A	p.R241C	c.721C>T	Yes	464	45	Yes positive
IVS12+1G>A‡	c.1315+1G>A	p.R243*‡	c.727C>T	Yes	588	21	No
IVS12+1G>A‡	c.1315+1G>A	p.R261P¶	c.782G>C	Yes	591	-4	No
IVS12+1G>A‡	c.1315+1G>A	p.R261Q¶	c.782G>A	No	852	-1	No
IVS12+1G>A‡	c.1315+1G>A	p.R261Q¶	c.782G>A	No	408	16	No
IVS12+1G>A‡	c.1315+1G>A	p.R261Q¶	c.782G>A	Yes	709	22	No
IVS12+1G>A‡	c.1315+1G>A	p.R261Q¶	c.782G>A	No	577	21	No
IVS12+1G>A‡	c.1315+1G>A	p.Y277D¶	c.829T>G	No	557	-18	No
IVS12+1G>A‡§	c.1315+1G>A	p.P281L‡	c.842C>T	Yes	950	22	No
IVS12+1G>A‡§	c.1315+1G>A	p.P281L‡	c.842C>T	No	650	19	No
IVS12+1G>A‡	c.1315+1G>A	p.P281L‡	c.842C>T	Yes	469	7	No
IVS12+1G>A‡	c.1315+1G>A	p.A300S	c.898G>T	Yes	321	62	Yes positive
IVS12+1G>A‡	c.1315+1G>A	p.L348V	c.1042C>G	Yes	593	33	Yes positive
IVS12+1G>A‡	c.1315+1G>A	p.L367Pfs*27‡	c.1099_1100insC	Yes	397	-5	No
IVS12+1G>A‡	c.1315+1G>A	p.L367Pfs*27‡	c.1099_1100insC	No	934	10	No
IVS12+1G>A‡	c.1315+1G>A	p.A403V	c.1208C>T	Yes	314	85	Yes positive
IVS12+1G>A‡	c.1315+1G>A	p.R408W‡	c.1222C>T	No	890	-1	No
IVS12+1G>A‡	c.1315+1G>A	p.Y414C	c.1241A>G	No	896	63	Yes positive
IVS12+1G>A‡	c.1315+1G>A	p.Y414C	c.1241A>G	Yes	681	72	Yes positive
IVS12+1G>A‡	c.1315+1G>A	p.Y414C	c.1241A>G	Yes	576	83	Yes positive
IVS12+1G>A‡§	c.1315+1G>A	p.Y414C	c.1241A>G	No	535	56	Yes positive
IVS12+1G>A‡§	c.1315+1G>A	p.Y414C	c.1241A>G	No	390	45	Yes positive
IVS12+1G>A‡	c.1315+1G>A	p.Y414C	c.1241A>G	No	465	38	Yes positive
p.L48S	c.143T>C	p.R158Q¶	c.473G>A	Yes	316	52	No
p.L48S §	c.143T>C	p.R241C	c.721C>T	Yes	365	49	Yes positive
p.L48S §	c.143T>C	p.R241C	c.721C>T	Yes	532	59	Yes positive

Allele 1		Allele 2		Phe load†	T=0	Max Phe ↓ (%)	Extension phase	
(Continued from previous page)								
p.L48S	c.143T>C	p.R261Q¶	c.782G>A	No	530	28	No	
p.L48S	c.143T>C	p.P281L‡	c.842C>T	Yes	525	63	Yes	positive
p.L48S	c.143T>C	p.R408W‡	c.1222C>T	No	815	36	Yes	positive
p.F55Lfs*6 ‡	c.165delT	p.P281L‡	c.842C>T	No	411	-7	No	
p.I65T	c.194T>C	p.L311P‡	c.932T>C	Yes	530	33	Yes	negative
p.I94del‡	c.283_285delATC	p.P314H‡‡	c.941C>A	Yes	436	65	Yes	positive
p.I94del‡	c.283_285delATC	p.Y414C	c.1241A>G	No	521	72	Yes	positive
p.A104D††	c.311C>A	p.A104D††	c.311C>A	Yes	660	57	Yes	positive
p.A104D††	c.311C>A	p.R158Q¶	c.473G>A	No	646	31	Yes	positive
p.A104D††	c.311C>A	p.R241C	c.721C>T	Yes	574	42	No	
p.A104D††	c.311C>A	p.R408W‡	c.1222C>T	Yes	486	34	Yes	positive
p.R111*‡	c.331C>T	p.R408W‡	c.1222C>T	No	766	14	No	
p.R158Q¶	c.473G>A	p.R158Q¶	c.473G>A	No	633	34	Yes	negative
p.R158Q¶	c.473G>A	p.R158Q¶	c.473G>A	No	535	5	No	
p.R158Q¶	c.473G>A	p.V190A	c.569T>C	No	327	53	Yes	positive
p.R158Q¶	c.473G>A	p.V190A	c.569T>C	Yes	511	52	Yes	positive
p.R158Q¶	c.473G>A	p.R243*‡	c.727C>T	No	323	4	No	
p.R158Q¶	c.473G>A	p.R261P¶	c.782G>C	No	1348	6	No	
p.R158Q¶	c.473G>A	p.Y277D¶	c.829T>G	Yes	257	40	Yes	negative
p.R158Q¶	c.473G>A	p.R408W‡	c.1222C>T	Yes	655	-1	No	
p.R158Q¶	c.473G>A	p.Y414C	c.1241A>G	No	366	68	Yes	positive
p.R158Q¶	c.473G>A	p.Y414C	c.1241A>G	Yes	362	74	Yes	positive
p.I174N¶§	c.521T>A	p.P211T††	c.631C>A	Yes	360	67	Yes	positive
p.I174T¶	c.521T>C	p.F299C‡	c.896T>G	No	1049	-24	No	
p.E178G	c.533A>G	p.Y343C¶	c.1028A>G	Yes	353	64	Yes	positive
p.W187*‡	c.561G>A	p.W187*‡	c.561G>A	No	505	17	No	

Allele 1		Allele 2		Phe load†	T=0	Max Phe ↓ (%)		Extension phase	
(Continued from previous page)									
p.W187*‡	c.561G>A	p.T1931††	c.578C>T	No	365	62		Yes	positive
p.W187*‡	c.561G>A	p.L367Pfs*27‡	c.1099_1100insC	Yes	689	-2		No	
p.V190A	c.569T>C	p.S231F‡	c.692C>T	Yes	448	41		Yes	positive
p.V190A	c.569T>C	p.L311P‡	c.932T>C	Yes	592	76		No	
p.L197Lfs‡	c.590_611del	p.D415N	c.1243G>A	Yes	302	73		Yes	positive
p.C203Y¶	c.608G>A	p.L348V	c.1042C>G	Yes	486	25		No	
p.P211Hfs‡	c.632delC	p.S231P‡	c.691T>C	Yes	474	-8		No	
p.T238A††	c.712A>G	p.P281L‡	c.842C>T	Yes	549	59		Yes	positive
p.R243*‡	c.727C>T	p.R261Q¶	c.782G>A	No	620	39		Yes	negative
p.R243*‡§	c.727C>T	p.S349P‡	c.1045T>C	Yes	719	37		No	
p.R243*‡§	c.727C>T	p.S349P‡	c.1045T>C	Yes	693	22		No	
p.R243*‡§	c.727C>T	p.S349P‡	c.1045T>C	Yes	352	31		No	
p.R243*‡§	c.727C>T	p.A403V	c.1208C>T	Yes	515	87		Yes	positive
p.R243*‡§	c.727C>T	p.A403V	c.1208C>T	Yes	420	74		No	
p.R243X‡	c.727C>T	p.Y414C	c.1241A>G	Yes	634	49		Yes	positive
p.R243*‡	c.727C>T	p.E440*‡	c.1318G>T	No	560	21		No	
p.R252W‡	c.754C>T	p.P281L‡	c.842C>T	No	882	-2		No	
p.R261P¶	c.782G>C	p.Y414C	c.1241A>G	No	572	30		Yes	positive
p.R261Q¶§	c.782G>A	p.R261Q¶	c.782G>A	No	758	25		No	
p.R261Q¶§	c.782G>A	p.R261Q¶	c.782G>A	Yes	510	7		No	
p.R261Q¶	c.782G>A	p.E280K‡	c.838G>A	Yes	654	25		No	
p.R261Q¶	c.782G>A	p.E280K‡	c.838G>A	No	1476	25		No	
p.R261Q¶	c.782G>A	p.P281L‡	c.842C>T	No	1237	29		No	
p.R261Q¶	c.782G>A	p.P281L‡	c.842C>T	Yes	488	79		Yes	positive
p.G272*‡	c.814G>T	p.Y414C	c.1241A>G	Yes	462	84		Yes	positive
p.Y277D¶§	c.829T>G	p.P314H††	c.941C>A	Yes	206	33		Yes	positive

Allele 1		Allele 2		Phe load†	T=0	Max Phe ↓ (%)	Extension phase
(Continued from previous page)							
p.Y277D¶§	c.829T>G	p.P314H††	c.941C>A	Yes	213	68	No
p.E280K‡	c.838G>A	p.Y414C	c.1241A>G	Yes	481	73	Yes positive
p.P281L‡§	c.842C>T	p.P281L‡	c.842C>T	No	581	-6	No
p.P281L‡§	c.842C>T	p.P281L‡	c.842C>T	No	1317	4	No
p.P281L‡	c.842C>T	p.P281L‡	c.842C>T	No	1020	15	No
p.P281L‡	c.842C>T	p.A300S	c.898G>T	Yes	480	66	Yes positive
p.P281L‡	c.842C>T	p.Y356*‡	c.1068C>G	Yes	1181	38	Yes negative
p.P281L‡§	c.842C>T	p.R413P‡	c.1238G>C	No	921	9	No
p.P281L‡§	c.842C>T	p.R413P‡	c.1238G>C	No	640	-1	No
p.A300S	c.898G>T	p.R408W‡	c.1222C>T	Yes	260	42	Yes positive
p.G352Vfs*48 ‡	c.1055delG	p.G352Vfs*48 ‡	c.1055delG	No	570	60	Yes negative
p.K363Nfs*37 ‡§	c.1089delG	p.K363Nfs*37 ‡	c.1089delG	No	817	3	No
p.K363Nfs*37 ‡§	c.1089delG	p.K363Nfs*37 ‡	c.1089delG	Yes	576	19	No
p.L367Pfs*27 ‡	c.1099_1100insC	p.Y414C	c.1241A>G	No	458	78	Yes positive
p.R408Q††	c.1223G>A	p.R408Q††	c.1223G>A	Yes	315	36	Yes positive
p.Y414C	c.1241A>G	p.Y414C	c.1241A>G	Yes	339	49	Yes positive

† Phe supplementation, ‡Putative null mutation, § siblings/family, ¶ unclassified mutation, || mutation associated with long-term BH4 responsiveness, †† mutation in this study newly associated with long-term BH4 responsiveness, novel mutations are in bold.

THE FIRST EUROPEAN GUIDELINES ON PHENYLKETONURIA: ITS USEFULNESS AND IMPLICATIONS FOR BH4 RESPONSIVENESS TESTING

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Journal of Inherited Metabolic Disease. 2020;
43(2): 244-250

Abstract

Objective: This study aimed to investigate and improve the usefulness of the 48-hour BH₄ loading test and to assess genotype for BH₄ responsiveness prediction, using the new definition of BH₄ 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 (Anjema et al 2013), we firstly assessed the positive predictive value (PPV) of the 48-hour BH₄ loading test using a cutoff value of 30%. Then, we tried to improve this PPV by using different cutoff values and separate time points. Lastly, using the BIOPKU database, we compared predicted BH₄ responsiveness (according to genotype) and genotypic phenotype values (GPVs) in BH₄-responsive and BH₄-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 BH₄ responsiveness. Higher cutoff values led to a higher PPV, but resulted in an increase in false-negative tests. Parameters for genotype overlapped between BH₄-responsive and BH₄-unresponsive patients, although BH₄ responsiveness was not observed in patients with a GPV below 2.4.

Conclusion: The 48-hour BH₄ loading test is not as useful as previously considered and cannot be improved easily, whereas genotype seems mainly helpful in excluding BH₄ responsiveness. Overall, the definition of BH₄ responsiveness and BH₄ responsiveness testing require further attention.

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 (BH₄), which can increase residual phenylalanine hydroxylase activity leading to better metabolic control and/or an increase in natural protein tolerance. However, different definitions of BH₄ responsiveness exist.¹⁻³

Recently, the first European guidelines on PKU were published.^{4,5} In these guidelines, BH₄ responsiveness is defined as “establishing an increase in natural protein tolerance of $\geq 100\%$ 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 BH₄ treatment”. Since these criteria are stricter than previously used in the Netherlands,² some patients in our population might no longer be considered BH₄ 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 BH₄. Since these patients clearly benefit from BH₄ treatment, we felt that the definition of BH₄ responsiveness from the European guidelines may need to be amended to include patients who can tolerate a safe natural protein intake due to BH₄.

The European PKU guidelines also give recommendations on the method of BH₄ responsiveness testing. With the exception of patients with a genotype consisting of two null mutations, in whom BH₄ responsiveness does not need to be further considered, it is recommended that BH₄ responsiveness testing is performed by a 48-hour BH₄ 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 BH₄ responsive. Although the 48-hour BH₄ loading test with a cutoff value of 30% is often cited as a reliable way to predict BH₄ responsiveness,^{2,6} its predictive value has not been assessed using the expert-based definition of BH₄ 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 BH₄ loading test, but this study defined BH₄ responsiveness as an increase in natural protein intake of $\geq 50\%$ or ≥ 4 g/day, which is a much lower threshold.² Therefore, the present study aimed to investigate and improve the usefulness of the 48-hour BH₄ loading test, and to assess the predictive value of genotype, firstly using the new definition of BH₄ responsiveness from the European guidelines, and secondly using an amended definition that also includes patients who can tolerate a safe natural protein intake due to BH₄.

Methods

Patients and protocol

We used data that were collected for a previous study on BH₄ responsiveness testing.² Detailed information on the data collection, subjects and protocol were described by Anjema et al.² Here, the most relevant methodological aspects are summarized.

Data were collected retrospectively from 183 pediatric and adult patients who performed the 48-hour BH₄ loading test between November 2009 and December 2010. For the 48-hour BH₄ loading test, baseline Phe concentrations were required to be over 400 µmol/L and Phe was supplemented if this was not the case. Patients received 20 mg/kg BH₄ for two 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 $\geq 30\%$ compared to t = 0 at any moment during the loading test were invited for a BH₄ treatment trial. Three-day dietary records were taken before and after the treatment trial to assess natural protein intake. During this treatment trial, BH₄ 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 BH₄ dose was finally decreased if possible. In the original protocol, true BH₄ responsiveness was defined according to guidelines previously used 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 BH₄ loading test with the same diet, and/or an increase in dietary Phe tolerance of $\geq 50\%$ 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 BH₄ 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

Genotype

Using the BIOPKU database (<http://www.biopku.org>, accessed on January 29, 2019), we assessed two ways for using genotype to predict BH₄ responsiveness. Firstly, we used the BIOPKU database to collect the percentage of BH₄-responsive patients (including “slow” responders) with a corresponding genotype, when information on BH₄ responsiveness was available for ≥ 5 cases. Secondly, 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 in PKU patients, ranging from 0 (lowest PAH activity) to 10 (highest PAH activity).⁸ Since BH₄ responsiveness is associated with higher levels of residual PAH activity, it was hypothesized that GPVs could be helpful in predicting BH₄ responsiveness. Patients who had a 48-hour BH₄ loading test that was considered positive (e.g. $\geq 30\%$ reduction in Phe levels) but did not perform a treatment trial were not included in these analyses.

Table 1

Demographic and clinical details of the study cohort

48-hour BH ₄ loading test result	No potential BH ₄ responsiveness ¹ (n = 97)	Potential BH ₄ responsiveness ¹ (n = 78)		
		BH ₄ responsive ² (n = 31)	BH ₄ unresponsive ² (n = 34)	Other ³ (n = 13)
Treatment trial result				
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) ⁴	663 (530 – 915)			
Phe supplementation (%)	458 (362 – 549)***	509 (408 – 623)**	485 (386 – 590)*	
Phe supplementation (mg/day) ⁵	29.9			
Natural protein intake prior to treatment trial (g/kg bw)	71.0***	58.8*	92.3***	

Data are presented as median (IQR) or as percentage of patients. ¹Based on a decrease in Phe levels of 30% as cutoff value. ²Based on an increase in natural protein tolerance of 100% as cutoff value. ³No treatment trial despite a positive 48-hour BH₄ loading test. ⁴At t = 0 during the 48-hour BH₄ loading test. ⁵Amount of Phe supplementation in patients who were supplemented with Phe. * p < 0.05; ** p < 0.01; *** p < 0.001 compared to patients with no potential BH₄ responsiveness (Mann-Whitney U test, corrected for multiple comparisons according to Bonferroni). § p < 0.05 compared to BH₄-unresponsive patients (Mann-Whitney U test).

Analyses

No analyses were copied from the original study. BH₄-responsiveness was assessed based on natural protein intake. The positive predictive value (PPV) of the 48-hour BH₄ loading test was calculated as the number of BH₄-responsive patients (based on the results of the treatment trial, using different definitions) divided by the number of potentially BH₄-responsive patients (based on the results of the 48-hour BH₄ loading test, using different cutoff values). Similarly, the negative predictive value (NPV) was calculated as the number of BH₄-unresponsive patients divided by the number of not potentially BH₄-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 < 0.05 was considered statistically significant. Statistical analyses were performed using IBM SPSS Statistics version 23 and GraphPad Prism version 7 for Windows.

Results

BH₄ responsiveness as defined by the European guidelines: the 48-hour BH₄ 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 BH₄ responsiveness testing following the recommendations of the European guidelines. All three patients were not considered to be BH₄

responsive in the original protocol. The positive predictive value (PPV) of the 48-hour BH₄ loading test was 50.0% when using the recommended cutoff value of 30% and the definition of BH₄ 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 negative predictive values (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, 51.6%, 29.0%, 16.1% and 6.5% of BH₄-responsive patients showed no Phe decrease $\geq 30\%$ at t = 8, t = 16, t = 24, and t = 48, respectively.

Table 2

Predictive values using different cutoff values for a decrease in Phe levels during the 48-hour BH₄ 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%

^aMaximum Phe decrease compared to the baseline value. ^bPositive predictive value (PPV) using the corresponding cutoff value. ^cNegative predictive value (NPV) for a decrease in Phe in the corresponding range

In total, 58 patients had a genotype for which the percentage of BH₄-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 BH₄ responsive and BH₄ unresponsive in this study. Significant differences were found between BH₄-responsive patients versus a total group of BH₄-unresponsive patients (including patients with no potential responsiveness after the loading test), as well as between patients considered BH₄ unresponsive after the loading test versus patients considered BH₄ unresponsive after the treatment trial. However, within the group of patients who performed the treatment trial, there was no difference between BH₄-responsive and BH₄-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 BH₄-responsive patients was 2.4.

BH₄ responsiveness using an amended definition: the 48-hour BH₄ loading test and genotype

For a second set of analyses, we defined BH₄ 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 (WHO/FAO/UNU 2007). Using this definition, the number of BH₄-responsive patients in our cohort increased from 31 to 43, which is a significant increase of 38.7% ($p < 0.001$, McNemar

test). Equally, again excluding the three patients with two null mutations, the PPV of the 48-hour BH4 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 BH4-responsive patients, 44.2%, 23.8%, 19.0% and 11.6% 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 BH4-responsive and BH4-unresponsive patients who performed the treatment trial (Supplemental material 4). With the amended definition, the lowest GPV in a BH4-responsive patient again was 2.4.

Figure 1 Association between genotype and BH₄ responsiveness. **(A).** Boxplots (median, 25th – 75th percentile, min, max) of the percentage of BH₄-responsive patients with a similar genotype in the BIOPKU database in BH₄-unresponsive and BH₄-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 BH₄-unresponsive and BH₄-responsive patients according to the definitions of the European PKU guidelines. Differences are tested with the Mann-Whitney U test.

Discussion

Following the publication of the first European guidelines for PKU, this study assessed the usefulness of the 48-hour BH₄ loading test and genotype for BH₄ responsiveness prediction. Our results indicate that the 48-hour BH₄ loading test is not useful for predicting BH₄ responsiveness as defined by the European PKU guidelines, whereas genotype seems mainly helpful in excluding BH₄ responsiveness. Additionally, we introduced an amended definition of BH₄ responsiveness, that in our opinion better defines which patients benefit from BH₄ treatment. This definition also leads to an increase in BH₄-responsive patients and results in a somewhat more effective 48-hour BH₄ 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 BH₄ loading test did not perform a treatment trial, and the number of false negative tests is therefore unknown. Furthermore, we determined BH₄ 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 BH₄ treatment, as shown by longer-term follow-up studies of BH₄-treated patients.⁹⁻¹¹ Besides these points, it is important to note that this protocol of the 48-hour BH₄ 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 BH₄ as a new treatment option for PKU, different definitions of BH₄ responsiveness have been proposed.¹⁻⁴ The definition of BH₄ 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 BH₄-responsive patients in this study compared to the original publication (33 versus 58 BH₄-responsive patients). As mentioned in the introduction, we noticed that some patients who did not meet the criteria of BH₄ responsiveness set by the European guidelines could tolerate a safe natural protein intake due to BH₄. Liberalization of the diet to such an extent has major advantages, including not needing amino acid supplementation and low-Phe medical food products. Therefore, in our opinion, the consensus-based definition in the European guidelines could be improved by defining BH₄ responsiveness as “an increase in natural protein tolerance $\geq 100\%$ 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 BH₄-responsive and BH₄-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 BH₄ responsiveness

that is evidence-based rather than consensus-based, long-term follow up studies comparing outcomes in BH₄-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 BH₄ treatment in PKU. Since these data are not yet available, developing a (better) consensus-based definition of BH₄ responsiveness may be the best alternative. However, different definitions of BH₄ responsiveness may require different BH₄ responsiveness testing methods, as shown by the results in this study.

Our results show a low PPV of the 48-hour BH₄ loading test when applying the definition of BH₄ 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 BH₄ testing and may also lead to an increase in disappointed patients with a false-positive BH₄ 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 BH₄ 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 BH₄ loading test cannot be improved easily, and its shortcoming should be kept in mind, especially when using the definition for BH₄ responsiveness stated in the European PKU guidelines.

To improve testing for potential BH₄ responsiveness, the simple 48-hour BH₄ 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 BH₄ loading test to 7 days¹² or even longer may be helpful in differentiating between daily Phe variation and a BH₄-induced decrease in Phe. On this topic, a comparison between the outcomes of the BH₄ testing regime in Europe (using a 48-hour BH₄ loading test) and the United States (using a 28-days BH₄ loading test) would be interesting. Alternatively, it could be argued that completely different testing methods are simply superior. Potential examples of this include comparing simple Phe loading with Phe + BH₄ loading,¹³ using a [¹³C]Phe breath test,¹⁴ 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) BH₄ responsiveness, which is in line with the report of Garbade et al. that mentions a cutoff value of 2 as a minimum for (potential) BH₄ responsiveness.⁸ Nevertheless, in line with that study, we found considerable overlap between BH₄-responsive and BH₄-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 BH₄-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, GPs seem to be primarily helpful to exclude BH₄ responsiveness in patients in case of low residual PAH activity. Predicting BH₄ responsiveness using data on BH₄ testing in the BIOPKU database from patients with the same genotype shows similar results. Importantly, the BIOPKU database defines BH₄ responsiveness as a decrease in Phe levels of at least 30% during a short-term BH₄ loading test only, regardless of the specific protocol used. While genotype may be strongly related to the results of 48-hour BH₄ loading test,⁸ our results show that the 48-hour BH₄ loading test is not a good predictor for BH₄ responsiveness. Therefore, although genotype is helpful in excluding BH₄ responsiveness, it is not an overall good predictor of BH₄ responsiveness. These results show the shortcomings of both genotype and the 48-hour BH₄ loading to predict BH₄ responsiveness, thereby underlining the importance of a treatment trial.

To conclude, the 48-hour BH₄ loading test with a cutoff value of 30% does not effectively predict long-term BH₄ responsiveness as defined by the European PKU guidelines, whereas genotype seems mainly helpful to exclude BH₄ responsiveness. We recommend amending the definition of BH₄ responsiveness from the European PKU guidelines to include patients who can tolerate a safe natural protein intake as a result of treatment with BH₄. Methods to predict BH₄ responsiveness require further attention, although a sound definition of BH₄ responsiveness may be even more important to optimize BH₄ responsiveness testing as well as treatment.

Acknowledgements

We thank everyone who was involved with the original publication, including patients, physicians, dietitians, researchers and laboratory technicians.

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Supplemental material 1. PPVs and NPVs for different cutoff values at separate time points during the 48-hour BH₄ loading test, using the definition of BH₄ responsiveness from the European PKU guidelines.

	Phe decrease ^a	PPV ^b	Phe decrease ^a	NPV ^c
T = 8	≥ 30%	51.7%		
	≥ 35%	50.0%	30-35%	44.4%
	≥ 40%	40.0%	30-40%	35.7%
	≥ 45%	40.0%	30-45%	35.7%
	≥ 50%	45.5%	30-50%	44.4%
T = 16	≥ 30%	53.7%		
	≥ 35%	54.3%	30-35%	50.0%
	≥ 40%	53.3%	30-40%	45.5%
	≥ 45%	52.0%	30-45%	43.8%
	≥ 50%	47.6%	30-50%	40.0%
T = 24	≥ 30%	59.1%		
	≥ 35%	59.5%	30-35%	42.9%
	≥ 40%	60.6%	30-40%	45.5%
	≥ 45%	56.0%	30-45%	36.8%
	≥ 50%	57.1%	30-50%	39.1%
T = 48	≥ 30%	54.7%		
	≥ 35%	62.2%	30-35%	87.5%
	≥ 40%	63.9%	30-40%	64.7%
	≥ 45%	65.6%	30-45%	61.9%
	≥ 50%	66.7%	30-50%	57.7%

^aMaximum Phe decrease compared to the baseline value. ^bPositive predictive value (PPV) using the corresponding cutoff value. ^cNegative predictive value (NPV) for a decrease in Phe in the corresponding range.

Supplemental material 2. Predictive values of the 48-hour BH₄ loading test using an amended definition of BH₄ responsiveness ("an increase in natural protein tolerance ≥ 100% or tolerating a safe natural protein intake"), and using different cutoff values.

	Phe decrease ^a	PPV ^b	Phe decrease ^a	NPV ^c
	≥ 30%	69.4%		
	≥ 35%	75.9%	30-35%	75.0%
	≥ 40%	80.4%	30-40%	62.5%
	≥ 45%	82.5%	30-45%	54.5%
	≥ 50%	83.8%	30-50%	52.0%

^aMaximum Phe decrease compared to the baseline value. ^bPositive predictive value (PPV) using the corresponding cutoff value. ^cNegative predictive value (NPV) for a decrease in Phe in the corresponding range.

Supplemental material 3. PPVs and NPVs for different cutoff values at separate time points during the 48-hour BH₄ loading test, using an amended definition of BH₄ responsiveness ("an increase in natural protein tolerance $\geq 100\%$ or tolerating a safe natural protein intake").

	Phe decrease ^a	PPV ^b	Phe decrease ^a	NPV ^c
T = 8	$\geq 30\%$	82.8%		
	$\geq 35\%$	90.0%	30-35%	33.3%
	$\geq 40\%$	93.3%	30-40%	28.6%
	$\geq 45\%$	93.3%	30-45%	28.6%
	$\geq 50\%$	90.9%	30-50%	22.2%
T = 16	$\geq 30\%$	78.0%		
	$\geq 35\%$	80.0%	30-35%	33.3%
	$\geq 40\%$	83.3%	30-40%	36.4%
	$\geq 45\%$	88.0%	30-45%	37.5%
	$\geq 50\%$	85.7%	30-50%	30.0%
T = 24	$\geq 30\%$	77.3%		
	$\geq 35\%$	78.4%	30-35%	28.6%
	$\geq 40\%$	81.8%	30-40%	36.4%
	$\geq 45\%$	80.0%	30-45%	26.3%
	$\geq 50\%$	85.7%	30-50%	30.4%
T = 48	$\geq 30\%$	71.7%		
	$\geq 35\%$	77.8%	30-35%	62.5%
	$\geq 40\%$	77.8%	30-40%	58.8%
	$\geq 45\%$	81.3%	30-45%	42.9%
	$\geq 50\%$	81.5%	30-50%	38.5%

^aMaximum Phe decrease compared to the baseline value. ^bPositive predictive value (PPV) using the corresponding cutoff value. ^cNegative predictive value (NPV) for a decrease in Phe in the corresponding range.

Supplemental material 4. Association between genotype and BH₄ responsiveness, using an amended definition of BH₄ responsiveness (“an increase in natural protein tolerance $\geq 100\%$ or tolerating a safe natural protein intake”).

A. Boxplots (median, 25th – 75th percentile, min, max) of the percentage of BH₄-responsive patients with a similar genotype in the BIOPKU database in BH₄-unresponsive and BH₄-responsive patients according to an amended definition. Differences are tested with the Mann-Whitney U test. **B.** Boxplots (median, 25th – 75th percentile, min, max) of the genotypic phenotype value (GPV) in BH₄-unresponsive and BH₄-responsive patients according to an amended definition. Differences are tested with the Mann-Whitney U test.

THE NEONATAL TETRAHYDROBIOPTERIN LOADING TEST IN PHENYLKETONURIA: WHAT IS THE PREDICTIVE VALUE?

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Abstract

Background: It is unknown whether the neonatal tetrahydrobiopterin (BH4) loading test is adequate to diagnose long-term BH4 responsiveness in PKU. Therefore we compared the predictive value of the neonatal (test I) versus the 48-h BH4 loading test (test II) and long-term BH4 responsiveness.

Methods: Data on test I (>1991 , 20 mg/kg) at T = 8 (n = 85) and T = 24 (n = 5) were collected and compared with test II and long-term BH4 responsiveness at later age, with ≥ 30 % Phe decrease used as the cut-off.

Results: The median (IQR) age at hospital diagnosis was 9 (7–11) days and the age at test II was 11.8 (6.6–13.7) years. The baseline Phe concentrations at test I were significantly higher compared to test II (1309 (834–1710) versus 514 (402–689) $\mu\text{mol/L}$, respectively, $P = 0.000$). 15/85 patients had a positive test I T = 8. All, except one patient who was not tested for long-term BH4 responsiveness, showed long-term BH4 responsiveness. In 20/70 patients with a negative test I T = 8, long-term BH4 responsiveness was confirmed. Of 5 patients with a test I T = 24, 1/5 was positive at both tests and showed long-term BH4 responsiveness, 2/5 had negative results at both tests and 2/5 showed a negative test I T = 24, but a positive test II with 1/2 showing long-term BH4 responsiveness.

Conclusions: Both a positive neonatal 8- and 24-h BH4 loading test are predictive for long-term BH4 responsiveness. However, a negative test does not rule out long-term BH4 responsiveness. Other alternatives to test for BH4 responsiveness at neonatal age should be investigated.

Introduction

Phenylketonuria (PKU, MIM 261600) is an autosomal recessive disorder caused by a deficiency of the enzyme phenylalanine-4-hydroxylase (PAH, EC 1.14.16.1), which is mainly active in the liver. PAH converts the essential amino acid phenylalanine (Phe) into tyrosine using the cofactor tetrahydrobiopterin (BH4). A deficiency of the enzyme leads to increased Phe and normal to decreased tyrosine concentrations in blood and tissues. Untreated, this results in progressive and irreversible neurological damage (among others mental retardation and epilepsy).^{1,2} Early and continuous treatment by reducing blood Phe concentrations to a large extent prevents patients from mental disability. Adequate blood Phe concentrations can be achieved by means of a Phe restricted diet and/or the relatively new BH4 treatment that has an effect in approximately 20-50 % of PKU patients.³

In most parts of the world, BH4 is prescribed to patients from four years of age onwards, because no large studies on safety of BH4 treatment in patients under the age of four years are available. Yet, also at this age treatment with BH4 is promising.^{4,5} If safety is proven, starting BH4 treatment at a young age can be beneficial for a healthy dietary pattern as some patients find it hard to learn to eat new foods after being restricted for many years.^{6,7} Also advantages of a diet containing more natural protein intake have been suggested.^{8,9}

If BH4 treatment is started at an early age, accurate tests to distinguish patients that are or are not responsive are of great importance. In recent years, several methods have been developed to predict long-term BH4 responsiveness. The most used method is the BH4 loading test. Historically this test was performed at neonatal age, immediately after neonatal screening and aimed to distinguish hyperphenylalaninemic patients with PAH deficiency and patients with a BH4 deficiency.^{10,11} For this purpose an eight hour test is sufficient,¹² although a 24 h test may be more effective in detecting dihydropteridine reductase deficiency.

In Europe, the standard test to evaluate BH4 responsiveness in PAH deficiency is a BH4 loading test taking at least 48 h and is for the greater part performed in patients of at least four years of age.¹³ This elongation of the test seems to be important as it has been shown that a positive response to BH4 frequently occurs after the eight or even 24 h time point.¹⁴⁻¹⁷ In the US even a 28-days method is used for all ages. Yet, these tests are inappropriate for the use in neonates as prolonged exposure to high Phe concentrations has a great impact on mental development at this age.^{18,19} BH4 loading tests postponing dietary treatment at neonatal age should be as short as possible to prevent unnecessary damage. This is extra important as the majority of the PAH deficient patients does not show BH4 responsiveness.

However, no study has investigated the predictive rate of the BH4 loading tests at neonatal age in comparison to later BH4 responsiveness. Therefore, our objective was to look at the discriminating ability of the historical neonatal BH4 loading test by comparing these tests to BH4 loading tests performed in the same patients at a later age.

Methods

Subjects

A historical cohort study was conducted comprising of patients with PKU and data available from both a neonatal BH4 loading test (test I) and a 48-h BH4 loading test (test II) at later age. Only patients born after 1991 who were tested with 20 mg/kg of BH4 during test I were included. The early diagnosed and treated patients were selected from six Dutch University Medical Centers and parts of the data have been reported before.¹⁷ Patients with blood samples missing that hinder comparison of the tests were excluded from analysis. All data were collected from electronic and paper patient records. The medical ethical committee of the university medical center Groningen has ascertained that the protocol is not a clinical research with test subjects as meant in the Medical Research involving human subjects act (WMO). Therefore the local medical ethical committee had no task in reviewing the protocol.

Neonatal BH4 loading test

The neonatal BH4 loading test (test I) was performed using 20 mg/kg BH4 (Schircks Laboratories), with 6R,S-BH4 until October 1999 and 6R-BH4 since October 1999. The BH4 was administered orally directly after taking a baseline blood sample. In most cases further blood samples were taken four and eight hours after BH4 administration and in some cases also after 24 h. The blood samples were collected as venous samples or with heel prick on filter paper, with the same sampling and analyzing method per patient. All patients were on a normal diet during the test.

48-h BH4 loading test

The 48-h BH4 loading test (test II) was conducted as described earlier,²⁰ with 2 days of 20 mg/kg/day BH4 (Kuvan®) and filter paper blood samples collected on T = 0, 8, 16, 24 and 48 h. Patients with blood Phe concentrations below 400 µmol/L where supplemented with Phe (L-Phe in powder, a protein rich supplement such as milk powder or an increase in natural protein intake) until the test was finished. In both tests a reduction in Phe concentration of at least 30 % (during at least one sampling moment) was considered as positive. Patients with 30 % or more decrease of blood Phe concentration during test II were invited to investigate whether they also showed long-term BH4 responsiveness as described previously.¹⁷

Statistical analysis

Data were tested for normality using the Shapiro-Wilk test. Data with a normal distribution are presented as mean $\pm$ SD, whereas data with a skewed distribution are presented as medians with interquartile ranges (IQR). To compare continuous non-parametric data the Wilcoxon Signed Rank test was used. For comparing categorical data the Fisher's exact test was used. The

independent samples t-test corrected for unequal variances in case the Levene's test was <0.05 was used for comparing parametric continuous data. All tests were performed two-tailed. The significance level was set at $p < 0.05$. Statistical analyses were performed using IBM Corp. SPSS Statistics for Windows, Version 20.0. Armonk, NY, USA.

Results

In total, data on 88 early diagnosed patients were collected on both test I and test II. One patient was excluded because the T = 8 and T = 24 h Phe measurements were missing in test II. In 85 patients data included a T = 8 blood sample in both test I and test II. Three of these patients and two more patients (without T = 8 blood sample) were tested for 24 h during test I. Table 1 shows the demographic and clinical characteristics of the included patients. Although 60 % of patients received Phe supplementation during the test II, the baseline Phe concentrations were significantly lower compared to test I ($P = 0.000$).

Table 1

Demographic and clinical characteristics

	Total n=87 patients
Sex	
Male	43 (49.4)
Female	44 (50.6)
Age at diagnosis (days)*	9 (7 – 11)
Phe at diagnosis ($\mu\text{mol/L}$)**	1319 (760 – 1820)
Phe at T=0 1 st test ($\mu\text{mol/L}$)	1309 (834 – 1710)
Age at 2 nd test (years)	11.8 (6.6 – 13.7)
Phe at T=0 2 nd test ($\mu\text{mol/L}$)	514 (402 – 689)

Data are n (%) or median (IQR). * Not known in 2 patients. ** at the first hospital visit (missing in 2 patients)

Of the 85 patients with a T = 8 h Phe measurement during the test I, 70 showed less than 30 % decrease in Phe concentration. An overview of test I, test II and long-term BH4 responsiveness results of all patients with a T = 8 Phe measurement is shown in Figure 1. Of 15 patients with 30 % or more Phe decrease at T = 8 during test I, fourteen had a positive test II, although not always within eight hours. All, except for one patient who did not continue BH4 treatment, showed long-term BH4 responsiveness. In 29/70 patients with less than 30 % Phe reduction at T = 8 during test I, 30 % or more Phe decrease at some moment during the entire test II was seen. Of these patients 26 were tested for their long-term response to BH4, which could be confirmed in twenty of them.

Of 5 patients with a T = 24 Phe measurement during the test I, two patients had the same negative result at T = 24 in test II and also less than 30 % Phe concentration reduction during the entire 48 h of test II. Two other patients had less than 30 % Phe concentration decrease in test I, but over 30 % in test II. Therefore both patients were tested for long-term BH4

responsiveness, during which one patient showed long-term BH4 responsiveness (6.5 g increase in natural protein with comparable Phe concentrations). One other patient had positive results at T = 24 in both tests and proved to show long-term BH4 responsiveness.

Figure 1 1st test, 2nd test and long-term BH4 treatment results in all patients with (at least) an 8-h neonatal BH4 loading test.

No significant difference was detected between the proportion of responders at T = 8 in test I and test II (15/85 and 18/85, respectively, $P = 0.699$). Patients with at least 30 % Phe reduction at T = 8 in test I had a mean baseline Phe concentration of $833 \pm 333 \mu\text{mol/L}$. This is significantly higher than the baseline Phe concentration of patients at least 30 % Phe reduction at T = 8 in test II ($410 \pm 119 \mu\text{mol/L}$, $P = 0.000$). For both test I and test II, the baseline Phe concentration as well as the Phe reduction at T = 8 is shown for all patients in Figure 2.

Discussion

The present study shows that a neonatal 8-h test is inadequate in determining BH4 responsiveness, as at least 29 % of the patients with a negative 8-h neonatal BH4 loading test showed long-term BH4 responsiveness at later age. Even in the small group of patients tested for 24 h at neonatal age, long-term BH4 responsiveness, proven at later age, can be missed. However, the frequency of response in an 8-h test is not different between test I and II. Patients with a positive test I showed this response despite the significantly higher baseline Phe concentration compared to test II.

Figure 2 Scatter plot of the phenylalanine concentration at T=0 and the percentage of Phe decrease at T=8 of (A) the 1st test (B) the 2nd test.

The first aim of neonatal screening always has been to start treatment as early as possible. This is very important as a delay in treatment has consequences for mental development. Treatment with BH4 could offer patients a diet with more or even a normal amount of natural protein. Consequently, it is desirable to know whether a patient is BH4 responsive or not at a young age. Historically, a short neonatal BH4 loading test was necessary for the distinction between PKU and BH4 deficiencies. Although a growing number of centers can discriminate between these deficiencies by measuring pterins and dihydropteridine reductase activity, the information gathered by the neonatal BH4 loading test is collected faster. Therefore, there still are a lot of centers for which the neonatal loading test is crucial. According to a study by Feillet et al., $n = 9$ PAH deficient patients with a 24-h neonatal BH4 loading test (20 mg/kg BH4) needed less time to reach Phe concentration under 300 $\mu\text{mol/L}$ compared to $n = 10$ patients who started with a Phe restricted diet immediately after diagnosis.²¹ This study nicely shows that neonatal use of BH4 in PAH deficient patients can effectively reduce the Phe concentration. Having said this, we need to stress that this does not take into account that the majority of patients is not responsive to BH4.³ More importantly, the patients that do not respond are often those with the highest Phe concentrations in which treatment is the most urgent.²²

One of the reasons explaining the lack of accurateness of the BH4 loading test at neonatal age in predicting long-term BH4 responsiveness is that at neonatal age Phe concentration is heavily influenced by the catabolic or anabolic state of the infant. Alternative methods to predict BH4 responsiveness could be the genotype, albeit that some inconsistent results exist.^{17,23} Another alternative could be to start BH4 treatment simultaneously with dietary Phe restriction and to stop administering BH4 when the metabolic control is stably within treatment ranges. A few studies have shown that in patients treated with BH4 Phe concentrations rise directly after missing a dose of BH4 or stopping the treatment.^{24,25} Therefore, a rise in Phe concentration is expected when BH4 treatment is stopped in patients with BH4 responsiveness. Hence, BH4

could be used first as emergency treatment, independent of whether BH4 responsiveness can be proven.

Limitations of this study include that the setup was not prospective and that not all patients were tested for long-term BH4 responsiveness. Additionally, conclusions were largely based on 8-h neonatal BH4 loading tests due to treatment urgency, where 24-h tests would be more informative. Furthermore, the mean baseline Phe concentration was significantly higher in test I compared to test II. Another important remark is that test I and test II were performed with BH4 from different companies of which some of the first tests with 6R,S-BH4 which is less active. Notwithstanding the increase in positive neonatal BH4 loading tests (test I) in patients born after 1999, using the more active form of BH4 (6R-BH4), even in that patient group, a substantial number of patients had a negative test I but positive test II later on and confirmed long-term BH4 responsiveness.

Conclusion

Although a positive neonatal 8-h BH4 loading test is predictive for long-term BH4 responsiveness, a negative 8-h or even a 24-h test can miss long-term BH4 responsive patients. As delays in treatment should be avoided, other alternatives to test for BH4 responsiveness at neonatal age, such as using genotype and starting BH4 treatment simultaneous with dietary treatment, should be investigated.

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LONG-TERM FOLLOW-UP AND OUTCOME OF PHENYLKETONURIA PATIENTS ON SAPROPTERIN: A RETROSPECTIVE STUDY

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Abstract

Objective: Sapropterin dihydrochloride, the synthetic form of 6R-tetrahydrobiopterin (BH4), is an approved drug for the treatment of patients with BH4-responsive phenylketonuria (PKU). The purpose of this study was to assess genotypes and data on the long-term effects of BH4/sapropterin on metabolic control and patient-related outcomes in 6 large European countries.

Methods: A questionnaire was developed to assess phenotype, genotype, blood phenylalanine (Phe) levels, Phe tolerance, quality of life, mood changes, and adherence to diet in PKU patients from 16 medical centers.

Results: One hundred forty-seven patients, of whom 41.9% had mild hyperphenylalaninemia, 50.7% mild PKU, and 7.4% classic PKU, were followed up over ≤ 12 years. A total of 85 different genotypes were reported. With the exception of two splice variants, all of the most common mutations were reported to be associated with substantial residual Phe hydroxylase activity. Median Phe tolerance increased 3.9 times with BH4/sapropterin therapy, compared with dietary treatment, and median Phe blood concentrations were within the therapeutic range in all patients. Compared with diet alone, improvement in quality of life was reported in 49.6% of patients, improvement in adherence to diet was reported in 47% of patients, and improvement in adherence to treatment was reported in 63.3% of patients. No severe adverse events were reported.

Conclusions: Our data document a long-term beneficial effect of orally administered BH4/sapropterin in responsive PKU patients by improving the metabolic control, increasing daily tolerance for dietary Phe intake, and for some, by improving dietary adherence and quality of life. Patient genotypes help in predicting BH4 responsiveness.

Introduction

Phenylketonuria (PKU) is the most common autosomal-recessive inherited disorder of amino acid metabolism and is caused by phenylalanine (Phe) hydroxylase (PAH) deficiency in the liver.¹ This reaction is dependent on the PAH cofactor 6R-tetrahydrobiopterin (BH4; sapropterin dihydrochloride).²

In PKU patients, neurotoxic Phe accumulates in the blood (hyperphenylalaninemia; HPA) and brain, and patients, if untreated, can suffer from severe mental retardation.^{3,4}

PKU is a phenotypically heterogeneous disorder ranging from very mild HPA to severe, classic PKU.⁵ The variability in the metabolic phenotypes in PAH deficiency is caused primarily by different mutations within the PAH gene. More than 830 variations in the PAH gene and almost 4200 patients with full genotypes, about 50% of them associated with results from BH4 challenge tests, are tabulated in the BIOPKU database (<http://www.biopku.org>; accessed April 8, 2013).⁶ It has been shown that genotypes are useful in predicting BH4 responsiveness in PKU patients.⁷⁻¹⁰

The aim of PKU treatment is to maintain Phe blood concentrations within the therapeutic range, which is mainly achieved by a natural protein-restricted diet and synthetic amino acid supplementation without Phe.¹¹ However, the recommended low-Phe diet potentially impairs quality of life by decreasing patients' flexibility and autonomy. Therefore, compliance is often poor, especially in adolescence.¹²

An alternative option to the low-Phe diet is to add pharmacologic treatment with BH4.¹³ It has been previously shown that, depending on the country, ~20% to 50% of all PKU patients (predominantly mild to moderate PKU) respond to oral administration of BH4, which lowers their blood Phe concentrations significantly.¹⁴⁻¹⁸ Sapropterin dihydrochloride (Kuvan; Merck Serono SA, Geneva, Switzerland) has been approved by the Food and Drug Administration in the United States and by the European Medicines Agency in Europe for the treatment of PKU patients in combination with diet.¹ In short- and long-term prospective and retrospective clinical studies, it has resulted in significant and sustained reductions in blood Phe concentrations in patients with PKU, even if administrated as monotherapy.¹⁹⁻²²

The objective of this multicenter study was to collect data on the long-term metabolic outcomes of PKU patients treated with BH4/sapropterin. We devoted special attention to the influence of genotype.

Methods

In this study, we retrospectively collected data from PKU patients who had been treated with BH4 (Schircks Laboratories, Jona, Switzerland) or with sapropterin (sapropterin dihydrochloride [Kuvan]) from 16 centers in 6 European countries (France, Germany, Italy, Netherlands, Spain,

and Switzerland). The active substance 6R-BH4 was identical in both of the compounds. A total of 147 patients who had previously been diagnosed with PKU and who had been treated with BH4 or sapropterin for at least 6 months were included in the study. The inclusion criteria were as follows: male and female patients without a limit of age, documented diagnosis of PKU, previous response to BH4 with a significant decrease in blood Phe concentrations, current treatment with sapropterin (or with previous BH4 treatment, if applicable), and patient-, parent-, and/or guardian-signed informed consent after the nature and content of the questionnaire had been explained. The exclusion criteria were subjects with BH4 deficiency, pregnancy, and severe illness with the use of additional medications. No diagnostic, therapeutic or experimental interventions were involved.

The questionnaire included age at diagnosis, laboratory newborn screening data, BH4/sapropterin loading test, highest blood Phe concentrations before initiation of treatment, and Phe tolerance before and during treatment with BH4/sapropterin. In addition, age at treatment initiation, changes in BH4/ sapropterin dosage, number of doses per day, frequency of blood testing, and blood Phe concentrations (minimum, maximum, median) were requested. A simple question (yes/no) about improvement in quality of life (QoL) was used to assess estimated clinical outcomes. Compliance with treatment and mood changes was also assessed; however, these outcomes were not among the primary aims of this study.

The methods used to assess QoL were as follows (open-answered questions): Kidscreen 52, World Health Organization QoL 100, patient and parent interviews, patient letters, pediatric and psychological assessments, and observation by the physician. To assess compliance with diet (open-answered question), index of dietary control, dietary records analyzed by a dietitian, and patient and parent interviews were used. The methods used to assess compliance with treatment in general were as follows (open-answered question): patient and parent interview, pediatric and psychological assessments, and clinical features. To assess changes in mood (open-answered question), patient and parent interviews, pediatric and psychological assessments, and observation by the physician were used. Changes in mood were classified as follows (open-answered question): better social integration, less emotional lability, better mood, and fewer depressive moods.

The central ethical committee of each country fully approved the protocol valid for all of the clinical centers. REGISTRAT-MAPI (Lyon, France), a clinical research organization, handled all of the legal documents and submissions to ethical committees. All of the data were fully anonymized and can no longer be retraced.

Statistical analyses

Statistical analysis was performed by using Microsoft Excel with the WinSTAT module (Fitch, Bad Krozingen, Germany). Descriptive data are presented as means $\pm$ SD and ranges or as medians and 5th to 95th percentiles.

Results

Data on 147 patients followed up in these centers between December 2011 and July 2012 were included. The mean age was 14.4 ± 8.8 years (range: 1–46 years). One hundred patients (68%) were minors, with a mean age of 9.5 ± 4.3 years (range: 1–17 years). Forty-seven patients were adults, with a mean age of 24.6 ± 6.7 years (range: 18–46 years). Newborn screening was completed in 130 patients (88.4%). Sixteen patients were diagnosed late: 12 (8.2%) within the first month of life, 2 (1.4%) within the first year of life, and 2 (1.4%) at the ages of 4 and 6 years. For 1 patient, no information regarding the time of diagnosis was available. One hundred forty-five patients (98.6%) underwent a BH4 or sapropterin-loading test: 27 patients were tested in the newborn period, 118 patients were tested after the first month of life. The loading test dosages were 5 mg/kg ($n = 4$; 3.1%), 10 mg/kg ($n = 6$; 4.6%), and 20 mg/kg ($n = 121$; 92.3%). For 14 patients, no information regarding the loading test dosage was available. Sixty-seven patients (45.6%) underwent a Phe challenge before the BH4 or sapropterin test. The maximal reduction in median blood Phe concentration during the test was 62% (range: 28%–89%) and thus significant ($P < .001$). There were no differences in the outcome of the test between the newborns and older patients. The median blood Phe concentration (5th–95th percentiles) before the loading test was $604 \mu\text{mol/L}$ (301–1267 $\mu\text{mol/L}$); after the test, it was $247 \mu\text{mol/L}$ (61–720 $\mu\text{mol/L}$).

Reductions in blood Phe concentration were between 31% and 40% in 20 patients (13.8%), between 41% and 49% in 18 patients (12.5%), and .50% in 99 patients (68.8%). In 7 patients, the reduction in blood Phe was between 20% and 30%, but BH4 administration resulted in an increase of daily Phe tolerance (data not shown). For 1 patient, no information was available regarding the reduction in blood Phe concentration after the test.

The test duration ranged from 8 hours to 3 weeks, with most centers using 24-hour (52.9%) or 48-hour (32.6%) protocols.

Phenotype

Phenotype was based on newborn screening Phe concentrations (58 patients; 42.6%), the highest blood Phe concentrations before starting treatment (64 patients; 47.1%), or Phe tolerance between the first and eighth years of life (14 patients; 10.3%). Taking all of the criteria into consideration, 67 patients (41.9%) were classified into the mild HPA group (120–600 $\mu\text{mol/L}$), 69 patients (50.7%) into the mild PKU group (600–1200 $\mu\text{mol/L}$), and 11 patients (7.4%) into the classic PKU group ($> 1200 \mu\text{mol/L}$).

Genotype

Eighty-five different genotypes were reported in 109 patients. In 8 patients, a mutation was detected on only 1 allele, and in 23 patients (16.4%) the genotype was not available. Of 85 genotypes, 2 genotypes (c.1066-11G>A/p.Y414C and c.1315+1G>A/p.Y414C) occurred in 4 patients (genotype frequency: 3.4%), 17 genotypes occurred in 2 patients, and 66 genotypes were identified in only 1 patient (Supplemental Table 3). A total of 69 different mutations were reported. The most frequent in our patient population were as follows: p.Y414C (allele frequency [AF] = 8.2%), p.R261Q (AF = 7.8%), c.1066-11G>A (AF = 6.9%), p.L48S (AF = 6.5%), p.V388M (AF = 5.7%), p.E390G (AF = 5.3%), c.1315+1G>A (AF = 3.7%), and p.A300S (AF = 3.3%) (Table 1). All of these mutations, with the exception of the splice variants c.1066-11G.A and c.1315+1G.A, were reported to be associated with substantial (>30% enzyme activity compared with the wild-type PAH) residual PAH activity (www.biopku.org). Supplemental Table 3 summarizes all of the genotypes and in vitro residual PAH activities.

Table 1

Most Common Mutations Found in Our BH4-Responsive PKU Patients

Variation	Nucleotide Aberration	AF, %
p.Y414C	c.1241A>G	8.2
p.R261Q	c.782G>A	7.8
IVS10-11G>A	c.1066-11G>A	6.9
p.L48S	c.143T>C	6.5
p.V388M	c.1162G>A	5.7
p.E390G	c.1169A>G	5.3
IVS12+1G>A	c.1315+1G>A	3.7
p.A300S	c.898G>T	3.3
p.P281L	c.842C>T	3.3

For details on complementary DNA level, see Supplemental Table 3.

Treatment

A total of 94 patients (63.9%) received treatment with sapropterin alone, and 53 patients (36.1%) were treated with sapropterin in combination with a low-Phe diet. Over the course of treatment, 42 patients (28.6%) were initially treated with the BH4 product, and 19 patients (12.9%) were treated with BH4 plus a low-Phe diet. They all changed treatment from BH4 to sapropterin.

The median (5th–95th percentiles) daily Phe tolerance before BH4 or sapropterin treatment in 38 patients with mild to classic PKU was 500 mg/d (200–800 mg/d). During sapropterin therapy, Phe tolerance increased significantly ($P < .001$) to 2500 mg/d (1500–3000 mg/d) (Fig 1).

Within this study, the effects of 4 different treatment protocols on blood Phe concentrations were investigated: (1) BH4, (2) sapropterin, (3) BH4 plus diet, and (4) sapropterin plus diet. Because there was no significant difference in median blood Phe concentrations between BH4 and

sapropterin alone (for BH4 and sapropterin, $P = .573$) or BH4 with diet (for BH4 and sapropterin, $P = .024$) (Table 2), we pooled the BH4 and sapropterin data for additional analysis (Table 2).

Figure 1 Daily Phe tolerance before starting treatment and with BH4/sapropterin in 38 BH4-responsive PKU patients (mild to classic phenotype). Median, 5th to 95th percentiles, and minimum and maximum values are shown.

Table 2

Blood Phe Concentrations After Treatment With BH4/Sapropterin With or Without Additional Dietary Supplementation

	Median Blood Phe With Treatment, $\mu\text{mol/L}^a$	Minimum Blood Phe With Treatment, $\mu\text{mol/L}^a$	Maximum Blood Phe With Treatment, $\mu\text{mol/L}^a$
BH4 ^b ($n = 42$)			
Median	277	124	536
5th percentile	134	12	180
95th percentile	531	344	1135
Range	46-690	11-348	178-1140
Sapropterin ^b ($n = 85$)			
Median	270	161	446
5th percentile	150	32	147
95th percentile	597	469	966
Range	32-966	15-930	65-1402
BH4 + diet ^c ($n = 17$)			
Median	241	93	500
10th percentile	110	56	290
90th percentile	482	153	780
Range	44-492	48-171	219-1157
Sapropterin + diet ^c ($n = 49$)			
Median	329	156	561
10th percentile	200	52	289
90th percentile	653	433	900
Range	83-826	18-642	180-1098

^a For each patient. ^b t Test for BH4 (Schircks Laboratories) and sapropterin, $P = .573$. ^c t Test for BH4 (Schircks Laboratories) + diet and sapropterin + diet, $P = .024$ (sample size too small for 5th-95th percentile calculation).

Treatment With BH4/Sapropterin Alone

Treatment was initiated at a mean age of 9.4 ± 7.8 years (range: 1 month to 35 years). The mean starting BH4/sapropterin dose was 11.1 ± 5.8 mg/kg per day (5.0–22.7 mg/kg per day), and the dosage was titrated for individual patients to reach a therapeutic range for blood Phe concentrations. The mean lowest BH4/sapropterin dose used was 9.4 ± 4.6 mg/kg per day (4.9–21.0 mg/kg per day), the mean highest dose was 12.1 ± 5.6 mg/kg per day (5.0–22.7 mg/kg per day), and the mean current daily dose of sapropterin (at the time of data collection) was 10.8 ± 5.3 mg/kg per day (4.9–21.0 mg/kg per day). Administration ranged between 1 dose (68%), 2 doses (20%), and 3 doses (12%) per day. Blood Phe concentrations were tested between 4 times per month and 9 times per year, depending on the patient's age.

In 127 patients (45% mild HPA, 53% mild PKU, 2% classic PKU), the median (5th–95th percentile) blood concentration during treatment was 276 $\mu\text{mol/L}$ (150–590 $\mu\text{mol/L}$) (Fig 2A). The minimum blood Phe concentration was 148 $\mu\text{mol/L}$ (28–372 $\mu\text{mol/L}$), and the maximum blood Phe concentration was 476 $\mu\text{mol/L}$ (168–1032 $\mu\text{mol/L}$) (Fig 2A). The mean treatment duration was 4.2 years (6 months to 8.8 years).

Treatment With BH4/Sapropterin Plus Low-Phe Diet

Treatment with BH4/sapropterin in combination with a low-Phe diet was started at a mean age of 10.4 ± 9.2 years (range: 1 month to 35 years). The initial mean BH4/sapropterin dose in all patients was 13.7 ± 5.0 mg/kg per day (5.0–22.2 mg/kg per day), the lowest BH4/sapropterin dose was 12.0 ± 4.6 mg/kg per day (2.6–20.0 mg/kg per day), and the highest dose was 14.9 ± 4.5 mg/kg per day (8.0–23.4 mg/kg per day). At the time of survey completion, the current dosage of BH4/sapropterin in all patients was 13.8 ± 4.8 mg/kg per day (2.6–23.4 mg/kg per day). Administration ranged between 1 dose (66%), 2 doses (27%), and 3 doses (5%) per day. Blood Phe was tested between 9 times per month and 4 times per year.

The median (5th–95th percentile) blood concentration during the treatment of 66 patients (27% mild HPA, 58% mild PKU, 15% classic PKU) was 308 $\mu\text{mol/L}$ (135–666 $\mu\text{mol/L}$) (Fig 2B). The minimum blood Phe concentration was 135 $\mu\text{mol/L}$ (38–471 $\mu\text{mol/L}$), and the maximum blood concentration during treatment was 549 $\mu\text{mol/L}$ (278–1045 $\mu\text{mol/L}$). The treatment duration was 5.7 ± 2.7 years (range: 2.0–12.0 years). All of the patients except for 5 (no access to Kuvan) terminated BH4 therapy and switched to sapropterin. For the 46 patients, the treatment regimen could be adapted from treatment with BH4/sapropterin plus a low-Phe diet to treatment with BH4/sapropterin alone. Thus, these patients were included in both groups.

Figure 2 Comparison of initial blood Phe concentrations in PKU patients (before any treatment initiation) with blood Phe concentrations (medians, 5th–95th percentiles, minimum and maximum values) after treatment with BH4/sapropterin alone (A) and after treatment with BH4/sapropterin in combination with a low-Phe diet (B). Max, maximum; Min, minimum.

Treatment Termination and Adverse Events

A total of 5 patients stopped sapropterin treatment for the following reasons: pregnancy, poor compliance of guardians, or poor compliance with treatment regulations regarding obesity. Adverse events, such as gastric pain, frequent urination during the treatment, and dizziness during the loading test, were reported in 3 patients (2%). All adverse events disappeared with lowering the doses of BH4/sapropterin.

Effect of BH4/Sapropterin Treatment on QoL

The investigators reported an improvement in QoL for 73 patients (49.6%). For 21 patients (14.3%), no improvement in QoL could be detected, and for 53 patients (36.1%) no information was available or the question was not answered (Fig 3A).

Effect of BH4/Sapropterin Treatment on Compliance With Diet

The investigators reported an improvement in compliance with diet in 69 patients (47%). For 39 patients (26.5%), no improvement in compliance with diet could be detected. For 39 patients (26.5%), no information was available or the question was not answered (Fig 3B).

Effect of BH4/Sapropterin Treatment on Compliance With Treatment

Investigators documented an improvement in compliance with sapropterin/BH4 treatment, compared with treatment with diet alone, in 93 patients (63.3%). For 29 patients (19.7%), no improvement could be found. For 25 patients (17%), no information was available or the question was not answered (Fig 3C).

Figure 3 Percentages of BH4-responsive PKU patients treated with BH4/sapropterin who reported improvement in quality of life (A), improvement in compliance to diet (B), and improvement in compliance to BH4/sapropterin treatment compared with diet (C).

Effect of Pharmacologic Treatment on Changes in Mood

The investigators documented positive changes in mood in 18 patients (12.2%). For 57 patients (38.8%), no changes in mood were documented. For 72 patients (49.8%), no information was available or the question was not answered.

Discussion

Sapropterin (BH4) is an alternative treatment option for ~20% to 50% of PKU patients. Primarily patients with mild to moderate PKU phenotypes, but also a few patients with classic PKU, have been shown to respond to oral administration of BH4.^{15,23} BH4 has been used in PKU patients since 1999, when Kure et al demonstrated for the first time that some PKU patients responded to its administration with decreased blood Phe concentrations.¹⁴ At that time, clinical centers used nonregistered BH4 (Schircks Laboratories). In the following years, a number of small local studies documented the effectiveness of BH4 in some PKU patients (for a review of the literature, see ref 24).²⁴ Since the approval of BH4 as sapropterin dihydrochloride (Kuvan) by the US Food and Drug Administration in 2007 and by the European Medicines Agency in 2008, sapropterin has become a part of the standard treatment of PKU patients who respond to its administration.^{25,26} Several placebo-controlled, double-blind studies have revealed that sapropterin administration reduces blood Phe concentrations, increases dietary tolerance for Phe, and is safe in PKU patients, with only few adverse events reported.^{17,21,22,27,28}

Our retrospective study with 147 PKU patients adds to the available knowledge and documents the long-term efficiency (up to 12 years) of BH4/sapropterin. In patients who were receiving

sapropterin >3 years, BH4 was used during the first years. Ninety-four patients (63.9%) received treatment with sapropterin only, and 54 patients (36.7%) were treated with sapropterin in combination with diet.

The 48-hour loading test with 20 mg/kg per day of sapropterin is recommended by the European working group for PKU²⁹ and has been adopted by most clinical centers.³⁰⁻³² In our study, most of the centers used the 24-hour (52.9%) or 48-hour (32.6%) protocols. The test dose ranged from 5 to 20 mg/kg, with most of the centers (92.3%) using 20 mg/kg.

The most widely accepted cutoff value to consider the test positive is a reduction in blood Phe of 30%.³³ In our study, the median reduction in blood Phe concentration was 62%, and all of the tested patients proved to be responders over longer periods of time. One should, however, bear in mind that the BH4/sapropterin challenge is only a screening test to detect potential responders and that long-term supplementation can confirm initial results indicating real responsiveness.

In addition to blood Phe reduction, an increase in tolerance for dietary Phe intake is another factor that determines true BH4 responsiveness. As previously shown^{23,34,35} and as documented in our survey, a significant increase in daily Phe tolerance was recorded with sapropterin treatment (Fig 1). In addition to reduction in blood Phe, an increase in Phe tolerance by at least a factor of 2 is now required by several protocols.^{20,21,36,37} Moreover, blood Phe concentrations were stable and within the therapeutic range in all patients (Fig 2), no severe adverse events were reported, and all of the side effects disappeared with reduced therapeutic sapropterin doses. However, treatment recommendations (blood Phe cutoff for starting treatment) are different in different countries, as recently documented by Blau et al.⁵

Today, it is generally accepted that only the full genotype determines both phenotype and BH4 responsiveness.^{7,9} A number of mutations are more frequently found in BH4 responders, whereas BH4 non-responders harbor more severe mutations, with less or little residual PAH activity.³⁸ All of the 85 genotypes identified within our survey and the 8 mutations detected on only 1 allele were reported to be associated with substantial (>30%) residual PAH activity (www.biopku.org). With the exception of the splice site mutations in introns 10 and 11, p.Y414C, p.R261Q, p.L48S, p.V388M, and p.E390G presented with substantial (>25%) residual PAH activity when expressed in recombinant eukaryotic cell systems. The same mutations were also found in the most common genotype among our patients (Supplemental Table 3), p.Y414C, with c.1066-11G.A and c.1315+1G.A being the 2 most common. Therefore, patient genotype is an important option for predicting BH4 responsiveness, although it should always be confirmed by the loading test.

For 49.6% of patients, an improvement in QoL with therapy with sapropterin was reported. Hennermann et al²³ reported similar results in patients with PKU. Ziesch et al³⁹ did not detect an improvement in health-related QoL in their study, at least not during the first 3 months of

BH4/sapropterin treatment. In our survey, this question was not answered in 36% of patients, and the assessment to evaluate QoL was rather heterogeneous (ie, subjective observation by the physician, patient and parent interviews, and questionnaires). Thus, our data are an estimate rather than fully quantitative, and a more topic-focused study is needed to document the real value and the cost-benefit effects of BH4/sapropterin on QoL in PKU patients.

A high percentage of investigators (63.3%) have reported improvements in compliance with general treatment. It is not clear whether it is a problem for patients to continue a relaxed Phe-free diet, and it has been suggested that specialized questionnaires (for patients and physicians) should be developed to answer this question.^{39,40}

Conclusions

Our survey emphasizes that sapropterin is a safe and effective alternative to conventional dietary treatment of PKU patients who respond to long-term treatment by stabilizing their blood Phe concentrations in the therapeutic range, thus possibly lowering the burden of the strict low-Phe diet. A sapropterin loading test should be performed under standardized conditions and, if possible, in combination with genotyping,⁴¹ but this requirement could change, depending on the patient's current blood Phe concentrations.⁴²

Acknowledgments

This work was supported by an investigator-sponsored trial from Merck Serono SA, Geneva, Switzerland, a branch of Merck Serono SA, Coinsins, Switzerland, an affiliate of Merck KGaA, Darmstadt, Germany. Merck Serono has reviewed the publication; however, the views and opinions described in the publication do not necessarily reflect those of Merck Serono. We are grateful to the European Phenylketonuria Group (EPG) for valuable discussions and to the many patients and parents who provided their data.

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Supplementary Table

List of Full Genotypes and Corresponding Relative *In Vitro* PAH Activity

Allele 1	Nucleotide aberration 1	Allele 2	Nucleotide aberration 2	No of patients	Allele 1 Activity, %	Allele 2 Activity, %	Total Activity, %
IVS10-11G>A	c.1066-11G>A	p.Y414C	c.1241A>G	4		57	28.5
IVS12+1G>A	c.1315+1G>A	p.Y414C	c.1241A>G	4		57	28.5
p.E178G	c.533A>G	p.S349P	c.1045T>C	2	39	1	20.0
p.I65T	c.194T>C	p.D222G	c.665A>G	2	33	?	16.5
p.I65T	c.194T>C	p.V388M	c.1162G>A	2	33	28	30.5
IVS10-11G>A	c.1066-11G>A	p.A403V	c.1208C>T	2		66	33.0
IVS10-11G>A	c.1066-11G>A	p.E390G	c.1169A>G	2		62	31.0
IVS10-11G>A	c.1066-11G>A	p.L48S	c.143T>C	2		39	19.5
IVS10-3C>T	c.1066-3C>T	p.L48S	c.143T>C	2		39	19.5
IVS12+1G>A	c.1315+1G>A	p.E390G	c.1169A>G	2		62	31.0
IVS2+5G>A	c.168+5G>A	p.R261Q	c.782G>A	2		44	22.0
p.L48S	c.143T>C	p.Y277D	c.829T>G	2	39	0	19.5
p.L48S	c.143T>C	p.E390G	c.1169A>G	2	39	66	52.5
p.L48S	c.143T>C	p.R261Q	c.782G>A	2	39	44	41.5
p.L48S	c.143T>C	p.R241C	c.721C>T	2	39	25	32.0
p.P362T	c.1084C>A	p.V388M	c.1162G>A	2	?	28	14.0
p.R243Q	c.728G>A	p.V388M	c.1162G>A	2	13	28	20.5
p.R261Q	c.782G>A	p.Y414C	c.1241A>G	2	44	57	50.5
p.R261Q	c.782G>A	p.R261Q	c.782G>A	2	44	44	44.0
p.R261Q	c.782G>A	p.P281L	c.842C>T	2	44	?	22.0
p.Y168H	c.502T>C	p.V388M	c.1162G>A	2	?	28	14.0
p.R243*	c.727C>T	p.Y414C	c.1241A>G	2	0	57	28.5
p.A104D	c.311C>A	p.A104D	c.311C>A	1	27	27	27.0
p.A104D	c.311C>A	p.R158Q	c.473G>A	1	27	10	18.5
p.A300S	c.898G>T	p.G352Vfs	c.1055delG	1	31	?	15.5

Allele 1	Nucleotide aberration 1	Allele 2	Nucleotide aberration 2	No of patients	Allele 1 Activity, ^a %	Allele 2 Activity, ^a %	Total Activity, ^b %
(Continued from previous page)							
p.A300S	c.898G>T	p.R408W	c.1222C>T	1	31	2	16.5
p.A300S	c.898G>T	p.S349P	c.1045T>C	1	31	1	16.0
p.C203Y	c.608G>A	p.A300S	c.898G>T	1	?	31	15.5
p.E178G	c.533A>G	p.Y343C	c.1028A>G	1	39	?	19.5
p.E218V	c.653G>T	p.Y414C	c.1241A>G	1	?	57	28.5
p.E390G	c.1169A>G	p.R408W	c.1222C>T	1	62	2	32.0
p.Ex3del	c.169-?_352+?del	p.Y414C	c.1241A>G	1	0	57	28.5
p.F39del	c.115_117delTTC	p.F55Lfs	c.165delT	1	20	?	10.0
p.F39del	c.115_117delTTC	p.Y414C	c.1241A>G	1	20	57	38.5
p.F39L	c.117C>G	p.V388M	c.1162G>A	1	49	28	38.5
p.G272*	c.814G>T	p.Y414C	c.1241A>G	1	0	57	28.5
p.G46S	c.136G>A	p.S303A	c.907T>G	1	16	?	8.0
p.I65T	c.194T>C	p.R261Q	c.782G>A	1	33	44	38.5
p.I94del	c.283-285delATC	p.R261Q	c.782G>A	1	27	44	35.5
p.I94del	c.283-285delATC	p.R111X	c.331C>T	1	27	0	13.5
IVS1+5G>A	c.60+5G>A	p.D129G	c.386A>G	1		?	-
IVS10-11G>A	c.1066-11G>A	p.R68S	c.204A>T	1		68	34.0
IVS10-11G>A	c.1066-11G>A	p.E178G	c.533A>G	1		39	19.5
IVS10-11G>A	c.1066-11G>A	p.P211T	c.631C>A	1		72	36.0
IVS10-11G>A	c.1066-11G>A	p.D338Y	c.1012G>T	1		?	-
IVS10-11G>A	c.1066-11G>A	p.R243Q	c.728G>A	1		13	6.5
IVS10-11G>A	c.1066-11G>A	p.D415N	c.1243G>A	1		72	36.0
IVS12+1G>A	c.1315+1G>A	p.A403V	c.1208C>T	1		66	33.0
IVS12+1G>A	c.1315+1G>A	p.A300S	c.898G>T	1		31	15.5
IVS2+19T>C	c.168+19T>C	p.Y414C	c.1241A>G	1		57	28.5
IVS2+19T>C	c.168+19T>C	p.R241Q	?	1		?	0.0

Allele 1	Nucleotide aberration 1	Allele 2	Nucleotide aberration 2	No of patients	Allele 1 Activity,* %	Allele 2 Activity,* %	Total Activity,* %
(Continued from previous page)							
IVS4-5C>G	c.442-5C>G	p.P281L	c.842C>T	1		2	1.0
IVS4+5G>T	c.441+5G>T	p.Y414C	c.1241A>G	1		57	28.5
IVS4+5G>T	c.441+5G>T	p.E76G	c.227A>G	1		85	42.5
IVS4+6C>T	c.441+2T>G	p.R261Q	c.782G>A	1		44	22.0
IVS7-4delT	c.843-4delT	p.L48S	c.143T>C	1		39	19.5
IVS7+3G>C	c.842+3G>C	p.A300S	c.898G>T	1		31	15.5
IVS7+5G>A	c.842+5G>A	p.Y414C	c.1241A>G	1		57	28.5
K274Nfs*5	c.822_832del11	p.E390G	c.1169A>G	1		62	31.0
p.L348V	c.1042C>G	p.E390G	c.1169A>G	1	35	62	48.5
p.L48S	c.143T>C	p.W326S	c.977G>C	1	39	?	19.5
p.L48S	c.143T>C	p.P281L	c.842C>T	1	39	2	20.5
p.P147S	c.439C>T	p.S349P	c.1045T>C	1	?	1	0.5
p.P243Q	c.728G>A	p.E390G	c.1169A>G	1	?	62	31.0
p.P244L	c.731C>T	p.R261Q	c.782G>A	1	51	44	47.5
p.P275R	c.824C>G	p.L348V	c.1042C>G	1	?	35	17.5
p.P275S	c.823C>T	p.R408W	c.1222C>T	1	?	2	1.0
p.P281L	c.842C>T	p.D338Y	c.1012G>T	1	2	?	1.0
p.P281L	c.842C>T	p.A300S	c.898G>T	1	2	31	16.5
p.R158Q	c.473G>A	p.A300S	c.898G>T	1	10	31	20.5
p.R158Q	c.473G>A	p.A403V	c.1208C>T	1	10	66	38.0
p.R158Q	c.473G>A	p.V190A	c.569T>C	1	10	110	60.0
p.R176L	c.527G>T	p.Q304Q	c.912G>A	1	42	?	21.0
p.R241H	c.722G>A	p.V388M	c.1162G>A	1	23	28	25.5
p.R241H	c.722G>A	p.R261*	c.781C>T	1	23	0	11.5
p.R243Q	c.728G>A	p.A403V	c.1208C>T	1	13	66	39.5
p.R243*	c.727C>T	p.A403V	c.1208C>T	1	0	66	33.0

Allele 1	Nucleotide aberration 1	Allele 2	Nucleotide aberration 2	No of patients	Allele 1 Activity, ^a %	Allele 2 Activity, ^a %	Total Activity, ^b %
(Continued from previous page)							
p.R252W	c.754C>T	p.E390G	c.1169A>G	1	0	62	31.0
p.R252W	c.754C>T	p.A403V	c.1208C>T	1	0	66	33.0
p.R261Q	c.782G>A	p.Y387H	c.1159 T>C	1	44	?	22.0
p.R261Q	c.782G>A	p.Y343C	c.1028A>G	1	44	?	22.0
p.R261Q	c.782G>A	p.P211T	c.631C>A	1	44	72	58.0
p.R408W	c.1222C>T	p.Y414C	c.1241A>G	1	2	57	29.5
p.S349P	c.1045T>C	p.E390G	c.1169A>G	1	1	62	31.5
p.S349P	c.1045T>C	p.V388M	c.1162G>A	1	1	28	14.5
p.T238A	c.712A>G	p.P281L	c.842C>T	1	?	2	1.0
p.V190A	c.569T>C	p.S231F	c.692C>T	1	110	?	55.0
p.V388M	c.1162G>A	p.V388M	c.1162G>A	1	28	28	28.0
p.V388M	c.1162G>A	p.E390G	c.1169A>G	1	28	62	45.0
p.W187*	c.561G>A	p.T193I	c.578C>T	1	0	?	0.0
p.Y204*	c.612T>G	p.P211T	c.631C>A	1	0	72	36.0
p.Y277D	c.829T>G	p.P314H	c.941C>A	1	0	?	0.0

?, not known.

^a Data from the BIOPKU database (www.biopku.org).

^b (Allele 1 Activity + Allele 2 Activity)/2; percentage of the wild-type enzyme.

**PKU:
BEHAVIOR, NEUROTRANSMITTERS
AND BH4**

PART

2

PKU: HIGH PLASMA PHENYLALANINE CONCENTRATIONS ARE ASSOCIATED WITH INCREASED PREVALENCE OF MOOD SWINGS

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Abstract

Introduction: In phenylketonuria, knowledge about the relation between behavior and plasma phenylalanine is scarce. The aim of this study was to determine whether high phenylalanine is associated with disturbed behavior noticed by the patient and or close environment (parents or partners).

Methods: 48 early treated PKU patients (median age 8.5, range 0–35 years) participated (median phenylalanine concentration in total sample 277 (range 89–1171) $\mu\text{mol/l}$; and in patients <12 years 238 (range 89–521) $\mu\text{mol/l}$). After sending blood samples, patients or close environment were interviewed with a standardized questionnaire whether they noticed hyperactivity, annoying behavior, mood swings and introvert or extravert behavior. The interviewer as well as the respondents were blinded with regard to the phenylalanine concentration.

Results: Patients reported less deviant behavior compared to close environment. Mood swings were positively associated with phenylalanine concentrations in the total group ($P=0.039$) and patients <12 years ($P=0.042$). The relationships between temporary high phenylalanine concentrations and hyperactivity, annoying behavior, introvert and extravert behavior were not statistically significant.

Conclusion: there is a positive association between phenylalanine concentrations and mood swings.

Introduction

Phenylketonuria (PKU) is an inborn error of metabolism resulting in high levels of phenylalanine (Phe) in blood and brain, which lead to irreversible and reversible damage to the brain. Untreated, this disease causes severe mental retardation, epilepsy, and behavioral problems.¹ Early and continuous treatment has reduced the loss of intelligence enormously. Still, some difference in IQ remains in comparison to the normal population.²⁻⁵ Notwithstanding mild mean deviation in IQ, PKU patients have more school achievement problems.^{6,7} Possibly, these problems in school performance could be attributed to deficits in executive function.⁸ Apart from subtle intellectual deficits, behavior problems have been reported frequently in early treated PKU patients. Behavior reported is to be more distractible, impulsive, hyperactive, fluctuating in their work performance, less conscientious and self-reliant, more anxious, unhappy, solitary, and unresponsive.⁹⁻¹¹ Disturbed behavior seems to be more prevalent in those with higher Phe concentrations,⁹ but research data are scarce. In daily practice patients with PKU and their parents regularly report that high Phe concentrations are affecting behavior. That raises the question if Phe concentrations change behavior. Therefore, we conducted a questionnaire to determine patients' behavior and compared them with the measured Phe concentration. The hypothesis was that high Phe concentrations are related to more disturbed behavior.

Methods

Patients

The medical ethical committee of the University Medical Centre of Groningen (UMCG) concluded that this study did not need judgment by them because the study consisted only of short interviews and use of blood Phe concentrations of samples taken by routine. From all 92 treated patients with PKU treated in the UMCG, those who were early (<21 days) diagnosed and continuously treated (N=81), were sent a written invitation to participate in the study. When they answered positively, informed consent was asked during the later contact by telephone. Eight patients did not participate. Reasons not to participate were language problems (3), pregnancy (3), "always hyperactive and always low Phe concentrations" (1) and without argumentation (1). Of the 73 patients who did participate nine patients could not be reached by telephone frequently enough during the study period and 14 patients did not send sufficient numbers of blood samples during the study period. Data collected during special circumstances, e.g. fever, a cold, birthday party were excluded from analysis. Therefore, for two additional patients we did not obtain the minimum criterion of participation of three questionnaires. In total, data from 48 patients were collected during November 2007 to November 2008.

Procedure

After sending blood samples for measurement of blood Phe concentrations in blood spot, patients, parents or partners were contacted by telephone. Participants were interviewed with a standardized questionnaire. Both participants (patients, parents or partners), and the interviewer did not know the Phe concentration at the time of the questionnaire. The maximum allowed time between taking the blood sample and the questionnaire was set at 5 days. The behavior questionnaire consisted of five questions: 1. Are you more hyperactive than usual? 2. Are you more annoying, aggressive or less willing to listen to other people? 3. Do you experience more mood swings? 4. Is your behavior more introvert? (more reserved, less outgoing) 5. Is your behavior more extravert (talk a lot, draw attention)? All questions could be answered by no, a little, yes or a lot. Participants were asked to recall the behavior at the time of the blood draw and to compare this behavior to the usual behavior of the patient. Blood phenylalanine concentrations from dried blood specimens were measured by the AccQ Tag method using high-performance liquid chromatography (Waters BV, Breda, The Netherlands).

Statistical analysis

Since all patients participated a different number of times, the median Phe concentration was calculated from the individual medians. Due to the scarcity of the answers 'a little' and 'a lot', the answers 'no' and 'a little', and 'yes' and 'a lot' were combined. The behavior questions versus the Phe concentrations (as natural logarithm) were analyzed with a linear mixed effects model, a model that takes into account the correlation between observations within the same patient. In this analysis maximum likelihood estimation and the covariance structure compound symmetry was used. The natural logarithm of Phe was used to approach normal distribution. A p-value ≤ 0.05 was considered to be statistically significant. Statistics were performed with PASW statistics 18.0; SPSS, Inc., Chicago, IL, USA.

Results

Data on the median age and Phe concentrations of the participants are shown in Table 1. The youngest patient was four months old. The age of the other patients varied from 1.4 to 35.0 years of age. Below 12 years of age (N=31) median Phe concentrations were 238 $\mu\text{mol/l}$ (range 89–521 $\mu\text{mol/l}$). In patients 12 years and older Phe concentrations were 558 $\mu\text{mol/l}$ (range 221–1171 $\mu\text{mol/l}$). Regarding the questions of the survey, 84% of the answers were given by parents (for <12 years 100%, and ≥ 12 years 39%), so questionnaires of younger patients were more often answered by the patient's close environment than in older patients. In patients ≥ 12 years, 61% of the answers were self-reports and only 1% was given by a partner. All questionnaires of one patient were answered by the same person in 38 cases, in the ten

remaining cases this was done by two different persons. The mean period between blood sampling and the questionnaire was 2.8 days $\pm$ 0.8 (min 1–max 5 days).

Table 1

Clinical data of the studied PKU patients (N=48)

	Total N	Median	Min-Max
Gender			
- Female	26		
- Male	22		
Age (years)		8.5	0 - 35
Number of Phe measurements (and behavior questionnaires)			
- Total Group	515	8.0	3 - 37
- < 12 years	380	12.0	4 - 34
- $\geq$ 12 years	135	4.0	3 - 37
Phe-level (μ mol/l)			
- Total Group		277	89 - 1171
- < 12 years		238	89 - 521
- $\geq$ 12 years		558	221 - 1171
Participant			
- Parent	431 (83.7%)		
- Patient	83 (16.1%)		
- Partner	1 (0.2%)		

Behavior

The frequencies of deviant behavior (questions answered with yes or a lot) are shown in Table 2. Patients reported less deviant behavior compared to parents or partners (close environment). Of 432 questionnaires answered by the close environment, deviant behavior was reported 81 times (18.8%), whereas patients reported deviant behavior in seven of 83 questionnaires (8.4%). Deviant behavior was reported in 3 of 34 questionnaires in the 4 months old patient. Question three (Do you experience more mood swings?) was significantly associated with the natural logarithm of Phe in the total group according to the linear mixed effects model (Estimate 0.28 $P=0.039$, age 0.05 $P=0.000$ and intercept 5.52) and in patients <12 years (Estimate 0.31 $P=0.042$, intercept 5.74).

Discussion

This paper adds to the scarce information on the relationship between Phe and behavior, given by papers of Smith⁹ and Ten Hoedt.¹² We found that some behavioral aspects, especially mood swings, are related to Phe concentrations in PKU. Since it was not possible to reach participants by telephone after every blood sample, numbers of participation do not reflect the actual adherence of the patients. The relatively small number of participants (N=48) could be explained by the relatively short study period of one year in which we required a minimum of three

participations, the delay in the response that patients were willing to participate and not being able to reach often enough. The questionnaire was designed with four possible answers. In reality the answers 'a little' and 'a lot' were hardly used. Therefore, for analysis, answers were combined.

Table 2

Numbers of deviant behavior (yes & a lot)

	whole group (515)	< 12 years (380)	≥ 12 years (135)	Environment (432)	Patient (83)
1. Are you more hyperactive then usual?	42	40	2	41	1
2. Are you more annoying, aggressive or less willing to listen to other people?	30	30	0	30	0
3. Do you experience more mood swings?	33	30	3	30	3
4. Is your behavior more introvert?	16	14	2	14	2
5. Is your behavior more extravert?	41	40	1	40	1

Behavior

This study showed that more mood swings are seen in combination with high Phe concentrations. In our study the participating patients reported less disturbing behavior in comparison to the patients' close environment (parents, partners). These results might indicate that the patients' close environment is better able to notice behavioral changes when blood Phe concentrations are high. Another possible explanation could be that patients are less willing to admit disturbing behavior, which results in underreporting. Furthermore it should be taken into account that age could also be a confounder. Unfortunately, data on patients ≥ 12 years could not be analyzed separately since this group was too small. Most data are of children of 2.7 to 12.3 years of age. The data of one child, who started the study at the age of 4 months, were also included. The parents of this child reported deviant behavior a number of times, showing that the parents were able to observe differences in behavior.

The presence of mood swings during high Phe concentrations has not been described before. Mood swings can be considered as a derivative of irritability, which has been described mainly in untreated PKU.¹³

Annoying or aggressive behavior or being less willing to listen has not been reported more in PKU patients.⁹ Also in this study there was no association with high Phe concentrations found in patients < 12 years. In patients ≥ 12 years annoying behavior was never reported. In contrast to

Stemerding et al., we did not find an association between introvert behavior and Phe concentration.¹⁴ Stemerding et al. showed a relationship between introvert behavior and high Phe concentrations, but only in the first 2 years of life.¹⁴

The present study also showed no evidence that high Phe concentrations lead to hyperactive behavior. This finding did not correlate with most parents' ideas, expecting of a relation between high Phe concentration and hyperactive behavior. Literature with regard to ADHD and PKU showed the same absence of hyperactivity. A study by Antshel et al. showed mainly inattentive symptoms associated with ADD.¹⁵ In this study we did not focus on inattentive symptoms.

Recently, ten Hoedt et al. also showed that elevated Phe levels are associated with a negative effect on the mood of patients in a double-blind cross-over trial.¹² In the study of ten Hoedt et al., less favorable mood was reported by the patient as well as a friend or relative. It is possible that patients in this trial were willing to admit deviant behavior despite desirable social behavior, because the high Phe concentrations were raised artificially.

A strong point in this research is the blinding of the Phe concentrations for the respondents as well as the interviewer, but there are some possible deficits in this study. The results are based on a questionnaire, giving a risk of over- or under reporting of the behavioral issues. However, by asking these questions by routine, the risk of giving socially accepted responses by patients or their close environment is decreased, so that the answers are considered to reflect the actual opinion of the respondents. Another inevitable problem of the study was the time gap between blood sampling and the telephone call. In most cases this time gap was 3 days at maximum, data on a period longer than 5 days were excluded. This time gap between blood sampling and questioning may have influenced the answers and in consequence may have had an effect on the reliability of the data. In this study design patients were contacted after the arrival of their blood sample at the laboratory.

Since both the interviewer and the respondents were blinded with regard to the Phe concentration these two forms of bias are likely to lead to non differential misclassification. Non differential misclassification will lead to a bias to the null value of effect estimates. Therefore, it is likely that we have underestimated the strength of the relationship between Phe and mood swings. To exclude the possibility that the data on behavior do refer to the day of calling rather than the day of blood sampling, future questionnaires could try to contact at the day of blood sampling.

In conclusion, we found a positive association between Phe concentrations and mood swings. We did not find more annoying behavior, introvert and extravert behavior, and hyperactive behavior with high Phe concentrations. These observations partly confirm our hypothesis that high Phe concentrations are related to more disturbed behavior, and provide us with information to help guide patients and parents in the most optimal way.

Acknowledgments

The authors would like to thank P. Modderman for the measurements of phenylalanine and providing blinded participant lists. This work was in part financially supported by Nutricia The Netherlands. The authors confirm independence from the sponsor; the content of the article has not been influenced by the sponsor. Dr. FJ van Spronsen is a member of scientific advisory boards for both Merck Serono and Nutricia.

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BH4 TREATMENT IN BH4-RESPONSIVE PKU PATIENTS: PRELIMINARY DATA ON BLOOD PROLACTIN CONCENTRATIONS SUGGEST INCREASED CEREBRAL DOPAMINE CONCENTRATIONS

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Abstract

In phenylketonuria (PKU), cerebral neurotransmitter deficiencies have been suggested to contribute to brain dysfunction. Present treatment aims to reduce blood phenylalanine concentrations by a phenylalanine-restricted diet, while in some patients blood phenylalanine concentrations also respond to cofactor treatment with tetrahydrobiopterin (BH4). Recently, a repurposing approach of BH4 was suggested to increase cerebral neurotransmitter synthesis.

To investigate whether BH4 may improve cerebral dopamine concentrations in PKU patients beyond its effect through lowering blood phenylalanine concentrations, we investigated blood prolactin concentrations — as a parameter of brain dopamine availability. We retrospectively compared blood prolactin in relation to blood phenylalanine concentrations of nine (male) BH4-responsive PKU patients, when being treated without and with BH4.

Blood prolactin concentrations positively correlated to blood phenylalanine concentrations ($p = 0.002$), being significantly lower with than without BH4 treatment ($p = 0.047$). In addition, even in this small number of male patients, blood prolactin concentrations tended to be lower at increasing BH4 dose ($p = 0.054$), while taking blood phenylalanine concentrations into account ($p = 0.002$). In individual BH4-responsive patients, median blood prolactin concentrations were significantly lower while using BH4 than before using BH4 treatment ($p = 0.024$), whereas median blood phenylalanine concentrations tended to be lower, but this did not reach statistical significance ($p = 0.107$).

Therefore, these data show that high blood phenylalanine in BH4-responsive PKU male patients seems to be associated with increased blood prolactin concentrations, suggesting reduced cerebral dopamine availability. Moreover, these data suggest that BH4 treatment in itself could decrease blood prolactin concentrations in a dose-responsive way, independent of blood phenylalanine concentrations. We conclude that these preliminary data indicate that BH4 treatment may improve cerebral dopamine concentrations in PKU patients beyond its effect through lowering blood phenylalanine concentrations, possibly in a dose-dependent manner, but further research would be warranted.

Introduction

In Phenylketonuria (PKU, OMIM 261600), deficiency of the hepatic enzyme phenylalanine hydroxylase (PAH) impairs the conversion of phenylalanine (Phe) into tyrosine (Tyr), resulting in strongly increased blood Phe concentrations and normal to slightly reduced blood Tyr concentrations. Left untreated, especially high blood Phe concentrations have been associated with classical PKU symptomatology: severe mental retardation, seizures, and depressive and anxiety disorders. Neonatal screening and immediate institution of a Phe-restricted diet aim to reduce the blood Phe concentrations as early as possible. Moreover, in some PKU patients, blood Phe concentrations respond to treatment with tetrahydrobiopterin (BH4), primarily by its activities as cofactor and natural chaperone for PAH.^{1,2} These current treatment strategies can prevent severe mental retardation, but outcome remains suboptimal. Even early diagnosed and continuously treated PKU patients show impaired neuropsychological functioning and are prone to depressive and anxiety problems.³

These neuropsychological impairments may at least in part be related to a cerebral deficiency of dopamine and serotonin.^{4,5} This consideration is in line with previous data in living and deceased PKU patients, showing reduced dopaminergic and serotonergic metabolites in cerebrospinal fluid (CSF) as well as decreased catecholamine and serotonin concentrations in the brain.^{6,7} Moreover, increased blood prolactin concentrations have been observed at increasing blood Phe concentrations,⁸ indicating reduced brain concentrations of its natural inhibitor dopamine.⁹

BH4 is not only a cofactor for hepatic PAH, but is also involved in cerebral neurotransmission by its cofactor and/or chaperone activities for Tyr hydroxylase, tryptophan hydroxylase, and nitric oxide synthase,^{10,11} while BH4 is known to cross the blood–brain barrier (BBB).¹¹ Therefore, it is hypothesized that BH4 may improve cerebral functioning beyond its effect through lowering blood Phe concentrations in BH4-responsive PKU patients. This hypothesis is substantiated by fMRI investigations in PKU patients showing improved neural activation after 4 weeks of BH4 treatment (20 mg/kg) even when blood Phe concentrations had not decreased.¹² To investigate whether BH4 might increase cerebral dopamine concentrations beyond its effect through lowering blood phenylalanine concentrations, this study compared blood prolactin in relation to blood phenylalanine concentrations in BH4-responsive PKU patients, who were subsequently treated without and with BH4.

Methods

Patients

In total, data of 9 BH4-responsive PKU males were collected retrospectively of whom both values of blood prolactin concentrations without and with BH4 treatment could be retrieved. Female patients were excluded because of too much possible variation by confounders. BH4-

responsiveness was defined as $\geq 30\%$ decrease of blood Phe concentrations during the 48-hour loading test and/or having a genotype with at least one DNA mutation suggesting BH4 responsiveness. Patients received BH4 treatment at individually-tailored dose (2–24 mg/kg/day), based on blood Phe concentrations. All patients were treated in the University Medical Center Groningen. Blood sampling was performed at variable time points throughout the day. Samples collected between June 2008 and March 2014, in which both Phe and prolactin were measured from the same blood draw, were used for analysis. All patients gave written informed consent for analysis of their data.

Biochemical analyses

Blood prolactin concentrations were determined by electrochemiluminescence immunoassay (ECLIA) on the Roche Modular analytics E170 (Elecsys module) immunoassay analyzer (Roche Diagnostics GmbH, D-68298 Mannheim, Germany), with a reference value for males of <300 mU/l. The detection limit for this analysis is 1–10,000 mU/l, with a coefficient of variation of 3.4% and 3.2% at 127 mU/l and 928 mU/l, respectively.

Phe concentrations were quantified in deproteinized plasma samples by cation-exchange high-performance liquid chromatography followed by post-column ninhydrin derivatization, using norleucine as an internal standard, on a Biochrom 20 or 30 analyzer (Pharmacia Biotech, Cambridge, UK).

Statistical analyses

All data were tested for normal distribution by Shapiro–Wilk tests, and for homogeneity of variances by Levene's test. All tests were performed two-sided at a significance level of $\alpha = 0.05$. Effect sizes were reported as Cohen's D values (d).

Comparison of blood prolactin concentrations exceeding the reference value in samples without and with BH4 treatment was performed by Fisher's exact test. Longitudinal comparisons for individual PKU patients without and with BH4 treatment were performed by paired samples T tests in case of normal distribution. Otherwise, related samples Wilcoxon signed rank tests were used.

To assess whether blood prolactin concentrations correlated with blood Phe concentrations, while taking into account the correlation between observations within the same patient, a univariate linear mixed effects model with unstructured covariance type was used. In addition, to investigate a possible correlation between BH4 treatment and blood prolactin concentrations, while correcting for blood Phe concentrations, multivariate linear mixed effects models with unstructured covariance type were used: consisting of blood Phe, and either BH4 treatment (yes/no) or BH4 dose (in mg/kg/day) as independent variables.

Results

Patient and biochemical characteristics

Patient characteristics of BH4-responsive PKU patients who were subsequently treated without and with BH4 are shown in Table 1. Of one patient, DNA analysis has not revealed any PAH mutations thus far. BH4 deficiency has been excluded biochemically and with the use of DNA analysis, while clinically, this patient does not show any signs suggestive of BH4 deficiency. Further investigations are currently being performed.

In total, 45 measurements were obtained from 9 patients. Of these, 16 samples (1–4 per patient) had been taken while patients did not receive BH4 treatment, and 29 samples (1–5 per patient) while patients received BH4 treatment at individually-tailored dose. Blood prolactin concentrations exceeded the reference value of 300 mU/L in 25% of measurements when patients did not receive BH4 treatment and in 10% of samples when patients received BH4 treatment ($p=0.235$).

Table 1

Patient characteristics

Patient	Genotype	Age	
		Before BH4	On BH4
1	p.Y414C/c.842 + 5G > A	28 y, 4 m 28 y, 2 m–28 y, 6 m	30 y, 7 m 29 y, 5 m–31 y, 7 m
2	^a	6 y, 7 m	10 y, 0 m 8 y, 6 m–10 y, 10 m
3	p.R243*/p.A403V	13 y, 6 m	17 y, 0 m
4	p.R243*/p.Y414C	30 y, 3 m	32 y, 11 m 31 y, 4 m–34 y, 5 m
5	c.143 T > C/c.842C > T	8 y, 2 m	10 y, 6 m 9 y, 6 m–11 y, 6 m
6	p.A300S/c.1066-11G > A	9 y, 0 m 8 y, 8 m–9 y, 10 m	11 y, 11 m 10 y, 10 m–13 y, 1 m
7	p.Y414C/c.1315 + 1G > A	15 y, 6 m	17 y, 8 m 16 y, 8 m–18 y, 9 m
8	p.V190A/p.R243*	10 y, 1 m 9 y, 6 m–10 y, 7 m	12 y, 9 m 11 y, 7 m–13 y, 7 m
9	c.842 + 1G > A/IVS65Thr	11 y, 6 m	12 y, 11 m 12 y, 1 m–13 y, 8 m

Data are expressed as median and range.

^a DNA analysis has not revealed any PAH mutations thus far. BH4 deficiency has been excluded both clinically, biochemically and by DNA analysis.

Longitudinal blood prolactin data in individual patients

Fig. 1 shows longitudinal data on blood prolactin and blood Phe concentrations in individual BH4-responsive PKU patients who were subsequently treated without and with BH4. Median blood prolactin concentrations while using BH4 were statistically significantly lower than before using BH4 treatment ($p = 0.024$, $t = 2.784$, $d = 0.928$).

Median blood Phe concentrations in PKU individuals while using BH4 tended to be lower, but were not statistically significantly different from concentrations prior to BH4 treatment ($p = 0.107$, $t = 1.814$, $d=0.605$).

Figure 1 Median blood prolactin (A) and phenylalanine (B) concentrations in BH4 responsive PKU males ($n = 9$) before BH4 treatment was used and while using BH4.

BH4 treatment and blood prolactin concentrations in relation to blood Phe concentrations

To investigate blood prolactin concentrations in response to BH4 treatment while taking into account blood Phe concentrations, mixed model analyses were performed to be able to correct for multiple measurements per patient. In the total of 45 blood measurements, taken while patients were either treated without or with BH4, blood prolactin concentrations positively correlated to blood Phe concentrations ($p=0.001$, $t=3.589$, $d=0.535$). In samples taken when patients were using BH4 treatment, blood prolactin concentrations were significantly lower than when not using BH4 treatment ($p = 0.047$, $t = -2.061$, $d = 0.307$), while taking into account blood Phe concentrations ($p = 0.002$, $t = 3.244$, $d = 0.484$). In addition, blood prolactin concentrations, while taking into account blood Phe concentrations ($p = 0.002$, $t = 3.329$, $d = 0.496$), showed a clear tendency to be lower at increasing BH4 dose, but this did not reach statistical significance ($p = 0.054$, $t = -1.991$, $d=0.297$).

Discussion

To investigate whether BH4 might improve brain dopamine concentrations beyond an effect through lowering blood Phe concentrations, this study compared blood prolactin in relation to blood Phe concentrations in BH4-responsive PKU males who were subsequently treated without and with BH4. Three main findings could be distinguished. Firstly, blood prolactin concentrations positively correlated to blood Phe concentrations. Secondly, blood prolactin concentrations were significantly lower when using BH4 than when not using BH4 treatment, while correcting for blood Phe concentrations. Thirdly, blood prolactin concentrations tended to be lower at

increasing BH4 dose. Before discussing these results in more detail, we will first address some methodological issues.

First, in females, time within the menstrual cycle, pregnancy, breastfeeding, and contraceptive medication are well-known to strongly influence blood prolactin concentrations. In this study, information on time within the menstrual cycle in PKU females was not obtained. So, although we knew more or less well the use of contraceptive medication and whether they were pregnant/giving breastfeeding, the most important confounding factor was unknown. Therefore, we restricted our data to PKU males only.

Second, blood Tyr concentrations and Phe:Tyr ratios are probably important parameters with respect to brain dopamine concentrations in PKU. It can be questioned, however, whether the use of Tyr concentrations as well as Phe:Tyr ratios taken at any time of the day are reliable enough to fully take these into account. Overnight fasting Tyr concentrations in PKU patients are known to be low,¹³ whereas postprandial responses are rather unpredictable, as blood Tyr concentrations show large diurnal fluctuations¹⁴. Therefore, we did just not assess blood Tyr concentrations and Phe:Tyr ratios.

Blood prolactin concentrations in BH4-responsive PKU patients were shown to positively correlate to blood Phe concentrations. Pituitary prolactin release is regulated by a complex connection to the central nervous system that predominantly involves the inhibitory action of hypothalamic dopamine.¹⁵ As pituitary prolactin synthesis is naturally under negative feedback control of brain dopamine, and the produced prolactin is transferred directly to the systemic circulation, blood prolactin concentrations have been found to reflect brain dopamine concentrations.⁹ Due to this correlation, blood prolactin has already been shown to be a peripheral biomarker of brain dopamine, used for monitoring patients with various neurological and oncological diseases. Among such neurological diseases are dihydropteridine reductase deficiency and 6-pyruvoyltetrahydropterin synthase deficiency. These are inborn errors of metabolism affecting the hydroxylating system of the aromatic amino acids Phe, Tyr, and tryptophan. In these disorders, it was found that, with peripheral prolactin quantification, the need of frequent CSF measurements could possibly be decreased.^{16,17} Normalizations of blood prolactin concentrations following introduction or adjustment of treatment were paralleled by clinical improvements.^{16,17} Our findings on blood prolactin concentrations in PKU patients without BH4 treatment are also in good agreement with the study by Schulpis et al. showing increased blood prolactin concentrations in PKU patients on a 'loose diet' (blood Phe 770 ± 450 $\mu\text{mol/L}$) compared to PKU patients on a strict diet (blood Phe 90 ± 39 $\mu\text{mol/L}$).⁸ Surprisingly, such correlation between blood prolactin and Phe concentrations was not observed in PKU patients as studied by Denecke et al., despite comparable group sizes and blood Phe concentrations.¹⁸

The present study is the first to report preliminary findings on a comparison between parameters relating to cerebral neurotransmitter concentrations in PKU patients without and with BH4 treatment. It should be noted that dopamine has been found to require ascorbic acid to exert its inhibiting effect on pituitary prolactin release. As BH4 tablets used in PKU treatment also include ascorbic acid to prevent the BH4 from oxidation allowing storage at room temperature, theoretically, the observed effect on blood prolactin in BH4-treated PKU patients might be due to the ascorbic acid rather than the BH4 itself. However, as a low concentration of ascorbic acid has been found to be sufficient for dopamine to maintain its inhibiting effect on pituitary prolactin release,¹⁹ and supplementing only ascorbic acid does not show such effect, it is questionable whether a small increase in ascorbic acid concentrations caused by BH4 treatment would have any effect on blood prolactin concentrations. Previous studies investigating the effects of BH4 treatment beyond lowering blood Phe concentrations in BH4-responsive PKU patients have not investigated the effects on measures relating to neurotransmitter metabolism in brain.^{20,21} Thus far, studies on the direct effects of BH4 on cerebral neurotransmitter synthesis have been restricted to animal research. In BH4 knock-out mice, chronic intraperitoneal BH4 administration (50 mg/kg) increased brain dopamine concentrations,^{22,23} while acute BH4 administration strongly increased brain serotonin concentrations²³. Moreover, oral BH4 administration (40 mg/kg) significantly increased brain serotonin but not dopamine concentrations in wild-type mice, whereas brain neurotransmitter concentrations in PKU mice remained unaltered. This latter finding could possibly be explained by either an inhibiting effect of strongly increased brain Phe concentrations which was too strong to be overcome by the BH4 dose used, or by insufficient brain availability of the precursors Tyr and tryptophan (Anjema et al., manuscript in preparation).

BH4 is known to cross the BBB and increase cerebral BH4 availability in mice in a dose dependent manner.¹¹ Further evidence that BH4 also in humans crosses the BBB is provided by increased biopterin concentrations in CSF following BH4 administration of ≥ 20 mg/kg.²⁴⁻²⁶ The present study showed a trend towards a correlation between blood prolactin concentrations and BH4 dose, when correcting for blood Phe concentrations. This suggests that the possible effect of BH4 on brain dopamine concentrations may be dose dependent and does not seem to relate to the responsiveness of the PAH enzyme to BH4, which differs for each individual PKU patient.

At present, the aim of BH4 treatment is to reduce blood Phe concentrations in BH4-responsive PKU patients. The unreported observations of various centers that some PKU patients experienced improved psychosocial functioning on BH4 treatment without a clear reduction of blood Phe concentrations, have led to the belief that BH4 may be advantageous for BH4-unresponsive PKU patients as well. Concerns to this idea, however, have been raised, stressing the possibility of a very expensive placebo effect, as real proof for such direct cerebral effect of BH4 has thus far been lacking.²⁷ In support of the previous unreported observations in PKU

patients, the present study suggests that high-dose BH4 may indeed improve brain function at least in some PKU patients beyond its effect on blood Phe concentrations. To such aim, patients may require a different dose than is used for its current purpose. Future prospective studies in a larger population should prove that high-dose BH4 could directly improve brain functioning in PKU. If so, high-dose BH4 treatment might be applied in all PKU patients rather than being restricted to the so-called BH4-responsive PKU patients.

In addition, it can be questioned whether a similar relationship exists between BH4 and cerebral serotonin concentrations in PKU patients. Platelet serotonin concentrations have often, though probably erroneously, been considered a parameter of cerebral serotonin synthesis. Other parameters, such as urinary melatonin metabolite concentrations, may prove to be a more adequate peripheral biomarker reflecting cerebral serotonin synthesis, which would allow for non-invasive assessment of cerebral serotonin status in PKU patients.²⁸ Although pineal melatonin synthesis is under the control of tryptophan hydroxylase type 1 instead of tryptophan hydroxylase type 2 as is found in the brain, probably, urinary melatonin metabolite concentrations are still a good proxy. Parameters of cerebral serotonin synthesis in relation to BH4 and tryptophan levels are therefore worth investigating.

Conclusion

In conclusion, this study suggests that BH4 treatment may increase cerebral dopamine concentrations at least in some PKU patients, beyond its effect through lowering blood Phe concentrations. Such a possible effect may be dose-dependent. Therefore, it can be hypothesized that high-dose BH4 treatment might improve neuropsychological functioning in both BH4-responsive and BH4-unresponsive PKU patients by improving cerebral neurotransmitter concentrations. The present study, however, was restricted to BH4-responsive male PKU patients, so further research is definitely warranted. Further research in the PKU mouse model should investigate the effects of different BH4 doses not only on cerebral dopamine but also on cerebral serotonin synthesis at different levels of Phe, Tyr, and tryptophan intake. Moreover, future clinical research in a larger PKU patient population should indicate whether BH4 treatment could improve neuropsychological outcome in both BH4-responsive and BH4-unresponsive PKU patients. Such a repurposing approach of BH4 in PKU might not only extend the PKU population benefitting from BH4 treatment, but may also require a different regimen than is needed for its current purpose.

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EFFECTS OF TETRAHYDROBIOPTERIN ON BRAIN NEUROTRANSMITTER CONCENTRATIONS IN C57BL/6 PKU MICE

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Abstract

Background: Tetrahydrobiopterin (BH4) is the co-factor for tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH-2), the rate-limiting enzymes for cerebral dopamine and serotonin synthesis. In phenylketonuria (PKU), brain concentrations of both dopamine and serotonin are decreased. BH4 treatment might increase neurotransmitter synthesis and cerebral function in PKU patients.

Methods: Wild type (WT) and *Pah^{enu2/2}* C57Bl/6 PKU mice were treated with 40 mg/kg/day BH4 or placebo by intraoral pipetting. Neurotransmitter and amino acid concentrations were measured in brain homogenates, while amino acid concentrations were also measured in plasma.

Results: Brain dopamine and serotonin concentrations were reduced in PKU mice compared to WT mice, both placebo-treated. Brain neurotransmitter concentrations of BH4-treated PKU mice were not significantly different from those of placebo-treated PKU mice. However, in WT mice, brain serotonin concentrations were significantly higher in the BH4-treated group (BH4: median 4.50 (IQR 4.36-4.60) versus placebo 3.88 (3.79-4.42) nmol/g wet weight, $P=0.021$). Brain dopamine concentrations were not statistically different between both WT groups.

Conclusion: Under the current conditions BH4 treatment does not affect brain neurotransmitter concentrations in PKU mice. However, in WT mice BH4 increased brain concentrations of serotonin, which could indicate increased cerebral TPH-2 activity. Possibly, too high concentrations of brain phenylalanine or too low concentrations of brain tryptophan inhibit proper function of this enzyme and therefore abolish the possible cerebral effect of BH4 in PKU.

Introduction

In phenylketonuria (PKU, OMIM 261600), deficiency of the hepatic phenylalanine hydroxylase (PAH) enzyme results in insufficient conversion of phenylalanine (Phe) to tyrosine (Tyr). Untreated, this deficiency results in markedly elevated plasma Phe concentrations, which are associated with severe mental retardation. Timely diagnosis and initiation of dietary Phe restriction lead to cognitive outcome within normal limits.^{1,2} Still, early and continuously treated patients show mild neuropsychological deficits³⁻⁵ and have an increased risk of developing anxiety and depressive disorders.⁶⁻⁸

Reduced cerebral concentrations of dopamine (DA), norepinephrine (NE), and serotonin (5-HT) are believed to contribute to the impairments of brain function in PKU.^{9,10} One study on neurotransmitters in deceased and untreated PKU patients has shown that concentrations of DA, NE and 5-HT were reduced by 60-70% in the caudate nuclei.¹¹ CSF obtained from both late-treated and early and continuously treated PKU patients shows decreased concentrations of the neurotransmitter metabolites homovanillic acid and 5-hydroxyindoleacetic acid (5-HIAA) compared to controls,¹²⁻¹⁴ in association with impaired neuropsychological performance.^{15,16} Phe restriction increases the CSF concentrations of these metabolites.^{11-13,17} The rate-limiting step of cerebral synthesis of DA, NE and epinephrine is the hydroxylation of Tyr to L-3,4-dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase (TH), whereas in 5-HT synthesis the rate-limiting step is the hydroxylation of tryptophan (Trp) to 5-hydroxytryptophan by tryptophan hydroxylase (TPH-2).

Recently, the PAH co-substrate and co-factor tetrahydrobiopterin (BH4) was found to increase PAH activity in a subset of PKU patients, who are described as BH4- responsive patients.¹⁸⁻²¹ Some patients being tested for BH4-responsiveness during several weeks reported feeling more relaxed and to have a better ability to concentrate during BH4 treatment, while their blood Phe concentrations did not change (unpublished observations). Possibly, these improvements relate to the fact that in addition to its effect on PAH in the liver, BH4 also serves as a co-factor for TH and TPH-2 in the brain. Although evidence in humans is scarce,²²⁻²⁴ studies in laboratory animals show that BH4 crosses the blood-brain barrier when dosed at 20 mg/kg/day or higher.²⁵⁻³⁰

Therefore, we hypothesized that BH4 treatment enhances cerebral TH and TPH-2 activity, thereby increasing brain neurotransmitter concentrations, independent of its effect on blood Phe concentrations. To investigate this hypothesis, we studied neurotransmitter and amino acid concentrations in the C57Bl/6 *Pah^{enu2}* PKU mouse model treated with 40 mg/kg/day BH4.

Materials and Methods

Animals

The reported experiment was approved by the ethics committee for the use of experimental animals of the University of Groningen, in accordance with national and international laws and standards for animal protection. A breeding colony was initiated using heterozygous founders, generously provided by Prof. B. Thöny (Department of Clinical Chemistry and Biochemistry, University Children's Hospital, Zurich, Switzerland). Wild-type (WT, C57Bl/6) and PKU (C57Bl/6 *Pah^{enu2/2}*) mice of both genders were used for the experiment. Animals were weaned at the age of four weeks and individually housed two weeks before the beginning of the experiment under a 12 hour light/dark cycle at 21 ± 1 °C. Animals had *ad libitum* access to water and normal chow (RMH-B food pellets, Arie Block BV, Woerden, The Netherlands).

Genotyping

Genetic characterization was performed on DNA extracted from tail tissue using quantitative PCR analysis. Primers were based on GenBank and designed to replicate an amplicon on exon 7 of the PAH gene according to the guidelines of Eurogentec. We used double-dye probes tagged with a non-fluorescent Black-Hole Quencher1TM (Eurogentec) that transforms the absorbed excitation energy of the reporter into heat (absorbance maximum at 435 nm). WT and *Pah-enu2* probes were tagged with a FAM fluorophore and a Yakima Yellow fluorophore (Epoch Biosciences), respectively. Both probes contained locked nucleic acids according to the guidelines of Exiqon to enhance mismatch discrimination. Reactions were carried out on an ABI Prism 7500 sequence detection system, using Applied Biosystems' standard thermal cycling parameters (10 minutes 95 °C followed by 40 cycles of 15 minutes at 95 °C and 1 minute at 60 °C).

Study design

Mice were divided into four groups: Placebo WT, BH4 treated WT, Placebo PKU and BH4 treated PKU, with $n=8$ animals per group. (6R)-5,6,7,8-Tetrahydro-L-biopterin dihydrochloride was purchased at Schircks Laboratories (Jona, Switzerland). BH4 was administered at a dose of 40 mg/kg/day in two doses by intra-oral pipetting two hours after the beginning of the light phase and two hours after the beginning of the dark phase (under red light conditions). Both the BH4 solution and the placebo solution contained 40 mg/kg ascorbic acid and 20 mg/kg *N*-acetyl-L-cysteine. Treatment started at age 2.5-4.5 months. At day 31 of the study, approximately three hours after the last morning dose, the mice were anesthetized with isoflurane. Next, blood was obtained by cardiac puncture, collected in heparinized tubes and centrifuged shortly after collection (12.000 rpm x 10 min). Mice were sacrificed by cervical dislocation, brains were removed and snap-frozen by freeze-clamping in liquid nitrogen. Plasma and brain samples were stored at -80 °C until further processing.

Biochemical analyses

Mouse brains were crushed in liquid nitrogen and divided into aliquots. Frozen brain aliquots for amino acid measurements were processed to 20% (weight:volume, mg:µl) homogenates in phosphate-buffered saline (pH 7.4). For neurotransmitter analyses, brain aliquots were processed to 20% (weight:volume) homogenates in acetic acid (0.08 M). Brain homogenates were sonified on ice at 11-12 W for approximately 30 s per sample and centrifuged at 12,800 rpm for 10 min at 4 °C. The supernatant was used for further analyses.

For amino acid concentration measurements, brain homogenate supernatants and plasma samples were processed using the same method. Norleucine was used as an internal standard (1:1, volume:volume), with 60 mg/ml sulfosalicylic acid to precipitate the dissolved proteins. Samples were vortexed and centrifuged at 20,800 rcf for 4 min. The supernatant was pipetted into capsules and measured by high-performance liquid chromatography (HPLC) using a cation exchange resin followed by post-column ninhydrin derivatization on a Biochrom 30 apparatus (Pharmacia Biotech, Cambridge, UK).

For neurotransmitters, neurotransmitter metabolites and Trp concentration measurements, the supernatants of the 20% brain homogenates were further diluted to 2% homogenates in acetic acid (0.08 M). An antioxidative solution containing 400 mg/l ascorbic acid and 1,616 g/l ethylenediaminetetraacetic acid was prepared in demineralised water. For catecholamine concentrations measurements, 10 µl of each 2% homogenate was pipetted in a 96 wells plate with 40 µl anti oxidative solution. For indole concentration measurements (including the indole derivative Trp), 25 µl of each 2% homogenate was pipetted in a 96 wells plate with 25 µl antioxidative solution. Analysis was performed using isotope dilution mass spectrometry, essentially as described by Van de Merbel et al.³¹

Statistical analyses

Data with a normal distribution are presented as mean $\pm$ SD, whereas data with a skewed distribution are presented as medians with interquartile ranges (IQR). The Shapiro-Wilk test was used to test whether the variables in each experimental group were normally distributed. To test whether body weight changed during the experiment, the Wilcoxon signed rank test was used. To compare normally distributed independent data the two-sample t-test was used. With skewed independent data, the Mann-Whitney U test was used. Statistical analyses were performed using IBM Corp. SPSS Statistics for Windows, Version 20.0. Armonk, NY, USA. A two-tailed P-value < 0.05 was considered to be statistically significant.

Results

Baseline characteristics and general health

Gender and age at the start of the experiment were equally distributed among the treatment groups (Table 1). The Kruskal-Wallis test revealed that body weight at the beginning of the experiment differed significantly among treatment groups ($P=0.004$). Comparison by Mann-Whitney U test showed that PKU mice weighed significantly less than WT mice ($P=0.000$), while within mice of the same genotype no significant differences were found between the treatment groups (PKU $P=0.536$ and WT $P=0.798$). One mouse (PKU, placebo-treated) was excluded from the experiment prematurely, as the animal developed seizures during daily handling. In the remaining mice, treatment as well as the procedures were well tolerated and did not affect general health. In WT mice, median weight did not significantly change during the experiment. In PKU mice, median weight increased slightly (PKU Placebo 0.9 g $P=0.018$, PKU BH4 0.7 g $P=0.017$).

Table 1

Baseline characteristics of experimental groups.

	WT placebo n=8	WT BH4 n=8	PKU placebo n=7	PKU BH4 n=8
Gender, M:F	4:4	4:4	3:4	3:5
Age at start (months) ^a	3.6 (3.0 – 4.4)	4.1 (3.0 – 4.1)	3.1 (3.1 – 4.1)	3.5 (3.1 – 4.1)
Weight at start (g) ^a	22.0 (21.5–26.9)	24.1 (22.1–26.4)	19.6 (18.1–22.7)	19.6 (17.4–20.3)

^aValues are median and interquartile range

(M: male, F: female, WT: wild-type, PKU: phenylketonuria)

Plasma phenylalanine and tyrosine concentrations

Median (IQR) plasma Phe concentration in placebo-treated PKU mice was significantly higher than in WT mice (1459 (1392 – 1646) versus 66 (58 – 79) $\mu\text{mol/l}$, respectively, $P=0.000$). In both PKU and WT mice, plasma Phe concentrations did not differ between placebo-treated and BH4-treated mice ($P=0.241$ and $P=0.161$). Median plasma Tyr concentration was lower in placebo-treated PKU mice compared to placebo-treated WT mice (37 (35 – 38) versus 79 (64 – 106) $\mu\text{mol/l}$, $P=0.001$). Both in PKU and WT mice, no differences in plasma Tyr concentration were found between placebo-treated and BH4-treated groups ($P=0.613$ and $P=0.574$).

Brain phenylalanine, tyrosine and tryptophan concentrations

Brain homogenate concentrations of Phe, Tyr and Trp are shown in Figure 1. Brain Phe concentrations were significantly higher in placebo-treated PKU mice compared to placebo-treated WT mice ($P=0.000$). Brain concentrations of both Tyr and Trp were reduced in placebo-treated PKU mice compared to placebo-treated WT mice ($P=0.002$ and $P=0.002$). No differences in Phe, Tyr and Trp were found between the treatment groups in both PKU as WT mice.

Figure 1 Brain homogenate concentrations of (A) phenylalanine, (B) tyrosine and (C) tryptophan, in wild-type and ENU2/2 PKU mice, either placebo or BH4 treated. Bar graphs show means $\pm$ SD. The whiskers in the box plots represent the 5th and 95th percentiles.

Brain neurotransmitter and metabolite concentrations

Regarding DA and NE, brain homogenate concentrations were significantly lower in PKU mice compared to WT mice (Figure 2). This was also true for DA metabolite normetanephrine (PKU 0.10 ± 0.02 and WT 0.15 ± 0.06 nmol/g wet weight, $P=0.033$). The concentration of 3-methoxytyramine (another DA metabolite) did not significantly differ between PKU and WT (0.54 ± 0.08 and 0.62 ± 0.21 nmol/g wet weight, respectively, $P=0.352$). In both PKU and WT mice, no differences were found between placebo-treated and BH4-treated groups for DA (PKU $P=0.683$, WT $P=0.464$), NE (PKU $P=0.465$, WT $P=0.215$), normetanephrine (PKU $P=0.450$, WT $P=0.730$) and 3-methoxytyramine (PKU $P=0.540$, WT $P=0.927$).

Regarding brain homogenate concentrations of 5-HT and its metabolite 5-HIAA, both were found significantly lower in PKU mice compared to WT mice. In PKU mice, no differences were found between placebo and BH4 treated animals for 5-HT ($P=0.714$) and 5-HIAA ($P=0.961$). However, in WT mice the median (IQR) 5-HT concentration was significantly higher in the BH4 treated group versus placebo (4.51 (4.36 – 4.60) versus 3.88 (3.79 – 4.42) nmol/g wet weight, $P=0.002$). For 5-HIAA, this difference was not found ($P=0.489$).

Figure 2 Brain homogenate concentrations of (A) serotonin, (B) 5-HIAA, (C) dopamine and (D) norepinephrine in wild-type and PAH deficient mice, either placebo or BH4 treated. Bar graphs show means $\pm$ SD. The whiskers in the box plots represent the 5th and 95th percentiles

Discussion

The main finding of this study is that BH4-treated WT mice showed higher brain concentrations of 5-HT than their placebo-treated counterparts, and that this effect was not seen in PKU mice, contrary to our hypothesis. This finding is important as it shows that BH4 may indeed increase neurotransmitter synthesis (currently only shown for 5-HT), while additional interventions may be required to observe a similar effect in PKU.

Before discussing the results in more detail, we will address the methodological issues of the study. First, brain BH4 concentrations were not measured. In theory, BH4 could have sufficiently reached the brain of the WT mice, but not of the PKU mice. Although the mechanism of transport of BH4 across the blood-brain barrier is not known, BH4 transport does not seem to be influenced by blood Phe concentrations, as BH4 has been shown to reach the brain in BH4 deficient mice with hyperphenylalaninemia.²⁹ Second, it is not known if cerebral BH4 is influenced by high Phe concentrations, for example by oxidation.

The C57Bl/6 *Pah^{enu2}* mouse is homozygous for a null-mutation obtained by chemical mutagenesis. Our results show that blood Phe concentrations in C57Bl/6 *Pah^{enu2}* mice do not respond to BH4 administration as previously shown for BTBR *Pah^{enu2}* mice.^{32,33} Therefore, any

observed effects of BH4 on neurotransmitter concentrations in *Pah^{enu2}* mice are unrelated to changes of blood Phe concentrations. Thus, these animals provide an excellent model to study the brain-specific actions of BH4 treatment in PKU.

Regarding the results of the present study, two issues deserve attention, i.e. 1) the difference in response to BH4 treatment regarding brain 5-HT concentration in WT and PKU mice, and 2) the different responses of 5-HT and dopamine to BH4 in WT mice. With regard to the different response to BH4 in WT mice compared to PKU mice, there may be several explanations. First, BH4 may have reached the brain in insufficient amounts to exert cerebral effects in PKU mice, in contrast to WT mice. However, as reasoned above, differences in transport of BH4 across the blood-brain barrier in PKU mice and WT mice are unlikely. In addition, both the clinical side-effects and the reports of BH4-non-responsive PKU patients using BH4 during longer time periods to test their BH4 responsiveness suggest that BH4 does reach the brain in these patients.^{34,35} Second, elevated brain Phe concentrations are suggested to decrease the activity of both TH and especially TPH-2 by inhibition,³⁶ although discussion on this topic remains.³⁷ This possible inhibiting effect of high brain Phe concentrations on cerebral neurotransmitter synthesis may have been too strong for the current dose of BH4 to overcome under the conditions of our experiment. Third, elevated blood Phe concentrations decrease the transport of the neurotransmitter precursors Tyr and Trp across the blood-brain barrier,^{38,39} supported by the reduced brain concentrations of these precursors in our PKU mice. Therefore, reduced brain Tyr and Trp concentrations may have been limiting factors for the improvement of cerebral neurotransmitter synthesis on BH4 treatment in PKU mice. This is in line with studies of Joseph and Dyer⁴⁰ and Pascucci et al,⁴¹ which showed that brain neurotransmitter concentrations were not (fully) restored by only decreasing brain Phe concentrations. Therefore, future studies in PKU mice should examine the combined effects of increasing the cerebral availability of BH4 as well as the precursors of DA and 5-HT, possibly in combination with decreasing the Phe concentration in (blood and) brain.

The observation that BH4 increased brain 5-HT concentrations in WT mice, while brain DA concentrations were unaffected, may be explained by different effects of BH4 on the TH and TPH-2 enzymes. Kinetic analyses on these enzymes have shown that Phe acted as an inhibitor more strongly against TPH-2 than against TH.³⁶ Also, in urine of PKU patients, excretion of 5-HT is inhibited at lower Phe concentrations compared to dopamine.⁴² In BTBR *PAH^{enu2}* mice, reduced precursor cerebral availability seems to be more limiting to TH than TPH-2, whereas inhibition by high Phe concentrations is more distinct for TPH-2.^{41,43} In line with our current findings, previous studies in WT mice showed that BH4 did not influence brain DA concentrations. In contrast, BH4 did increase the amounts of TH protein and its activity.³⁰ Possibly, this finding can be attributed to feedback inhibition mechanisms by catecholamines^{44,45} and post-translational regulation of TH by phosphorylation by serine kinases.⁴⁶

It has been a matter of debate whether disturbed myelination or neurotransmitter abnormalities are the most important in the pathogenesis of brain dysfunction in PKU.^{9,10} Very recent studies showed that white matter integrity is important in PKU pathogenesis, even in early and continuously treated PKU patients.⁴⁷⁻⁴⁹ These studies support the theory that neuropsychological dysfunction in PKU is not only related to executive function and maybe even 'just' a matter of speed of neural transmission.⁵⁰⁻⁵² Those white matter abnormalities have previously been thought to be primarily due to high blood and brain phenylalanine concentrations, but recent studies showed that the white matter abnormalities might be explained by problems in neurotransmitter metabolism rather than high phenylalanine,^{40,53} suggesting that both processes are involved in cognitive dysfunction in PKU, and interact with one another. Therefore, neurotransmitter metabolism likely has a central role in the pathogenesis of PKU brain dysfunction one way or the other and improving this metabolism in PKU might be the primary treatment goal.

Conclusions

In conclusion, 40 mg/kg/day of orally administered BH4 seems to improve brain 5-HT concentrations in WT mice, but not in PKU mice. In both WT and PKU mice, BH4 treatment did not affect brain dopamine concentrations. Possibly, changes in the BH4 treatment regime and/or additional interventions are required to observe BH4-mediated treatment effects in PKU mice. Probably, too low cerebral concentrations of Trp and/or too high cerebral concentrations of Phe inhibit proper function of TPH-2 and therefore abolish the possible effect of BH4. Further studies are needed to investigate the cerebral effects of higher doses of BH4 treatment alone or the present dose in combination with other interventions.

Acknowledgements

This work was financially supported by the Dutch brain foundation (hersenchichting), grant number KS2011(1)-135. KA was financially supported by a MD/PhD grant from the Junior Scientific Master Class of the University of Groningen. BT received research funding from BioMarin. EAvdZ is a member of advisory boards of ARLA. FJvS received research funding from Alexion and Actelion, research funding and honoraria from Nutricia, SOBI, and Merck Serono, was a member of the Sapropterin Advisory Board (Merck Serono international), is a member of advisory boards for Biomarin, ARLA and Danone-Nutricia, and received speaking honoraria from Vitaflo international.

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GENERAL DISCUSSION AND FUTURE PERSPECTIVES

10

General discussion and future perspectives

In this thesis I aimed at revealing several aspects of phenylketonuria (PKU) treatment. Firstly, analyzing the tetrahydrobiopterin (BH4) loading test and thereby gaining knowledge in predicting BH4 responsiveness and secondly, analyzing clinically relevant matters of PKU patients, namely behavior and the effect of BH4 on neurotransmitters.

Prediction of BH4 responsiveness

In **Chapter 2**, the 48 hour BH4 loading test from 186 patients from almost all Dutch university medical centers was analyzed. This study showed that a substantial number (46%) of patients had a response to BH4 and that the 48 hour time point added 8% of potential responders compared to the 24 hour test. Interestingly, the number of potential responders was a lot higher than the 20% identified by Burton et al.¹ One of the differences in the protocol is the duration of the test (48 hours compared to one week), but another important difference is the dosage used; 10 mg/kg/day in the American study instead of the 20 mg/kg/day used in our study and in current European protocols. A study by Fiege et al,² also showed 46% potential responder in a 24 hours test with 20 mg/kg/day BH4. Therefore, we recommend 20 mg/kg/day of BH4 during BH4 testing.

The question whether potential BH4 responders are also true responders was addressed in **chapter 3**. It was shown that 87% of those patients who tested positive in the 48 hour BH4 loading test were also true responders, defined as: long-term $\geq 30\%$ reduction in mean Phe concentration and/or ≥ 4 g/day and/or $\geq 50\%$ increase of natural protein intake. Patients with a higher percentage Phe decrease during the 48 hour BH4 loading test were more often true responders. Additionally, late responders in the 48 hour BH4 loading test had a lower positive predictive value, but the majority does respond to long-term treatment (seven out of eleven patients were true responders). Surprisingly, our data showed that the late responders who were true responders had a comparable increase in tolerance for natural protein compared to the other responders. Therefore, late responders are worth detecting and should be allowed to test for true responsiveness. Probably, circumstances (e.g. altered intake or increased stress) resulting in higher Phe concentrations during the first 24 hours of the 48 hour BH4 loading test result in a late response (or in other words a false negative 24 hour test).

Chapter 3 also showed that genotyping can help predicting BH4 responsiveness and is at least comparably informative as the BH4 loading test. Our study confirmed other studies stating that patients carrying two null-mutations are no candidates for BH4 treatment.³⁻⁵ Our study also showed that having two so called BH4 responsive mutations makes the BH4 loading test unnecessary. Therefore, a BH4 loading test should be performed in any PKU patient who does

not carry two zero or two BH4 responsive mutations, acknowledging that the last group can directly start with a treatment trial with BH4.

In retrospect, two patients included in the database used in chapter 3 turned out to have compound heterozygous variants of the at that time not known DNJC12 gene,⁶ and therefore should be withdrawn from the study, resulting in a small decrease in BH4 efficacy. Recently, autosomal recessive deficiency of DNAJC12, which is thought to act as a co-chaperone for phenylalanine-hydroxylase (PAH), tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH-2), has been described as a cause for hyperphenylalaninemia. DNAJC12 deficiency results in a heterogeneous neurological phenotype and the few cases reported show a marked Phe decrease after a BH4 challenge.⁶⁻⁹ The discovery of this disease has changed the strategy of evaluation of newly diagnosed patients with a high Phe to include the determination of the DNAJC12 gene if pterins, DHPR activity and the PAH gene determination are normal.¹⁰

In 2017 the first European guidelines on PKU were published.^{11,12} In these guidelines a stricter definition of BH4 responsiveness is given compared to the definition used in chapter 3. The guideline definition states: 'establishing an increase in natural protein tolerance of $\geq 100\%$ with blood Phe concentrations remaining consistently within the target range' or by improved metabolic control '>75% of blood Phe levels remaining within target range without any decrease in natural protein intake associated with BH4 treatment', proven by a BH4 treatment trial. Therefore, in **Chapter 4**, we re-evaluated the usefulness of the 48 hour BH4 loading test using the new definition. As predicted the positive predictive value (PPV) of the 48 hour BH4 loading test using the European guidelines definition was a lot lower (50%) compared to the original study definition (PPV 87%). We noticed that patients with a higher baseline natural protein intake did not always meet the European guidelines definition of BH4 responsiveness, because less than a hundred percent increase in natural protein intake already meant a safe/recommended protein intake. Therefore, also a new 'amended' definition was introduced in this article: "an increase in natural protein tolerance $\geq 100\%$ or tolerating a safe natural protein intake". Using this amended definition, the PPV of the 48 hour BH4 loading test increased from 50% to 69%. As this is still suboptimal, the relation between BH4 responsiveness and genotype was also re-evaluated. The results show that genotype is mainly useful in excluding patients rather than demonstrate BH4 responsiveness.

As BH4 treatment is now also available for patients under four years of age, a crucial question is how to detect BH4 responsiveness in neonates. Aiming for the smallest delay in starting dietary treatment in newborns with PKU who are not BH4 responsive, the neonatal BH4 loading should be as short as possible. Historically, the neonatal BH4 loading test was designed to distinguish patients with a defect in BH4 metabolism compared to those with a defect in the PAH enzyme. Protocols used for that purpose lasted eight to 24 hours.

In **Chapter 5** the neonatal BH4 loading test was compared to the 48-hour BH4 loading test performed in (the same) patients after the age of four years and to long-term BH4 responsiveness. This showed that a positive neonatal BH4 loading test (8- or 24-hours) is predictive for long-term BH4 responsiveness. However, frequently, patients with less than 30% decrease in Phe concentration in the neonatal BH4 loading test showed potential BH4 responsiveness in the 48-hour BH4 loading test and also proved to be long-term responders. Therefore, the 8- or 24-hour neonatal BH4 loading test as performed in this study is not sensitive enough. This can be explained by various factors. First of all, the duration of the test, which in this neonatal test in most cases only lasted for 8 hours (normal duration at that time). Secondly, the higher Phe concentrations at the beginning of the neonatal BH4 loading test could result in altered PAH activity. This hypothesis is based on research that showed that the Phe-to-BH4 ratio is one of the factors determining the availability of active PAH enzyme and this can be higher or lower depending on the mutation.¹³ Third, BH4 from different companies was used in this study and some of the first tests were performed with 6R,S-BH4, which is less active than pure 6R-BH4, as 6R-BH4 is the diastereoisomer with the highest affinity with the PAH enzyme. Another study on the neonatal BH4 loading test showed a $\geq 30\%$ decrease in Phe concentration after 24 hours in 38% of the patients, and $\geq 20\%$ in 49% of the patients, with no additional long-term BH4 responsiveness results.¹⁴ Interestingly, this study also presented a new pharmacodynamics model taking into account a more complex kinetics effect that seems to be sensitive using 24-hour BH4 loading test data. However, no long-term BH4 responsiveness data are available to judge its (additional) value.

Chapter 6 addressed the question what the long-term effects of BH4 are on metabolic control and dietary tolerance in PKU patients. In this chapter, we worked together with Swiss colleagues Prof Blau and dr Keil who initiated this study among 16 centers in six European countries. A beneficial effect was shown on metabolic control and an increase in tolerance for dietary Phe intake. Another important part of this study was the inventory of adverse events in BH4 users. There were no severe adverse events reported and the adverse events that were mentioned disappeared with reduced therapeutic BH4 doses. This suggests that BH4 treatment is safe in patients over four years of age and can be used as a regular treatment option (data up to 12 years). This was also confirmed in a recent publication by Longo et al, which showed a 6% rate of tetrahydrobiopterin-related adverse events with 91% being non-serious in a population of 1189 patients.¹⁵ Drug related adverse events reported were gastrointestinal (diarrhea, unspecified gastrointestinal disorder); respiratory (dysphonia and rhinorrhea), and nervous system (amnesia, dizziness, memory impairment, headaches, migraines, poor quality sleep, and psychomotor hyperactivity). Ten serious adverse events were reported possibly-related to BH4 treatment, being: arrhythmia (N=1), abdominal discomfort and gastroesophageal reflux (N = 2), cholecystitis (N = 1), diabetes mellitus (N = 1), spontaneous abortions (N = 3), premature labor (N = 1) and

conversion disorder ($N = 1$). More data are needed to determine whether these adverse events are indeed drug related. One patient in our own population stopped BH4 during an extended BH4 loading test after 5 days because of a severe headache, after which the headache disappeared. The patient had been prescribed 20 mg/kg of BH4, but seems to have taken 40 mg/kg. Another two patients reported headache a few years after starting BH4 treatment. In both cases, this was shown not to be related to the BH4 therapy as the headache did not disappear after temporarily stopping BH4 treatment. Furthermore, one patient stopped BH4 treatment after some years because of pain symptoms in several joints which could be related to BH4 treatment as the complaints decreased after stopping the medication.

Behavior, neurotransmitters and BH4

As mentioned in chapter 1, the pathophysiology of PKU has not yet been unraveled. Several mechanisms have been proposed and probably a combination of factors contribute to the disease phenotype. A shortage/ imbalance of specific neurotransmitters is seen in untreated PKU patients¹⁶ and in early treated patients on a (relaxed) diet or who discontinued their diet.^{17,18} Also, in treated patients, neurocognition, social cognition, mood and behavior can be affected. This indicates that current treatment is still not optimal. As BH4 is not only the cofactor of the PAH enzyme, but also for TH and TPH-2,¹⁹ this compound could influence neurotransmitter levels irrespective of the effect on the plasma Phe. As neurotransmitters also affect mood and behavior this triggered our interest.

First, to ascertain the effects of Phe in relation to behavior, a questionnaire was applied in **Chapter 7**. In this study, a group of 48 PKU patients (not on BH4 therapy) was interviewed after sending in a blood sample for Phe analysis. A positive association between mood swings and Phe concentrations was found. Interestingly, the study of ten Hoedt et al showed that high plasma Phe concentrations have a negative effect on mood.²⁰ Apart from that, especially internalizing behavior is reported in PKU,²¹⁻²⁴ while poorer executive function and again internalizing problems were seen in more recent studies.^{25,26} A study by Stemerding et al showed more introversion in girls compared to boys with early treated PKU.²⁷ It was surprising that introvert behavior was not found in our present study. A possible explanation could be that internalizing problems are due to chronic exposure to high Phe concentrations. However, it has also been associated with the stress evoked by the burden of the diet itself.²⁸

In **Chapter 8** of this thesis an effort was made to study the effect of BH4 on cerebral dopamine concentration by measuring blood prolactin concentrations in patients. As pituitary prolactin synthesis is naturally under negative feedback control of brain dopamine, and the produced prolactin is transferred directly to the systemic circulation, blood prolactin concentrations have been found to reflect brain dopamine concentrations.²⁹ After correction for blood Phe concentrations, the results showed that blood prolactin concentrations were significantly lower in

patients using BH4 compared to the same patients not on BH4 treatment. The correction for blood Phe concentrations was applied because higher blood Phe concentrations are associated with increased blood prolactin concentrations in our and other reports.^{30,31} The study in chapter 8 also suggests that the association is stronger with higher doses of BH4. Animal studies and high-dose BH4 studies in patients are necessary to confirm this data and whether this results in changes in intracerebral dopamine concentrations and brain function.

To investigate the effect of BH4 directly on brain neurotransmitters, a study was performed in mice, presented in **Chapter 9**. Earlier publications have shown significant reduction of serotonin, dopamine and their metabolites in multiple brain areas of PKU mice compared to controls.³² To study the effect of BH4 on a brain level it is critical that effects are not to be attributed to confounding factors such as Phe concentrations. The *Pah^{enu2}* PKU mouse model has no PAH residual activity and therefore does not convert Phe to tyrosine.^{33,34} These mice do not show a reduction in plasma Phe when treated with BH4.^{33,34} In our study wild type and *Pah^{enu2/2}* C57Bl/6 PKU mice were treated with 40 mg/kg/day BH4 or placebo by intraoral pipetting for 30 days. No significant effect on neurotransmitters was seen between placebo or BH4 treated PKU mice. However, in wild type mice increased concentrations of brain serotonin were found in the BH4 treated group.

Speculations why this difference between wild type and PKU mice exists include an inhibition of TPH-2 enzyme activity by high Phe concentrations in the brain and thereby inhibiting the effect of BH4. It could be that higher doses of BH4 are needed. Another possibility could be that brain concentrations of the serotonin precursor tryptophan are too low because of reduced transport across the blood-brain barrier due to high Phe concentrations. Following this line of reasoning, BH4 may need to be combined with additional treatment strategies that aim to reduce brain Phe and/or increase brain tyrosine and tryptophan to be able to increase brain neurotransmitters by BH4 in PKU. In this respect, a previous study showed that increasing brain tyrosine and tryptophan availability rather than reducing brain Phe concentrations improved brain neurotransmitter concentrations in PKU mice that were not treated with BH4, while the combination of both strategies was most effective.³⁵

Only a few other studies were performed that investigated the effect of BH4 on neurotransmitter synthesis. In a study by Thöny et al, only an increase in TH enzyme and its activity was found after oral supplementation of BH4 (100 mg/kg/day) in wild type mice.³⁶ No effect on dopamine concentrations was found. Possibly, this can be attributed to maximal saturation of the TH protein or very tight control.³⁷ Feedback inhibition mechanisms by catecholamines^{38,39} and phosphorylation by serine kinases⁴⁰ have been described. We did not measure TH enzyme concentrations or activity therefore results cannot be compared.

Another study regarding effects of BH4 on brain neurotransmitter concentrations was performed by Winn et al.¹⁹ This study showed an increase in dopamine and serotonin metabolites

(respectively, homovanillic acid and 5-HIAA) after 100 mg/kg/day BH4 in both WT and PKU mice, but no increase in the absolute amounts of dopamine and serotonin.¹⁹ The authors speculate that BH4 did not affect the de novo dopamine or serotonin synthesis, but instead stimulates synaptic neurotransmitter release and subsequent metabolism to HVA and 5-HIAA respectively. This could be explained because there is some evidence that BH4 besides being a TH cofactor also stimulates monoamine release.⁴¹

Studies in humans on the effect of BH4 on the brain are rare. White et al, showed in twelve BH4 responsive PKU patients that white matter integrity, evaluated by mean diffusivity from diffusion tensor imaging improved during the 6 months that BH4 was used.⁴² Based on this observation the authors concluded that BH4 had resulted in lower blood Phe concentrations and consequently in improvement of the white matter abnormalities. The study does not include imaging data of BH4 treatment in BH4 non-responders, thus a direct effect of BH4 on white matter metabolism cannot be examined. The effect in white matter was not accompanied by an improvement in executive functions. This is of interest as a decrease in blood Phe concentrations is previously shown to result in an improvement of (white matter related speed in) executive functions.⁴³ Possibly, this is related to the younger age in the study by Huijbrechts et al. In a study by Burton et al, improvements in ADHD inattentive symptoms and aspects of executive functioning in response to BH4 was seen in a placebo controlled study (including both children and adults, with a significant effect for children but not adults in separate analysis). However, again no data are available in BH4 non responders.²⁵ In a small subgroup of PKU patients with measurable maladaptive behaviors there are data in BH4 non responders. Although this study does not include controls it shows a decrease in maladaptive behaviors in BH4 treated patients, suggesting a direct effect of BH4 on the central nervous system, independent of lowering blood Phe.⁴⁴

Future perspectives

Regarding the prediction of BH4 responsiveness in general, some more issues need to be discussed.

Duration of the BH4 loading test

The optimal length of the BH4 loading test is a subject of international debate in which Europe started to expand the previously used tests lasting a couple of hours to a few days whereas in the USA a BH4 challenge is being used, which could last up to 28 days.⁴⁵ As shown in chapter 3, we believe 48-hours is the shortest time necessary to detect a reasonable number of potential BH4 responders, but an actual comparison between USA and European strategies has never been done. Probably, prolongation of the test will detect even more responders. However, as the test elongates, more confounders will begin to play a role and the test gets less reliable.

Furthermore, a long test is not suitable for neonates. More studies are necessary to determine the optimal length of the BH4 loading test. Future studies could concern elongating the BH4 loading test, for example to seven days, and to establish its additional value compared to the 48 hour BH4 loading test. Furthermore, an evaluation of the USA versus the European strategy would be useful.

Predicting BH4 responsiveness with genotype

As mentioned in chapter 3 and 4, genotype can help discriminate between responders and non-responders. This is especially true for genotypes consisting of two null-mutations as at least some residual PAH activity is required for BH4 to have an effect.^{46,47} However, prediction of BH4 responsiveness in patients with other mutations is more complicated. First of all, there are a lot of different mutations reported. Second, most patients are compound heterozygous, resulting in a large number of possible mutation combinations. Compound heterozygous genotypes are relevant as the PAH enzyme is tetrameric (existing out of four monomers) and therefore heterozygous patients probably have heteromeric PAH enzymes. Probably, the two mutant subunits could interact with each other, a process called interallelic complementation. Therefore, a combination of two alleles could result in a more or less severe phenotype than expected from residual PAH activity studies with homotetrameric proteins.⁴⁸⁻⁵⁰ This may lead to inconsistencies between genotype and BH4 responsiveness. This is especially seen in some mutations like R261Q. Furthermore, recent publications have shown that PAH has a resting-state and an active structure. This suggests that mutations (other than those resulting in PAH variants prone to aggregation and/or degradation), result in a defect in the ability to stabilize active PAH or a defect in the ability to transition from the “off” state to the “on” state.⁵¹ Interestingly, BH4 seems to stabilize resting-state PAH and could act as an inhibitor. The response to BH4 differs with different PAH mutations. This could explain the observation that some PKU patients have lower Phe concentrations when treated with a lesser dose of BH4.⁴⁷ Future studies are needed to see whether it is possible to provide optimal personalized advice based on genotype.

Alternative tests for BH4 responsiveness

Other alternatives for testing BH4 responsiveness might be the ¹³C-phenylalanine breath test and the BH4 withdrawal-loading test. The first test is probably the most representative of in vivo phenylalanine oxidation. In this test phenylalanine with a ¹³C stable isotope is supplied orally to patients. After intestinal absorption ¹³C-phenylalanine is converted into ¹³C-tyrosine in the liver, after which ¹³C-tyrosine is degraded by three major pathways. In all of the main three routes, ¹³CO₂ is exhaled.⁵² The amount of ¹³CO₂ in expired air can be measured by isotope ratio mass spectrometry. As the conversion from phenylalanine to tyrosine is considered to be the rate-limiting step, the test represents the PAH activity.⁵³ Therefore, testing with and without BH4

could show whether BH4 increases PAH activity and thereby increase residual activity.^{54,55} As the test protocol needs to be optimized and asks for sophisticated laboratory equipment, the measurements are not (yet) performed routinely and the test has not been compared to long-term BH4 responsiveness, at this stage this test is used only in a research setting.

The BH4 withdrawal-loading test could be another alternative, especially in neonates. As a long BH4 loading test in neonates poses the risk of prolonged exposure to high Phe concentrations, an alternative is desirable. Particularly because the majority does not show BH4 responsiveness and these are predominantly the patients with the highest levels of Phe. Also the very high concentrations of Phe itself could impact the test outcome, as different genotypes respond differently at high or low baseline Phe concentrations.¹³

Therefore, the BH4 withdrawal-loading test is proposed. In this test, newly diagnosed patients from newborn screening start simultaneously with the Phe restricted diet and BH4 treatment. After a few weeks, when Phe concentrations are within target range and stable, BH4 is stopped and restarted again after 72 hours, while Phe concentrations are closely monitored. When Phe concentrations rise when BH4 is stopped and then Phe concentrations decrease on 're-loading', this is probably attributed to the BH4 responsiveness. If Phe concentrations do not change, the neonate is BH4 non-responsive and still optimally treated. Another possibility, as already suggested above, is the sophisticated mathematical approach of the 24 hour BH4 loading test,¹⁴ but for both methods long-term BH4 responsiveness data are still required. Also, it would be interesting to look at Phe to Tyr ratio's in BH4 responsive patients and to see whether this ratio could be used to make the loading test more reliable.

BH4 in pregnancy

Questions on BH4 treatment also include those on the safety of BH4 use during pregnancy. At the moment BH4 is -officially- not indicated for the treatment of PKU patients wanting to become pregnant or who are pregnant unless dietary treatment fails. However, a growing number of case studies substantiated the earlier suggestion that BH4 does not harm the fetus development.⁵⁶⁻⁶¹ Indirectly, it might even improve the growth and development of the fetus as Teissier et al, showed that the Phe restricted diet during pregnancy could result in intra uterine growth restriction. More natural protein intake therefore could have a better outcome.⁶² Registration of pregnancies in PKU patients with and without the use of BH4 need to be kept to assure the safety of BH4 use in this vulnerable patient category. Especially since the registration by Longo et al did mention abortion and premature labor.¹⁵

Effects of BH4 on brain

As results in studies on the effect of BH4 administration on neurotransmitters are inconsistent further research is needed. Aspects worth investigating are for example the BH4 dose, the

technique of neurotransmitter measurement or enzyme (activity) measurement and the methods of retrieving brain material. In literature there has been a long debate whether BH4 crosses the blood-brain-barrier or not. Although evidence in humans is limited,⁶³⁻⁶⁵ studies in laboratory animals suggest that BH4 crosses the blood-brain barrier when dosed at 20 mg/kg/day or higher,^{19,36,66-70} with more evidence using higher doses.¹⁹ Although animal studies with high doses of BH4 would be helpful to see if any consistent (and relevant) effect is seen, it remains the question whether this will be applicable in humans because of safety and cost issues. Regarding measurement techniques it would be interesting to look at microdialysis as this will allow to look at an effect in specific brain regions as well as making a distinction between increased synthesis or an increase in synaptic release of neurotransmitters.

In animal studies there is some evidence that BH4 can be (neuro)toxic administered at a very high dose.^{69,71} In a study with sepiapterin, a stable precursor of BH4, toxicity was seen on immature developing brain cells.⁷² Consequently, studies need to be performed on toxicity of BH4 in higher concentrations in various age periods, starting on neurons.

Who can benefit from BH4?

Selecting patients to be considered for BH4 treatment includes multiple aspects. On the one hand any relief of the strict diet is an improvement for many patients. However, on the other hand BH4 therapy is expensive and therefore justifies a minimally required effect, and furthermore false expectations are easily made.

Since the introduction of BH4 treatment, different definitions of BH4 responsiveness have been proposed.^{12,73,74} Especially the definition used in the European guidelines is stricter than the definition used in our population. Applying the European definition, some patients in our clinic would no longer be considered BH4 responsive. Besides patients with a lower baseline natural protein intake that are not able to double their intake, there are patients where doubling the protein intake would result in a more than recommended amount of protein intake. In our opinion, the latter patients would incorrectly be discarded as BH4 non-responder. Therefore, the amended definition of BH4 responsiveness including those patients who reach safe amounts of protein intake by BH4 treatment was formulated in this thesis.

Of course, a lot of the above mentioned patients would also show effects on metabolic control. Since we did not analyze those data in detail, it is not possible to determine whether they would meet the criterion of having 75% of blood Phe concentrations within target range. Indeed, probably most patients have a combination of increased natural protein intake and an improved metabolic control, making a definition for BH4 responsiveness more complex. Therefore, I agree with the European guideline that also mentions that BH4 responsiveness should be determined on an individual case basis.

If BH4 could help restore low neurotransmitter concentrations in PKU patients, the treatment could also be helpful in patients who show no response to BH4 regarding increased protein tolerance or blood Phe concentrations. If such an effect can be proven, the question which patients qualify for this treatment indication arises. Again considerations have to be made regarding the minimal effect gained and the cost-effectiveness. As the evidence is marginal at the moment, first more research is needed to look at the clinical effects.

Alternative treatment options

Apart from BH4, alternative pharmacological treatment options are currently under investigation and are certainly worth mentioning. First, sepiapterin, a natural precursor of BH4, is thought to be more stable and possibly transported more efficiently across cellular membranes. It has been shown to result in increased BH4 concentrations in healthy volunteers.⁷⁵ Second, other PAH chaperones are being developed which could replace BH4 or be used in addition to BH4 treatment.⁷⁶ A third treatment strategy could be supplementation of large neutral amino acids (LNAA). These amino acids compete with Phe at the blood-brain barrier and could therefore reduce brain Phe concentrations and improve neurotransmitter concentrations.³⁵ Fourth, trials with Phe ammonia lyase conjugated with polyethylene glycol (PEG) have been performed. This drug now being called Pegvaliase contains an enzyme derived from bacteria which converts Phe to ammonia and trans-cinnamic acid. As this bypasses the need for residual PAH activity, this helps a wider group of PKU patients. Substantial and sustained blood Phe reductions have been shown in adult PKU patients. There are however some concerns about adverse events and efficacy mainly due to the immune response. According to the latest phase III trials these adverse events were manageable and declined over time.^{77,78} Gene therapy could be another therapeutic option. Studies in animals have shown promising results, for example minicircle naked-DNA vectors have been shown to have an effect for over one year in PKU mice.⁷⁹ Development of trials in humans are started now, but so far no patients have yet been included. An alternative to gene therapy could be the use of synthetic mRNA for PAH given intravenously or subcutaneously to target the liver.⁸⁰ This still is in the preclinical setting, but also here human trials are about to start. Finally, there are efforts made to produce a probiotic organism to help digest Phe in the gut.

Conclusion

In conclusion, it can be said that BH4 treatment is a valuable addition to the PKU treatment and studies on this treatment and patient selection has given more insight. In a way, the introduction of BH4 has given the PKU world a boost and the wish to develop more and even better treatment strategies to further improve the quality of life of patients of various ages with PKU. Despite

these new developments, much more is still to be investigated regarding the pathophysiology of the disease and brain dysfunction in early and continuously treated PKU patients.

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SUMMARY

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Summary

Phenylketonuria (PKU) is a rare inborn error of metabolism affecting 1:10,000 (range 1:3,000-1:30,000) individuals in Europe, caused by a deficiency in the enzyme phenylalanine hydroxylase (PAH) that converts phenylalanine (Phe) into tyrosine. If untreated, PKU results in high Phe concentrations in blood and brain resulting in severe mental retardation, epilepsy and severe behavioral issues. This can be (mostly) prevented by detecting PKU infants early by neonatal screening and starting dietary restriction of Phe by a natural protein-restricted diet combined with Phe free amino acids. This treatment is effective and should be continued lifelong.

Fortunately, previous studies showed that some PKU patients respond to tetrahydrobiopterin (BH4), showing the possibility for a less strict diet and/or lower blood Phe concentrations. BH4 was introduced on the market around 10 years ago. During the first years of introduction a lot of studies have been published mostly involving small patient numbers. Therefore, there was not a lot of evidence determining the best method of predicting BH4 responsiveness.

In this thesis, I evaluated diagnostic strategies to detect BH4 responsiveness and thereby tried to determine which patients benefit from BH4 treatment as well as search for its effects on a brain level.

In **Chapter 1** an introduction on PKU in general is given and some issues on BH4 responsiveness in PKU are discussed in more detail. Furthermore, the scope of the thesis is outlined; the key issues being: determining the predictive rate of the BH4 loading test and genotype, and on the other end mood and behavior in PKU and the effect of BH4 on neurotransmitter levels.

Prediction of BH4 responsiveness

In **Chapter 2** we explored the question: “Does the 48 hour BH4 loading test reveal new long-term responders compared to the 24 hours?”. In this study, the 48 hour BH4 loading test from 186 patients from six Dutch university medical centers was analyzed. This study showed that 46% of patients showed a $\geq 30\%$ decrease in blood Phe concentration after supplementations of BH4. Furthermore, it showed that the 48 hour time point added a substantial group of potential responders. Despite adding Phe to the diet in some patients, still 21% of patients had baseline Phe concentrations under 400 $\mu\text{mol/l}$ (recommended lower limit). Notwithstanding, in these patients a response was frequently seen. Therefore 400 $\mu\text{mol/l}$ should not be a hard limit. As this study does not show whether the response in the BH4 loading test is also clinically relevant, a follow-up study is necessary and was performed.

Chapter 3 answered more than one question. Most importantly, 87% of those patients who tested positive in the 48 hour BH4 loading test were also true responders. We saw that patients with a higher percentage of Phe decrease and a faster Phe decrease during the 48 hour BH4 loading test were more often true responders. However, also patients with just over 30% decrease in Phe and only responding at the 48 hour time point indeed could be true responsive. Regarding genotype, we saw that a genotype comprising of two so-called BH4 responsive mutations makes the BH4 loading test unnecessary because these patients were always responders. On the other hand, I could confirm earlier predictions that patients carrying two putative null (zero) mutations never show a response to BH4. Therefore, a BH4 loading test should be performed in any PKU patient who does not carry two null or two BH4 responsive mutations. Patients carrying one mutation associated with true BH4 responsiveness are very likely to respond as well, but as this is not known for a lot of mutations, there are some mutations with an inconsistent response to BH4 and the interplay between two mutations can influence the response, a BH4 loading test is advised.

In **Chapter 4** we re-evaluated the predictive ability of the 48 hour BH4 loading test by using the new definition of true BH4 responsiveness as proposed in the European guidelines ($\geq 100\%$ increase in natural protein tolerance). As this definition is more strict compared to the one we used in Chapter 3 (increase in natural protein intake of $\geq 50\%$ or ≥ 4 g/day) we anticipated that the positive predictive value would decrease and that assumption was indeed confirmed. However, we also noticed that some patients with a clear response to BH4 would (in our eyes unjustly) no longer be considered BH4 responsive. Analysis showed that these patients often started out with a high baseline natural protein intake and therefore could not achieve a 100% increase as this would result in a more than recommended intake. Therefore, an amended definition incorporating this phenomenon was introduced: “an increase in natural protein tolerance $\geq 100\%$ or tolerating a safe natural protein intake”. Furthermore, analysis of using another cut-off value than 30% was performed, and although this results in a higher positive predictive value, it also results in an increase in false-negative tests. These data show that, overall, the 48-hour BH4 loading test cannot be improved easily.

In **Chapter 5** I compared the neonatal BH4 loading test (performed at diagnosis) to the 48-hour BH4 loading test (the same children at median 11.8 years old). This showed that a positive neonatal BH4 loading test (8- or 24 hours) is indeed predictive for true BH4 responsiveness. But in contrast, a negative neonatal BH4 loading test does not rule out long-term BH4 responsiveness. In this chapter I suggest to investigate other alternatives to determine BH4 responsiveness in neonates, such as a BH4 withdrawal test.

Chapter 6 addressed the question about the long-term effects of BH4 on metabolic control and dietary tolerance. This was addressed in a study initiated by our Swiss colleagues Prof Blau and dr Keil assessing the long-term (positive and negative) effects of BH4 treatment among 16

centers in six European countries. A beneficial effect was shown on metabolic control and dietary Phe intake, while this study showed no severe adverse events and only some adverse events that disappeared with reduced therapeutic BH4 doses suggesting that BH4 treatment is both safe and effective in at least some patients (data up to 12 years).

Behavior, neurotransmitters and BH4

In **chapter 7** we tried to ascertain the effects of Phe in relation to behavior using a questionnaire. Indeed it was shown that PKU patients had more mood swings at higher Phe concentrations.

As BH4 may pass the blood-brain barrier and is not only the co-substrate of PAH but also of the (cerebral) tyrosine and tryptophan hydroxylating enzyme. As these enzymes are the rate-limiting step in the synthesis of dopamine and serotonin, BH4 could influence cerebral neurotransmitter levels irrespective of its effect on plasma Phe.

In **Chapter 8**, we studied the effect of BH4 treatment on prolactin concentrations. As prolactin is under negative feedback control of dopamine, blood prolactin concentrations have been found to reflect brain dopamine concentrations. As prolactin is also influenced by female hormones we studied the effect exclusively in men. In this preliminary study in nine patients we saw that prolactin concentrations were significantly lower on BH4 treatment. Also, when correcting for phenylalanine concentrations this association subsisted.

Another way to investigate the effect of BH4 more directly on brain neurotransmitter metabolism is to use a PKU mouse model as was done in **chapter 9**. Our study was performed in *Pah^{enu2/2}* PKU mice, a model for BH4 unresponsive PKU, thereby results are not influenced by changes in Phe concentrations. PKU mice and wild type mice were treated with 40 mg/kg/day of BH4 or placebo during 30 days. As expected the brain neurotransmitter concentrations were notably lower in the PKU mice compared to wild type mice. However, we did not see an effect of BH4 on restoring this deficit. Interestingly in wild type mice we did see higher serotonin concentrations in BH4 treated mice compared to placebo treated mice. Searching for an explanation for this difference in reaction between wild type and PKU mice among others include too high concentrations of brain Phe that may inhibit the tryptophan hydroxylase enzyme activity or shortage of brain tryptophan by selected transport across the blood-brain barrier.

Finally, in **Chapter 10**, the main findings of the studies in this thesis are discussed. Based on the findings in these chapters and its discussion, I have the idea that PKU research has not reached its ultimate goal but just started another chapter of its journey (after Scriver, PKU: The Journey: not the Arrival... yet. In: Blau N. PKU and BH4–Advances in Phenylketonuria and Tetrahydrobiopterin. 1st ed. SPS Publications;2006.).

NEDERLANDSE SAMENVATTING

Nederlandse samenvatting

Wat is Phenylketonurie (PKU)?

Phenylketonurie, kortweg PKU, is een zeldzame ziekte waarbij de eiwitstofwisseling verstoord is. Bij deze ziekte lukt het niet om één van de bouwstenen van eiwit namelijk phenylalanine af te breken. Dit komt omdat het enzym phenylalanine hydroxylase onvoldoende of helemaal niet aanwezig is. Normaal gesproken zet dit enzym het aminozuur phenylalanine om in het aminozuur tyrosine. Doordat het enzym dit onvoldoende doet is het phenylalanine gehalte in het bloed te hoog en het tyrosine gehalte vaak aan de lage kant. Als deze situatie onbehandeld blijft bestaan leidt dit onder andere tot een ernstige ontwikkelingsachterstand, epilepsie en ernstige gedragsproblemen.

De ziekte werd voor het eerst beschreven in 1934 door Asbjorn Følling, een Noorse arts en chemicus die bij twee kinderen met een verstandelijke handicap een hoge concentratie phenylpyruvaat in de urine vond. Deze stof wordt gevormd als het phenylalanine gehalte in het bloed erg hoog is. Tien jaar na de ontdekking van Følling werd er voor het eerst een behandeling voor PKU ontwikkeld door Horst Bickel en zijn collega's. Hierbij werd gebruik gemaakt van een dieet dat weinig phenylalanine bevat. Dit betekent dat de hoeveelheid eiwit in de voeding streng beperkt moet worden omdat phenylalanine voorkomt in alle voedingseiwitten. Omdat een patiënt de andere bouwstenen, genaamd aminozuren, uit het eiwit wel nodig heeft werd een speciaal mengsel van aminozuren gemaakt zonder phenylalanine dat in plaats van eiwit moet worden ingenomen.

Al snel bleek dat een reeds bestaande verstandelijke beperking niet teruggedraaid kan worden, maar dat vroeg behandelen dit wel (grotendeels) kan voorkomen. Om de ziekte zo vroeg mogelijk te ontdekken en behandelen werd door Robert Guthrie een test ontwikkeld die op grote schaal kon worden uitgevoerd. Hieruit is de neonatale screening ontstaan met de hielprik. Sinds 1974 wordt in Nederland door middel van de hielprik gescreend op PKU. Tegenwoordig is het aantal ziekten waarop getest wordt met de hielprik uitgebreid tot 22, waarvan 16 stofwisselingsziekten.

PKU is een erfelijke ziekte die autosomaal recessief overerft. Dat wil zeggen dat een kind pas ziek wordt als op beide chromosomen een fout zit op de plek van het PKU gen. Vaak zijn beide ouders drager van de ziekte. Zij hebben een gen met een fout en een gen zonder een fout. Doordat zij een gen hebben zonder fout hebben zij zelf geen klachten. De ziekte komt in Nederland voor bij 1 op de 18.000 personen, dat betekent dat er per jaar in Nederland ongeveer 10 kinderen met PKU worden geboren.

De behandeling door middel van het dieet is gelukkig erg effectief. Echter het dieet vormt een strenge beperking en is daardoor erg belastend. Om een idee te geven, voor veel patiënten zijn voedingsmiddelen als, vlees, kaas, ei, melkproducten en gewone graanproducten verboden terrein en bestaat het dieet uit veel eiwitarme dieetproducten (brood, pasta's, biscuits) en fruit, groente en beperkt aardappelproducten.

Onderzoek naar andere behandelmogelijkheden heeft uitgewezen dat sommige PKU patiënten baat lijken te hebben van behandeling met BH4. BH4 (volledige naam tetrahydrobiopterine) is een stof die samenwerkt met het enzym phenylalanine hydroxylase dat niet goed werkt bij PKU. Op het moment dat mijn onderzoek begon was BH4 net verkrijgbaar in Nederland en waren onderzoeken beperkt tot kleine patiënten aantallen.

Het onderzoek

In dit proefschrift wordt met name gekeken hoe we patiënten die baat kunnen hebben bij BH4 het beste kunnen selecteren, maar ook wat de effecten kunnen zijn op het brein. In **Hoofdstuk 1** wordt een algemene introductie over PKU en BH4 gegeven. Hierin wordt onder andere vermeld dat BH4 helaas niet bij alle patiënten effect heeft. Het lijken vooral PKU patiënten te zijn met een mildere vorm die er wel baat van hebben, maar op basis hiervan is de patiëntselectie niet goed te maken. Om te onderzoeken bij welke patiënten het zinvol is om te starten met BH4 therapie wordt in Europa gebruik gemaakt van een relatief korte belastingstest (geef BH4 en kijk wat het effect is op de phenylalanine bloedspiegel). De meeste studies gebruiken een test met een duur van 24 uur, maar kleine studies tonen aan dat langer testen mogelijk wel zinvol kan zijn. In Nederland is bij de introductie van BH4 behandeling een test gedurende 48 uur gedaan.

In **Hoofdstuk 2** worden de gegevens die zijn verzameld van de 48 uren BH4 belastingstest in Nederland weergegeven. In totaal zijn de testen van 186 patiënten uit zes universitair medische centra geanalyseerd. Hierbij werd gezien dat 46% van de patiënten een daling van phenylalanine lieten zien groter dan of gelijk aan de afkapwaarde van 30%. Van de patiënten die deze daling van phenylalanine lieten zien was dit bij ongeveer 1 op de 6 pas na 48 uur. Deze groep noemen we late responders.

In **Hoofdstuk 3** werden ook de gegevens van patiënten die naar aanleiding van een positieve test voor langere tijd met BH4 zijn behandeld geanalyseerd. Er is gekeken of deze personen inderdaad baat hadden bij BH4 en of dit voorspeld kon worden aan de hand van de 48 uren test uitslag. Ook is gekeken of de 'fouten' in de genen een voorspeller kunnen zijn.

Resultaten toonden dat 87% van de mensen met een positieve test uiteindelijk baat hadden bij BH4 therapie. Personen met een snellere of grotere daling in phenylalanine hadden vaker baat

bij BH4 therapie. Echter ook enkele “late responders” hadden baat bij de therapie, derhalve lijkt het wel zinvol om 48 uur lang te testen. Een voorspelling doen aan de hand van de genen is complex omdat er veel verschillende foutjes bestaan. Wel werd gezien dat patiënten met op elk van de chromosomen een ernstige fout nooit baat hadden bij BH4 therapie. Het is dus ook niet nodig om bij deze patiënten een BH4 belastingstest te doen.

In **Hoofdstuk 4** is gekeken of de nieuwe Europese PKU richtlijn ons anders naar de bovenstaande gegevens doet kijken. In deze richtlijn is de definitie van baat hebben bij BH4 therapie strenger dan de definitie die wij in hoofdstuk 3 hebben gebruikt. Logischerwijs hebben er dan minder patiënten ‘baat bij BH4’. Helaas is het niet makkelijk om de 48 uren belastingstest aan te passen zodat deze een betere voorspelling kan maken.

In **Hoofdstuk 5** is gekeken naar oude neonatale BH4 belastingstesten. In het verleden werden patiënten waarbij een positieve hielprik werd gevonden voor PKU getest op een andere stofwisselingsziekte met een tekort aan BH4 (BH4 deficiëntie). Om het onderscheid te maken tussen PKU en een BH4 deficiëntie werd een korte BH4 belastingstest gedaan, waarbij de patiënten met een BH4 deficiëntie over het algemeen een snelle daling van phenylalanine laten zien. Tegenwoordig is dit onderscheid op een andere manier in het laboratorium te maken. In dit hoofdstuk hebben we de testen van dezelfde patiënt op zuigelingenleeftijd (neonataal) en de 48 uren belastingstest op latere leeftijd met elkaar vergeleken. Hieruit kunnen we concluderen dat een positieve neonatale BH4 belastingstest voorspellend is voor baat hebben bij BH4, maar een negatieve neonatale BH4 belastingstest niet altijd correct is. Dit laatste kan deels verklaard worden omdat in veel gevallen de neonatale BH4 belastingstest maar 8 uur duurde, maar ook zijn er op die jonge leeftijd andere invloeden op de stofwisseling die de test kunnen beïnvloeden.

In **Hoofdstuk 6** zijn in Europees verband gegevens verzameld van een grote groep PKU patiënten die langdurig met BH4 is behandeld om te kijken naar lange termijn effecten. Deze studie, geïnitieerd door onze Zwitserse collega’s Prof Blau en dr Keil, laat zien dat er een gunstig effect is van BH4 op ziektecontrole en dieet. Verder werden er geen ernstige bijwerkingen gevonden, wel een aantal minder ernstige bijwerkingen die over het algemeen verdwenen bij een verlaging van de dosis BH4.

In **Hoofdstuk 7** stappen we over naar het volgende onderwerp van het proefschrift namelijk gedrag, hersenen en BH4. In dit hoofdstuk hebben we getracht te kijken naar het effect van phenylalanine spiegels op gedrag. Hierbij zagen we een associatie tussen een wisselende stemming en een hoog phenylalanine. Bij andere gedragingen zoals druk gedrag konden we deze associatie niet vinden.

In **Hoofdstuk 8** hebben we een begin gemaakt met het onderzoek naar het effect van BH4 op de hersenen. BH4 is naast een hulpstof voor het PKU enzym phenylalanine hydroxylase ook hulpstof voor andere enzymen betrokken bij de aanmaak van neurotransmitters. Neurotransmitters zijn signaalstoffen in de hersenen. We weten dat bij onbehandelde PKU een tekort is aan deze neurotransmitters. Mogelijk heeft BH4 een effect op deze neurotransmitters. Eén van deze stoffen is dopamine, maar helaas is deze stof niet betrouwbaar in bloed te meten. Daarom is in dit hoofdstuk prolactine gemeten, dit is een stof die onder invloed van dopamine staat. In deze zeer kleine studie zagen we wel een associatie tussen prolactine en BH4 gebruik.

In **Hoofdstuk 9** is met behulp van PKU muizen gekeken naar het effect van BH4 op de hersenen. Ook PKU muizen hebben een verlaagde hoeveelheid van bepaalde neurotransmitters in de hersenen vergeleken met gezonde muizen. In deze studie werd geen effect gezien op de hoeveelheid neurotransmitters in de BH4 behandelde groep PKU muizen. Wel werd in de groep gezonde muizen een stijging gezien van één van de neurotransmitters. Theorieën waarom dit bij de gezonde muizen wel wordt gezien en bij de PKU muis niet zijn o.a. dat de hoge phenylalanine spiegel bij de PKU muis mogelijk een tegenwerkend effect heeft.

Als laatste worden in **Hoofdstuk 10** de belangrijkste resultaten van dit proefschrift bediscussieerd en wordt er een idee geschetst van de vooruitzichten.

DANKWOORD

Dankwoord

Dit proefschrift was niet tot stand gekomen zonder de hulp van anderen. Ik wil dan ook iedereen die betrokken is geweest bij de realisatie van mijn proefschrift ontzettend bedanken. Samen kom je zo veel verder dan alleen. Zonder iemand te kort te willen doen, wil ik een aantal mensen in het bijzonder noemen.

Allereerst wil ik graag mijn promotor, prof. dr. F.J. van Spronsen, bedanken. Beste Francjan, jouw enthousiaste begeleiding heeft er voor gezorgd dat ik dit proefschrift heb kunnen schrijven. Al vanaf het begin toen ik als 3^e jaars student, geïnspireerd door een college, een afspraak had om een project op te zetten waren er een hele hoop ideeën. Toen ik mij in 2010 bezig mocht houden met het net geïntroduceerde BH4 was het duidelijk dat we hier samen genoeg mogelijkheden in zagen om er een promotietraject van te maken. Ik heb in deze periode erg prettig met je kunnen overleggen en de vele ideeën zijn altijd gebleven. Ook grondige revisies van manuscripten tegenover bondige mails zoals 'vooral niet te moeilijk doen... :)' of 'brrrrr' heb ik altijd erg op prijs gesteld. Ook was het zeer leerzaam betrokken te raken bij internationale projecten en de vele congressen waar ik mijn resultaten mocht presenteren. Dank voor al deze ervaringen en jouw begeleiding hierin.

Zeergeleerde copromotor dr. M.R. Heiner-Fokkema, beste Rebecca, ik ben erg dankbaar voor jouw fijne begeleiding. Jij hebt mij wegwijs gemaakt in de biochemie en een heleboel laboratoriumzaken, een voor mij onbekende wereld. Ik kan jouw enthousiaste manier van overleggen erg waarderen. Als vrouw tussen de mannen ook erg verfrissend.

Zeergeleerde copromotor dr. M. van Rijn, beste Greet, dankjewel dat je mijn copromotor wilt zijn. Ik weet dat jij dat altijd als feestje/cadeautje hebt gezien, maar ik meen het oprecht dat jouw begeleiding van onschatbare waarde is geweest. In het begin om mij wegwijs te maken in het PKU-dieet en wat dat praktisch inhoudt en later met het doorwerken van manuscripten, data beoordelen (heerlijk SPSS'en) en besprekingen op je kamer in de P-gang. Blijf vooral je tomeloze energie inzetten voor alles wat je niet wilt laten.

The members of the Assessment Committee, dr. F.K. Trefz, prof. dr. F.A. Wijburg, and prof. mr. dr. A.A.E. Verhagen, thank you for reading my thesis critically and judging this thesis.

Verder wil ik alle medeauteurs van mijn manuscripten bedanken voor hun medewerking, te beginnen met iedereen die mij heeft geholpen om in de verschillende centra data te verzamelen en vervolgens te publiceren: Floris Hofstede en Ems Carbasius Weber van het Wilhelmina Kinderziekenhuis te Utrecht, Annet Bosch, Nienke ter Horst, Carla Hollak en Cora Jonkers van het

Amsterdam UMC locatie AMC, Estela Rubio-Gozalbo en Liesbeth van der Ploeg van het Maastricht Universitair Medisch Centrum, Maaikje de Vries, Renske Janssen-Regelink, Annemiek van Wegberg, Mirian Janssen en Heidi Zweers-van Essen van het Radboud Universitair Medisch Centrum en Carolien Boelen en Ada van der Herberg-van de Wetering destijds van het Leids Universitair Medisch Centrum. Natuurlijk ook de collega's van het UMCG die mij daarbij geholpen hebben, nog niet eerder genoemd Gineke Venema, Ninja ter Borg en Esther van Dam. Dank ook voor Roeland Evers die mijn werk deels heeft voorgezet, erg leuk om jou hierbij te mogen helpen.

Dear Professor Blau and Stefanie Keil, thank you for initiating the European project and the effort you have made to make it a successful publication. I was delighted to be a part of it. Also, many thanks for participating in the other studies, it has been very helpful to receive so much knowledge and I appreciate the thorough review of the manuscripts.

Verder wil ik de volgende personen bedanken voor de extra ondersteuning bij het onderzoek: Paul Verkerk en Johannes Burgerhof voor het beoordelen van de methodologie en uitvoeren van een aantal complexe analyses.

Met het onderzoek naar het effect van BH4 op de hersenen ben ik een totaal andere wereld binnengestapt. Graag bedank ik Martijn de Groot als mijn voorganger/collega onderzoeker, voor de ideeën en het helpen bij het opzetten van het nieuwe onderzoeksplan. Ik vind het erg prettig om te horen dat je het naar je zin hebt bij de klinische genetica. Verder Vibeke Bruinenberg en Els van der Goot voor het helpen met de daadwerkelijke muizenstudie. Dankzij jullie handigheid hebben jullie mij er doorheen geloodst. Prof. dr. E.A. van der Zee, beste Eddy, dank voor het delen van jouw uitgebreide kennis van de neuro- en gedragswetenschappen. Erg fijn dat ik af en toe ook bij jouw onderzoeksgroep op het Zernike mocht aansluiten. Danique van Vliet, ik noem je straks ook als paranimf, maar hier alvast inhoudelijk voor je werk ontzettend veel dank. Muizen typeren, helpen verwerken van de monsters op het lab, data-analyse, ik vond het erg gezellig om dit grotendeels samen met jou te doen. Martijn van Faassen, hartelijk dank voor jouw hulp bij het analyseren van de neurotransmittermonsters. Tevens bedank ik Professor Ido Kema voor het mede-interpreteren van de neurotransmitterdata en het opdoen van ideeën voor vervolgstappen.

Dear Tanja Scherer and Professor Thöny, thank you for sharing your experiences with the PKU mouse model and working with BH4. Your input has lifted the research plan to a higher level.

Veel dank aan de analisten van het lab metabole ziekten en bijzondere chemie. In het bijzonder Pim, Pim, Fjodor en Albert voor jullie hulp, geduld en expertise bij het bepalen van aminozuren, regelen van geanonimiseerde namenlijsten, uitleggen van de PCR en het maken van niet-standaard oplossingen.

Een speciaal woord van dank voor mijn paranimfen, Marian en Danique. Wat fijn dat jullie mijn paranimfen willen zijn en mij bij willen staan op deze speciale dag.

Lieve Marian, al vanaf de middelbare school zij wij vriendinnen. Jij mocht al een jaar eerder geneeskunde gaan studeren, maar ik volgde je op de voet. Toen ik uiteindelijk onderzoek ging doen bleek al snel dat jij ook een promotietraject (naast de huisartsenopleiding) ging doen. Wat fantastisch om naast onze vriendengroep ook dat te kunnen delen. Ik hoop dat jouw promotie gauw gaat volgen!

Lieve Danique, naast collega's zijn wij ook vriendinnen geworden. Als medeonderzoeker bij 'metabole ziekten' was er altijd genoeg inhoudelijk te bespreken, maar ook daarbuiten hebben we heel wat afgeketst, geluncht en gezelligheid gehad tijdens de reizen naar buitenlandse congressen. Ontzettend bedankt ook voor jouw hulp met al het labwerk en mentale ondersteuning tijdens de muizenonderzoeken. Wat ben jij een harde werker! Heel erg fijn om je nu ook nog als collega te hebben tijdens onze opleiding tot kinderarts.

Nienke, als Metabolo van het eerste uur, hebben we veel inhoudelijke discussies gehad, maar ook zeker buiten het werk veel gezelligheid gehad. Ik vond het fantastisch om jouw paranimf te mogen zijn. Je was bij mij ook van harte welkom geweest ware het niet dat jij inmiddels wat verder weg woont (en alweer bijna klaar met je opleiding tot klinisch geneticus in Utrecht) en je handen vol hebt met jullie prachtige kinderen.

Natuurlijk bedank ik ook de overige Metabolo's! Wat was het fijn dat er altijd wel iemand was om mee te sparren of samen mee op buitenlands congres te gaan. Rianne ik vergeet nooit hoe hard jij kan werken op die ontzettend trage computer! En wat is het een mooi proefschrift geworden. Verder natuurlijk Esther, Wiggert (Wiggle ;)) en Priscila. En ook de nieuwere generatie metabolo's bij wie ik de laatste tijd soms ook nog op de kamer mocht zitten: Roeland, Fabian, Irene, Geertje, Willemijn, Kimber en Iris.

Ook Annemiek en Marrit, mijn ex-medebewoners van KZ00.23, ben ik erg dankbaar. Weggestopt in de kelders van het triade gebouw heb ik het maar getroffen met zulke geweldige kamergenoten als jullie! Ondanks dat jullie elkaar al zo goed kenden heb ik me altijd welkom gevoeld. Met onze bijna volledige keukenuitrusting kwamen wij de dag wel door en ik heb nog altijd goede herinneringen aan de wijncursusavonden. Binnenkort maar weer eens proosten!

Ook de andere ganggenoten van de triade-kelder wil ik niet overslaan: Martijn, Menno, Mark-Jan, Djoeke, Michelle, Jozien, Janyte, Chris-Peter en Nicole. In de loop van de tijd is er aardig wat gewisseld in de samenstelling (dus ik hoop heel erg dat ik nu niemand ben vergeten), maar desondanks stonden jullie altijd open voor een kopje koffie op de stoep (via het raam naar buiten), lunch in het brugrestaurant of een taartje als er iemand jarig was. Dank hiervoor.

Beste kinderartsen van het Medisch Spectrum Twente in Enschede, Isala in Zwolle en Beatrix kinderziekenhuis UMC Groningen, mijn promotie afmaken tijdens het klinisch werken was niet gemakkelijk, maar wel veel dank voor jullie steun, belangstelling en leermeesterschap. ANIOS en AIOS collega's uit Enschede, Zwolle en Groningen, bedankt voor de goede samenwerking, borrels en gezelligheid.

De secretaresses, zowel van de poli als op het secretariaat, Mirjam, Anneke, Tineke, Fay, Heidi en Georgette, dank voor jullie hulp bij het maken van afspraken in overbezette agenda's, het opvragen van dossiers (ja die dikke "niet altijd even leesbare" dossiers uit het archief met de ingeplakte labblaadjes) en ruimte voor een gezellig praatje.

De Junior Scientific Masterclass wil ik bedanken voor de mogelijkheid die ik heb gekregen om dit onderzoek in de vorm van een MD/PhD-traject uit te voeren.

Ontspanning naast je werk is zeer belangrijk. Wat een geluk heb ik dan ook dat ik zoveel lieve en begripvolle vrienden om mij heen heb. Als speciale vermelding de vriendenclub die ik aan de middelbare school heb overgehouden, inmiddels aangevuld met aanhang en via via toevoegingen die inmiddels ook alweer jaren meegaan. Wat een plezier om af en toe een weekend weg te gaan of te gaan skiën, spontaan te gaan barbecueën, elkaar te helpen verhuizen, creatief bezig te zijn tijdens de babytheebijeenkomsten of een goed gesprek te hebben met een bakje koffie of een borrel. Ook mijn muziekmaatjes van Harmonie 67 en de andere orkesten waar ik tijdelijk werd opgevangen, tijdens mijn verblijf in Enschede en Zwolle: bedankt voor de nodige afleiding en prachtige muzikale intermezzo's.

Lieve (schoon)familie, allemaal bedankt voor jullie steun en aanmoediging. Het zal niet voor iedereen altijd even duidelijk zijn geweest waarmee ik me bezighield, maar druk had ik het wel. Gelukkig is het niet voor niets geweest: zie hier het prachtige resultaat!

Lieve Judith en Gilby. Wat fijn om zo'n lieve ambitieuze zus te hebben. Na een tijdje samen in Groningen gestudeerd te hebben woon jij inmiddels al weer enige tijd een eindje verder weg. Ik vind het erg leuk om te volgen wat er bij jou op je pad komt net als dat jij altijd geïnteresseerd bent in mijn bezigheden en dat we dat over de telefoon of live met een kop thee erbij bespreken. Fijn ook dat Gilby erbij is gekomen. Jullie huwelijksdag was prachtig en ik hoop op veel geluk nu de uitbreiding van jullie gezin eraan zit te komen.

Leave heit en mem, wat ben ik ontzettend blij om jullie als ouders te hebben. Dankzij jullie heb ik zelf mogen doen en kiezen wat ik met mijn toekomst wilde. Ik kan ook erg genieten van jullie

‘grutsk’ zijn op de momenten dat ik een mijlpaal heb bereikt. Jullie staan altijd voor ons klaar en daar ben ik jullie ontzettend dankbaar voor.

Tot slot lieve Douwe, myn skat, vader van onze prachtige zoon Jelte, bedankt voor al je steun en zorgzaamheid tijdens mijn onderzoeksperiode, maar vooral ook toen ik als arts aan de slag ging en het afmaken van dit proefschrift wat minder van een leien dakje ging. Ik vind het heel fijn hoe jij altijd weer iets interessant vindt en verhalen kan vertellen, maar ook de zaken soms kunt relativeren. Blijf dat vooral altijd doen.

ABOUT THE AUTHOR

About the author

Karen Anjema was born on May 22th 1985 in Giekerk, Tietjerksteradeel, The Netherlands. She grew up in Drachten and surroundings as the oldest of two children. She attended the atheneum at the CSG Liudger school in Drachten. After graduation she completed the first year (propedeuse) of 'life science and technology' at the University of Groningen. In 2004 she started medical school at the university of Groningen. In 2007 she was a member of the daily board of ISCOMS, an international student congress for medical sciences which inspired her to do research herself. Therefore, in 2007 she started a research project under supervision of prof. dr. F.J. van Spronsen which eventually resulted in a successful application for a MD/PhD trajectory in 2011. Afterwards she moved to Enschede to work as an intern (ANIOS) in pediatrics at the Medisch Spectrum Twente hospital. In 2015 she moved back to Groningen to continue her work as intern at the University Medical Center Groningen. In 2016 she started her traineeship in pediatrics in Groningen and Zwolle (Isala hospital), meanwhile finishing her PhD thesis.

LIST OF PUBLICATIONS

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