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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.



## 1. 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

*Abbreviations:* BBB, blood–brain barrier; BH4, tetrahydrobiopterin; PAH, phenylalanine hydroxylase; Phe, phenylalanine; PKU, phenylketonuria; Tyr, tyrosine.

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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 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.

## 2. Methods

### 2.1. 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 ≥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.

**Table 1**
Patient characteristics.

| Patient | Genotype | Age | |
|---|---|---|---|
| | | Before BH4 | On BH4 |
| 1 | p.Y414C/c.842 + 5G > A | 28 y, 4 m | 30 y, 7 m |
| | | 28 y, 2 m–28 y, 6 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 | 11 y, 11 m |
| | | 8 y,8 m–9 y, 10 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 | 12 y, 9 m |
| | | 9 y,6 m–10 y, 7 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.

### 2.2. 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).

### 2.3. 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.

## 3. Results

### 3.1. 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 ($p = 0.235$).

### 3.2. 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 ($p = 0.024$, $t = 2.784$, $d = 0.928$).

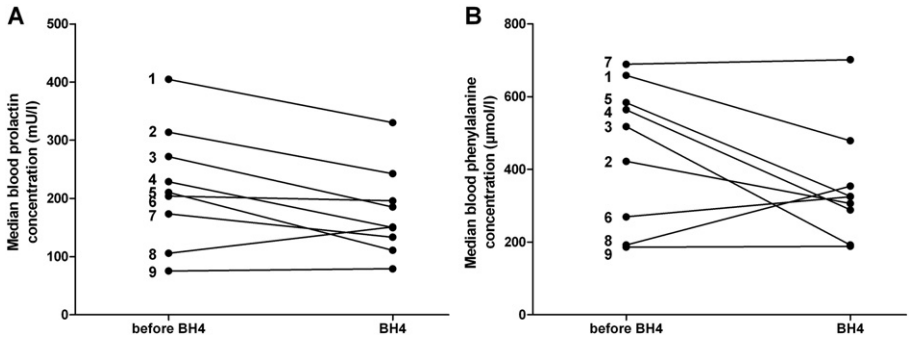


**Fig. 1.** Median blood prolactin (A) and phenylalanine (B) concentrations in BH4-responsive PKU males ($n = 9$) before BH4 treatment was used and while using BH4.

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 ($p = 0.107$, $t = 1.814$, $d = 0.605$).

### *3.3. 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 ($p = 0.054$, $t = -1.991$, $d = 0.297$).

## 4. 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 fluctuations [14]. 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 µmol/L) compared to PKU patients on a strict diet (blood Phe 90 ± 39 µmol/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 concentrations [23]. 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.

## 5. 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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