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Effect of BH4 on blood phenylalanine and tyrosine variations in patients with phenylketonuria

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ABSTRACT

Background: In patients with phenylketonuria, stability of blood phenylalanine and tyrosine concentrations might influence brain chemistry and therefore patient outcome. This study prospectively investigated the effects of tetrahydrobiopterin (BH4), as a chaperone of phenylalanine hydroxylase on diurnal and day-to-day variations of blood phenylalanine and tyrosine concentrations.
Methods: Blood phenylalanine and tyrosine were measured in dried blood spots (DBS) four times daily for 2 days (fasting, before lunch, before dinner, evening) and once daily (fasting) for 6 days in a randomized cross-over design with a period with BH4 and a period without BH4. The sequence was randomized. Eleven proven BH4 responsive PKU patients participated, 5 of them used protein substitutes during BH4 treatment. Natural protein intake and protein substitute dosing was adjusted during the period without BH4 in order to keep DBS phenylalanine levels within target range. Patients filled out a 3-day food diary during both study periods. Variations of DBS phenylalanine and Tyr were expressed in standard deviations (SD) and coefficient of variation (CV).
Results: BH4 treatment did not significantly influence day-to-day phenylalanine and tyrosine variations nor diurnal phenylalanine variations, but decreased diurnal tyrosine variations (median SD 17.6 μmol/l, median CV 21.3%, $p = 0.01$) compared to diet only (median SD 34.2 μmol/l, median CV 43.2%). Consequently, during BH4 treatment diurnal phenylalanine/tyrosine ratio variation was smaller, while fasting tyrosine levels tended to be higher.
Conclusion: BH4 did not impact phenylalanine variation but decreased diurnal tyrosine and phenylalanine/tyrosine ratio variations, possibly explained by less use of protein substitute and increased tyrosine synthesis.

1. Introduction

Phenylketonuria (PKU; OMIM #261600) is an autosomal recessive inborn error caused by a deficiency of the enzyme phenylalanine hydroxylase (PAH), which normally converts phenylalanine (Phe) into tyrosine (Tyr). Left untreated, PAH deficiency results in high concentrations of Phe in blood and tissues, resulting in severe intellectual disability, seizures and behavioral problems as well as musty odor and light skin, eyes and hair [1]. Despite the success of newborn screening and early treatment, suboptimal neurocognitive and psychosocial outcomes still exist [1,2].

Besides the hypothesis that brain dysfunction is caused by a neurotoxic effect of high Phe concentrations, it is also hypothesized that cerebral protein and neurotransmitter synthesis are impaired by insufficient availability of other large amino acids that are all indispensable in PKU [3,4]. Tyr is important for protein synthesis and is normally converted into dopamine, which is an important neurotransmitter. Phe, Tyr and other large amino acids compete at the blood-brain barrier (BBB) as they use the same exchange system, the so-called Large neutral amino acid transporter type 1 (LAT1). LAT1 is already fully activated at physiological concentrations of the amino acids so that every increase of one amino acid results in a decrease of the influx of another amino acid. LAT1 shows high affinity for Phe. This in combination with increased blood Phe concentrations, leads to efficient brain Phe influx, which results in increased brain Phe at the expense of Tyr and other large neutral amino acids [5,6]. Possibly, this is also the reason why not only (high) Phe but also high Phe/Tyr ratios have been reported to correlate to aspects of cognitive functioning, executive functioning and depressive symptoms [7–10]. In addition to increased blood Phe and Phe/Tyr ratios, the variability of blood Phe and Tyr concentrations might also influence

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brain influx and brain chemistry as data indicate a correlation of Phe variation to IQ, executive functioning and motor control [7,11–14].

While dietary Phe restriction is still the main treatment to prevent high blood Phe levels, a subset of PKU patients is responsive to tetrahydrobiopterin (BH4, prescribed as sapropterin dihydrochloride). Using BH4, these BH4 responsive PKU patients are able to decrease their blood Phe concentrations and/or liberalize dietary treatment [15,16]. In recent years, a lot of knowledge has been gained on BH4 responsiveness-testing, genotype-phenotype correlation and treatment efficacy. However, data on items like the effect of BH4 on Phe and Tyr variation is still limited. There are some data indicating that BH4 does not only decrease blood Phe concentrations (in BH4 responsive PKU patients) but also results in less variability in blood Phe concentrations [17–19]. However, these data were retrospectively collected. Furthermore, there are no data on the effect of BH4 on variability in blood Tyr concentrations. Therefore, the variation of blood Phe and Tyr concentrations under BH4 is largely unknown. For that reason, this study investigated the effects of BH4 on diurnal and day-to-day variations of blood Phe and Tyr concentrations in proven BH4 responsive PKU patients.

2. Methods

This multicenter cross-over trial was conducted between 2014 and 2018 in the University Medical Center Groningen and the Radboudumc Nijmegen, the Netherlands. PKU patients treated with BH4, aged 4 to 12 years and ≥ 18 years under good metabolic control (defined as 67% of blood Phe levels within target range during the last year) were invited to participate in the study. BH4 responsiveness was proven in a treatment trial in all BH4 treated PKU patients as reported by Anjema et al. [20]. During this treatment trial of several months, patients were considered to be true-responders when they were able to decrease their Phe levels with ≥30% compared to baseline and/or increase their natural protein tolerance with ≥50% or 4 g. The study was approved by the medical ethical committee of the University Medical Center Groningen, and all participants signed a written informed consent.

2.1. Study periods

The study consisted of two study periods: a strict diet period without BH4 (regimen 1) and patient's usual treatment with BH4 (regimen 2). Each patient underwent both study regimens. The sequence was randomized by a computer program. During regimen 1, patients returned to their former protein restricted diet with Phe-free protein substitute. The amount of the protein substitute was prescribed according to national guidelines, based on age, bodyweight and the amount of natural protein intake [21].

2.2. Stabilization period

Both study periods consisted of a stabilization and a testing period. A stabilization period of 2–4 weeks preceded the 8 testing days to ensure that Phe concentrations were within target levels during each treatment regimen (0–12 years: 120–360 μmol/l, ≥12 years 120–600 μmol/l [22,23]). During this stabilization period blood samples were collected every other day, and patients could continue to the test period when 67% of the Phe concentrations were within target range with a minimum of 8 blood samples.

2.3. Test period

During the 8 testing days, blood Phe and Tyr were measured four times daily (7–8 am, 12–1 pm, 5–6 pm and bedtime) during the first two days, and once per day (7–8 am) on the 6 days thereafter. The morning samples (7–8 am) were under fasting conditions. The samples at noon and dinner time were taken before the meal.

In addition, patients filled out detailed food records of testing days 1 and 2 and the preceding day, for analyzing total protein, natural protein, energy, and meal (including protein substitute) frequency and timing. The food records were analyzed in the program Madows, version VWB.2.2.16R1 (NEVO 2016).

2.4. Analysis of Phe and Tyr in dried blood spots

All blood samples were retrieved by finger puncture on dried blood spots (DBS). The blood samples from both participating centers were taken on the same filtration paper (Grade 179 g/m2, Sartorius Stedim DEU). The DBS samples were analyzed by Liquid chromatography-tandem mass spectrometry (LC-MS/MS) in the laboratory of Metabolic Diseases in the University Medical Center Groningen [24].

2.5. Statistical analysis

Normality of continuous data was assessed using histograms, QQ-plots and the Shapiro-Wilk test. The primary outcome measures were Phe and Tyr variation. Absolute variations of DBS Phe, Tyr and Phe/Tyr ratios were expressed as standard deviations (SD) in μmol/l. Relative variations were expressed as coefficient of variation (CV). The CV corrects for differences in the mean. For diurnal variation the SD of the average and the CV of all 9 samples from the first 48 h was calculated for each single patient. For the day-to-day variation the SD of the average and CV from the fasting morning samples for 8 days was calculated for each single patient. Comparison of the SD and CV were tested with the non-parametric Sign Rank test. Comparison of the other data was tested with the non-parametric Wilcoxon Sign Rank test. Mixed modeling was considered not to be appropriate due to the number and nature of the collected data. A two-tailed p-value <0.05 was assumed statistically significant. All statistical analyses were performed using IBM SPSS Statistics version 22.

3. Results

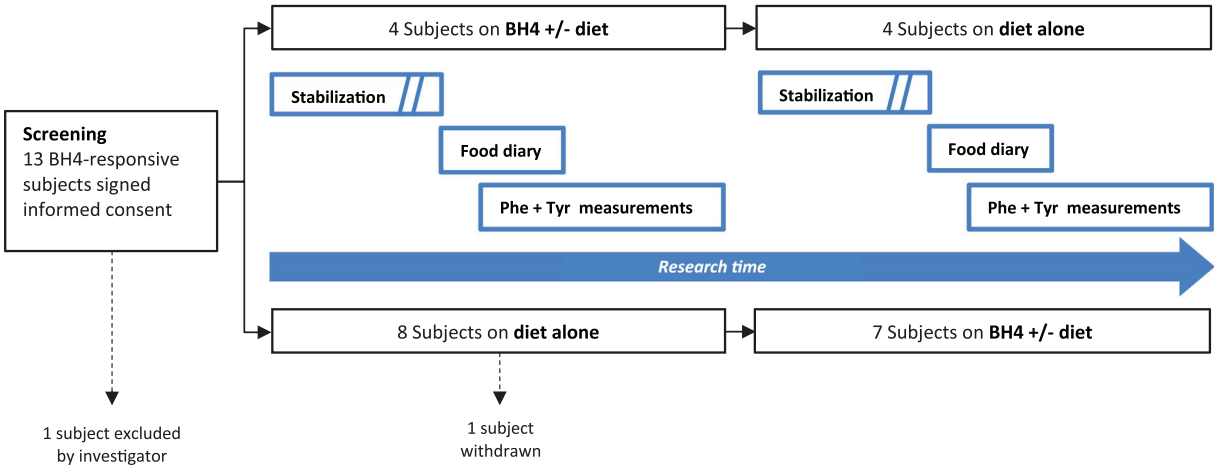
A total of 13 PKU patients were included in the study (Fig. 1). One child withdrew during the first stabilization phase as it was too difficult given the patient's circumstances to follow the strict diet without BH4. One adult was excluded from the study because of non-compliance with study procedures in the judgment of the investigator.

3.1. Patient characteristics

Eleven patients completed the study, of whom four were adults (patient 8–11). The median age of the children and adults were 8.9 (range 5.1–11.6) years and 40.7 (range 31.9–41.8) years, respectively. Most participants were female ($n = 7$). The median dose of BH4 was 18 mg/kg (2–21 mg/kg). BH4 was taken twice daily by one patient (patient 6). Five patients took BH4 in evening, four patients took BH4 in the morning and one during lunch. Five patients (1 adult) used a mild natural protein-restricted diet necessitating protein substitute when using BH4, while the other six patients (3 adults) followed a liberalized diet without protein substitute when using BH4. During dietary treatment without BH4, all patients distributed their protein substitute over three times a day except for one adult who distributed the protein substitute twice daily (patient 8). Dietary data are presented in Table 1. Genotype and phenotype characteristics of the patients are presented in Table 2.

3.2. Phe and Tyr concentrations and Phe/Tyr ratio

Fasting Phe, Tyr and Phe/Tyr ratio levels are presented in Table 1. Fasting Tyr concentrations tended to be higher during BH4 treatment without reaching statistical significance ($p = 0.09$).



Randomized cross-over trial. Participants underwent both study regimens of which the sequence was randomized. Each test-period was preceded by a stabilization phase to ensure that Phe concentrations were within target levels.

Fig. 1. Study flowchart. Randomized cross-over trial. Participants underwent both study regimens of which the sequence was randomized. Each test-period was preceded by a stabilization phase to ensure that Phe concentrations were within target levels.

3.3. Phe variation

For both diurnal and day-to-day Phe variations, no significant differences were found between the groups (Table 1, Fig 2ab). Individual data is shown in supplemental Fig. 1–4.

3.4. Tyr variation

Day-to-day Tyr variations did not differ significantly between BH4 and dietary treatment (Table 1, Fig. 2 cd). However, diurnal Tyr SD and CV were significantly smaller during BH4 treatment ($p = 0.01$). Individual data is shown in supplemental Fig. 1–4.

3.5. Phe/Tyr ratio

Consequently to the combined findings of Phe and Tyr, the SD and CV of diurnal Phe/Tyr ratios were smaller during BH4 treatment, however only CV was statistically significant ($p = 0.01$, Table 1). The variation of the day-to-day ratios did not differ significantly between dietary and BH4 treatment.

4. Discussion

This randomized cross-over trial is the first study that thoroughly investigated the diurnal and day-to-day Phe and Tyr variability, during treatment with and without BH4. The first main finding is that the data did not reveal a statistical difference in Phe fluctuations. On the other hand, the data did show a statistically significant lower variation of diurnal Tyr and diurnal Phe/Tyr ratio variation during BH4 treatment. A third finding was that patients tended to have higher fasting Tyr concentrations during BH4 treatment.

Before discussing our findings in more detail, some limitations of this study need to be addressed. One of the main limitations of this study is the low number of participants. Several potential candidates declined the study invitation as they did not want to stop the BH4 treatment. Secondly, the timing of BH4 intake was not controlled, as this was according to usual care of the patients. Since patients were their own controls, we do not expect a major influence. Another limitation could be the use of DBS of which accuracy results have recently been discussed [25]. However, at Phe concentrations >50 umol/l, recent studies on DBS and plasma Phe and Tyr in our laboratory showed no bias [24]. Given the importance of both the neurotransmitters dopamine and serotonin in the pathogenesis of PKU brain dysfunction we would have liked to also study the day-to-day and diurnal variation of tryptophan (Trp) concentrations and Phe/Trp ratio, but as yet we cannot measure Trp in DBS in a trustworthy way [26]. Another limitation is that 2 of the participants (number 3 and 4) later turned out to have DNAJC12 deficiency, after the data was collected [27]. Before and after this diagnosis they were treated as PKU patients, not necessitating other drugs. In theory, patients with DNAJC12 deficiency may be more responsive to BH4 than patients with PAH deficiency, possibly influencing Phe and Tyr variability. However, leaving these patients out did not influence our results for either Phe, Tyr or Phe/Tyr ratio variations (Supplemental Table 1). The final limitation of this study is that BH4 responsiveness was defined according to a milder definition for true-BH4 responsiveness (natural protein increase of ≥50% or 4 g) compared to the definition of the European PKU Guidelines (natural protein increase of ≥100%) [22,23]. However, when we returned to the data, there was only one borderline case (Table 2), the rest of the patients were able to increase their natural protein intake with 100% or more.

Regarding the Phe results, we found no statistical difference for diurnal and day-to-day Phe variation. As we initially expected to find a difference in Phe variation, we examined our data for a potential confounding effect of the level of responsiveness by separating the data according to the degree of Phe reduction during their initial 48-h BH4 loading tests (<70% Phe reduction in 6 patients versus >70% Phe reduction in 5 patients). No confounding effect was found, possibly due to small samples. Our Phe results are in concordance with the findings of Thiele et al., but are in conflict with the findings of Burton et al. and Leuret et al. [17,18,28]. Comparing these studies is difficult as Phe variation is expressed differently, patients groups differ and studies were retrospectively not knowing what happened in real life (Table 3). SD values cannot be easily compared as they do not take differences in their respective means into account. However, looking at Phe variation during BH4 treatment, the variability seems to be in the same range as Thiele et al. and Leuret et al. The variability of Burton et al. appears to be much higher. The reason for this difference is not

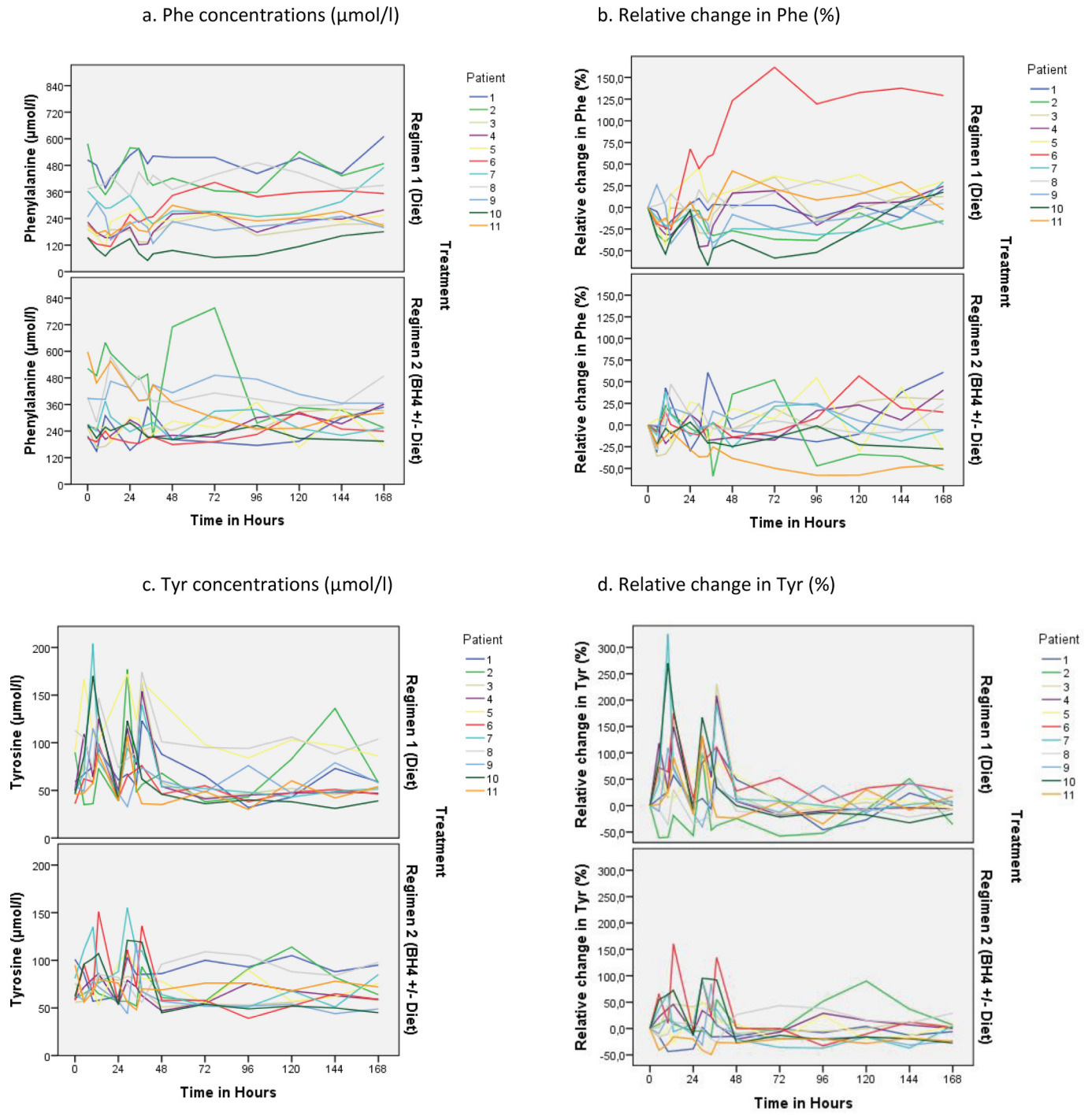


Fig. 2. Absolute and relative blood Phe and Tyr concentrations. All diurnal and fasting data collected during the test period are included in this figure. Fig. 2a shows all diurnal and fasting absolute Phe concentrations of individual patients with dietary only treatment (upper box) compared to BH4 (+/− diet) treatment (lower box). Fig. 2b shows the relative change in Phe, calculated from baseline ($t = 0$) in individual patients. In Figure, 2c all absolute diurnal and fasting Tyr concentrations are presented of individual patients. Fig. 2d shows the relative change in Tyr, calculated from baseline ($t = 0$) in individual patients.

clear. Phe variation during dietary treatment, seems to be lower in our study than reported previously (Table 3). These findings might be explained by the design of this study. Patients were aware they were studied and may have really aimed for Phe levels within target ranges.

For diurnal Tyr variation, we found statistically significant smaller variations during BH4 treatment. This could perhaps be explained by using less protein substitute in combination with an increased synthesis from Phe. From former studies with patients on dietary treatment only, we know that Tyr concentrations are usually low but increase immediately after the intake of protein substitute and can even reach concentrations over 200 µmol/l [29,30]. If less Tyr-enriched protein substitute is used throughout the day, the peak level and, consequently, the fluctuation of blood Tyr will be lower. Our individual Tyr results (Supplemental Table 2 and Supplemental Fig. 1–2) show not only lower Tyr fluctuations during the day in patients without protein substitute, but also in some patients using lower amounts of protein substitute during BH4 treatment (patient 1, 7, 10). When we analyzed groups separately according to their use of protein substitute, there was only a

Table 1
Comparison of regimen 1 and regimen 2.

	Regimen 1 (Diet)	Regimen 2 (BH4 +/− diet)
Fasting Phe (μmol/l)*	243 (124–509)	280 (222–468)
Fasting Tyr (μmol/l)*	49.1 (39.5–96.9)	67.5 (51.4–92.3) $
Fasting Phe/Tyr ratio*	5.0 (2.6–9.1)	4.5 (2.5–7.8)
SD diurnal Phe (0-48 h)	47.6 (28.3–88.3)	44.0 (18.9–137.9)
CV diurnal Phe (0-48 h)	0.202 (0.09–0.39)	0.179 (0.08–0.34)
SD fasting Phe (day 1–8)	43.4 (22.5–86.4)	48.9 (34.5–202.0)
CV fasting Phe (day 1–8)	0.141 (0.10–0.35)	0.186 (0.11–0.43)
SD diurnal Tyr (0-48 h)	34.2 (19.5–51.3)	17.6 (12.0–36.4) $$
CV diurnal Tyr (0-48 h)	0.432 (0.26–0.64)	0.213 (0.17–0.41) $$
SD fasting Tyr (day 1–8)	7.4 (3.5–33.3)	9.0 (5.6–20.7)
CV fasting Tyr (day 1–8)	0.125 (0.07–0.48)	0.148 (0.11–0.28)
SD diurnal Phe/Tyr ratio (0-48 h)	1.9 (0.5–3.4)	1.0 (0.6–2.5)
CV diurnal Phe/Tyr ratio (0-48 h)	0.504 (0.26–0.78)	0.269 (0.15–0.49) $$
SD fasting Phe/Tyr ratio (day 1–8)	1.3 (0.3–3.2)	1.0 (0.5–4.2)
CV fasting Phe/Tyr ratio (day 1–8)	0.199 (0.13–0.41)	0.231 (0.11–0.59)
Natural protein intake (g/kg/day)	0.3 (0.1–0.5)	0.9 (0.5–1.7) $$$
Protein equivalent intake of protein substitute (g/kg/day)	1.0 (0.4–1.6)	0 (0–1.0) $$$
Energy intake (kcal)	2177 (1312–3459)	2007 (1006–2301)

$ P = 0.09 (Related-Samples Wilcoxon Signed Rank Test), $$ Statistically significant $P < 0.05$ (Sign Rank test), $$$ Statistically significant P < 0.05 (Related-Samples Wilcoxon Signed Rank Test). Data are expressed as median (min-max). Phe: phenylalanine, Tyr: Tyrosine, *Median of individual means was calculated.

significant difference between dietary treatment and BH4 treatment for the ones not using protein substitute ($n = 6, p = 0.03$). However, as the patient numbers are low, these results should be interpreted with caution.

Regarding day-to-day Tyr variations we found no statistically difference between the groups, although fasting Tyr levels tended to be higher during BH4 treatment, without reaching statistical significance ($p = 0.09$). Leaving out the patients with DNAJC12 deficiency, being a child or adult, and the use of protein substitutes did not seem to influence this tendency (Table 4, Supplemental Table 2). The tendency of increased fasting Tyr levels can probably be explained by the chaperone effect of BH4 positively influencing the tyrosine synthesis. In general, BH4 responsive patients are able to increase the dietary Phe intake, while maintaining the blood Phe levels in the target range [31]. The proportion of Phe converted to Tyr by PAH is higher, resulting in lower Phe/Tyr ratios. This may have a positive effect on Tyr transport into the brain. Although there is no data available regarding the relationship between variation of Phe/Tyr ratios and neurocognitive outcome, Phe/Tyr ratios negatively correlate to aspects of cognitive functioning, executive functioning and depressive symptoms [7–10]. Previously, Tansek et al. compared plasma blood levels of 2 years before and 2 years during BH4 treatment in a group of 9 patients of which 7 discontinued their protein substitute. They found no significant change in median Phe and Tyr levels, but lower Phe/Tyr ratios during BH4 treatment [32]. These findings support some kind of effect to Phe/Tyr ratio. However, our data did not find a significant decrease in fasting Phe/Tyr ratio and variation during BH4 treatment.

In order to be able to draw more robust conclusions, further research is necessary with a higher number of patients, ideally in a multicenter trial.

5. Conclusion

BH4 treatment did not seem to influence Phe variation in this small cohort of BH4 responsive PKU patients. Diurnal Tyr and diurnal Phe/Tyr ratio variation were smaller during BH4 treatment, which might be explained by less use of the Tyr enriched protein substitute and/or the chaperone effect of BH4. Such an effect could also be responsible for the tendency of an increase of the fasting Tyr levels. Therefore, BH4 treatment and the following liberalization of the diet influences Tyr synthesis and variability rather than Phe concentrations itself, and by that support improvement of Phe/Tyr ratios that could help to improve outcome of PKU patients.

Table 2
Genotype and phenotype characteristics of participants.

	Mutation 1	Mutation 2	GPV	Maximum Phe Decrease % 48 h BH4 test	Natural protein increase %[1]
1	p.Y414C c.1241A > G	IVS12 + 1G > A c.1315 + 1G > A	5.1 (mPKU)	83	200–300
2	p.Y414C c.1241A > G	IVS12 + 1G > A c.1315 + 1G > A	5.1 (mPKU)	74	375
3	c.85delC DNAJC12	c.596G > T DNAJC12	/	73	225
4	c.85delC DNAJC12	c.596G > T DNAJC12	/	68	325–450
5	IVS12 + 1G > A c.1315 + 1G > A	p.P211T c.631C > A	10 (MHP)	81	875
6	p.G272* c.814G > T	p.Y414C c.1241A > G	5.1 (mPKU)	84	150–200
7	p.L367Pfs*27 c1099dupC	p.Y414C c.1241A > G	Not reported	53	225
8	p.A104D c.311C > A	p.R241C c.721C > T	5.5 (mPKU)	42	125
9	p.I174N c.521 T > A	p.P211T c.631C > A	4.2 (mPKU)	67	1000–1275
10	p.A104D c.311C > A	IVS12 + 1G > A c.1315 + 1G > A	2.6 (cPKU)	69	50–100
11	p.V190A c.569 T > C	p.R158Q c.473G > A	7.1 (mHPA)	53	125

GPV: Genotypic Phenotype Value. Definition of GPV: 0–2.7 is classic PKU, 2.8–6.6 is mild PKU and 6.7–10.0 is mild HPA (www.biopku.org; last accessed 17-02-2021).
[1] natural protein increase was calculated from a food diary before and after BH4 treatment trial. When these data were complete a combination of prescribed diet and calculated intake was presented.

Table 3
Comparison of Phe levels with other studies.

	Study characteristics					Mean Phe ± SD (μmol/l)		Within subject variation (μmol/l)	
	Design	N	Age	BH4 dose mg/kg/day	Follow-up on BH4	Before BH4	With BH4	Before BH4	With BH4
Burton 2010	Retro-spective	37	mean 12.6 years (min-max 1.5–32.0)	mean 20.1	mean 19 months (min-max 12–31)	404 ± 254[1,2]	312 ± 229[1,2]	417 ± 159[1,3 4]	290 ± 132[1,3,4]
Leuret 2012	Retro-spective	8	median 4.6 months (min-max 0.3–35)	median 20 (min-max 8–24)	median 23 months (min-max 7–80)	352 ± 85 331 ± 76 (only 1 year)	254 ± 64 243 ± 75 (year 1 only)	130 ± 21[5]	93 ± 27[5]
Thiele 2015	Retro-spective	8	mean 10.5 years (min-max 6.0–16.6)	median 18 (min-max 10–19)	3 years	262.2 ± 129.4	337.1 ± 129.6 (year 1) 382.7 ± 148.1 (year 2) 371.7 ± 119.8 (year 3)	77.9 ± 35.9[6]	87.0 ± 24.7[6] 84.4 ± 24.9[6] 80.6 ± 29.3[6]
Our study	Cross-over trial	11	median 11.4 years (min-max 5.1–41.8)	median 18 (2−21)	8 days	299 ± 119 (fasting) 278 ± 122 (all)	311 ± 85 (fasting) 311 ± 93 (all)	48 ± 22[6,7] 55 ± 21[6,7]	70 ± 49[6,7] 68 ± 41[6,7]

N = number of patients. [1]Before BH4 6.67 mg/dl ± 4.20 and on BH4 5.16 mg/dl ± 3.78; [2]1mg /dl Phe ≈ 60.5 μmol/l Phe; [3]Before BH4 6.897 ± 2.62 and on BH4 4.799 ± 2.19; [4] Linear mixed modeling was utilized to estimate variances of phenylalanine before and after starting sapropterin therapy; [5]The variance of Phe levels before and during BH4 treatment was estimated from the standard deviations for Phe levels of each patient during the corresponding periods. [6]Phe variability was evaluated by averaging the SDs from the single patients. [7]To be consistent with the other papers, the mean was reported here instead of the median as in Table 1.

Table 4
Fasting Tyr levels according to mutation, use of protein substitute and age.

	Regimen 1 Diet	Regimen 2 BH4 (+/− diet)
All participants (n = 11)	49.1 (39.5–96.9)	67.5 (51.4–92.3)
Only PAH deficient patients (n = 9)*	58.8 (39.5–96.9)	73.3 (51.4–92.3)
Using protein substitute during BH4 (n = 5)	49.1 (39.5–69.4)	67.5 (51.4–91.3)
Not using protein substitute during BH4 (n = 6)	53.5 (44.4–96.9)	66.8 (53.6–92.3)
Children (n = 7)	49.1 (45.1–93.2)	67.5 (55.5–91.3)
Adults (n = 4)	51.6 (39.5–96.9)	64.9 (51.4–92.3)

Data are expressed as median (min-max), the median of individual means was calculated. Tyr: Tyrosine, *Two patients with DNAJC12 deficiency were excluded.

Contributions of individual authors

AMJ van Wegberg collected the data, analyzed the data, interpreted the results and was the lead writer of the manuscript. RAF Evers interpreted the results and was the second lead writer of the manuscript. FJ van Spronsen designed the study, interpreted the results, co-wrote the manuscript and supervised the project. E van Dam designed the study, collected the data and co-wrote the manuscript. JGM Burgerhof analyzed the data, interpreted the results and co-wrote the manuscript. MR Heiner-Fokkema was responsible for DBS Phe and Tyr analysis, interpreted the results and co-wrote the manuscript. MC de Vries and MCH Janssen co-wrote and approved the manuscript.

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Declaration of competing interest

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E van Dam was a member of a scientific advisory board of Merck-Serono/Biomarin.

JGM Burgerhof, MC de Vries, MCH Janssen and MR Heiner-Fokkema declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymgme.2021.02.008.

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