Chaperone-Like Therapy with Tetrahydrobiopterin in Clinical Trials for Phenylketonuria: Is Genotype a Predictor of Response?

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Abstract Prospectively enrolled phenylketonuria patients ($n = 485$) participated in an international Phase II clinical trial to identify the prevalence of a therapeutic response to daily doses of sapropterin dihydrochloride (sapropterin, KUVAN®). Responsive patients were then enrolled in two subsequent Phase III clinical trials to examine safety, ability to

reduce blood Phenylalanine levels, dosage (5–20 mg/kg/day) and response, and bioavailability of sapropterin. We combined phenotypic findings in the Phase II and III clinical trials to classify study-related responsiveness associated with specific alleles and genotypes identified in the patients. We found that 17% of patients showed a response to sapropterin. The patients harbored 245 different genotypes derived from 122 different alleles, among which ten alleles were newly discovered. Only 16.3% of the genotypes clearly conferred a sapropterin-responsive phenotype. Among the different *PAH* alleles, only 5% conferred a responsive phenotype. The responsive alleles were largely but not solely missense mutations known to or likely to cause misfolding of the *PAH* subunit. However, the metabolic response was not robustly predictable from the *PAH* genotypes, based on the study design adopted for these clinical trials, and accordingly it seems prudent to test each person for this phenotype with a standardized protocol.

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Introduction

Phenylketonuria (PKU) is an autosomal recessive disorder affecting L-phenylalanine oxidation (OMIM #261600). In PKU, mutations in the phenylalanine hydroxylase (*PAH*) gene impair function of the hepatic enzyme (*PAH*; E.C. 1.14.16.1). Persistent hyperphenylalaninemia (HPA) has a neurotoxic effect resulting in mental retardation. The PKU/HPA phenotype can be ameliorated by restricting the dietary supply of the essential amino acid, phenylalanine (Phe) (Donlon et al. 2010).

Prevention of this form of mental retardation in the population (prevalence $\sim 10^{-4}$) begins with newborn screening for early detection of PKU/HPA followed by early and continuing treatment with a diet low in Phe. This

regimen became an epitome of human biochemical genetics (Donlon et al. 2010; Scriver 2007; Scriver and Clow 1980a, b). However, dietary treatment of PKU is difficult to maintain (Burgard et al. 1999) and is easily compromised, with three out of four PKU patients becoming noncompliant by late adolescence. Accordingly, discovery of a subset of PKU/HPA patients (Kure et al. 1999), who respond to daily pharmacological oral doses of tetrahydrobiopterin (BH₄), the catalytic cofactor for the PAH enzyme, has attracted interest (Bernegger and Blau 2002; Panel NIOHCD 2001; Phenylketonuria MRCWPO 1993). For such patients, treatment with oral BH₄ in pharmacological doses improves enzyme activity, enhances Phe oxidation, and leads to reduction in blood Phe levels (Burton et al. 2010; Elsas et al. 2011; Levy et al. 2007; Muntau et al. 2002).

The potential for a simple and effective oral therapy that improves control of blood Phe levels in PKU/HPA led to the development of sapropterin, a synthetic 6R-epimer of BH₄ [(containing 6R)-L-erythro-5,6,7,8 tetrahydrobiopterin]. Safety and efficacy of sapropterin have been demonstrated in various studies including Phase II and III clinical trials (Burton et al. 2007; Lee et al. 2008; Levy et al. 2007; Trefz et al. 2009a), and other studies in selected populations have offered estimates of the BH₄-responsive phenotype (Blau and Trefz 2002; Dobrowolski et al. 2009a; Dobrowolski et al. 2011; Karacić et al. 2009; Lindner et al. 2001; Muntau et al. 2002; Spaapen and Rubio-Gozalbo 2003; Trefz et al. 2001; Trefz et al. 2010; Weglage et al. 2002). Here, we offer an estimate (17%) of the sapropterin-responsive phenotype in a large multi-national clinical study. *PAH* locus genotypes were also obtained in this study and did not reveal a robust genotype–phenotype correlation. Our findings have relevance for the management of PKU patients as well as indicating a need for improved protocol design to generate more comprehensive data sets in related clinical trials.

Methods

Clinical Trials Study Design, Measure of Efficacy and Analysis

Recruitment criteria and patient selection, test product (sapropterin dihydrochloride-available under the trade name KUVAN[®]) description, dose and mode of administration, study design and treatment schedules as well as method of serum [Phe] analysis were described in the clinical trials protocols (Burton et al. 2007; Lee et al. 2008; Levy et al. 2007). The three clinical trials (designated as PKU-001, PKU-003 and PKU-004) are registered with ClinicalTrials.gov.: NCT00104260, NCT00104247, NCT00225615. As part of the PKU-004 protocol, a population pharmacokinetics

analysis was performed, and some subjects participated in an adjunct open-label sub-study to evaluate the effect of once-daily dosing of sapropterin on Phe levels over a 24-h period (not reported by Lee et al. 2008).

The primary efficacy outcome variable was the metabolic response to sapropterin or placebo. It was defined as the percent change in blood Phe levels at a pre-determined observation point of treatment compared with baseline, prior to sapropterin treatment (for PKU-003, the baseline Phe level used was a calculated level using several pre-dose levels).

In screening study PKU-001, subjects who had a reduction in blood Phe level of at least 30% in study were defined as responders. If the observed response rate was 30%, the 95% confidence interval (CI) was expected to be 26–35% (Burton et al. 2007).

In the randomized placebo-controlled study PKU-003, the mean change in blood Phe levels, for 6 weeks, in each group was compared using an analysis of covariance model, with baseline Phe levels and treatment as the only covariates (Levy et al. 2007). The models utilized a last observation carried forward (LOCF) imputation approach to address missing data. The mean change in weekly blood Phe levels during the 6 weeks of treatment was evaluated using a longitudinal model. The proportion of subjects who had blood [Phe] ≤ 600 μ M at week 6 was assessed using a Fisher's Exact Test.

In the safety and efficacy study PKU-004, blood Phe values for each subject, corresponding to the end of each 2-week dosing period (5, 20, and 10 mg/kg/day) over 6 weeks, were used to estimate the effect of dose on blood Phe concentration. Long-term persistence of response to sapropterin was measured up to week 22. The short-term effect of once-a-day dosing of sapropterin was measured by comparing blood Phe concentrations at set intervals over a 24-h period, in the PKU-004 sub-study (Lee et al. 2008). Analysis of variance for crossover designs was used to estimate average within-person changes in blood Phe concentration for the three dose levels. False positives, incorrectly assigned as responders in PKU-001, were identified using these findings.

PAH Gene Mutation Analysis

Informed consent was received for mutation analysis (conducted on DNA from blood samples collected in protocol PKU-001) or for the use of data obtained from archival clinical records documenting previous mutation analysis. Coded samples were submitted to the Molecular Genetics Laboratory of the Montreal Children's Hospital/McGill University Health Centre for *PAH* gene sequencing. Genomic DNA was isolated from EDTA-blood from each participant using a blood kit (Qiagen). Polymerase chain reaction (PCR) amplification was performed on genomic

DNA for all exons and adjacent intronic splice regions. Amplicons were sequenced using BigDye terminator cycle sequencing kit (version 3.1) and run on the ABI 3100 Genetic Analyzer (Applied Biosystems). Sequence changes were detected using Mutation Explorer software (Version 2.41, SoftGenetics).

Primary Data

All data collected on participants, including site of study, race-ethnicity, mutation-genotype identification and measure of serum [Phe] throughout each of the clinical trials that the subject participated in, were recorded.

Allele or Genotype Relationships with Phenotype

PAH alleles were classified as (a) Responsive: when the mutation present in a homozygous and/or functionally hemizygous genotype was associated with responsiveness in PKU-001, in PKU-003 (if sapropterin was administered) and PKU-004. If a mutation occurring in subjects with homozygous genotypes was always associated with responsiveness, and yet some variation existed with hemizygous subjects carrying the mutation in question, this mutation was still considered responsive; (b) Ambiguously Responsive: when the mutation was associated with both responsiveness and unresponsiveness in different subjects with homozygous or equivalently hemizygous genotypes. A final verdict on responsiveness was not possible in individuals carrying mutations that did not fulfill the criteria of homozygosity or functional hemizygosity; (c) Unresponsive: when the mutation present in a homozygous and/or functionally hemizygous genotype was not associated with responsiveness in the clinical protocol PKU-001, or showed responsiveness in PKU-001 but not in PKU-003 (if sapropterin was administered) or PKU-004. Mutations that did not belong to any of the above categories and/or occurred only in a genotype carrying three mutations remained unclassified.

Genotypes were classified in a similar manner as either (a) Responsive: when they demonstrated responsiveness in all three clinical protocols; (b) Ambiguously responsive: when the same genotype was associated with a variation in responsiveness between subjects throughout the protocols; or (c) Unresponsive: when they were not associated with responsiveness in clinical protocol PKU-001 or demonstrated responsiveness in PKU-001 but not in PKU-003 (if sapropterin was administered) and/or PKU-004. Note: (a) If a genotype proved to be associated with responsiveness in PKU-001, and on subsequent evaluation the subject displayed a blood [Phe] reduction of $\geq 27\%$ in PKU-003 and/or PKU-004, the genotype was considered responsive. (b) Genotypes of participants that proved to be responsive or

ambiguously responsive only in PKU-001, and did not participate in further studies or participated only in the placebo series in PKU-003 and/or dropped out of the extended study (PKU-004) early before generating further comprehensive data, were accordingly assigned to the separate groups titled responsive or ambiguously responsive according to PKU-001 findings only. (c) One subject who participated in PKU-001, 003, and 004 had very low Phe levels that prevented proper interpretation of the change with sapropterin treatment and adequate classification of responsiveness. Although this subject was still classified as unresponsive, this observation was also identified in the results as an exception.

Results

Participants

Four hundred and eighty-five subjects completed protocol PKU-001 of which the majority (>95%) were Caucasian; the remainder were of Hispanic, Arabic, Middle Eastern, African and Asian Pacific origins; 88 of the putative sapropterin-responsive group discovered in protocol PKU-001 then participated in PKU-003. Forty-seven of these subjects were assigned to the placebo group, and 41 to the sapropterin treatment group; 87 of these subjects actually completed the protocol. Eighty subjects from the PKU-003 cohort continued on to PKU-004; one noncompliant subject withdrew; 12 subjects joined the PKU-004 subgroup in which 24-h response to a single dose of sapropterin was evaluated.

Primary Data

The complete set of data for protocols PKU-001, 003, 004 and 004 (sub-study) were recorded and are available online in Supplementary Table 1.

Clinical Results

Ninety-six subjects (19.8% of cohort) from Protocol PKU-001 were responsive to sapropterin according to the criteria ($\geq 30\%$ decline in the level of blood Phe).

The randomized, double-blind, placebo-controlled trial (PKU-003) revealed that sapropterin was the effective therapeutic agent and the therapeutic effect was persistent, week by week.

Protocol PKU-004 showed a dosage response to sapropterin at three concentrations (5, 10, and 20 mg/kg/day) (Supplementary Table 2). There was an inverse relationship between drug dosage and blood Phe concentration. The average reduction of Phe levels for responders over the doses 5, 10, and 20 mg/kg/day was 16% ($-156 \mu\text{M}$), 28% ($-265 \mu\text{M}$), and 39% ($-329 \mu\text{M}$), respectively.

Supplementary Table 2 describes the responsive and unresponsive groups of patients participating in PKU-004. Of the 80 patients that participated in PKU-004, 69 responded to sapropterin treatment during the long-term protocol; the remaining 11 patients were identified as nonresponders; however, they were originally and falsely identified as responders in PKU-001. Thus, a more accurate estimation of the overall responders participating in these clinical trials is 17%.

The PKU-004 sub-study, which was designed to measure daily fluctuations in the middle of the long-term treatment, demonstrated stable blood Phe levels throughout the day. The fluctuations were minimal with an initial drop of approximately 16% 8 h postdosing and a stable and gradual rise in Phe levels by 16 h postdosing (12 midnight).

Mutation Analysis and Genotype–Phenotype Relationships

Responsiveness, based on data collected from PKU-004, identified false positives in patients participating in post-PKU-001 protocols. These findings were incorporated into the mutation and genotype assignments presented in Tables 1 and 2.

Among those enrolled in Protocol PKU-001, we obtained complete *PAH* genotypes (both alleles) in 424 subjects and partial genotypes (one allele) in 34 subjects; two patients carried three mutations. Among the fully genotyped subjects, only 61 (14.4%) harbored a homozygous genotype, while 363 subjects (85.6%) were compound heterozygotes.

We detected 122 different *PAH* mutant alleles (Table 1) harbored in 245 different genotypes. We classified the alleles as sapropterin-responsive ($n = 6$), ambiguous in their response (different response in different patients harboring the same allele) ($n = 10$), or unresponsive ($n = 73$) (Table 1). We could not classify 33 different alleles due to lack of homozygosity or pairing with a null allele. We mapped the point mutations and the associated state of responsiveness to sapropterin on to the molecular model of the *PAH* subunit (Fig. 1).

The most prevalent allele, identified in 145 of 458 completely or partially genotyped patients, was the null allele c.1222C>T (p.R408W). Ten alleles (Table 3) had not been previously reported in the literature or catalogued in the *PAH* gene mutation database (<http://www.pahdb.mcgill.ca>).

Among the 245 different genotypes (Table 2), 40 were responsive to sapropterin, 26 were ambiguously responsive and 179 were unresponsive.

Discussion

Successful dietary treatment of PKU/HPA due to deficient *PAH* enzyme function is now seen as a landmark and

paradigm shift in our overall view of human biochemical genetics (Donlon et al. 2010; Scriver 2007). The enzyme deficiency is now known to reflect a large array of mutant alleles and genotypes at the human *PAH* locus. The associated metabolic phenotype in some patients is ameliorated in response to pharmacologic oral doses of a synthetic 6*R* epimer of tetrahydrobiopterin (6*R*-BH₄). The epimer is sapropterin dihydrochloride, marketed under the name KUVAN®. Many benefits would accrue to the patient if oral sapropterin could be integrated with dietary control (Blau et al. 2009); hence, our interest in knowing the prevalence of sapropterin-responsive PKU/HPA, and the likelihood of predicting a responsive or unresponsive phenotype from the associated genotype.

It is not surprising that many new reports, not all cited here, have appeared on this and related topics such as: the pharmacokinetic features of the oral agent; a defined metabolic response to the agent under a standardized protocol; whether dietary tolerance of Phe is improved in the responsive patient; and whether the agent is safe for long-term use and without adverse effects. However, it is already apparent that not all PKU/HPA patients can or will respond to oral sapropterin therapy. The responders are to some extent dependent on drug dosage, they tend to have more benign metabolic phenotypes in the untreated state, they are not equally distributed in human populations (Guldborg et al. 1998; Kayaalp et al. 1997; Lindner et al. 2003), and the distribution reflects that of mutant genotypes in the population. Moreover, the beneficial response to sapropterin is a complex person-specific process (Trefz et al. 2009b).

We examined responsiveness to sapropterin in a large international randomly selected group of PKU patients. Efficacy was measured by the decline in blood Phe concentration in the PKU patients after receiving sapropterin. Our analysis used the combined results of the three different components of the clinical trials, and draws conclusion on genotype-phenotype relationships based solely on the entire population that participated. It examines the associations of responsiveness with the *PAH* mutation and genotype.

Each protocol in the sapropterin project served a different purpose: Protocol PKU-001 yielded the apparent prevalence of sapropterin responsiveness in the sample (Burton et al. 2007); protocol PKU-003, confirmed that sapropterin is an agent conferring responsiveness (Levy et al. 2007); protocol PKU-004 revealed a dose-related response to sapropterin (Lee et al. 2008), showing that once-daily dosing is an effective regimen and provided evidence of false positive classifications in protocol PKU-001. All data for all subjects and all components of these clinical trials are available in Supplementary Table 1.

BH₄ is the catalytic cofactor for several enzymes, including *PAH*, and mutations exist that impair the enzymes

Table 1 Classification of mutant *PAH* alleles, in the present study, grouped by the phenotypic response to sapropterin (ordered 5'–3')

<i>Responsive</i>			
p.I65T	p.Q226H	p.A309D	
p.A104D	p.R241H	p.Y414C	
<i>Ambiguously responsive</i>			
p.F39L	p.G218V	p.A345S	c.1315+1G>A
p.L48S	p.R261Q	p.L348V	
p.R68S	p.A309V	c.1066-3C>T	
<i>Unresponsive</i>			
c.47_48delCT (p.S16>XfsX1)	c.503delA (p.Y168>Sfs)	p.R252W	c.969+5G>A
c.60+5G>T	p.G171R	p.L255S	p.Y325X
c.115_117delTTC (p.F39del)	p.R176X	p.A259V	p.G344R
p.S40L	p.V177L	p.R261X	p.S349P
c.165delT (p.F55>Lfs)	p.Y179N	p.R270G	p.G352R
p.F55L	p.Y179H	p.G272X	c.1066-11G>A
p.E56D	p.W187X	c.822_832del11 (p.K274_T278>Nfs)	p.Y356X
c.168+5G>C	p.C203W	p.Y277D	p.K371R
c.206_207insC (p.P69>Pfs)	p.Y204C	p.T278N	p.Y386C
p.T81P	p.Y204X	p.E280K	p.V388M
c.266_267insC (p.P89>Pfs)	c.632delC (p.P211>Hfs)	p.P281L	p.A395P
c.283_285delATC (p.I94del)	p.L213P	c.842+3G>C	c.1200-1G>A
p.R111X	p.Y216X	c.842+5G>A	p.R408W
c.398_401delATCA (p.N133_Q134>Rfs)	c.663_664delAG (p.E221_D222>Efs)	p.F299C	p.R408Q
c.441+2T>G	p.P225T	c.912+1G>A	p.A447D
c.441+5G>T	p.G239V	c.913-7A>G	c.1355_1356insA (p.K452>Kfs)
p.R158W	p.R243X	p.I306V	
p.R158Q	p.R243Q	p.S310F	
p.N167I	p.L249F	c.967_969delACA (p.T323del)	
<i>Unclassified</i>			
p.Q20H	p.G188D	p.R243L	p.D338Y
p.I65N	p.V190A	p.R261P	p.Y343C
p.E78K	c.580_581delCT (p.L194>Efs)	p.T266P	c.1055delG (p.G352>Vfs)
p.D84Y	p.L194P	p.I269N ^a	p.Y386D
c.284_286delTCA (p.I95_K96delinsK)	p.P211T	c.806delT (p.I269>Tfs)	p.Y387H
c.441+1delGT	p.L212P	c.842+1G>A	p.E390G
c.441+1G>A	p.D222G	p.D282N	p.R413P
c.442-5C>G	p.S231P	c.919delG	
	p.R241C	p.Y325C	

^a p.I269N was only present in a subject classified as having three mutations

serving synthesis and recycling of BH₄ (Thony and Blau 2006); these Mendelian disorders of BH₄ metabolism can be treated by sapropterin replacement therapy. The metabolic responsiveness of our subset of sapropterin-responsive HPA/PKU patients is explained by a different process, namely enhancement of residual PAH enzyme activity and

opening of the oxidative pathway (Muntau et al. 2002). Pharmacological doses of sapropterin appear to achieve this effect, mainly in association with *PAH* missense alleles, either by a kinetic effect to overcome unfavorable binding of cofactor, or a chaperone-like effect on a misfolding enzyme subunit (Bechtluft et al. 2007; Dobrowolski et al.

Table 2 Classification of mutant *PAH* Genotypes, in the present study, grouped by the phenotypic response to sapropterin (ordered from 5' to 3' according to the mutation closest to the 5')

<i>Responsive</i>		
c.115_117delTTC (p.F39del): p.A345S p.F39L: p.I65T p.L48S: p.I65T p.L48S: p.F299C p.L48S: p.S349P p.L48S: p.Y387H c.165delT (p.F55>Lfs): p.Q226H p.I65T: p.I65T p.I65T: c.442-5C>G p.I65T: p.Y277D p.R68S: p.R252W	p.R68S: c.1066-11G>A p.R68S: c.1315+1G>A c.283_285delATC (p.I94del): p.R261Q c.283_285delATC (p.I94del): p.P281L p.A104D: p.A104D p.A104D: p.D222G p.A104D: p.Y414C p.P211T: p.R261Q p.G218V: p.P281L p.R241H: p.R252W p.R241H: p.G272X	p.R243L: p.R261Q c.806delT (p.I269>Tfs): p.Y414C p.G272X: c.1066-3C>T p.Y277D: p.Y343C p.E280K: p.L348V p.F299C: p.Y414C p.A309D: p.R408W p.L348V: p.L348V c.1066-3C>T: c.1315+1G>A p.V388M: p.E390G
<i>Ambiguously responsive</i>		
p.F39L: p.R408W p.L48S: p.R261Q p.L48S: c.1066-11G>A p.L48S: p.R408W p.I65T: p.R243X p.I65T: p.P281L p.I65T: p.L348V	p.I65T: p.R408W p.I65T: p.Y414C p.I65T: c.1315+1G>A c.441+5G>T: p.R158Q p.R243X: p.R408W p.R261Q: c.842+1G>A p.R261Q: c.1066-11G>A	p.G272X: p.Y414C p.E280K: p.R408W p.A309V: p.R408W p.L348V: p.R408W c.1066-11G>A: p.Y414C p.R408W: p.Y414C c.1315+1G>A: c.1315+1G>A
<i>Classified as responsive according to PKU-001 findings only</i>		
p.L48S: p.R158Q p.L48S: p.P281L p.I65T: p.R252W	p.E78K: p.R408W p.R158Q: p.R241C p.R261Q: p.Y414C	p.A309V: p.Y414C p.E390G: p.R408W
<i>Classified as ambiguously responsive according to PKU-001 findings only</i>		
p.F39L: p.F39L p.I65T: p.E280K	p.R261Q: p.R261Q p.P281L: p.Y414C	p.D282N: p.Y414
<i>Unresponsive</i>		
c.47_48delCT (p.S16>XfsX1):c.47_48delCT (p.S16>XfsX1) c.47_48delCT (p.S16>XfsX1): p.A345S c.47_48delCT(p.S16>XfsX1) ^a : p.V388M ^a p.Q20H: p.R158Q c.60+5G>T: p.R243Q c.60+5G>T: c.1066-11G>A c.60+5G>T: p.R408W c.60+5G>T: p.Y414C c.60+5G>T: c.1315+1G>A c.115_117delTTC (p.F39del): c.822_832del11 (p.K274_T278>Nfs) c.115_117delTTC (p.F39del): p.S349P p.F39L: p.R252W p.F39L: p.E280K p.F39L: c.1066-11G>A p.F39L: c.1315+1G>A p.S40L: c.842+1G>A p.S40L: p.R408W	c.283_285delATC (p.I94del): c.1315+1G>A p.A104D: c.822_832del11 (p.K274_T278>Nfs) p.A104D: p.R408W p.R111X: p.R111X p.R111X: p.R408W p.R111X: p.R408Q c.398_401delATCA (p.N133_Q134>Rfs): p.R408W c.441+1delGT: p.R158Q c.441+2T>G: p.R408W c.441+5G>T: p.V388M p.R158W: p.R261P p.R158W: p.R408W p.R158Q: c.580_581delCT (p.L194>Efs) p.R158Q: p.L212P p.R158Q: p.L213P p.R158Q: p.R243X p.R158Q ^a : p.R261Q ^a	p.R241H: p.R408W p.R243X: p.R243X p.R243X: p.Y414C p.R243Q: p.R243Q p.R243Q: p.R261Q p.R243Q: p.L348V p.R243Q: p.R408W p.L249F: p.L249F p.L249F: p.R408W p.R252W: p.P281L p.R252W: c.1066-11G>A p.R252W: c.1315+1G>A p.L255S: p.L255S p.A259V: c.1066-11G>A p.R261X: p.R261X p.R261X: p.Y325X p.R261X: c.1066-11G>A

Table 2 (continued)*Unresponsive*

p.L48S: p.I65N	p.R158Q: p.P281L	p.R261X: p.R408W
p.L48S: p.R111X	p.R158Q: p.F299C	p.R261P: p.Y414C
p.L48S: p.R252W	p.R158Q: c.1066-11G>A	p.R261Q ^{a,b} : p.G272X ^{a,b}
c.165delT (p.F55>Lfs): c.441+5G>T	p.R158Q: p.Y356X	p.R261Q: p.P281L
c.165delT (p.F55>Lfs): p.R158Q	p.R158Q: p.Y386D	p.R261Q: p.F299C
c.165delT (p.F55>Lfs): p.R252W	p.R158Q: p.V388M	p.R261Q: c.913-7A>G
c.165delT (p.F55>Lfs): p.R261X	p.R158Q: p.R408W	p.R261Q: p.L348V
c.165delT (p.F55>Lfs): p.R261Q	p.R158Q: p.Y414C	p.R261Q ^a : p.R408W ^a
c.165delT (p.F55>Lfs): p.G272X	p.R158Q: c.1315+1G>A	p.R261Q: c.1315+1G>A
c.165delT (p.F55>Lfs): p.P281L	p.N167I: p.G272X	p.R270G: p.R408W
c.165delT (p.F55>Lfs): p.R408W	c.503delA (p.Y168>Sfs): p.R408W	p.G272X: p.S349P
p.F55L: c.1200-1G>A	p.G171R: p.R408W	p.G272X: c.1066-11G>A
p.E56D: p.E56D	p.R176X: p.R176X	p.G272X: p.R408W
c.168+5G>C: c.168+5G>C	p.V177L: p.R408W	p.G272X: c.1315+1G>A
c.168+5G>C: p.I65T	p.Y179N: c.1066-11G>A	p.Y277D: c.1066-11G>A
c.168+5G>C: p.R408W	p.Y179H: p.R408W	p.Y277D ^a : c.1315+1G>A ^a
p.I65T ^a : p.R68S ^a	p.W187X: c.632delC (p.P211>Hfs)	p.T278N: p.P281L
p.I65T: p.E78K	p.V190A: p.T266P	p.T278N: c.1315+1G>A
p.I65T: c.441+5G>T	c.580_581delCT (p.L194>Efs): p.L348V	p.E280K: p.E280K
p.I65T: p.R158W	p.L194P: p.Y325C	p.E280K: p.P281L
p.I65T: p.R158Q	p.C203W: c.1315+1G>A	p.E280K: c.1066-11G>A
p.I65T: p.F299C	p.Y204C: c.663_664delAG (p.E221_D222>Efs)	p.E280K: c.1315+1G>A
p.I65T: c.912+1G>A	p.Y204X: p.Y414C	p.P281L: p.P281L
p.I65T: c.1066-11G>A	p.Y204X: c.1315+1G>A	p.P281L: p.S349P
p.I65T: p.R408Q	p.L213P: p.L213P	p.P281L: c.1066-11G>A
p.R68S: p.G272X	p.L213P: p.E280K	p.P281L: p.Y356X
p.R68S: p.R408W	p.L213P: p.R408W	p.P281L: p.R408W
c.206_207insC (p.P69>Pfs): c.1066-11G>A	p.Y216X: c.967_969delACA (p.T323del)	p.P281L: c.1315+1G>A
p.T81P: c.822_832del11 (p.K274_T278>Nfs)	p.G218V: p.S349P	c.842+1G>A: p.Y414C
p.T81P: p.R408W	c.663_664delAG (p.E221_D222>Efs): p.P281L	c.842+3G>C: p.R408W
p.D84Y: p.P281L	p.P225T: p.R243X	c.842+5G>A: p.Y414C
c.266_267insC (p.P89>Pfs): c.1066-11G>A	p.S231P: p.R261Q	p.F299C: c.1066-11G>A
c.283_285delATC (p.I94del): p.R408W	p.G239V: c.1066-11G>A	p.F299C: p.R408W
p.F299C: c.1315+1G>A	p.S349P: c.1315+1G>A	p.R408W ^a : p.R408W ^a
c.912+1G>A ^a : c.1055delG (p.G352>Vfs) ^a	p.G352R: c.1315+1G>A	p.R408W ^a : c.1315+1G>A ^a
c.912+1G>A: p.R408W	c.1066-11G>A: c.1066-11G>A	p.R408W: p.A447D
p.I306V: c.1066-11G>A	c.1066-11G>A: p.K371R	p.R408W: c.1355_1356insA (p.K452>Kfs)
		p.R408W: p.L48S-p.I269N ^c
p.S310F: c.1066-3C>T	c.1066-11G>A: p.R408W	p.R408W: p.R413P-p.Y414C ^c
c.969+5G>A: c.969+5G>A	c.1066-11G>A: c.1315+1G>A	p.R408Q: c.1315+1G>A
p.A345S: c.1066-11G>A	p.Y356X: p.Y414C	p.R413P: p.Y414C
p.L348V: c.1066-11G>A	p.Y386C ^a : c.1315+1G>A ^a	p.Y414C: c.1315+1G>A
p.L348V: c.1315+1G>A	p.A395P: p.R408W	
p.S349P: c.1066-11G>A	c.1200-1G>A: c.1315+1G>A	

^a These genotypes were classified as unresponsive based on findings of PKU-001 and PKU-003 and/or PKU-004

^b This genotype was classified as unresponsive according to the rules stated in the protocol; however, the levels were so low in the beginning of PKU-003 and PKU-004 that a true measure was not possible

^c Double mutant; mutation is not completely classified

2009b; Erlandsen et al. 2004; Pey et al. 2007; Scavelli et al. 2005; Scriver and Waters 1999; Waters et al. 1999, 2000). Accordingly, it is relevant to know which among the several hundred *PAH* alleles (see www.pahdb.mcgill.ca) are associated with sapropterin responsiveness.

We classified the *PAH* mutant alleles (Table 1) and genotypes (Table 2), identified in the participants of the Phase II and III clinical trials, as either responsive, ambiguously responsive and unresponsive to sapropterin or unclassifiable, by using a simple metabolic (phenotypic) response as measured in these studies; while recognizing that other methods of response classification exist (Langenbeck 2008), which may influence the estimates of response prevalence. We used homozygous or hemizygous: null genotypes to classify the alleles, as was done earlier to describe PKU/HPA phenotypes (Guldberg et al. 1998; Kayaalp et al. 1997). We noticed that the proportion of homozygous genotypes in the present study (14.4%) is less than the frequency (~25%) observed previously in the earlier studies (Guldberg et al. 1998; Kayaalp et al. 1997), a finding that suggests selective sampling of the patient population in these studies. The most prevalent allele (p.R408W, c.1222C>T) is a nonresponsive null allele identified in 145 of 458 patients that were completely or partially genotyped; it reflects the predominance of subjects of northern European origins enrolled in the clinical study (<http://www.pahdb.mcgill.ca>). The frequency of sapropterin-responsive patients has varied between our own and other reports, a feature that could be explained in part by population genetics and the nonrandom distribution of mutant sapropterin-responsive *PAH* alleles (Guldberg et al. 1998; Kayaalp et al. 1997).

We mapped the responsive, ambiguously responsive and unresponsive point mutations onto the PAH crystal structure (Fig. 1a–c, respectively) according to the finding of these clinical trials. The responsive mutations seldom mapped to the cofactor binding site. One responsive allele, p.Y414C, maps to the dimer–dimer interfaces of the tetramer, suggesting that sapropterin enhances stabilization of the tetramer (Erlandsen et al. 2004; Pey et al. 2004). Among the other responsive mutations, p.I65T and p.A104D are located in the regulatory domain, while p.Q226H, p.R241H, and p.A309D are part of the catalytic domain (Fig. 1a). Of the mutations identified as ambiguously responsive, p.F39L, p.L48S, and p.R68S are located in the regulatory domain and p.G218V, p.R261Q, p.A309V, p.A345S, and p.L348V in the catalytic domain (Fig. 1b). Because our study is not a comprehensive sample of the human PKU/HPA population, a limitation compounded by a relaxed protocol design, we could not readily reveal the true prevalence of responsive mutations; for example, E390G in the catalytic domain is strongly associated with

Fig. 1 Mapping of the identified point mutations on one enzyme subunit of the PAH crystal structure composite model (PDB ID code 1PAH): (a) responsive mutations, (b) ambiguously responsive mutations, and (c) unresponsive mutations

a chaperone-like effect (Erlandsen et al. 2004; Pey et al. 2004) yet did not readily disclose this property here.

Genotypes classified as responsive in these clinical trials have at least one mutation with residual PAH activity (see www.pahdb.mcgill.ca for data on activity of mutant alleles,

Table 3 Novel alleles not previously catalogued in www.pahdb.mcgill.ca

Mutation name	Nucleotide name	Responsiveness
p.Q20H	c.60G>C	Unclassified
p.P69>Pfs	c.206_207insC	Unresponsive
IVS4+1delGT	441+1delGT	Unclassified
IVS4+2T>G	c.441+2T>G	Unresponsive
p.Y179N	c.535T>A	Unresponsive
p.G188D	c.563G>A	Unclassified
p.C203W	c.609C>G	Unresponsive
p.T266P	c.796A>C	Unclassified
p.R270G	c.808A>G	Unresponsive
p.Y386D	c.1156T>G	Unclassified

e.g. the responsive allele p.R241H: paired with the null allele p.G272X). There was also unexpected responsiveness with genotypes classically associated with null alleles (e.g. p.G272X: c.1066-3C>T); and there were inconsistencies in the response of patients carrying previously characterized genotypes (e.g., p.I65T:p.R68S which were formerly described as responsive (Lindner et al. 2003), but in our study were unresponsive). Some of these anomalies may be explained by unmonitored dietary escape during clinical trials.

Genotypes bearing mutant alleles: p.I65T, p.A104D, p.Q226H, p.R241H, p.A309D, or p.Y414C (Table 1) were usually responsive to sapropterin (Table 2) in these clinical trials. However, these alleles were not necessarily predictive of responsiveness because genotypes harboring p.I65T, p.A104D, p.R241H, and p.Y414C were found in at least one sapropterin-unresponsive patient, leading us to conclude that factors other than *PAH* genotype contribute to sapropterin responsiveness. Inconsistencies in predicting phenotype from genotype is a recognized problem at the *PAH* locus (Guldberg et al. 1998; Kayaalp et al. 1997) and confounds earlier hopes to the contrary (Okano et al. 1991; Scriver 1991).

The response of certain PKU patients to BH₄ treatment has been postulated to occur through a variety of mechanisms (Erlandsen et al. 2004), one of which may involve overcoming suboptimal in vivo BH₄ concentrations (Kure et al. 2004). Consistent with this proposed mechanism, and other findings (Fiori et al. 2005; Hennermann et al. 2005; Matalon et al. 2004), we found that some putatively severe PKU phenotypes were partially responsive to sapropterin treatment in our clinical trials. We are aware that classification of responsiveness is dependent not only on the mutant allele at the *PAH* locus, but also on the genotype at that locus and on modifiers, yet to be identified, in the genome (Scriver and Waters 1999; Waters et al. 1999).

Other mechanisms to explain a positive or ambiguous sapropterin response may exist in mutations that affect splicing (Desviat et al. 2004) or modulate *PAH* gene expression (Blau and Trefz 2002). Splice-site mutations may generate multiple forms of transcripts and, thus, multiple forms of the encoded enzyme, a subset of which may be active. In this situation, positive response to sapropterin would depend on efficiency in generating a transcript encoding an enzyme with some activity. If the splice-site mutation increases the variability of the generation of specific transcripts, then the allele may show ambiguous or inconsistent response to sapropterin. In this study, the only intronic mutations classified as showing ambiguous sapropterin response were c.1066-3C>T and c.1315+1G>A, whereas others remained unresponsive or unclassifiable (Table 1).

Other factors that could contribute to an inconsistency in our findings could include once again the limited methodology with unmonitored changes in diet or other external factors such as intercurrent illness (Burton et al. 2007) and variability between individuals that may reflect sapropterin absorption and pharmacokinetics or bioavailability of the cofactor in the individual patient (Fiege et al. 2004; Leuzzi et al. 2006; Nielsen et al. 2010; Shintaku et al. 2005). Meanwhile, studying the residual PAH activity arising from the interaction of the mutant PAH subunits, carried by the PKU patients, is essential and may also influence BH₄-responsiveness. In this regard, a measure of predictability is enhanced when studying patients with homozygous or functionally hemizygous genotypes (Dobrowolski et al. 2009b, 2011; Fiori et al. 2005; Kure et al. 1999). The inconsistencies between genotype and BH₄/sapropterin response lead us to conclude that the nature of the response to BH₄ treatment is still not fully understood.

Our evidence that responsiveness to sapropterin is influenced by dosage of the agent (PKU-004) suggests a chemical response of the mutant protein in the presence of either a chaperone-like molecule or mass action to overcome impaired binding, as proposed elsewhere (Kure et al. 2004). The findings also highlight the individuality of the response to sapropterin therapy, the extent of which will be revealed in continuing studies that will measure, among others, the metabolic, cognitive, and psychological responses. While noting that our initial classification process for responsiveness in these clinical trials was based on response to sapropterin in the short-term protocol (PKU-001), we also recognize that there might be a different rate of responsiveness to be revealed when other more stringent short-term protocols and long-term exposures to sapropterin are considered. This also accounts for the minor inconsistencies of the rate of response reported here, as compared to what was reported in the prior and related clinical trial findings.

Once again, and in concordance with earlier findings of many others, we show that sapropterin responsiveness is not robustly predicted from genotype alone. In addition, though we acknowledge that the protocol parameters adapted in these clinical trials were designed to accommodate every day patient behavior, given the inconsistencies found between the clinical trial results reported here and other related studies, we recommend further review and application of more stringent and universally approved protocols and methodologies during upcoming drug trials. In the interim, we propose that it currently remains safer to identify the phenotype by direct observation of the response to sapropterin than to rely on prediction by genotype. Once again, we return to the thought that we should investigate and treat the patient and not just the genotype.

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Synopsis

Some *PAH* mutations causing misfolding of the PAH protein respond to pharmacological doses of sapropterin dihydrochloride (BH₄) acting as a chaperone; however, to know genotype is not a robust predictor of therapeutic response, an assumption corroborated by findings reported in Phase II and III clinical trials.

Author Contributions

All co-authors participated in various aspects of the study. CNS and AG organized, corrected, analyzed, and interpreted the data, PS did the genotyping, JD completed the analysis of genotypes, AD compiled the data, CNS, AG and CRS drafted the manuscript, and CNS, AG, AD, RCS, and CRS reviewed it. The final version was seen and approved by all authors. CNS and AG are co-first authors who contributed equally to this work.

Guarantor

Raymond C. Stevens

Competing Interests Statement

The authors report commercial affiliations and competing financial interests: this study was supported by BioMarin Pharmaceutical Inc., the manufacturer of KUVAN[®]. AD was an employee of BioMarin Pharmaceutical Inc., and owns stock or stock options in the company. RCS and CRS have consulted (or are consultants) for BioMarin Pharmaceutical Inc. regarding their development of treatments for PKU/HPA.

	CNS	AG	PS	JD	AD	CRS	RCS
1. Have you in the past 5 years accepted the following from an organization that may in any way gain or lose financially from the results of your study or the conclusions of your review, editorial, or letter:							
Reimbursement for attending a symposium?	Yes	No	No	No	Yes	Yes	No
A fee for speaking or for organizing education?	No	No	No	No	Yes	No	No
Funds for research or for a member of staff?	Yes	No	No	No	Yes	Yes	Yes
A fee for consulting?	Yes	No	No	No	Yes	Yes	Yes
2. Have you in the past 5 years been employed by an organization that may in any way gain or lose financially from the results of your study or the conclusions of your review, editorial, or letter?	Yes	Yes	No	Yes	Yes	Yes	Yes
Do you hold any stocks or shares in such an organization?	No	No	No	No	Yes	No	No
3. Have you acted as an expert witness on the subject of your study, review, editorial, or letter?	No	No	No	No	No	No	No
4. Do you have any other competing financial interests?	No	No	No	No	No	No	No

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data, drafting of the manuscript and review. BioMarin Pharmaceutical Inc. did not influence the analysis process or outcome of the project.

Ethics Approval

- Ethics approval for this research study was covered as a component of the clinical trials.
- Patient consent for this research study was covered as a component of the clinical trials.
- No vertebrate animals were used.

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