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journal homepage: www.elsevier.com/locate/ymgmeMolecular genetics and impact of residual *in vitro* phenylalanine hydroxylase activity on tetrahydrobiopterin responsiveness in Turkish PKU populationSteven F. Dobrowolski^{a,1}, Caroline Heintz^{b,1}, Trent Miller^c, Clinton Ellingson^d, Clifford Ellingson^e, Işıl Özer^f, Gulden Gökçay^f, Tolunay Baykal^f, Beat Thöny^{b,g,h}, Mübeccel Demirkol^{f,*}, Nenad Blau^{b,g,h,*}^a Department of Pathology, University of Utah School of Medicine, Salt Lake City, UT, USA^b University Children's Hospital, Division of Clinical Chemistry and Biochemistry, Zürich, Switzerland^c Idaho Technology, Salt Lake City, UT, USA^d Department of Medicine, Pennsylvania State University, College of Medicine, Hershey, PA, USA^e Case Western Reserve University, School of Medicine, Cleveland, OH, USA^f Istanbul University, Istanbul Faculty of Medicine, Children's Hospital, Division of Nutrition and Metabolism, Istanbul, Turkey^g Zürich Center for Integrative Human Physiology (ZIHP), Zürich, Switzerland^h Pediatric Research Center (PRC), Zürich, Switzerland

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ABSTRACT

Background: The prevalence of phenylalanine hydroxylase (PAH)-deficient phenylketonuria (PKU) in Turkey is high (1 in 6500 births), but data concerning the genotype distribution and impact of the genotype on tetrahydrobiopterin (BH₄) therapy are scarce.

Objective: To characterize the phenotypic and genotypic variability in the Turkish PKU population and to correlate it with physiological response to BH₄ challenge.

Methods: We genotyped 588 hyperphenylalaninemic patients and performed a BH₄ loading test (20 mg/kg bw) in 462 patients. Residual PAH activity of mutant proteins was calculated from available *in vitro* expression data. Data were tabulated in the BIOPKU database (www.biopku.org).

Results: Eighty-eight mutations were observed, the most common missense mutations being the splice variant c.1066-11G>A (24.6%). Twenty novel mutations were detected (11 missense, 4 splice-site, and 5 deletion/insertions). Two mutations were observed in 540/588 patients (91.8%) but in 9 patients atypical genotypes with >2 mutations were found (8 with p.R155H *in cis* with another variant) and in 19 patients mutations were found in BH₄-metabolizing genes. The most common genotype was c.1066-11G>A/c.1066-11G>A (15.5%). Approximately 22% of patients responded to BH₄ challenge. A substantial *in vitro* residual activity (average >25% of the wild-type enzyme) was associated with response to BH₄. In homozygous genotypes (*n* = 206), both severity of the phenotype (*r* = 0.83) and residual PAH activity (*r* = 0.85) correlate with BH₄ responsiveness.

Conclusion: Together with the BH₄ challenge, these data enable the genotype-based classification of BH₄ responsiveness and document importance of residual PAH activity. This first report of a large-scale genotype assessment in a population of Turkish PKU patients also documents a high prevalence (47%) of the severe classic phenotype.

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1. Introduction

Phenylketonuria (PKU; OMIM# 261600) is an autosomal recessive disorder associated with deficient hepatic phenylalanine hydroxylase (PAH) activity [1]. PAH converts phenylalanine (Phe) to tyrosine in the

presence of the essential cofactor tetrahydrobiopterin (BH₄), molecular oxygen, and Fe²⁺. BH₄ is synthesized from guanosine triphosphate (GTP) in a biosynthetic pathway including the enzymes GTP cyclohydrolase I (GTPCH; gene *GCH1*), 6-pyruvoyl-tetrahydropterin synthase (PTPS; gene *PIS*), and sepiapterin reductase (SR; gene *SPR*). The oxidized cofactor is regenerated in two enzymatic steps involving pterin-4a-carbinolamine dehydratase (PCD; gene *PCBD1*) and dihydropteridine reductase (DHPR; gene *QDPR*) [2]. Mutations in genes coding for PAH and BH₄-metabolizing enzymes result in hyperphenylalaninemia (HPA) [3]. *SPR* deficiency and autosomal dominant *GCH1* deficiency present without HPA [4]. BH₄ deficiencies are more severe than PKU, and in addition to HPA present with catecholamines and serotonin deficiency [5].

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The overall prevalence of PKU in Europe and the United States is about 1 in 10,000 live births. Higher disease incidence is observed in cultures where consanguinity is practiced (e.g., Turkey, Saudi Arabia, or Gaza; ca. 1 in 3500–6500); however, in regions such as Finland the incidence is low (1 in >100,000). Prevalence of BH₄ deficiencies is about 1–2% of all HPAs [6].

Late-diagnosed, untreated PKU leads to severe neurological impairment including mental retardation, microcephaly, autistic behavior, eczema, and seizures [7], particularly in the most severe forms of PAH deficiency, “classic PKU” (blood Phe concentrations >1200 μmol/L). Less severe forms include mild PKU (blood Phe concentrations 600–1200 μmol/L) and mild HPA without any clinical findings (blood Phe concentrations <600 μmol/L). Hyperphenylalaninemic patients are identified through prospective newborn screening and follow-on diagnostic procedures will identify the defective gene, enabling early initiation of appropriate therapy [8]. Not every HPA patient is routinely tested for DNA mutations.

The observation that serum Phe concentration may be controlled in a subset of PKU patients through oral administration of synthetic 6R-BH₄ [9] and reports of a relatively high incidence (20–30%) of BH₄ responsiveness [10,11] provided an alternative to the traditional low Phe diet [14]. A number of studies documented that PAH-deficient patients with mild to moderate phenotypes are more likely to benefit from BH₄ therapy [10–14]. In some patients Phe concentration may be controlled with BH₄ monotherapy; however, others require a combination of BH₄ and dietary restrictions to maintain blood Phe in the therapeutic range while increasing daily Phe tolerance [15–18]. Mechanisms of BH₄ responsiveness are multifactorial [19]. Current data suggest the most common mechanism by which BH₄ rescues PAH function is by acting as a pharmacological chaperone promoting proper enzyme folding, which in turn reduces enzyme degradation and inactivation [20,21].

The PAHdb (www.pahdb.mcgill.ca/) has cataloged over 500 mutations in the PAH gene [22], while the BIOPKU database (<http://www.bh4.org/BH4DatabasesBiopku.asp>) describes an approximately equal number of PAH genotypes and their association with BH₄ response [23]. A systematic review of PKU in Europe identified 29 mutations that may be regarded as prevalent in European populations [24], but there are very few reports on the molecular basis of PKU in Turkey [25,26].

Herein are presented PAH genotypes of 588 Turkish PKU patients where 88 mutations were identified; among these are 20 novel mutations. Data from oral BH₄ challenge in 462 patients are reported. Comparisons are made relating BH₄ response with the genotype, residual *in vitro* PAH activity, and disease phenotype. The results extend the knowledge of the genotypic PKU variation in the Turkish PKU population and document a high prevalence of classical PKU (47%), a relatively high proportion (22%) of potential candidates for the BH₄ therapy, and the common occurrence of BH₄ deficiencies (2.4%) within this study.

2. Patients and methods

2.1. Patients and samples

A total of 588 hyperphenylalaninemic patients were investigated. At the time of diagnosis, 165 patients presented with mild HPA (blood Phe <600 μmol/L), 130 with mild PKU (blood Phe 600–1200 μmol/L), and 274 patients presented with classic PKU (blood Phe >1200 μmol/L). Nineteen patients with BH₄ deficiencies presented with a variable range of blood Phe (9 mild HPA, 7 mild PKU, 3 classic PKU). Forty-six percent of patients were the offspring of consanguineous mating (Table 1); however, an even higher percentage (48.7%) displayed mutation homozygosity, suggesting inbreeding (Suppl. Table 1). Nine pedigrees, where >2 mutations were identified, are included in this study.

Table 1
Consanguinity in Turkish PKU patients investigated in this study.

Related marriage	Number of families	%
No consanguinity	221	43.1
No consanguinity, but parents from same village	57	11.1
1st grade cousin	149	29.0
1.5 grade cousin	10	1.9
2 grade cousin	36	7.0
2.5 grade cousin	2	0.4
3 grade cousin	27	5.3
3.5 grade cousin	2	0.4
4 grade cousin	9	1.8

The majority of patients (~75%) were identified through prospective newborn screening, while the remainders were identified by selective screening. Blood specimens were collected on filter paper cards by finger or heel prick, and all tests were performed within routine clinical and biochemical investigation and in accordance with local regulations. Blood phenylalanine was measured using a fluorometric method until 2003, and tandem mass spectrometry was used afterwards. The first confirmatory quantitative phenylalanine was performed during clinical assessment when the child was provided a normal diet. Informed consent for genotype assessment was obtained from all subjects. The University of Utah Institutional Review Board approved the plan to receive de-identified specimens for assessment of the PAH gene and genes of the BH₄ synthesis/recycling pathways.

2.2. Loading test with BH₄

A single-dose BH₄ challenge (20 mg/kg body weight) was performed on 462 PKU patients (81%) (Schircks Laboratories, Switzerland). Three different protocols were used: A) Prior to 1999, a partially active formulation of BH₄, containing a mixture of the active R enantiomer and inactive S enantiomer (66.6% 6R-BH₄ and 33.3% 6S-BH₄), was used to challenge 166 patients. Thus a 20 mg tablet contained 13.3 mg of biologically active BH₄. In this subset, serum Phe was monitored over 8 h. B) A fully active formulation of BH₄ (6R-BH₄) was utilized post-1999. Among the 296 patients challenged, Phe was monitored over an 8-h period (0, 4, and 8 h) in 104 patients and C) over a 24-h period (0, 4, 8, and 24 h) in 192 patients. Data from patients whose plasma Phe concentration was monitored over 24 h were used for genotype–phenotype correlation and determination of residual PAH activity. In all BH₄ challenge protocols, response was defined as a sustained reduction of blood Phe concentration by ≥30% from the pre-challenge baseline [27].

2.3. Assessment of the PAH, PTS, and QDPR genes

DNA was prepared from dried blood on filter paper as previously described [28]. The PAH gene was assessed utilizing a previously described system involving high-resolution melt profiling and follow-on DNA sequencing of regions displaying aberrant melting profiles [29,30]. DNA sequence data were analyzed using Mutation Surveyor software (Softgenetics, State College, PA, USA). The protocols utilized in assessment of PTS and QDPR also involved high-resolution melt profiling and follow-on DNA sequencing of regions displaying aberrant melting profiles. The specifics of these assessments will be included in a separate study.

In several instances, PAH-deficient patients were identified with >2 mutations in the PAH gene. When family participation could be recruited in such cases, blood samples were obtained from parents and other first-degree relatives for performance of pedigree studies to determine the *cis/trans* relationship between the mutations.

2.4. Relative residual PAH activity

Relative residual PAH activity ('PAH activity') was calculated from data provided from *in vitro* experiments using recombinantly expressed mutant proteins in eukaryotic cells. PAH activity is the average of the sum of activities of both alleles, and expressed as the percentage of the wild-type enzyme. Expression data were compiled from the PAHdb (www.pahdb.mcgill.ca/). Calculated *in vitro* 'PAH activity' is most probably different from *in vivo* enzyme activity. A splice-site mutation is estimated as having no 'PAH activity' if it is associated with classic phenotype in >95% of patients and is not recognized to facilitate a response to BH₄. Some splice-site mutations may, however, produce wild-type protein (albeit at a reduced level) and are thus associated with milder phenotypes.

2.5. Phenotype scoring

Phenotype scoring was utilized for patients with homozygous mutations. Phenotype severity was scored according to blood Phe levels assigning a score of 1 for the mildest HPA (lowest blood Phe levels) and a score of 10 for the severe classic PKU (highest blood Phe levels).

2.6. Statistical analysis

Statistical analysis was performed using WinSTAT 2007.1 for Excel (R. Fitch Software, Germany). Passing-Bablok regression analysis was used to compare the relative residual 'PAH activity' and phenotypes with BH₄ responsiveness.

3. Results

3.1. PAH genotypes

Among the patients genotyped two mutations were observed in 540/569 (94.9%) patients (Suppl. Table 1). A single mutation was observed in 29 patients (data not shown) and in no instances was there a PAH-deficient patient in which no mutation was observed. A total of 88 mutations were observed and Table 2 provides those mutations that were observed with ≥3% allele frequency. The most frequently encountered PAH genotypes and their association with BH₄ responsiveness are shown in Table 3.

Consanguinity and inbreeding are apparent in that 8 of the 12 most frequently observed genotypes involve homozygosity and furthermore the c.1066-11G>A mutation is represented in 5 of the most common genotypes. Twenty novel mutations were identified, with p.Y204X occurring in 15 alleles (Table 4). Table 5 shows that a high number of deletions (n = 54) and insertions (n = 5) were observed.

Table 2
Most common mutations (AF>3%) found in Turkish patients with PKU.

Sequence variation	Effect	Alleles	Allele frequency (%)	Metabolic phenotype*	PAH activity** (%)
c.1066-11G>A	Splice dysfunction	273	24.6	cPKU	nd
c.782G>A	p.R261Q	96	8.7	mPKU	38.5
c.842C>T	p.P281L	93	8.4	cPKU	<1
c.143T>C	p.L48S	78	7.0	mPKU	39.0
c.1222C>T	p.R408W	71	6.4	cPKU	1.0
c.898G>T	p.A300S	56	5.0	MHP	31.0
c.1169A>G	p.E390G	46	4.1	MHP	72.5
c.441+5G>T	Splice dysfunction	33	3.0	cPKU	nd

MHP: mild HPA; mPKU: mild PKU; cPKU: classic PKU; nd: not determined.

* Based on phenotype characteristics of functionally hemizygous individuals (BIOPKUdb).

** Of the wild-type activity when recombinantly expressed in eukaryotic cell system [23].

Table 3
Most common genotypes and association with BH₄ responsiveness.

PAH genotype		Frequency	Metabolic phenotype	PAH activity* (%)	Response to BH ₄ challenge
Allele 1	Allele 2				
c.1066-11G>A	c.1066-11G>A	84 (15.5%)	cPKU	nd	–
p.P281L	p.P281L	27 (5.0%)	cPKU	<1	–
p.R261Q	p.R261Q	27 (5.0%)	mPKU	38.5	+
p.R408W	p.R408W	15 (2.8%)	cPKU	<1	–
p.L48S	c.1066-11G>A	13 (2.4%)	mPKU	nd	+/-
p.R408W	c.1066-11G>A	13 (2.4%)	cPKU	nd	–
p.L48S	p.L48S	12 (2.2%)	mPKU	39.0	+
c.441+5G>T	c.441+5G>T	11 (2.0%)	cPKU	nd	–
p.A300S	c.1066-11G>A	10 (1.8%)	MHP	nd	+
p.P281L	c.1066-11G>A	10 (1.8%)	cPKU	nd	–
p.A300S	p.A300S	9 (1.7%)	MHP	31.0	+
p.E390G	p.E390G	8 (1.5%)	MHP	42.5	+

MHP: mild HPA; mPKU: mild PKU; cPKU: classic PKU; nd: not determined.

* Of the wild-type activity when recombinantly expressed in eukaryotic cell system [23].

Within the same group of genotypes, BH₄ responsiveness is equally distributed and frequently associated with mild HPA or mild PKU. In several instances, patients with 3 or even 4 mutations were identified (Table 6). Frequently the missense mutations p.R115H and p.A300S were observed together. To determine the inheritance phase of sequence variants when >2 mutations are observed, specimens from parents and other first-degree relatives were recruited. Pedigree studies demonstrate a relatively common compound mutation in Turkish PKU patients where p.R115H and p.A300S are present on a common chromosome. Although not all parents were tested for hyperphenylalaninemia, two mothers presented with mild HPA (blood Phe levels 170–255 μmol/L).

3.2. Loading test with BH₄

Prior to 1999, a formulation of BH₄ containing 66.6% 6R-BH₄ and 33.3% 6S-BH₄ was utilized in challenge studies; thus, a 20 mg dose contained 13.3 mg of active drug. One hundred sixty-six patients (19% mild HPA, 19% mild PKU, 62% classic PKU) were challenged with 20 mg/kg (that in actuality provided 13.3 mg/kg), and Phe was monitored over 8 h. Among this group 6 patients (3.5%) responded with blood Phe reduction of ≥30% (Fig. 1A). The residual 'PAH activity' of BH₄-responsive genotypes (4 mild HPA and 2 mild PKU) was between 25 and 72.5%. Additionally, three classic PKU patients

Table 4
Novel mutations detected in Turkish PKU patients.

PAH mutation	Nucleotide aberration	Location	Number of alleles
p.Q20H	c.60G>C	Exon 1	2
p.L37X	c.48-49insCT	Exon 2	3
p.F39del	c.113-115delTCT	Exon 2	1
p.E57K	c.169G>A	Exon 3	1
p.D75X	c.197-204del8	Exon 3	1
p.P119S	c.355C>T	Exon 4	1
p.W120fs	c.358delT	Exon 4	1
p.G148D	c.443G>A	Exon 5	2
p.Y204X	c.590-611del22	Exon 6	15
p.E280A	c.839A>C	Exon 7	7
p.P281R	c.842C>G	Exon 7	2
IVS7+4A>G	c.842+4A>G	Intron 7	2
p.L293S	c.878T>C	Exon 8	1
IVS9-7A>G	c.970-7A>G	Intron 9	2
IVS10-7C>A	c.1066-7C>A	Intron 10	1
p.F382L	c.1144T>C	Exon 11	2
p.K396R	c.1187A>G	Exon 11	2
p.V399A	c.1196T>C	Exon 11	2
IVS11-2A>G	c.1200-2A>G	Intron 11	1
p.Y417C	c.1250A>G	Exon 12	4

Table 5
Deletions and insertions found in Turkish PKU patients.

PAH variation	Effect	Alleles
c.1089delG	p.K363>Nfs	19
c.592_613del22	p.Y198Sfs	15
c.165delT	p.F55>Lfs	12
c.47_48delCT	p.S16>XfsX1	3
c.48_49insCT	p.L37X	3
c.266_267insC	p.P89>Pfs	2
c.1087_1088delAA	p.K363>Afs	2
c.197_204del8	p.D75X	1
c.358delT	p.F149X	1
c.113_115delTCT	p.F39del	1

responded to BH₄; however, their genotypes indicated that they had <1% 'PAH activity' and as such were classified as BH₄ non-responders.

Two hundred ninety-six patients were challenged with the fully active BH₄ formulation; thus a dose of 20 mg/kg was achieved. One hundred four patients were monitored for 8 h post-challenge; among these, 19 patients were responsive including 11 mild HPA (31–73% 'PAH activity'), 4 mild PKU (38–39% 'PAH activity'), and 4 classic PKU (<1% 'PAH activity') (Fig. 1B). One hundred ninety-two patients, challenged with the fully active BH₄ formulation, were assessed for 24 h post-challenge. Responsive patients included 45 mild HPA (16–56% PAH activity), 18 mild PKU (14–39% 'PAH activity'), and 4 classic PKU (<1% 'PAH activity') (Fig. 1C). Because the 8-h test may not identify some responsive patients, only data from the 24-h test were used for genotype–phenotype correlation.

3.3. BH₄ deficiency

Nineteen patients (8 female, 11 male) were diagnosed with BH₄ deficiency (11 with QDPR deficiency, 8 with PTS deficiency), all of them responding to BH₄ administration by lowering blood phenylal-

Table 6
Turkish PKU genotypes with additional mutations in cis.

Allele 1	Allele2	Mutations in cis	Metabolic phenotype	Number of patients
p.R176X	p.A300S	p.R155H	mPKU	2*
p.R155H	p.A300S	—	—	Mother
p.R176X	Wild-type	—	—	Father
IVS10-11G>A	IVS10-11G>A	p.L430P	cPKU	1
IVS10-11G>A	Wild-type	—	—	Mother
IVS10-11G>A	Wild-type	—	—	Father
p.P281L	p.P281L	p.R413P	cPKU	1
p.P281L	Wild-type	—	—	Mother
p.P281L	p.R413P	—	—	Father
p.L430P	p.A300S	p.R155H	MHP	1
p.R155H	p.A300S	—	—	Mother
p.L430P	Wild-type	—	—	Father
p.R413P	p.A300S	p.R155H	mPKU	1
p.R413P	Wild-type	—	—	Mother
p.R155H	p.A300S	—	—	Father
p.A300S	p.A300S	p.R155H	MHP	1
p.R155H	p.A300S	—	—	Father**
p.A300S	p.A300S	p.R155H/p.R155H	mPKU/cPKU	2
p.R155H	p.A300S	—	—	Mother
p.R155H	p.A300S	—	—	Father
p.R155H	p.R155H	p. R261Q	mPKU	1
p. R155H	Wild-type	—	—	Mother
p.R155H	p. R261Q	—	—	Father
IVS10-11G>A	IVS10-11G>A	p.R155H/ p.R155H	cPKU	1
p.R155H	IVS10-11G>A	—	—	Mother
p.R155H	IVS10-11G>A	—	—	Father

MHP: mild HPA; mPKU: mild PKU; cPKU: classic PKU.

* Siblings.

** Mother not tested.

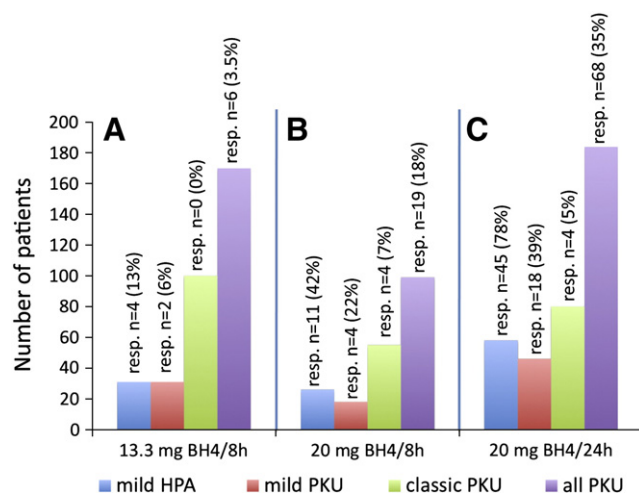


Fig. 1. Number of patients loaded with (A) 13.3 mg BH₄ (old product) over 8 h; (B) 20 mg BH₄ over 8 h; and (C) 20 mg BH₄ over 24 h and numbers of responsive patients (blood Phe reduction by >29.9%) defined by both the BH₄ challenge and genotype; resp. = BH₄-responder.

anine levels by more than 80% after 24 h (data not shown). These patients require a unique treatment regimen that includes neurotransmitter precursors and will be described in a separate study.

3.4. Phenotype, genotype, PAH activity, and BH₄ responsiveness

For the comparison between the genotype, phenotype, BH₄ responsiveness, and residual 'PAH activity,' the 10 most common homozygous genotypes (total n=206) with a frequency of >5 patients were compared, utilizing the severity of the phenotype and the residual 'PAH activity' calculated from *in vitro* experiments (Table 7).

Phenotype severity was scored according to highest blood Phe levels assigning a score of 1 for the mildest HPA and a score of 10 for the severe classic PKU. A good correlation (r=0.83) was observed for BH₄ responsiveness and 'PAH activity' (Fig. 2A) and with r=0.85 for BH₄ responsiveness and phenotype (Fig. 2B). Similar calculations with compound heterozygous genotypes yielded low correlation coefficients (data not shown).

For most homozygous genotypes distribution of the phenotype was clear (e.g., p.R408W/p.R408W with no 'PAH activity,' classic phenotype, and 100% non-responsiveness). However, some genotypes (e.g., p.L48S/p.L48S) show a high inconsistency with regard to both phenotype (42% mild HPA, 33% mild PKU, 25% classic PKU) and BH₄ responsiveness (44.4% responder) (Suppl. Table 1).

4. Discussion

The observed frequency of PAH-deficient PKU is higher in the Turkish population than in either Europe or the United States. Consanguinity, as a social norm in some communities within Turkish culture, has led to an increased frequency of PKU; furthermore, inbreeding within ethnic groups has also contributed to an increased disease frequency. An additional problem is delayed diagnosis and the quality of dietary management, both issues are a consequence of a paucity of PKU centers and the long distance patients must often travel to obtain care. The need for effective newborn screening and follow-on diagnostic procedures to categorize prospectively identified HPA newborns in the Turkish population is evident. We report a large-scale assessment of Turkish PKU patients and critically examine both PAH genotypes and response to challenge with BH₄.

Genotypes observed among the patient cohort showed homozygosity at a rate of ~48%. A high rate of homozygosity is not unique to

Table 7
Ten most common homozygous genotypes occurring in more than 5 Turkish PKU patients with different phenotypes, residual *in vitro* 'PAH activity,' percentage of responders, and calculated phenotype score. Phenotype score was calculated for genotypes presented with different phenotypes.

PAH genotype		Number of patients	PAH activity (%) [*]	BH ₄ responder (%)	Phenotypes (%)			Phenotype score ^{**}
Allele 1	Allele 2				MHP	mPKU	cPKU	
p.R408W	p.R408W	15	2.0	0.0	0	7	93	9.7
IVS4+5G>T	IVS4+5G>T	11	<1	0.0	0	9	91	9.5
p.P281L	p.P281L	27	1.0	3.7	4	11	85	9.1
IVS10-11G>A	IVS10-11G>A	84	<1	3.6	1	18	81	9.0
p.A300S	p.A300S	9	31.0	25.0	0	22	78	8.9
p.R252W	p.R252W	7	1.0	20.0	29	29	43	6.0
p.R261Q	p.R261Q	27	38.5	39.1	11	67	22	5.7
p.L48S	p.L48S	12	39.0	44.4	42	33	25	4.6
p.R241C	p.R241C	6	25.0	40.0	50	33	17	3.8
p.E390G	p.E390G	8	72.5	40.0	100	0	0	1.0

MHP: mild HPA; mPKU: mild PKU; cPKU: classic PKU.

^{*} Compared with the wild-type enzyme.

^{**} Lowest score (1) for mild HPA and highest score (10) for classic PKU.

Turkish PKU patients as we previously reported a high rate of homozygosity for the p.R408W mutation (43%) along with a limited spectrum of mutations among patients in western Poland [29]. As this study identified 88 different PAH mutations, including 20 novel mutations, the spectrum of PAH mutations in the Turkish population is relatively diverse. A unique genotypic feature of Turkish PKU patients is the number of *in cis* compound mutations (see Table 6). The p.R155H mutation was identified *in cis* with p.A300S in several patients. Upon reevaluation of a previously reported patient, we found p.R155H

to be *in cis* with p.D143G (data not shown) [31]. Previously we determined that p.R155H is a mild mutation with minimal impact upon characteristics of the PAH enzyme; however, the combined influence of two missense changes in the same polypeptide chain could impact the enzyme more so than either mutation individually [31].

The mutation spectrum in Turkish PKU patients reveals c.1066-11G>A (24.6%), p.R261Q (8.7%) and p.P281L (8.4%) to be frequently associated with mild to classic PKU. This finding is in accordance with previous reports from small studies involving 44 Turkish PKU patients [25,26] and patients of Turkish origin in Germany [32]. Four of the 10 most common mutations (p.R261Q, p.L48S, p.A300S, and p.E390G) present with substantial residual activity (31–72.5% of the wild-type PAH) when expressed in eukaryotic cell systems, and are associated with mild HPA or mild PKU. It has been suggested that p.E390G has only a modestly deleterious impact on the PAH enzyme [26] and the same may apply to the p.A300S. In contrast, p.L48S with 39% residual 'PAH activity' may be associated with both mild and classic PKU [23,33]; thus, the mutation should be classified as equivocal in regard to being BH₄-responsive. However, classifying alleles as BH₄-responsive or non-responsive has limited utility, particularly when compound heterozygosity is involved. Inter-allelic complementation between unique PAH missense enzymes may exert a dominant-negative effect in regard to BH₄ response. Also, *in vitro* expressed activity may not necessarily represent the PAH activity in hepatocytes. Bartholomé et al. [34] showed that patients with classic PKU had no PAH activity in liver needle biopsies, patients with mild to moderate PKU showed up to 6% residual activity, and patients with mild HPA showed 8–35% of the normal activity. This and other studies [35,36] have shown that HPA occurs at *in vivo* PAH activities below 10–15% and that residual activity is essential for maintaining normal hepatic phenylalanine homeostasis. Thus, *in vitro* data should be interpreted cautiously, particularly with regard to BH₄ responsiveness. As liver needle biopsy is no longer justified in PKU patients, *in vitro* assessment of mutant PAH proteins is the primary source of information concerning residual enzyme activity which may be applied to patient phenotypes.

Our study documents, in contrast to a previous report [23], that some mutations (e.g., p.R158Q) with <20% 'PAH activity' should not be classified as BH₄-responsive. Alternatively, some splice-site variants (e.g., c.1066-3C>T) are clearly associated with response to BH₄. It is possible that a BH₄-responsive splicing mutation may not be fully penetrant and the gene may produce multiple mRNAs, including some wild-type PAH-mRNA message. This hypothesis would explain a mild phenotype and BH₄ responsiveness in one patient from our cohort who is homozygous for c.1066-3C>T (Suppl. Table 1).

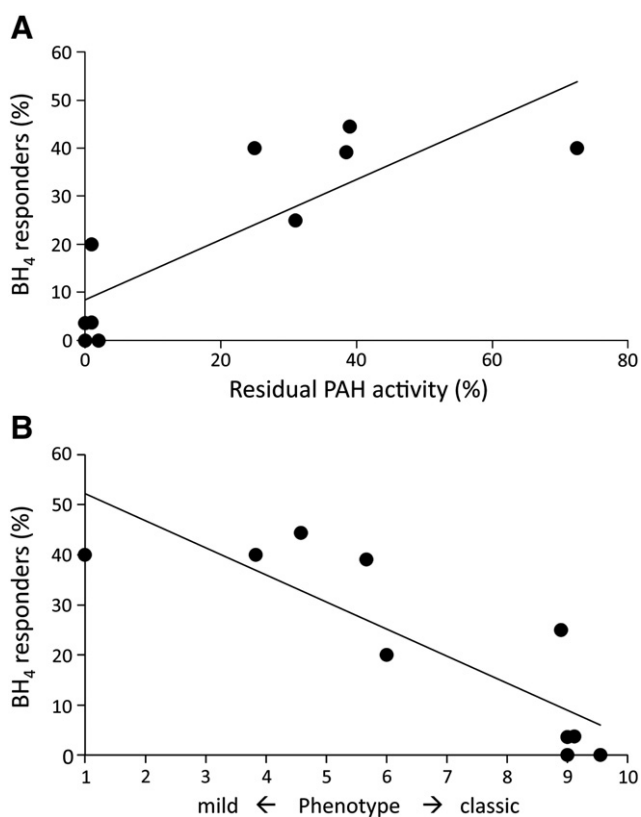


Fig. 2. Correlation between (A) BH₄ responsiveness and residual 'PAH activity,' and (B) BH₄ responsiveness and phenotype in patients with 10 most common ($n \geq 6$) homozygous mutations. Phenotype was scored according to the number of mild HPA, mild PKU or classic PKU patients within the same genotype and with the lowest score (1) for mild HPA and highest score (10) for classic PKU. For details see Table 7.

There is a single report on BH₄ response among 20 Turkish PKU patients (4 mild HPA, 16 mild to moderate PKU, no classic PKU) that estimates the prevalence of responsive patients to be 45%. The authors conclude that predicting BH₄ response based solely on the genotype is difficult owing to a small sample size and compound heterozygous genotypes [37]. In our study, we compared genotypes in which the residual 'PAH activity' of the mutated proteins was previously investigated with the outcome of the BH₄ test. As expected and reported in previous studies [11,14], mild HPA and mild to moderate PKU patients are most likely to elicit a physiological response to BH₄.

For the first time, we are able to compare a large number of homozygous genotypes with the outcome of the BH₄ loading test and with the residual 'PAH activity.' Genotypes that lack residual 'PAH activity' (e.g., p.R408W/p.R408W or IVS4+5G>T/IVS4+5G>T) can be considered non-responsive to BH₄, eliminating the need for a clinical BH₄ challenge. Calculating the residual 'PAH activity' from the information available from *in vitro* experiments may be useful for the prediction and/or exclusion of potential candidates for BH₄ therapy. This method is demonstrably more powerful than calculations based on a single mutation only.

Supplementary data to this article can be found online at doi:10.1016/j.jmgme.2010.11.158.

Competing interests

none

Acknowledgments

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