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ORIGINAL ARTICLE

Molecular epidemiology, genotype–phenotype correlation and BH_4 responsiveness in Spanish patients with phenylketonuria

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Phenylketonuria (PKU), the most common inborn error of amino acid metabolism, is caused by mutations in the phenylalanine-4-hydroxylase (*PAH*) gene. This study aimed to assess the genotype–phenotype correlation in the PKU Spanish population and the usefulness in establishing genotype-based predictions of BH_4 responsiveness in our population. It involved the molecular characterization of 411 Spanish PKU patients: mild hyperphenylalaninemia non-treated (mild HPA-NT) (34%), mild HPA (8.8%), mild-moderate (20.7%) and classic (36.5%) PKU. BH_4 responsiveness was evaluated using a 6R-BH_4 loading test. We assessed genotype–phenotype associations and genotype–BH_4 responsiveness in our population according to literature and classification of the mutations. The mutational spectrum analysis showed 116 distinct mutations, most missense (70.7%) and located in the catalytic domain (62.9%). The most prevalent mutations were c.1066-11G>A (9.7%), p.Val388Met (6.6%) and p.Arg261Gln (6.3%). Three novel mutations (c.61-13del9, p.Ile283Val and p.Gly148Val) were reported. Although good genotype–phenotype correlation was observed, there was no exact correlation for some genotypes. Among the patients monitored for the 6R-BH_4 loading test: 102 were responders (87, carried either one or two BH_4-responsive alleles) and 194 non-responders (50, had two non-responsive mutations). More discrepancies were observed in non-responders. Our data reveal a great genetic heterogeneity in our population. Genotype is quite a good predictor of phenotype and BH_4 responsiveness, which is relevant for patient management, treatment and follow-up.
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INTRODUCTION

Phenylalanine hydroxylase (PAH) deficiency (MIM #261600) is the most common inborn error of amino acid metabolism, with an average incidence of 1 in 10 000 births.[1,2] The disease results from a deficiency of the enzyme phenylalanine-4-hydroxylase (PAH, EC 1.14.16.1), which catalyzes the irreversible conversion of phenylalanine (Phe) to tyrosine, in the presence of (6R)-L-erythro-5,6,7, 8-tetrahydrobiopterin (6R-BH_4), the cofactor of the PAH enzyme.[1,2] Depending on the plasma Phe levels at the time of diagnosis and the Phe tolerance and blood Phe levels, patients are classified into five phenotypic categories: mild hyperphenylalaninemia non-treated (mild HPA-NT), which does not require further treatment, mild HPA, mild phenylketonuria (PKU) (mPKU), moderate PKU (MPKU) and classic PKU (CPKU).[3]

Patients with PKU are typically recommended to follow a specific diet to prevent the accumulation of Phe and its metabolites in physiological fluids, which leads to severe neurological damage.[4,5] For decades, the mainstay of treating PKU in order to maintain

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optimal serum Phe levels has mainly been a vegan-like diet that consists of a marked reduction in Phe intake, and supplementation with Phe-free protein substitutes and specially manufactured low-protein products.[6] This diet has undoubtedly proven effective in preventing the developing brain from being severely injured. However, effects secondary to protein-restricted diets, such as growth retardation, reduced bone mineral density, malnutrition or subtle neurological differences, have been described in the early stages of PKU treatment.[7–9] In an attempt to ameliorate the effects secondary to eating low-protein diets, several approaches have emerged, such as treatment with 6R-BH_4 and the use of large neutral amino acids and glycomacropeptide.[10,11]

The first BH_4-based therapy (using an unregistered formulation) for the treatment of PKU, caused by defective or deficient PAH, was described in 1999,[12] notwithstanding preceding evidence in several PAH-deficient patients of a reduction in Phe concentration levels after 6R-BH_4 loading test. This cofactor-based therapy opened up novel treatment approaches for a significant proportion of patients with mild or moderate PKU who followed vegan-like diets and responded to 6R-BH_4 loading tests. In many cases, the 6R-BH_4 therapy allows patients with PKU to partially or totally liberalize their diet, which in turn should increase dietary compliance and improve the quality of life of patients and their families.[13] In addition, 6R-BH_4 treatment effectively reduces the fluctuation of blood Phe levels, a phenomenon usually observed in PKU patients exclusively and continuously treated with Phe-restricted diets.[14–16] Although, the mechanism underlying treatment with the cofactor is not fully understood, it has been proposed that 6R-BH_4 could act as a chemical chaperone which stabilizes some mutant PAH forms.[17]

The *PAH* gene is located in chromosome 12 (12q22–q24.2), spans about 80 kbp and consists of 13 exons.[18,19] More than 900 mutations has been identified in the *PAH* gene (PAHvdb; http://www.biopku.org/pah/ and HGMD; http://www.biobase-international.com/product/hgmd and http://www.hgmd.ac.uk/ac/index.php), including missense and nonsense mutations, small and large deletions, small insertions and splicing defects. The analyzed populations present great variability in their mutational spectrum, which is characterized by the presence of few prevalent and public mutations and a large number of private pathogenic alterations. Moreover, since each mutation exhibits different levels of residual PAH activity and most patients are compound heterozygotes, a wide variability of phenotypic severity could be observed.[20,21]

Genotype–phenotype correlations in PKU and HPA are assessed based on residual enzyme activity data, which are obtained from *in vitro* expression and analysis of recombinant mutant proteins.[22] However, some discrepancies between the *in vitro* residual activity and patients' clinical phenotype have been reported, and reports showed that siblings with the same genotype exhibited different clinical phenotype.[23–25] Bearing in mind that genotype–phenotype correlations have been proven to be a reliable predicting tool, patients' genotype may help physicians predict phenotype, prescribe individually tailored diets and assess BH_4 responsiveness.

Herein, we present the molecular analysis of the *PAH* gene, the evaluation of genotype–phenotype relationships and the predicted rate of BH_4 responsiveness in a HPA and PKU Spanish population.

MATERIAL AND METHODS

Patients

Patients from 14 Spanish reference metabolism units (see author list), which represented 12 out of a total of 17 regions in Spain, were enrolled in this study. The patients included in the study were diagnosed through the Newborn Screening Programmes and a definitive diagnosis was obtained by mutation analysis of the *PAH* gene. The current cutoff value for Phe is 120 μM. A defect in the synthesis or regeneration pathways of 6R-BH_4 was ruled out by analyzing urinary pterin levels as well as by measuring the dihydropteridine reductase activity.

Patients were classified into one of four phenotypic categories according to blood Phe levels, which were measured at diagnosis: mild HPA-NT (<360 μmol l^{-1}), which does not require further treatment; mild HPA (360–600 μmol l^{-1}), mild-moderate PKU (600–1200 μmol l^{-1}) and classic PKU (>1200 μmol l^{-1}).

The study protocol was approved by Clinical Research Ethics Committees at each hospital involved in this study. Written informed consent was obtained from patients and parents/legal guardians of all the children included.

Genotype analysis

Genetic analyses were performed at Centro de Diagnóstico de Enfermedades Moleculares (Madrid, Spain) by direct sequencing using the BigDye Terminator v.3.1 kit (Applied Biosystems, Foster City, CA, USA) and capillary electrophoresis with a ABI Prism 3700 Genetic Analyser (Applied Biosystems). Patient samples in which only one causative mutation was identified were subjected to multiplex ligation probe amplification (MLPA) analysis (SALSA MLPA Probemix P055 PAH; MRC-Holland, Amsterdam, The Netherlands) to exclude large deletions/duplications. Patients' and their families' genomic DNA was obtained from whole-blood samples and/or dried blood spots. Total genomic DNA was isolated using MagnaPure System (Roche Applied Science, Indianapolis, IN, USA). The primers used for cDNA and genomic DNA amplifications were designed using ENSEMBL database (http://www.ensembl.org/index.html) and GenBank accession number NM_000277. The entire coding sequence and intronic flanking intronic sequences were amplified and sequenced. Segregation analysis was done to rule out the presence of large genomic rearrangements.

PAH residual activity for each mutant protein was assessed from data compiled in the PAHdb (www.pahdb.mcgill.ca), BIOPKUdb (http://www.biopku.org/biopku/) and PAHvdb (http://www.biopku.org/pah/) databases, which provide values calculated from *in vitro* expression of recombinant mutant proteins. PAH activity is defined as the average sum of activities of both individual mutant alleles, and expressed as the percentage of the wild-type enzyme.

BH_4 loading tests and genotype–BH_4-responsiveness correlation

Usually, BH_4 responsiveness is evaluated using a 6R-BH_4 loading test. The 6R-BH_4 loading test used in this study was based on recommendations in the Spanish protocol for treating and monitoring patients with PKU (for a full description of these recommendations, see references 26–28). Briefly, participants were loaded with Phe (100 mg kg^{-1}; Nutricia S.R.L., Madrid, Spain) before 6R-BH_4 administration. In general, an individual was considered to be a primary responder when blood Phe levels fell by at least 30% within 8 h after 6R-BH_4 administration. Patients who showed a reduction in blood Phe levels of at least 30% within 12 h after administration were classified as late responders.

From 2005 onwards, BH_4 responsiveness was evaluated based upon the 50% criterion at Virgen del Rocío University Hospital (for a full description of the protocol, see reference 29). This loading test was designed as a two-stage protocol. In the first stage (the 24-h test), participants were loaded with Phe before 6R-BH_4 administration, as above. With this initial stage of the protocol, an individual was considered 6R-BH_4 responsive when blood Phe levels fell by at least 50% within 24 h after 6R-BH_4 administration. Patients who did not meet the aforementioned criterion underwent the therapeutic test. In this second stage, patients were administered a 6R-BH_4 dose of 20 mg kg^{-1} per day for 1 week together with a daily intake of protein, set on a case-by-case basis in line with age- and sex-specific recommended dietary allowances. The results of the therapeutic test were considered to be positive when Phe levels remained below an established threshold (<360 μmol l^{-1}, for individuals <6 years of age; <480 μmol l^{-1} for those from 6 to $\leqslant 10$ years of age, and <600 μmol l^{-1} for those >10 years of age). Patients who met this criterion were classed as late responders.

Side effects were assessed by asking about headache, vomiting, abdominal pain, rhinorrhoea and any other symptoms after the initiation of 6R-BH_4 treatment.

In addition, we examined the rate of BH_4-resposiveness within our population, and compared with theoretical genotype–BH_4 responsiveness correlation: (a) 'non-responsive', when both alleles carried null mutations; (b) 'responsive', when patients consistently harbored BH_4-responsive mutations on at least one allele; (c) 'probably responsive', when patients harbored at least one mutation with known residual activity, but with inconsistent information on response to BH_4; (d) 'unknown', when information on residual activity or BH_4 responsiveness of at least one allele was pending.

Calculation of homozigosity (*j*)

Homozigosity (*j*) at the *PAH* locus in a given population is determined by $j = \sum x_i^2$, where x_i is the frequency of the allele. In our population, where ascertainment of mutations was not 100%, each of the uncharacterized alleles was defined as having a frequency of 1/*N*, where *N* is the total number of mutant chromosomes investigated.

RESULTS

Mutational spectrum

This study involved the molecular characterization of 411 patients living in Spain, including 24 siblings. The results revealed a mutational spectrum encompassing 116 distinct mutations which were distributed along the *PAH* gene sequence (Table 1). Most mutations were nucleotide substitutions corresponding to 82 missense mutations (70.7%), 15 mutations at splicing sites (12.9%), 11 deletions (9.4%) and 8 nonsense mutations (6.9%). No mutation was detected in exon 13. The majority of the mutations (73) were located in the catalytic domain (62.9%), 22 in the regulatory domain (19%), 6 in the tetramerization domain (5.2%) and 15 in the intronic regions (12.9%).

The most prevalent mutation in our population was c.1066-11G>A (p.Gln355_Tyr356insGlyLeuGln), which displayed a relative frequency of 9.73%, and together with the two mutations c.1162G>A (p.Val388Met) and c.782G>A (p.Arg261Gln), each of them with a relative frequency >6%, represented 22.74% of the mutant alleles. While 22 mutations had a frequency between 0.97 and 3.77% (Table 1), 44 mutations had a frequency between 0.85 and 0.24%, and the remaining mutations (47) were present in only one mutant allele (0.12% each).

A detection rate of 95% was achieved, with complete genotyping of 372 patients, while in the remaining 39 individuals only one causative mutation was identified. Among the fully genotyped patients, 53 (14.2%) were homozygous (Table 2). In addition, we observed 249 different genotypic combinations, 70 of them appeared in more than one patient (Table 2). Homozygous for c.1066-11G>A (p.Gln355_Tyr356insGlyLeuGln) were detected in 13 patients, the combination of this mutation with c.782G>A (p.Arg261Gln) and c.728G>A (p.Arg243Gln) were found in eight and five patients respectively and the combination of c.1162G>A (p.Val388Met) and c.441+5G>T (p.?) in six patients. Sixty-six genotypes were found between two and four times and 179 were observed in single patients.

Furthermore, we detected three novel sequence variants: c.61-13del9 (p.?), c.487A>G (p.Ile283Val) and c.443G>T (p.Gly148Val). Using the computational algorithms SIFT (http://sift.jcvi.org/www/SIFT_enst_submit.html) and Polyphen2 software (http://genetics.bwh.harvard.edu/pph2/), c.487A>G (p.Ile283Val) mutation is predicted to be a benign mutation while c.443G>T (p.Gly148Val) is predicted to be probably damaging. The splice site predictors used, Human Splice Finder (www.umd.be/HSF/) and NNSPLICE (www.fruitfly.org/seq_tools/splice.html), indicated that the novel variant c.61-13del9 (p.?) dramatically disrupt the acceptor splice site of intron.

Homozigosity rate (*j*) in Spain was 0.029, which revealed a high genetic heterogeneity.

Genotype–phenotype correlation

One hundred and fifty patients were classified as classic PKU (36.5%), 85 as mild-moderate PKU (20.7%), 36 as mild HPA (8.8%) and 140 as mild HPA-NT (34%) (Table 2). The patients with classic PKU were mainly homozygous or compound heterozygous of two null mutations (stop gain, frameshift or missense mutations without residual activity).

With regard to the homozygous patients, discrepancies were observed in those who harbored severe or null mutations with no residual activity (c.838G>A (p.Glu280Lys) and c.1066-11G>A (p.Gln355_Tyr356insGlyLeuGln)); the c.838G>A/c.838G>A (p.Glu280Lys/p.Glu280Lys) genotype in our group was related to mild HPA ($n=1$), in addition to the expected classic PKU ($n=3$). Two patients with c.1066-11G>A (p.Gln355_Tyr356insGlyLeuGln)/c.1066-11G>A (p.Gln355_Tyr356insGlyLeuGln) genotype showed mild HPA while the others were mild-moderate ($n=2$) or classic ($n=10$) PKU, consistent with the type of mutation (severe). Discrepancies were also observed in patients who harbored mutations with *a priori* some residual enzymatic activity (c.1241A>G (p.Tyr414Cys), c.782G>A (p.Arg261Gln), c.386A>G (p.Asp129Gly) and c.1162G>A (p.Val388Met)), who displayed a more severe phenotype than expected. Hence, one of the patients homozygous for c.1241A>G (p.Tyr414Cys), one patient homozygous for c.386A>G (p.Asp129Gly) and all patients homozygous for c.782G>A (p.Arg261Gln) mutation displayed mild-moderate PKU instead of the expected mild HPA phenotype and those homozygous for c.1162G>A (p.Val388Met) showed mild-moderate or classic PKU. In the case of the mutation c.1169A>G (p.Glu390Gly), which has been described as MHP (mild HPA-NT), it exhibited a slightly different phenotype (mild HPA) according to the standard classification in homozygosis (c.1169A>G/c.1169A>G (p.Glu390Gly/p.Glu390Gly)).

Although a good genotype–phenotype correlation was observed (Figure 1) in the subgroup of compound heterozygous patients, we observed inconsistencies with regard to genotype–phenotype correlations in 50 patients. Most of them involved the c.194T>C (p.Ile65Thr), c.1162G>A (p.Val388Met), c.143T>C (p.Leu48Ser), c.1241A>G (p.Tyr414Cys), c.204A>T (p.Arg68Ser), c.1223G>A (p.Arg408Gln), c.136G>A (p.Gly46Ser), c.1012G>T (p.Asp338Tyr), c.386A>G (p.Asp129Gly), c.117C>G (p.Phe39Leu) and c.1042C>G (p.Leu348Val) mutations, which in our population were related to phenotypes more severe than expected, as it has been previously reported. On the other hand, three patients with a combination of mutations that has been classified as severe had milder PKU phenotypes (mild HPA-NT or mild HPA) (c.1066-3C>T (p.?)/c.1222C>T (p.Arg408Trp); c.1315+1G>A (p.?)/c.165T>G (p.Phe55Leu) and c.165T>G (p.Phe55Leu)/c.116_118delTCT (p.Phe39del)). In addition, three patients with mutations classified as mild PKU (c.194T>C (p.Ile65Thr), c.722G>A (p.Arg241His) and c.890G>A (p.Arg297His)) in combination with severe mutations had a mild HPA-NT phenotype. Furthermore, some patients with mutations (such as c.898G>T (p.Ala300Ser), c.1169A>G (p.Glu390Gly) and c.1208C>T (p.Ala403Val)) that have been associated to MHP (mild HPA-NT) phenotype had mild HPA and others with mutations (c.183C>G (p.Asn61Lys) and c.527G>A (p.Arg176Leu)) had more severe phenotypes: mild-moderate or classic PKU. In addition to some of the above-mentioned patients, we found that there was no exact

Table 1 Mutational spectrum in the Spanish HPA population, indicating the frequency of alleles and their relative residual PAH activity *in vitro* (mutations are numbered with respect to the reference sequence NM_000277.1)

Nucleotide change	*Amino acid change*	*Type*	*Location*	*Domain*	*Activity (%)*[a]	*Alleles N/%*	*Classification*[a]
c.3G>A	p.?	Miss	E1	Regulatory	NS	2/0.24	U
c.47_48delCT	p.Ser16*	Del	E1	Regulatory	0	1/0.12	Severe
c.58C>T	p.Gln20*	Non	E1	Regulatory	0	1/0.12	Severe
c.61-13del9	p.?	Del	I1	—	NS	1/0.12	U
c.60+5G>A	p.?	Splic	I1	—	NS	1/0.12	U
c.60+5G>T	p.?	Splic	I1	—	NS	4/0.48	Severe
c.116_118delTCT	p.Phe39del	Del	E2	Regulatory	NS	11/1.33	Severe
c.117C>G	p.Phe39Leu	Miss	E2	Regulatory	49	1/0.12	Mild-Moderate
c.124A>G	p.Lys42Glu	Miss	E2	Regulatory	NS	1/0.12	U
c.136G>A	p.Gly46Ser	Miss	E2	Regulatory	16	8/0.97	Moderate
c.140C>T	p.Ala47Val	Miss	E2	Regulatory	13	2/0.24	MHP
c.143T>C	p.Leu48Ser	Miss	E2	Regulatory	39	17/2.06	Mild
c.158G>A	p.Arg53His	Miss	E2	Regulatory	79	1/0.12	MHP
c.161T>C	p.Leu54Ser	Miss	E2	Regulatory	NS	1/0.12	U
c.165T>G	p.Phe55Leu	Miss	E2	Regulatory	NS	7/0.85	Severe
c.165delT	p.Phe55Leufs*6	Del	E2	Regulatory	NS	8/0.97	Severe
c.168+5G>A	p.?	Splic	I2	—	NS	2/0.24	Severe
c.183C>G	p.Asn61Lys	Miss	E3	Regulatory	NS	1/0.12	MHP
c.194T>C	p.Ile65Thr	Miss	E3	Regulatory	33	31/3.77	Mild
c.196G>A	p.Glu66Lys	Miss	E3	Regulatory	NS	1/0.12	U
c.204A>T	p.Arg68Ser	Miss	E3	Regulatory	68	19/2.31	Mild
c.209C>T	p.Ser70Phe	Miss	E3	Regulatory	NS	1/0.12	U
c.261C>A	p.Ser87Arg	Miss	E3	Regulatory	25	6/0.72	U
c.331C>T	p.Arg111*	Non	E3	Regulatory	0	1/0.12	Severe
c.353-1G>C	p.?	Splic	I3	—	NS	1/0.12	Severe
c.364C>T	p.Pro122Ser	Miss	E4	Regulatory	NS	1/0.12	U
c.386A>G	p.Asp129Gly	Miss	E4	Regulatory	NS	3/0.36	Mild
c.434A>T	p.Asp145Val	Miss	E4	Catalytic	NS	1/0.12	MHP
c.439C>T	p.Pro147Ser	Miss	E4	Catalytic	NS	1/0.12	Severe
c.440C>T	p.Pro147Leu	Miss	E4	Catalytic	NS	1/0.12	U
c.441+5G>T	p.?	Splic	I4	—	NS	27/3.28	Severe
c.442-?_509+?del	p.?	Del	E5	Catalytic	NS	1/0.12	U
c.442-5C>G	p.?	Splic	I4	—	ND	1/0.12	Mild-Moderate
c.443G>T	p.Gly148Val	Miss	E5	Catalytic	NS	1/0.12	U
c.463C>T	p.Arg155Cys	Miss	E5	Catalytic	NS	1/0.12	U
c.473G>A	p.Arg158Gln	Miss	E5	Catalytic	10	11/1.33	Severe
c.490A>G	p.Ile164Val	Miss	E5	Catalytic	NS	1/0.12	U
c.520A>G	p.Ile174Val	Miss	E6	Catalytic	NS	1/0.12	Moderate-Severe
c.526C>T	p.Arg176*	Non	E6	Catalytic	0	1/0.12	Severe
c.527G>T	p.Arg176Leu	Miss	E6	Catalytic	42	22/2.67	MHP
c.527G>A	p.Arg176Gln	Miss	E6	Catalytic	NS	1/0.12	U
c.529G>A	p.Val177Met	Miss	E6	Catalytic	NS	4/0.48	MHP
c.533A>G	p.Glu178Gly	Miss	E6	Catalytic	39	4/0.48	MHP
c.586_608del23	p.Ser196Leufs*2	Del	E6	Catalytic	NS	1/0.12	Severe
c.593_614del22	p.Tyr198Cysfs*136	Del	E6	Catalytic	NS	3/0.36	Severe
c.608G>A	p.Cys203Tyr	Miss	E6	Catalytic	NS	1/0.12	U
c.612T>G	p.Tyr204*	Non	E6	Catalytic	0	4/0.48	Severe
c.613G>A	p.Glu205Lys	Miss	E6	Catalytic	NS	2/0.24	Severe
c.618C>G	p.Tyr206*	Non	E6	Catalytic	0	2/0.24	Severe
c.631C>A	p.Pro211Thr	Miss	E6	Catalytic	72	2/0.24	MHP
c.649T>G	p.Cys217Gly	Miss	E6	Catalytic	NS	2/0.24	U
c.688G>A	p.Val230Ile	Miss	E6	Catalytic	63	4/0.48	MHP
c.722G>A	p.Arg241His	Miss	E7	Catalytic	23	3/0.36	Mild
c.727C>T	p.Arg243*	Non	E7	Catalytic	0	22/2.67	Severe
c.728G>A	p.Arg243Gln	Miss	E7	Catalytic	14	23/2.79	Severe
c.734T>C	p.Val245Ala	Miss	E7	Catalytic	50	3/0.36	MHP
c.754C>T	p.Arg252Trp	Miss	E7	Catalytic	0	3/0.36	Severe
c.755G>A	p.Arg252Gln	Miss	E7	Catalytic	3	1/0.12	Severe
c.773T>C	p.Leu258Pro	Miss	E7	Catalytic	NS	2/0.24	MHP
c.781C>T	p.Arg261*	Non	E7	Catalytic	1	4/0.48	Severe

Table 1 (Continued)

Nucleotide change	*Amino acid change*	*Type*	*Location*	*Domain*	*Activity (%)*[a]	*Alleles N/%*	*Classification*[a]
c.782G>A	p.Arg261Gln	Miss	E7	Catalytic	44	52/6.32	Moderate
c.782G>C	p.Arg261Pro	Miss	E7	Catalytic	NS	2/0.24	Moderate
c.805A>C	p.Ile269Leu	Miss	E7	Catalytic	NS	1/0.12	MHP
c.809G>A	p.Arg270Lys	Miss	E7	Catalytic	NS	2/0.24	Severe
c.814G>T	p.Gly272*	Non	E7	Catalytic	0	3/0.36	Severe
c.824C>G	p.Pro275Arg	Miss	E7	Catalytic	NS	4/0.48	U
c.827T>A	p.Met276Lys	Miss	E7	Catalytic	NS	1/0.12	U
c.829T>G	p.Tyr277Asp	Miss	E7	Catalytic	0	6/0.72	Mild
c.838G>A	p.Glu280Lys	Miss	E7	Catalytic	2	20/2.43	Severe
c.841C>T	p.Pro281Ser	Miss	E7	Catalytic	NS	1/0.12	Severe
c.842C>T	p.Pro281Leu	Miss	E7	Catalytic	2	14/1.70	Severe
c.842+1G>A	p.?	Splic	I7	—	NS	4/0.48	Severe
c.842+3G>C	p.?	Splic	I7	—	NS	4/0.48	Severe
c.847A>G	p.Ile283Val	Miss	E8	Catalytic	NS	1/0.12	U
c.865G>A	p.Gly289Arg	Miss	E8	Catalytic	NS	2/0.24	U
c.889C>T	p.Arg297Cys	Miss	E8	Catalytic	NS	3/0.36	Mild
c.890G>A	p.Arg297His	Miss	E8	Catalytic	21	2/0.24	Mild
c.898G>T	p.Ala300Ser	Miss	E8	Catalytic	31	22/2.67	MHP
c.907T>G	p.Ser303Ala	Miss	E8	Catalytic	NS	2/0.24	Mild
c.912G>A	p.(=)	Splic	E8	Catalytic	NS	6/0.72	U
c.912+1G>A	p.?	Splic	I8	—	ND	4/0.48	Severe
c.913-7A>G	p.?	Splic	I8	—	ND	7/0.85	Severe
c.916A>G	p.Ile306Val	Miss	E9	Catalytic	39	1/0.12	MHP
c.926C>T	p.Ala309Val	Miss	E9	Catalytic	42	6/0.72	Mild-Moderate
c.932T>C	p.Leu311Pro	Miss	E9	Catalytic	1	1/0.12	Severe
c.938C>T	p.Ala313Val	Miss	E9	Catalytic	NS	2/0.24	MHP
c.964G>A	p.Ala322Thr	Miss	E9	Catalytic	NS	1/0.12	MHP
c.1012G>T	p.Asp338Tyr	Miss	E10	Catalytic	NS	1/0.12	Mild
c.1027T>G	p.Tyr343Asp	Miss	E10	Catalytic	NS	1/0.12	U
c.1042C>G	p.Leu348Val	Miss	E10	Catalytic	35	7/0.85	Moderate
c.1045T>C	p.Ser349Pro	Miss	E10	Catalytic	1	28/3.40	Severe
c.1055delG	p.Gly352Valfs*48	Del	E10	Catalytic	ND	4/0.48	Severe
c.1056delT	p.Glu353Asnfs*47	Del	E10	Catalytic	ND	4/0.48	Severe
c.1066-3C>T	p.?	Splic	I10	—	NS	1/0.12	Severe
c.1066-11G>A	p.Gln355_Tyr356ins3 p.Gln355_Tyr356insGlyLeuGln	Splic	I10	—	5	80/9.73	Severe
c.1081A>C	p.Lys361Gln	Miss	E11	Catalytic	NS	1/0.12	U
c.1084C>A	p.Pro362Thr	Miss	E11	Catalytic	NS	3/0.36	Severe
c.1089G>T	p.Lys363Asn	Miss	E11	Catalytic	NS	1/0.12	U
c.1139C>T	p.Thr380Met	Miss	E11	Catalytic	NS	9/1.09	MHP
c.1162G>A	p.Val388Met	Miss	E11	Catalytic	28	55/6.69	Mild-Moderate
c.1162G>C	p.Val388Leu	Miss	E11	Catalytic	NS	1/0.12	U
c.1169A>G	p.Glu390Gly	Miss	E11	Catalytic	62	16/1.94	MHP
c.1171_1172delAG	p.Ser391Phefs*2	Del	E11	Catalytic	NS	1/0.12	Severe
c.1184C>G	p.Ala395Gly	Miss	E11	Catalytic	NS	2/0.24	Mild
c.1199+88delC	p.?	Del	I11	—	NS	1/0.12	MHP
c.1208C>T	p.Ala403Val	Miss	E12	Catalytic	66	31/3.77	MHP
c.1217T>C	p.Ile406Thr	Miss	E12	Catalytic	NS	1/0.12	U
c.1222C>T	p.Arg408Trp	Miss	E12	Catalytic	2	22/2.67	Severe
c.1223G>A	p.Arg408Gln	Miss	E12	Catalytic	46	2/0.24	Mild
c.1241A>G	p.Tyr414Cys	Miss	E12	Tetramerization	57	20/2.43	Mild
c.1243G>A	p.Asp415Asn	Miss	E12	Tetramerization	72	8/0.97	MHP
c.1249T>C	p.Tyr417His	Miss	E12	Tetramerization	NS	2/0.24	Mild
c.1259G>T	p.Arg420Met	Miss	E12	Tetramerization	NS	1/0.12	U
c.1262T>C	p.Ile421Thr	Miss	E12	Tetramerization	NS	1/0.12	U
c.1314_1315+4del6	p.?	Splic	E12	Tetramerization	NS	1/0.12	Severe
c.1315+1G>A	p.?	Splic	I12	—	<1	8/0.97	Severe

Abbreviations: E, exon; Del, deletion; I, intron; Miss, missense; ND, not detectable; Non, nonsense; NS, not stated; Splic, splicing; U, undefined.
*, STOP CODON; MHP, mild hyperphenylalaninemia.
[a]Classification of PAH mutants into four phenotype categories (AV=1, severe; AV=2, moderate; AV=4, mild; AV=8, MHP. AV=arbitrary value) according to PAHvdb (Phenylalanine Hydroxylase Gene Locus-Specific Database; http://www.biopku.org/pah/), Guldberg *et al.*,[39] Pey *et al.*,[30] Jennings *et al.*[45] and Desviat *et al.*[46]

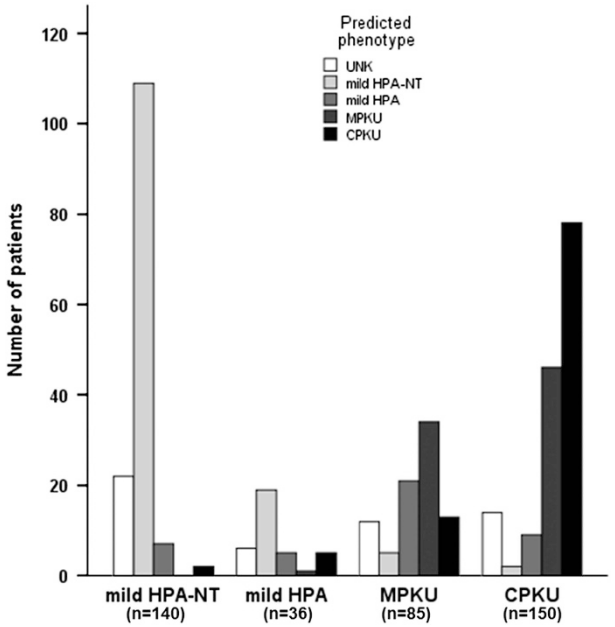

Figure 1 Genotype and phenotype correlation in the different severity groups of patients with phenylketonuria (PKU). The *X*-axis indicates the four phenotypic categories in our population: mild HPA-NT ($n=140$), mild HPA ($n=36$), mild-moderate PKU (MPKU) ($n=85$) and classic PKU (CPKU) ($n=150$). Each column represents the predicted phenotype in each group of patients. Predicted phenotype was determined according to the severity and residual enzymatic activity for each type mutation as previously described in the literature. Predicted phenotype based on the type of the two mutated alleles of the genotype is: □: UNK: unknown; □: mild HPA-NT; ■: mild HPA; ■: MPKU and ■: CPKU. The *Y*-axis indicates the number of patients with each predicted phenotype in each group. *n*: number of patients.

correlation for patients with these same genotypes: c.143T>C (p.Leu48Ser)/c.829T>G (Tyr277Asp); c.527G>T (p.Arg176Leu)/c.782G>A (p.Arg261Gln); c.1042C>G (p.Leu348Val)/c.1241A>G (p.Tyr414Cys); c.782G>A (p.Arg261Gln)/c.1169A>G (p.Glu390Gly); c.1162G>A (p.Val388Met)/c.1208C>T (p.Ala403Val); c.1139C>T (p.Thr380Met)/c.1066-11G>A (p.Gln355_Tyr356insGlyLeuGln); c.60+5G>T (p.?)/c.1169A>G (p.Glu390Gly); c.1208C>T (p.Ala403Val)/c.165delT (p.Phe55Leufs*6) and c.907T>G (p.Ser303Ala)/c.136G>A (p.Gly46Ser).

Genotype and BH_4 responsiveness

Within our group of 116 mutations, we identified 37 mutations that are known to be BH_4 responsive[30–32] (Table 2), the majority are located in the catalytic domain. We have detected 20 non-responsive mutations, and the remaining 59 has been classified as unclear.[30–32]

BH_4 responsiveness was evaluated using a 6R-BH_4 loading test in 296 patients. No 6R-BH_4 loading test was performed in the remaining patients (115), who were mild HPA-NT. Among those patients who were monitored for the 6R-BH_4 loading test, we observed 102 patients who responded to 6R-BH_4 therapy (34.45%). Out of these 102 BH_4-responder patients, 87 (85.29%) carried either one or two BH_4-responsive alleles and 13 patients' genotypes involved one allele with no detectable mutation or mutations with no information available for BH_4 responsiveness. In addition, there was one BH_4-responsive patient who carried mutations previously described as unresponsive to BH_4 in both alleles: c.842C>T (p.Pro281Leu)/c.1045T>C (p.Ser349Pro).

Moreover, we observed 194 patients unresponsive to 6R-BH_4. Among the non-responders, 50 patients (25.77%) had two non-responsive mutations and 42 patients (21.64%) carried either combinations of one non-responsive mutation with no detectable mutation or mutations with no information available for BH_4 responsiveness, or combinations of alleles with no detectable mutation or no information available. Genotype-based predictions of BH_4 responsiveness were assessed in almost all the patients, and some discrepancies were observed in 102 patients in the non-responder group. In this regard, 52.57% of non-responders carried at least one mutation previously reported to be related to BH_4 responsiveness (c.782G>A (p.Arg261Gln), c.1241A>G (p.Tyr414Cys), c.143T>C (p.Leu48Ser), c.1169A>G (p.Glu390Gly), c.194T>C (p.Ile65Thr), c.473G>A (p.Arg158Gln), c.1162G>A (p.Val388Met), c.116_118delTCT (p.Phe39del), c.117C>G (p.Phe39Leu), c.204A>T (p.Arg68Ser), c.1042C>G (p.Leu348Val), c.926C>T (p.Ala309Val), c.439C>T (p.Pro147Ser), c.527G>T (p.Arg176Leu), c.1208C>T (p.Ala403Val), c.1139C>T (p.Thr380Met), c.165delT (p.Phe55Leufs*6), c.442-5C>G (p.?))[31], whereas the others harbored mutations that were not associated with positive response to 6R-BH_4 therapy.

The analysis of the theoretical response to 6R-BH_4 therapy based on available *PAH* locus-specific mutation data (BIOPKUdb; http://www.biopku.org/biopku/ and PAHvdb; http://www.biopku.org/pah/) anticipated that 105 genotypes would be responders to BH_4, 109 genotypes would be BH_4-unresponsive, 89 genotypes would probably show a positive response to BH_4 and 108 genotypes would have an unknown response to BH_4 due to the lack of information on residual activity or BH_4 response. In general, there was a good correlation between the analysis of the theoretical BH_4 responsiveness and the results from the 6R-BH_4 loading tests (Table 2 and Figure 2). However, more discrepancies between both analyses were observed in the non-responders group.

DISCUSSION

In the present study, patients were classified according to the Spanish recommendations for patients with PKU[26,27] as well as the National Institutes of Health (NIH) Phenylketonuria Scientific Review Conference.[3] Our classification system may differ from the one used in north-European countries: non-PKU-HPA or MHP ($\leqslant$600 μmol l^{-1} Phe), mild PKU (600–1200 μmol l^{-1}) and classic PKU ($\geqslant$1200 μmol l^{-1}) groups.[1] This fact should be kept in mind when considering our findings. In this regard, our mild HPA-NT ($<$360 μmol l^{-1} Phe) and mild HPA (360–600 μmol l^{-1}) groups would correspond to the MHP group (180–600 μmol l^{-1}). However, we considered that the nomenclature system used in our study highlights the fact that treatment might be required at baseline Phe levels between 360 and 600 μmol l^{-1} (mild HPA group), whereas at Phe $\leqslant$ 360 μmol l^{-1} (mild HPA-NT) there is general consensus that no treatment is required.

The molecular characterization of a PAH deficiency population living in Spain confirmed its genetic heterogeneity. This heterogeneity is a common feature of South European populations[33,34] in contrast to the North-Eastern ones.[35] In our study, the most prevalent mutations are c.1066-11G>A (p.Gln355_Tyr356insGlyLeuGln) (9.73%), c.1162G>A (p.Val388Met) (6.69%) and c.782G>A (p.Arg261Gln) (6.32%). Mutations such as c.194T>C (p.Ile65Thr), c.1208C>T (p.Ala403Val), c.1045T>C (p.Ser349Pro), c.441+5G>T (p.?), c.728G>A (p.Arg243Gln), c.727C>T (p.Arg243*), c.898G>T (p.Ala300Ser), c.527G>T (p.Arg176Leu) and c.1222C>T (p.Arg408Trp) have also a considerably high percentage in our population

Table 2 Summary of genotypes, phenotypes and response to the BH_4 loading test in our Spanish study population

Genotype (allele 1);[allele 2)	*Phenotype*	*Predicted phenotype*	*Response BH_4 test*	*Predicted BH_4 responsiveness*[a] *(allele 1];[allele 2)*	*BIOPKUdb BH_4 responsiveness*[a]
p.[Val230Ile];[Arg408Trp]	mild HPA-NT	MHP	No test	Y];[N	Not tested
p.[Ala47Val];[Glu178Gly]	mild HPA-NT	—	No test	UNK];[Y	No records
p.[Ala300Ser];[Arg68Ser]	mild HPA-NT	—	No test	Y];[Y	No records
p.[Phe55Leu];[Phe39del]	mild HPA-NT	—	No test	UNK];[Y	No records
p.[Ser349Pro];[Ala403Val]	mild HPA-NT	MHP	No test	N];[Y	Not tested
p.[Arg243Gln];[Ala300Ser] (*n*=2)	mild HPA-NT	MHP	No test	N];[Y	Not tested
p.[Phe55Leu];c.[3G>A] (*n*=2)	mild HPA-NT	—	No test	UNK];[UNK	No records
p.[Ala300Ser];[Glu390Gly]	mild HPA-NT	—	No test	Y];[Y	No records
p.[Val245Ala];[Cys217Gly]	mild HPA-NT	—	No test	Y];[UNK	No records
p.[Ser349Pro];[?]	mild HPA-NT	—	No test	N];[?	No records
p.[Arg261Gln];[Asp415Asn]	mild HPA-NT	MHP	No test	Y];[Y	Not tested
p.[Glu178Gly];[Ala403Val]	mild HPA-NT	MHP	No test	Y];[Y	Y
p.[Arg176Leu];[Phe39del] (*n*=2)	mild HPA-NT	—	No test	Y];[Y	No records
p.[Ala403Val];[Arg420Met]	mild HPA-NT	—	No test	Y];[UNK	No records
p.[Ile269Leu];[Val388Met]	mild HPA-NT	—	No test	Y];[Y	No records
p.[Thr380Met];[Arg243Gln]	mild HPA-NT	—	No test	Y];[N	No records
p.[Ser87Arg];c.[1315+1G>A]	mild HPA-NT	—	No test	Y];[N	No records
p.[Val177Met];[Val388Met]	mild HPA-NT	—	No test	Y];[Y	No records
p.[Arg176Leu];[Leu48Ser] (*n*=2)	mild HPA-NT	—	No test	Y];[Y	No records
p.[Arg176Leu];[Tyr277Asp]	mild HPA-NT	—	No test	Y];[UNK	No records
p.[Ala313Val];c.[1066-11G>A]	mild HPA-NT	—	No test	UNK];[N	No records
p.[Arg243*];[Arg176Leu] (*n*=3)	mild HPA-NT	—	No test	N];[Y	No records
p.[Ala300Ser];[Ile174Val]	mild HPA-NT	—	No test	Y];[UNK	No records
p.[Arg176Gln];[Val388Met]	mild HPA-NT	—	No test	UNK];[Y	No records
p.[Tyr198Cysfs*136];[Val230Ile] (*n*=2)	mild HPA-NT	—	No test	UNK];[Y	No records
p.[Thr380Met];[Ala300Ser]	mild HPA-NT	—	No test	Y];[Y	No records
p.[Arg243*];[Lys361Gln]	mild HPA-NT	—	No test	N];[UNK	No records
p.[Ala300Ser];c.[1066-11G>A]	mild HPA-NT	MHP];[MPKU	No test	Y];[N	Y];[Not tested
c.[442-?_509+?del];p.[Ala313Val]	mild HPA-NT	—	No test	Y];[UNK	No records
p.[Tyr417His];[?]	mild HPA-NT	—	No test	Y];[?	No records
p.[Ser87Arg];[Arg261Gln] (*n*=3)	mild HPA-NT	—	No test	Y];[Y	No records
p.[Ala300Ser];[?]	mild HPA-NT	—	No test	Y];[?	No records
p.[Phe55Leu];[Lys42Glu]	mild HPA-NT	—	No test	UNK];[UNK	No records
p.[Ala300Ser];[Ala300Ser]	mild HPA-NT	MHP];[MPKU	No test	Y];[Y	Y];[N];[Slow]; [Not tested
p.[Glu280Lys];[Thr380Met] (*n*=2)	mild HPA-NT	—	No test	N];[Y	No records
p.[Glu390Gly];c.[1315+1G>A]	mild HPA-NT	MPKU];[MHP	No test	Y];[N	Y];[Not tested
c.[1066-11G>A];p.[Ala403Val] (*n*=2)	mild HPA-NT	MHP];[MPKU	No test	N];[Y	Y];[Not tested
p.[Pro211Thr];[Phe55Leu]	mild HPA-NT	—	No test	Y];[UNK	No records
c.[1066-11G>A];p.[Arg297His]	mild HPA-NT	—	No test	N];[Y	No records
p.[Arg297His];[?]	mild HPA-NT	—	No test	Y];[?	No records
p.[Val230Ile];[?]	mild HPA-NT	—	No test	Y];[?	No records
c.[1315+1G>A];p.[Phe55Leu]	mild HPA-NT	—	No test	N];[UNK	No records
p.[Arg241His];[?]	mild HPA-NT	—	No test	Y];[?	No records
c.[1066-11G>A];p.[Asp415Asn] (*n*=2)	mild HPA-NT	MHP];[MPKU	No test	N];[Y	Y];[Not tested
p.[Ala403Val];c.[1199+88delC]	mild HPA-NT	—	No test	Y];[UNK	No records
p.[Tyr206*];[Thr380Met]	mild HPA-NT	—	No test	N];[Y	No records
p.[Arg176Leu];[Arg243Gln]	mild HPA-NT	—	No test	Y];[N	No records
c.[913-7A>G];p.[Arg53His]	mild HPA-NT	—	No test	N];[UNK	No records
p.[Ser70Phe];[Ala300Ser]	mild HPA-NT	—	No test	UNK];[Y	No records
c.[60+5G>A];p.[Ala403Val]	mild HPA-NT	—	No test	UNK];[Y	No records
p.[Val245Ala];c.[1066-11G>A]	mild HPA-NT	MHP	No test	Y];[N	Y];[Not tested
p.[Thr380Met];[Ala403Val]	mild HPA-NT	MHP	No test	Y];[Y	Not tested
p.[Ala403Val];[Arg243Gln]	mild HPA-NT	MHP	No test	Y];[N	Y];[Not tested
c.[441+5G>T];p.[Ala300Ser]	mild HPA-NT	MPKU	No test	UNK];[Y	Y];[Not tested
p.[Ala300Ser];c.[842+3G>C]	mild HPA-NT	MPKU	No test	Y];[UNK	Y
p.[Asp415Asn];[Ile65Thr] (*n*=2)	mild HPA-NT	—	No test	Y];[Y	No records
p.[Arg243*];[Ala300Ser]	mild HPA-NT	MPKU];[CPKU	No test	N];[Y	Not tested
p.[Asp415Asn];[441+5G>T]	mild HPA-NT	MHP	No test	Y];[UNK	Not tested
p.[Ser349Pro];[Ala322Thr]	mild HPA-NT	—	No test	N];[UNK	No records

Table 2 (Continued)

Genotype (allele 1);[allele 2)	*Phenotype*	*Predicted phenotype*	*Response BH_4 test*	*Predicted BH_4 responsiveness[a] (allele 1];[allele 2)*	*BIOPKUdb BH_4 responsiveness[a]*
p.[Glu178Gly];[Ala300Ser] ($n=2$)	mild HPA-NT	—	No test	Y];[Y	No records
p.[Phe55Leufs*6];[Lys363Asn]	mild HPA-NT	—	No test	Y];[UNK	No records
p.[Ile65Thr];[Val245Ala]	mild HPA-NT	MHP	No test	Y];[Y	Not tested
p.[Arg176Leu];[Tyr414Cys] ($n=3$)	mild HPA-NT	—	No test	Y];[Y	No records
p.[Leu48Ser];[Ala395Gly] ($n=2$)	mild HPA-NT	—	No test	Y];[UNK	No records
p.[Ser87Arg];[Ala403Val]	mild HPA-NT	—	No test	Y];[Y	No records
p.[Glu353Asnfs*47];[Ala403Val]	mild HPA-NT	—	No test	UNK];[Y	No records
p.[Ala403Val];[Val177Met] ($n=2$)	mild HPA-NT	—	No test	Y];[Y	No records
p.[Arg297Cys];[Arg297Cys]	mild HPA-NT	—	No test	UNK];[UNK	No records
p.[Arg297Cys];[?]	mild HPA-NT	—	No test	UNK];[?	No records
p.[Ile164Val];[?]	mild HPA-NT	—	No test	UNK];[?	No records
p.[Ala403Val];[Ala403Val]	mild HPA-NT	MHP];[MPKU	No test	Y];[Y	Y];[Not tested
p.[Arg176Leu];[Glu390Gly]	mild HPA-NT	—	No test	Y];[Y	No records
p.[Arg176Leu];[Arg408Trp]	mild HPA-NT	MHP	No test	Y];[N	Not tested
p.[Ala403Val];[Tyr417His]	mild HPA-NT	—	No test	Y];[Y	No records
p.[Ala300Ser];[Val388Met]	mild HPA	MHP	No test	Y];[Y	Not tested
p.[Glu390Gly];[Glu390Gly]	mild HPA	MHP];[MPKU	No test	Y];[Y	Y];[Not tested
p.[Glu353Asnfs*47];[Ile306Val]	mild HPA	—	No test	UNK];[Y	No records
p.[Pro362Thr];[Ala300Ser]	mild HPA-NT	—	Y	UNK];[Y	No records
p.[Asp145Val];[?]	mild HPA-NT	—	Y	UNK;[?	No records
p.[Ala47Val];c.[1066-11G>A]	mild HPA-NT	—	Y	UNK];[N	No records
p.[Arg241His];[Ser349Pro]	mild HPA-NT	MPKU	Y	Y];[N	Y];[N];[Not tested
p.[Arg68Ser];[Asp415Asn]	mild HPA-NT	—	Y	Y];[Y	No records
p.[Tyr414Cys];[Gly46Ser] ($n=2$)	mild HPA-NT	MPKU];[MHP	Y	Y];[UNK	Y];[Not tested
p.[Arg176Leu];[Glu280Lys]	mild HPA-NT	—	Y	Y];[N	No records
p.[Asp415Asn];[Pro122Ser]	mild HPA-NT	—	Y	Y];[UNK	No records
p.[Arg241His];[Ile421Thr]	mild HPA-NT	—	Y	Y];[UNK	No records
p.[Ser87Arg];[Gly352Valfs*48]	mild HPA-NT	MHP	Y	Y];[UNK	Y
p.[Ile65Thr];[Phe55Leu]	mild HPA-NT	MHP	Y	Y];[UNK	Y
p.[Tyr414Cys];[Arg261Gln]	mild HPA-NT	MHP];[MPKU	Y	Y];[Y	Y
c.[1066-11G>A];p.[Glu390Gly]	mild HPA-NT	MHP];[MPKU	Y	N];[Y	Y];[Not tested
p.[Ala300Ser];[Leu48Ser]	mild HPA-NT	MHP];[MPKU	Y	Y];[Y	Not tested
p.[Val388Met];[Arg68Ser]	mild HPA-NT	MHP	Y	Y];[Y	Y];[Not tested
p.[Val177Met];[Arg261Gln]	mild HPA-NT	—	Y	Y];[Y	No records
p.[Arg68Ser];[Met276Lys]	mild HPA-NT	—	Y	Y];[UNK	No records
p.[Arg68Ser];[Ala403Val]	mild HPA-NT	MHP	Y	Y];[Y	Not tested
p.[Val388Met];[Ala403Val] ($n=2$)	mild HPA-NT ($n=1$) mild HPA ($n=1$)	MHP	Y	Y];[Y	Y
p.[Ser303Ala];[Gly46Ser] ($n=2$)	mild HPA-NT ($n=1$) mild HPA ($n=1$)	MHP	Y	UNK];[UNK	Y
p.[Ala300Ser];[Arg261Gln]	mild HPA	MHP	Y	Y];[Y	Y];[Not tested
p.[Tyr414Cys];c.[441+5G>T]	mild HPA	MPKU];[MHP];[CPKU	Y	Y];[UNK	Y];[Not tested
p.[Cys203Tyr];[Ala300Ser]	mild HPA	MHP	Y	UNK];[Y	Y
p.[Pro211Thr];c.[1066-11G>A]	mild HPA	MHP];[MPKU	Y	Y];[N	Y];[Slow
c.[1066-3C>T];p.[Arg408Trp]	mild HPA	MPKU];[CPKU	Y	Y];[N	N];[Not tested
p.[Arg68Ser];[Arg158Gln]	mild HPA	—	Y	Y];[Y	No records
p.[Ala309Val];[Glu390Gly]	mild HPA	—	Y	Y];[Y	No records
p.[Arg68Ser];[Arg68Ser] ($n=2$)	mild HPA	MPKU	Y	Y];[Y	Y
p.[Gly352Valfs*48];[Glu390Gly]	mild HPA	—	Y	UNK];[Y	No records
p.[Arg243Gln];[Arg408Gln]	mild HPA	MHP];[MPKU	Y	N];[Y	Y];[Not tested
p.[Val388Met];[Glu390Gly]	mild HPA	MHP];[MPKU	Y	Y];[Y	Y
p.[Cys217Gly];c.[1066-11G>A]	mild HPA	MHP	Y	UNK];[N	Y
p.[Arg155Cys];[Gly289Arg]	mild HPA	MHP	Y	UNK];[UNK	Y
p.[Arg68Ser];[Pro362Thr]	mild HPA	MHP	Y	Y];[UNK	Y
p.[Ala300Ser];c.[912G>A]	mild HPA	MHP	Y	Y];[UNK	Y
p.[Glu66Lys];[Ser349Pro]	mild HPA	MHP	Y	UNK];[N	Y
p.[Glu390Gly];c.[441+5G>T]	mild HPA	MPKU	Y	Y];[UNK	Not tested
p.[Leu48Ser];[Tyr277Asp] ($n=2$)	mild HPA-NT ($n=1$) MPKU ($n=1$)	MPKU];[MHP	Y	Y];[UNK	Y

Table 2 (Continued)

Genotype (allele 1);[allele 2)	*Phenotype*	*Predicted phenotype*	*Response BH_4 test*	*Predicted BH_4 responsiveness[a] (allele 1];[allele 2)*	*BIOPKUdb BH_4 responsiveness[a]*
p.[Leu348Val];[Tyr414Cys] (*n*=2)	mild HPA (*n*=1) MPKU (*n*=1)	MPKU	Y	Y];[Y	Y
p.[Tyr414Cys];[Gly272*]	MPKU	MPKU];[CPKU	Y	Y];[N	Y];[Slow];[Not tested
c.[1315+1G>A];p.[Arg68Ser]	MPKU	CPKU];[MPKU	Y	N];[Y	Y];[N];[Slow
c.[441+5G>T];p.[Tyr414Cys]	MPKU	MPKU];[MHP];[CPKU	Y	UNK];[Y	Y];[Not tested
p.[Arg261Gln];[Arg261Gln] (*n*=3)	MPKU	MHP];[MPKU];[CPKU	Y	Y];[Y	Y];[N];[Slow];[Not tested
p.[Ile65Thr];[Arg158Gln]	MPKU	MPKU];[CPKU	Y	Y];[Y	N];[Y];[Not tested
c.[1066-11G>A];p.[Asn61Lys]	MPKU	MHP	Y	N];[UNK	Y
p.[Arg408Trp];[Arg408Gln]	MPKU	MPKU	Y	N];[Y	Y];[Not tested
p.[Leu348Val];[Arg261Gln]	MPKU	MPKU];[CPKU	Y	Y];[Y	Y];[Slow];[Not tested
p.[Ile65Thr];[Arg243*]	MPKU	CPKU];[MPKU	Y	Y];[N	N];[Slow
c.[912G>A];p.[Arg176Leu]	MPKU	MKPU	Y	UNK];[Y	Y];[Not tested
p.[Arg158Gln];[Leu48Ser]	MPKU	MPKU];[CPKU];[MHP	Y	Y];[Y	Y];[N];[Slow];[Not tested
p.[Arg68Ser];c.[1066-11G>A]	MPKU	MPKU];[MHP	Y	Y];[N	Y];[Not tested
p.[Pro275Arg];[Arg158Gln] (*n*=2)	MPKU	—	Y	UNK];[Y	No records
p.[Arg261*];[Pro147Leu]	MPKU	—	Y	N];[Y	No records
c.[1066-11G>A];[?]	MPKU	—	Y	N];[?	No records
p.[Ser349Pro];[Arg68Ser]	MPKU	MHP];[MPKU	Y	N];[Y	Y];[Not tested
p.[Val388Met];c.[61-13del9]	MPKU	—	Y	Y];[UNK	No records
p.[Arg243Gln];[Arg261Gln] (*n*=2)	MPKU	MPKU];[CPKU	Y	N];[Y	N];[Slow];[Not tested
p.[Asp129Gly];[Asp129Gly]	MPKU	MPKU	Y	Y];[Y	No tested
p.[Pro281Leu];[Asp338Tyr]	MPKU	MPKU	Y	N];[Y	Y
p.[Arg243Gln];[Tyr277Asp]	MPKU	—	Y	N];[UNK	No records
c.[1066-11G>A];[Asp129Gly]	MPKU	—	Y	N];[Y	No records
p.[Val388Met];[Arg261Gln] (*n*=2)	CPKU	MPKU];[CPKU	Y	Y];[Y	Y];[Slow];[Not tested
p.[Ile65Thr];[Ala309Val]	CPKU	MPKU	Y	Y];[Y	Y
p.[Pro281Leu];[Ser349Pro]	CPKU	CPKU	Y	N];[N	N
c.[1066-11G>A];p.[Glu353Asnfs*47]	CPKU	—	Y	N];[UNK	No records
p.[Arg243Gln];[Val388Met]	CPKU	CPKU];[MPKU	Y	N];[Y	Y];[Not tested
p.[Ile65Thr];[Ser16*]	CPKU	CPKU	Y	Y];[UNK	Not tested
c.[441+5G>T];p.[Pro281Leu]	CPKU	CPKU	Y	UNK];[N	No tested
p.[Arg158Gln];[?]	CPKU	—	Y	Y];[?	No records
p.[Leu348Val];[Arg252Trp]	CPKU	CPKU	Y	Y];[N	N
c.[842+1G>A];p.[Arg261Gln]	CPKU	CPKU	Y	UNK];[Y	N];[Y];[Slow];[Not tested
p.[Arg261Gln];[Ala403Val] (*n*=4)	mild HPA-NT	MHP];[MPKU	Y (*n*=3) No test (*n*=1)	Y];[Y	Y];[Not tested
p.[Arg176Leu];[?] (*n*=2)	mild HPA-NT	—	No test (*n*=1) Y (*n*=1)	Y];[?	No records
c.[60+5G>T];p.[Glu390Gly] (*n*=3)	mild HPA-NT (*n*=2) mild HPA (*n*=1)	MHP	No test (*n*=1) Y (*n*=2)	UNK];[Y	Y
p.[Ala403Val];[Phe55Leufs*6] (*n*=3)	mild HPA-NT (*n*=2) mild HPA (*n*=1)	MHP];[MPKU	No test (*n*=2) Y (*n*=1)	Y];[Y	Y];[Not tested
c.[168+5G>A];p.[Ile65Thr]	mild HPA	MHP	N	UNK];[Y	N];[Slow
p.[Ile65Thr];[Ile406Thr]	mild HPA	MHP];[MPKU	N	Y];[UNK	Not tested
p.[Leu258Pro];[Leu258Pro]	mild HPA	MHP	N	UNK];[UNK	N];[Slow
c.[1066-11G>A];[1066-11G>A] (*n*=14)	mild HPA (*n*=2) MPKU (*n*=2) CPKU (*n*=10)	CPKU];[MPKU	N	N];[N	N];[Not tested];[Slow];[Y
p.[Arg176*];[Phe39del]	MPKU	CPKU];[MPKU	N	N];[Y	N
p.[Ile65Thr];[Pro275Arg]	MPKU	MPKU	N	Y];[UNK	N
p.[Arg68Ser];[Phe39del]	MPKU	MPKU	N	Y];[Y	Y];[N];[Slow
p.[Arg158Gln];[Arg158Gln]	MPKU	CPKU];[MPKU	N	Y];[Y	N];[Not tested];[Y
p.[Arg243*];[Leu48Ser]	MPKU	MPKU];[CPKU	N	N];[Y	N];[Not tested];[Slow
p.[Val388Met];c.[913-7A>G]	MPKU	—	N	Y];[N	N
p.[Leu54Ser];c.[912+1G>A]	MPKU	—	N	UNK];[UNK	No records
p.[Val388Met];[Pro281Leu]	MPKU	CPKU	N	Y];[N	Not tested
p.[Glu390Gly];[?]	MPKU	—	N	Y];[?	No records
p.[Val388Met];[Ala309Val]	MPKU	—	N	Y];[Y	No records

Table 2 (Continued)

Genotype (allele 1);[allele 2)	*Phenotype*	*Predicted phenotype*	*Response BH_4 test*	*Predicted BH_4 responsiveness[a] (allele 1];[allele 2)*	*BIOPKUdb BH_4 responsiveness[a]*
p.[Ser349Pro];[Tyr343Asp]	MPKU	—	N	N];[UNK	No records
p.[Arg408Trp];[Tyr414Cys]	MPKU	MPKU];[CPKU	N	N];[Y	Y];[Not tested];[N];[Slow
p.[Leu48Ser];[Pro275Arg]	MPKU	—	N	Y];[UNK	No records
p.[Val388Leu];[Pro281Leu]	MPKU	—	N	UNK];[N	No records
p.[Ile65Thr];c.[441+5G>T]	MPKU	CPKU	N	Y];[UNK	N];[Not tested
p.[Leu311Pro];c.[842+1G>A]	MPKU	—	N	N];[UNK	No records
p.[Ile65Thr];[Ser349Pro]	MPKU	CPKU	N	Y];[N	N];[Not tested
p.[Ala309Val];[Ala309Val]	MPKU	MPKU	N	Y];[Y	Not tested
p.[Arg270Lys];[Leu348Val]	MPKU	MPKU	N	N];[Y	Not tested
p.[Val388Met];[Arg408Trp]	MPKU	MPKU];[CPKU	N	Y];[N	Not tested];[N
p.[Arg243Gln];[Leu348Val]	MPKU	MPKU	N	N];[Y	N
c.[441+5G>T];p.[Arg243Gln] (*n*=3)	MPKU (*n*=1) CPKU (*n*=2)	CPKU	N	UNK];[N	N];[Not tested
p.[Arg261Gln];c.[1066-11G>A] (*n*=8)	MPKU (*n*=6) CPKU (*n*=2)	CPKU];[MPKU	N	Y];[N	N];[Not tested];[Slow];[Y
p.[Arg408Trp];[Arg408Trp] (*n*=2)	MPKU (*n*=1) CPKU (*n*=1)	CPKU];[MPKU	N	N];[N	N];[Not tested
c.[1066-11G>A];p.[Arg243Gln] (*n*=5)	CPKU (*n*=4) MPKU (*n*=1)	CPKU];[MPKU	N	N];[N	N];[Y];[Not tested
c.[1315+1G>A];[1315+1G>A] (*n*=2)	MPKU (*n*=1) CPKU (*n*=1)	CPKU];[MPKU	N	N];[N	N];[Not tested]; [Y];[Slow
p.[Glu280Lys];[Val388Met] (*n*=3)	MPKU (*n*=1) CPKU (*n*=2)	CPKU	N	N];[Y	N];[Slow];[Not tested
p.[Tyr204*];[Arg68Ser] (*n*=4)	MPKU (*n*=1) CPKU (*n*=3)	MPKU	N	N];[Y	N];[Not tested];[Y
p.[Ile65Thr];[Arg261Gln] (*n*=2)	MPKU (*n*=1) CPKU (*n*=1)	MPKU];[CPKU	N	Y];[Y	Y];[N];[Not tested];[Slow
p.[Glu205Lys];[Arg243*] (*n*=2)	MPKU (*n*=1) CPKU (*n*=1)	—	N	UNK];[N	No records
p.[Arg261Gln];[?] (*n*=2)	MPKU (*n*=1) CPKU (*n*=1)	—	N	Y];[?	No records
p.[Ile65Thr];c.[913-7A>G] (*n*=2)	MPKU (*n*=1) CPKU (*n*=1)	—	N	Y];[N	No records
p.[Arg261*];c.[1066-11G>A] (*n*=2)	MPKU (*n*=1) CPKU (*n*=1)	CPKU	N	N];[N	N];[Not tested
c.[1066-11G>A];p.[Arg243*] (*n*=3)	CPKU	CPKU	N	N];[N	N];[Slow];[Not tested
p.[Tyr206*];[Ala309Val]	CPKU	—	N	N];[Y	No records
p.[Gly272*];[Gly289Arg]	CPKU	—	N	N];[UNK	N
p.[Ser349Pro];c.[1066-11G>A] (*n*=2)	CPKU	CPKU	N	N];[N	N];[Not tested
p.[Ser349Pro];[Tyr198Cysfs*136]	CPKU	—	N	N];[UNK	No records
p.[Val388Met];c.[60+5G>T]	CPKU	MPKU	N	Y];[UNK	Not tested
c.[441+5G>T];[441+5G>T] (*n*=2)	CPKU	CPKU];[MPKU	N	UNK];[UNK	N];[Not tested
c.[1314_1315+4del6];p.[Arg158Gln]	CPKU	—	N	UNK];[Y	No records
p.[Tyr414Cys];[?]	CPKU	—	N	Y];[?	No records
p.[Phe55Leufs*6];[Arg261Gln] (*n*=4)	CPKU	CPKU];[MPKU	N	Y];[Y	Slow];[N];[Not tested
p.[Ser349Pro];[Ser349Pro] (*n*=3)	CPKU	CPKU	N	N];[N	N];[Not tested
c.[441+5G>T];[1066-11G>A] (*n*=2)	CPKU	CPKU	N	UNK];[N	N];[Not tested
p.[Val388Met];[Ser349Pro] (*n*=2)	CPKU	CPKU	N	Y];[N	N];[Y];[Slow
p.[Pro281Leu];[Pro281Leu] (*n*=2)	CPKU	CPKU];[MPKU	N	N];[N	N];[Not tested
c.[442-5C>G];[1315+1G>A]	CPKU	—	N	Y];[N	No records
c.[1066-11G>A];p.[Ile65Thr] (*n*=2)	CPKU	CPKU];[MPKU	N	N];[Y	N];[Not tested];[Y];[Slow
c.[1315+1G>A];p.[Gly272*]	CPKU	CPKU];[MPKU	N	N];[N	N];[Not tested
p.[Arg261Gln];[Arg408Trp] (*n*=3)	CPKU	MPKU];[CPKU	N	Y];[N	N];[Not tested];[Slow];[Y
p.[Pro281Leu];[Gln20*]	CPKU	CPKU	N	N];[UNK	N
p.[Arg261Gln];[Glu280Lys]	CPKU	CPKU	N	Y];[N	N];[Slow
p.[Ile65Thr];c.[912G>A] (*n*=2)	CPKU	—	N	Y];[UNK	N
p.[Arg261Pro];[Ile65Thr] (*n*=2)	CPKU	CPKU	N	N];[Y	N
c.[912G>A];[912G>A]	CPKU	CPKU	N	UNK];[UNK	N];[Not tested

Table 2 (Continued)

Genotype (allele 1);[allele 2)	*Phenotype*	*Predicted phenotype*	*Response BH_4 test*	*Predicted BH_4 responsiveness[a] (allele 1];[allele 2)*	*BIOPKUdb BH_4 responsiveness[a]*
p.[Ser349Pro];[Arg261Gln] (*n*=2)	CPKU	CPKU];[MPKU];[MHP	N	N];[Y	Not tested];[Y];[Slow];[N
p.[Arg408Trp];[Arg252Gln]	CPKU	CPKU];[MPKU	N	N];[UNK	N];[Slow];[Not tested
p.[Phe39Leu];[Arg408Trp]	CPKU	CPKU];[MPKU	N	Y];[N	N];[Not tested];[Y
c.[1066-11G>A];p.[Pro362Thr]	CPKU	CPKU	N	N];[UNK	N
c.[441+5G>T];[913-7A>G] (*n*=2)	CPKU	—	N	UNK];[N	No records
p.[Arg408Trp];[?]	CPKU	—	N	N];[?	No records
p.[Ser349Pro];[Arg243*]	CPKU	CPKU	N	N];[N	N];[Not tested];[Slow
c.[1066-11G>A];p.[Arg270Lys]	CPKU	CPKU	N	N];[N	N];[Not tested
p.[Gly46Ser];[Arg252Trp] (*n*=2)	CPKU	—	N	UNK];[N	No records
p.[Arg243*];[Val388Met]	CPKU	CPKU	N	N];[Y	N];[Not tested
p.[Phe39del];[Phe39del]	CPKU	CPKU	N	Y];[Y	Slow];[Not tested
p.[Arg243*];[Arg243*]	CPKU	CPKU	N	N];[N	N];[Not tested
p.[Arg261Gln];[Ser196Leufs*2]	CPKU	CPKU	N	Y];[UNK	Not tested
p.[Glu280Lys];c.[441+5G>T]	CPKU	CPKU	N	N];[UNK	N
p.[Arg408Trp];c.[912+1G>A]	CPKU	—	N	N];[UNK	No records
p.[Val388Met];c.[842+3G>C] (*n*=3)	CPKU	—	N	Y];[UNK	No records
c.[842+1G>A];[842+1G>A]	CPKU	CPKU	N	UNK];[UNK	N];[Not tested
p.[Arg243*];[Phe39del] (*n*=2)	CPKU	CPKU	N	N];[Y	Not tested
c.[913-7A>G];[912+1G>A]	CPKU	CPKU	N	N];[UNK	N];[Not tested
p.[Ser349Pro];[Ser391Phefs*2]	CPKU	—	N	N];[UNK	No records
p.[Ser349Pro];[Arg408Trp]	CPKU	CPKU	N	N];[N	N];[Not tested
p.[Ile65Thr];[Arg243Gln]	CPKU	MPKU];[CPKU	N	Y];[N	Not tested];[Slow];[N
p.[Pro281Leu];[Arg408Trp] (*n*=2)	CPKU	CPKU];[MPKU	N	N];[N	N];[Not tested
p.[Arg243*];[Arg408Trp]	CPKU	CPKU];[MPKU	N	N];[N	N];[Not tested
p.[Pro281Leu];[?]	CPKU	—	N	N];[?	No records
p.[Arg408Trp];[Phe39del]	CPKU	MPKU];[CPKU	N	N];[Y	N];[Not tested
c.[353-1G>C];p.[Ser349Pro]	CPKU	—	N	N];[N	No records
p.[Tyr277Asp];[Tyr277Asp]	CPKU	CPKU	N	UNK];[UNK	Not tested
c.[168+5G>A];p.[Ser349Pro]	CPKU	—	N	UNK];[N	No records
p.[Pro147Ser];[Arg243Gln]	CPKU	—	N	Y];[N	Not tested
p.[Leu48Ser];[Ile65Thr]	CPKU	HPA];[MPKU	N	Y];[Y	Y];[Not tested
p.[Arg261*];[Arg158Gln]	CPKU	CPKU	N	N];[Y	Not tested
p.[Arg243*];[Pro281Ser]	CPKU	—	N	N];[UNK	No records
p.[Glu280Lys];c.[1066-11G>A] (*n*=2)	CPKU	CPKU];[MPKU	N	N];[N	N];[Not tested
c.[1066-11G>A];p.[Phe39del]	CPKU	MPKU];[CPKU	N	N];[Y	N];[Not tested
p.[Ser349Pro];[Gly148Val]	CPKU	—	N	N];[UNK	No records
p.[Pro281Leu];c.[1066-11G>A]	CPKU	CPKU];[MPKU	N	N];[N	N];[Not tested];[Y
c.[912+1G>A];[?]	CPKU	—	N	UNK];[?	No records
p.[Arg158Gln];[Glu280Lys]	CPKU	CPKU	N	Y];[N	N];[Not tested
p.[Gly352Valfs*48];[Gly352Valfs*48]	CPKU	CPKU	N	UNK];[UNK	N];[Not tested
p.[Leu348Val];[Val388Met]	CPKU	CPKU	N	Y];[Y	Y];[Not tested
p.[Glu280Lys];[Glu353Asnfs*47]	CPKU	—	N	N];[UNK	No records
p.[Arg111*];[?]	CPKU	—	N	N];[?	No records
p.[Arg261Gln];[Glu390Gly] (*n*=2)	mild HPA-NT (*n*=1) mild HPA (*n*=1)	MHP];[MPKU	No test (*n*=1) N (*n*=1)	Y];[Y	Y];[N];[Not tested
p.[Thr380Met];c.[1066-11G>A] (*n*=3)	mild HPA-NT (*n*=2) mild HPA (*n*=1)	MHP	No test (*n*=2) N (*n*=1)	Y];[N	Not tested
p.[Leu48Ser];[?] (*n*=3)	mild HPA-NT (*n*=2) mild HPA (*n*=1)	—	No test (*n*=2) N (*n*=1)	Y];[?	No records
c.[441+5G>T];[?] (*n*=2)	mild HPA-NT (*n*=1) MPKU (*n*=1)	—	No test (*n*=1) N (*n*=1)	UNK];[?	No records
p.[Ile65Thr];[?] (*n*=2)	mild HPA-NT (*n*=1) CPKU(*n*=1)	—	No test (*n*=1) N (*n*=1)	Y];[?	No records
p.[Arg176Leu];[Arg261Gln] (*n*=4)	mild HPA-NT (*n*=3) CPKU(*n*=1)	—	No test (*n*=3) N (*n*=1)	Y];[Y	No records
p.[Arg243*];[?] (*n*=2)	mild HPA-NT (*n*=1) CPKU (*n*=1)	—	No test (*n*=1) N (*n*=1)	N];[?	No records
p.[Tyr414Cys];[Tyr414Cys] (*n*=2)	mild HPA (*n*=1) MPKU (*n*=1)	MPKU];[MHP];[CPKU	Y (*n*=1) N (*n*=1)	Y];[Y	Y];[Not tested];[N

Table 2 (Continued)

Genotype (allele 1);[allele 2)	*Phenotype*	*Predicted phenotype*	*Response BH_4 test*	*Predicted BH_4 responsiveness[a] (allele 1];[allele 2)*	*BIOPKUdb BH_4 responsiveness[a]*
p.[Glu280Lys];[Glu280Lys] (*n*=4)	mild HPA (*n*=1) CPKU (*n*=3)	CPKU];[MPKU	Y (*n*=1) N (*n*=3)	N];[N	N];[Not tested
p.[Ile65Thr];[Val388Met] (*n*=4)	MPKU	MPKU];[CPKU];[MHP	Y (*n*=3) N (*n*=1)	Y];[Y	Y];[Slow];[Not tested
p.[Val388Met];[Tyr414Cys] (*n*=3)	MPKU	MPKU	Y (*n*=1) N (*n*=2)	Y];[Y	Y];[Not tested
p.[Val388Met];[Leu48Ser] (*n*=3)	MPKU	MPKU	Y (*n*=1) N (*n*=2)	Y];[Y	Y
p.[Gly46Ser];[Arg243Gln] (*n*=2)	MPKU (*n*=1) CPKU (*n*=1)	CPKU	N (*n*=1) Y (*n*=1)	UNK];[N	Y
p.[Val388Met];[Val388Met] (*n*=3)	MPKU (*n*=2) CPKU (*n*=1)	MPKU];[CPKU	Y (*n*=1) N (*n*=2)	Y];[Y	Y];[N];[Slow];[Not tested
p.[Val388Met];c.[441+5G>T] (*n*=6)	MPKU (*n*=2) CPKU (*n*=4)	CPKU];[MPKU	Y (*n*=1) N (*n*=5)	Y];[UNK	Slow];[Not tested
c.[1066-11G>A];p.[Val388Met] (*n*=2)	CPKU	CPKU];[MPKU	Y (*n*=1) N (*n*=1)	N];[Y	N];[Slow];[Not tested
p.[Ala403Val];[?] (*n*=5)	mild HPA-NT (*n*=4) CPKU (*n*=1)	—	No test (*n*=3) Y (*n*=1) N (*n*=1)	Y];[?	No records
p.[Val388Met];[?] (*n*=4)	mild HPA-NT (*n*=1) CPKU (*n*=3)	—	No test (*n*=1) Y (*n*=1) N (*n*=2)	Y];[?	No records

Abbreviations: CPKU, classic PKU; MPKU, mild-moderate PKU; mild HPA, mild hyperphenylalaninemia; mild HPA-NT, mild hyperphenylalaninemia non-treated; MHP, mild hyperphenylalaninemia; *n*: number of patients.
*, STOP CODON; Y, yes; N, no; UNK, unknown; ?, not detected; —, not previously reported.
Previously predicted phenotypes and BH_4 responsiveness in the literature and BIOPKUdb is indicated.
[a]References 30–32.

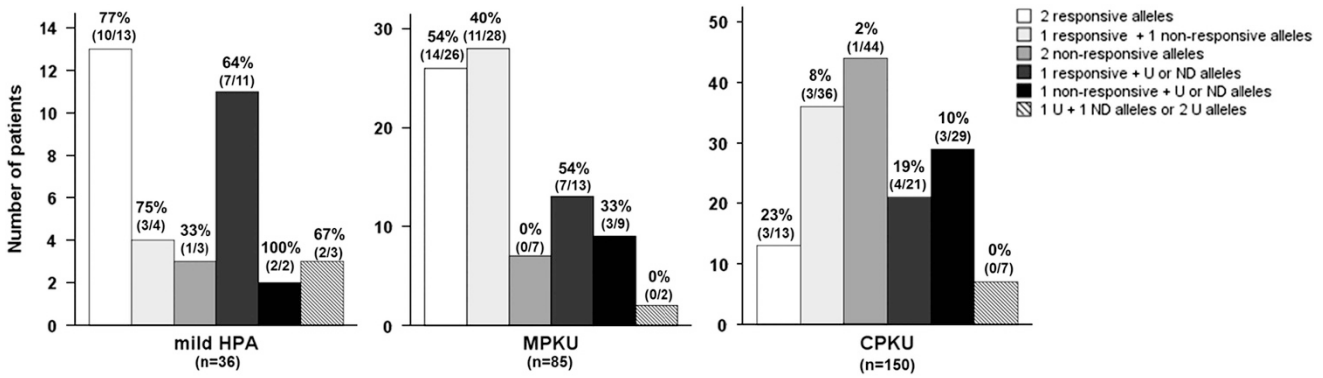

Figure 2 Genotype and BH_4 responsiveness correlation in the different severity groups of patients with phenylketonuria (PKU). The *X*-axis indicates the phenotypic categories in our population: mild HPA (*n*=36), mild-moderate PKU (MPKU) (*n*=85) and classic PKU (CPKU) (*n*=150). Percentage (%) of positive 6R-BH_4 loading tests as well as number of 6R-BH_4-responder patients are indicated on the top of each column in the different severity groups. Patients classified as mild HPA-NT are not displayed in the graph, since 6R-BH_4 loading test was not performed in this group. Theoretical BH_4 responsiveness was determined based on responsiveness of both alleles: □: 2 responsive alleles; □: 1 responsive allele+1 non-responsive allele; ■: 2 non-responsive alleles; ■: 1 responsive allele+1 U or ND allele; ■: 1 non-responsive allele+1 U or ND allele and ▨: 1 U allele+1 ND allele or 2 U alleles. The *Y*-axis indicates the number of patients with each combination of alleles in each phenotypic group. U: unknown, no available information on theoretical response to BH_4; ND: not detected, one of the alleles was not detected; *n*: number of patients. The graph shows the percentage (%) of 6R-BH_4 positive test in mild HPA patients carrying two BH_4-responsive alleles or only one responsive mutation is higher than in patients with MPKU and CPKU.

(2.67–3.70%). The most prevalent mutations found in two Spanish regions, Galicia and Andalucía, is the common Mediterranean mutation c.1066-11G>A (p.Gln355_Tyr356insGlyLeuGln) with frequencies of 13.8 and 10.9%, respectively, which is associated with classic PKU phenotype. On the other hand, in the Basque Country region, the mutations c.1162G>A (p.Val388Met) and c.194T>C (p.Ile65Thr), which are associated with a mild-moderate phenotype, were found with higher frequency.

The mutational spectrum involves 116 mutations; most of them were missense as it has been described in The Human Gene Mutation Database (HGMD) (http://www.biobase-international.com/product/hgmd and http://www.hgmd.ac.uk/ac/index.php). All the mutations that were identified in our patients have already been described in other populations, except for c.61-13del9 (p.?), c.847A>G (p.Ile283Val) and c.443G>T (p.Gly148Val). The patient who harbored the mutation c.61-13del9 (p.?) in heterozygosis (c.1162G>A

(p.Val388Met)/c.61-13del9 (p.?)) had a mild-moderate PKU phenotype, and was BH_4-responder. The effect of the presumable splicing mutation c.61-13del9 (p.?) would be a severe effect but functional analysis should be done to confirm the real effect. The mutation p.Val388Met has been classified as a mild-moderate mutation and as a BH_4 responsive.[31] The mutation c.847A>G (p.Ile283Val) was identified in heterozygosis in a patient with classic PKU, who was BH_4 non-responder (c.1045T>C (p.Ser349Pro)/c.847A>G (p.Ile283Val)). In this regard, the mutation c.1045T>C (p.Ser349Pro) is a BH_4 non-responsive mutation and is classified as severe mutation. The patient who harbored the mutation c.443G>T (p.Gly148Val) had a phenotype of classic PKU and was BH_4 non-responder; this mutation was found in heterozygosis with the mutation c.1045T>C (p.Ser349Pro). Taking into account the phenotype of these patients the three novel mutations would be severe non-responder mutations.

Although the majority of the pathogenic mutations were found in the coding region or canonical splice sites, the diagnosis rate was close to 95% in our populations. No mutations have been detected in 39 mutant alleles. The development of massive parallel sequencing with an immense capacity of sequencing of the entire coding sequence of the *PAH* gene or the transcriptomic analysis of hepatic cells by RNAseq combined with comprehensive functional analysis would provide new deep intronic mutations or regulator changes in these misdiagnosed PKU patients.[36,37]

It worth mentioning that 42.8% of our population has mild phenotypes (mild HPA-NT and mild HPA) in contrast to North-Eastern Europe where most of the phenotypes are severe;[35] even though the severe splicing variant c.1066-11G>A (p.Gln355_Tyr356insGlyLeuGln) was the most frequent mutation detected in our population. In this regard, 31 mutations out of the 116 mutations found in our study population display considerable residual enzymatic activity, which ranges from moderate to high, whereas 23 mutations had none or extremely low activity. On the other hand, 50 mutations are classified as severe and mild-moderate, 38 mutations as mild HPA and 28 mutations as undefined.

Genotype–phenotype correlation is the cornerstone in most studies on metabolic diseases. Nowadays, it relies on *in vitro* expression analysis of recombinant mutant proteins. Our study revealed that in general severe loss-of-function mutations (such as splicing, nonsense or severe missense mutations), which display *in vitro* null/reduced residual activity, are associated with the most severe forms of the disease.[38] Issues arise with mutant enzymes that are associated with moderate levels of residual activity. Patients harboring missense mutations retaining residual activity are usually associated with a milder form. In our group we had good genotype–phenotype correlation in 268 patients, but we detected inconsistencies in 23.9% of this cohort. In this regard, it has been suggested that *in vitro* expression analysis may ultimately be important in discriminating between mutations which allow a margin for variability in the enzymatic and metabolic phenotypes and those that do not.[39,40] In general, phenotype–genotype inconsistencies are related to destabilizing mutations (c.1162G>A (p.Val388Met), c.1241A>G (p.Tyr414Cys), c.194T>C (p.Ile65Thr), etc.) due to inter-individual differences on cellular folding and a different behavior depending of modifiers genes, such as genes involved in folding, due to the fact that 'single' gene disorders are not simple traits, but they are influenced by several modifiers genes.[41,42]

An accurate genetic diagnosis is important for 6R-BH_4 treatment, and in this study the evaluation of BH_4 responsiveness in our Spanish patients revealed encouraging results. In this regard, nearly half of the selected individuals (47.2%) harbor a genotype with a potential positive response to 6R-BH_4 therapy. Accordingly, one-third of the patients who were monitored with the 6R-BH_4 loading test (102 out of 296) were clear BH_4-responders. It should be pointed out that the 115 patients with no test done had a mild HPA-NT phenotype and a high probability of being BH_4-responders, increasing the proportion of patients who would be able to gain to be treated with 6R-BH_4. The remaining patients were non-responders, although some of them harbor previously described BH_4-responder mutations (c.782G>A (p.Arg261Gln), c.1241A>G (p.Tyr414Cys), c.143T>C (p.Leu48Ser), c.1169A>G (p.Glu390Gly), c.194T>C (p.Ile65Thr), c.473G>A (p.Arg158Gln), c.1162G>A (p.Val388Met), c.116_118delTCT (p.Phe39del), c.117C>G (p.Phe39Leu), c.204A>T (p.Arg68Ser), c.1042C>G (p.Leu348Val), c.926C>T (p.Ala309Val), c.439C>T (p.Pro147Ser), c.527G>T (p.Arg176Leu), c.1208C>T (p.Ala403Val), c.1139C>T (p.Thr380Met), c.165delT (p.Phe55Leufs*6), c.442-5C>G (p.?)). Bearing this in mind, the decision of 6R-BH_4 treatment must also consider some other factors, such as the possibility of negative inter-allelic complementation between some mutant alleles in compound heterozygous patients.[40,43] In addition, Staudigl *et al.*[44] evaluated the influence of substrate and cofactor concentrations on enzyme function and on response to 6R-BH_4 treatment in several genotypes. Indeed, 6R-BH_4 administration can cause short-term kinetic and long-term chaperone effects, which may be shifted depending on individual mutations. Moreover, the initial Phe levels seemed to be important, since a significant number of Spanish HPA patients harbor mutant PAH enzymes, whose activity strongly depends on cofactor and/or substrate concentrations (c.194T>C (p.Ile65Thr), c.782G>A (p.Arg261Gln) and c.1241A>G (p.Tyr414Cys)).

In summary, the present study represents the first multicentre study of patients with PKU in Spain and provides insight to design more personalized screening 6R-BH_4 loading tests based on each patient's genotype. We consider that genotype is quite a good predictor of the phenotype and of the BH_4 responsiveness, which is relevant for patient management, treatment and follow-up. Furthermore, this study will provide essential clues for more rationale evaluation of responders patients in historically related Latin America countries, where severe socioeconomic restrictions do not allow to perform a 6R-BH_4 loading test in every patient with PKU.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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ETHICAL STANDARD STATEMENT

This study has been approved by Clinical Research Ethics Committees at each hospital involved, and has therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. Written informed consent was obtained from patients and parents/legal guardians of all the children included in this study.

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