Mutation analysis of the PAH gene in phenylketonuria patients from Rio de Janeiro, Southeast Brazil

Eduardo Vieira Neto1,2 | Francisco Laranjeira3 | Dulce Quelhas3 | Isaura Ribeiro3 | Alexandre Seabra3 | Nicole Mineiro3 | Lilian d. M. Carvalho4 | Lúcia Lacerda3 | Márcia G. Ribeiro2

1Gerência de Monitoramento Assistencial, Agência Nacional de Saúde Suplementar, Rio de Janeiro, Brazil
2Serviço de Genética Médica, Instituto de Puericultura e Pediatria Martagão Gesteira, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil
3Centro de Genética Médica Doutor Jacinto Magalhães, Unidade de Bioquímica Genética, Porto, Portugal
4Serviço de Metabologia, Instituto de Diabetes e Endocrinologia Luiz Capriglione, Rio de Janeiro, Brazil

Abstract

Background: Phenylketonuria (PKU) is an autosomal recessive disease resulting from mutations in the PAH gene. Most of the patients are compound heterozygotes, and genotype is a major factor in determining the phenotypic variability of PKU. More than 1,000 variants have been described in the PAH gene. Rio de Janeiro’s population has a predominance of Iberian, followed by African and Amerindian ancestries. It is expected that most PKU variants in this Brazilian state have originated in the Iberian Peninsula. However, rare European, African or pathogenic variants that are characteristic of the admixed population of the state might also be found.

Methods: A total of 102 patients were included in this study. Genomic DNA was isolated from dried blood spots. Sanger sequencing was used for PAH gene variant identification. Deletions and duplications were also screened using MLPA analysis. Haplotypes were also determined.

Results: Nine (8.8%) homozygous and 93 (91.2%) compound heterozygous patients were found. The spectrum included 37 causative mutations. Missense, nonsense, and splicing pathogenic variants corresponded to 63.7%, 2.9%, and 22.6% of the mutant alleles, respectively. Large (1.5%), and small deletions, inframe (5.4%) and with frameshift (3.9%), comprised the remainder. The most frequent pathogenic variants were: p.V388M (12.7%), p.R261Q (11.8%), IVS10-11G>A (10.3%), IVS2+5G>C (6.4%), p.S349P (6.4%), p.R252W (5.4%), p.I65T (4.4%), p.T323del (4.4%), and p.P281L (3.4%). One novel variant was detected: c.934G>T (p.G312C) [rs763115697].

Conclusion: The three most frequent pathogenic variants in our study (34.8% of the alleles) were also the most common in other Brazilian states, Portugal, and Spain (p.V388M, p.R261Q, IVS10-11G>A), corroborating that the Iberian Peninsula is the major source of PAH mutations in Rio de Janeiro. Pathogenic variants that have other geographical origins, such IVS2+5G>C, p.G352Vfs*48, and IVS12+1G>A were also detected. Genetic drift and founder effect may have also played a role in the mutation spectrum we observed.

Keywords
Brazil, epidemiology, haplotypes, mutation analysis, PAH gene, phenylketonuria
1 | INTRODUCTION

Phenylketonuria (PKU; OMIM # 261600) is a hereditary autosomal recessive disease characterized by an accumulation of the amino acid phenylalanine (Phe) in blood (hyperphenylalaninemia) and other tissues (Donlon, Sarkissian, Levy, & Scriver, 2014). The disease is caused by mutations in the gene encoding the hepatic enzyme phenylalanine hydroxylase (PAH, EC 1.14.16.1), resulting in a decrease or even elimination of enzyme activity, responsible for the conversion of Phe into tyrosine (Tyr), in the presence of its cofactor tetrahydrobiopterin - BH₄ (van Wegberg et al., 2017). When untreated, the protracted high blood Phe levels cross the blood–brain barrier causing extensive structural damage to the central nervous system (Anderson & Leuzzi, 2010).

The human PAH gene (OMIM # 612349) covers approximately 100 kb of genomic DNA, consists of 13 exons and 12 introns, and has been mapped on chromosome 12, band region q23.2 (Donlon et al., 2014). The current number of PAH variants in the PAHdb - Phenylalanine Hydroxylase Gene Locus-Specific Database is 1041 (http://www.biopku.org/pah/search-results-browse.asp), of which circa 630 are disease-causing mutations, associated with various degrees of PAH deficiency (BIOPKU; http://www.biopku.org/biopku/search-start.asp). These mutations may result in clinical manifestations ranging from mild hyperphenylalaninemia (MHP), which does not require dietary therapy, to a severe and persistent phenotype, classic PKU (Guldberg et al., 1998). Certain mutations are correlated with a specific class of PKU, responsive to tetrahydrobiopterin cofactor - BH₄ (Trefz, Scheible, Gotz, & Frauroendienst-Egger, 2009).

Approximately 6-9 different alleles account for the majority of mutant chromosomes described in previous PKU population genetics studies done in Latin America – Brazil (Acosta, Silva, Carvalho, Gomes, & Zago, 2001; Santana da Silva et al., 2003; Santos et al., 2008), Chile (Hamilton et al., 2017), and Mexico (Vela-Amieva et al., 2015), and the Iberian Peninsula (Aldamiz-Echevarria et al., 2016; Couce et al., 2013; River et al., 2011); the remainder are minor, rare, and even private disease-causing alleles.

Additionally, the gene is rich in intragenic polymorphic markers, including seven biallelic restriction-fragment-length polymorphisms (RFLP), silent single-nucleotide polymorphism (SNP) alleles, a short tandem tetranucleotide repeat (STR) in intron 3, and a variable number of tandem repeats (VNTRs) (30-bp-length AT-rich cassettes) in the 3′ untranslated region (Donlon et al., 2014). The polymorphic sites are in linkage disequilibrium and describe a large series of extended and mini-haplotypes. These haplotypes have been extensively used as tools in population genetics studies to increase the knowledge of the historical and prehistorical movements of human populations, which can explain the contemporary geographic distribution of many PAH gene mutant alleles (Rivera et al., 2011).

In the 60s of the 20th century, Guthrie developed a bacterial inhibition test that could detect high amounts of Phe in a dried blood spot (Guthrie & Susi, 1963). This test made it possible to carry out newborn screening test for PKU, enabling early diagnosis and dietary treatment of the disease and the prevention of the development of intellectual disability (van Wegberg et al., 2017). In the next decade, newborn screening programs for PKU were routine in most developed countries.

In Brazil, newborn screening for PKU began in 1976 with the pioneering work of Benjamin Schmidt at a private institution for the intellectually disabled in São Paulo (de Carvalho, dos Santos, dos Santos, Vargas, & Pedrosa, 2007). Only in 2001, the Federal Government implemented the National Newborn Screening Program all over the country (de Carvalho et al., 2007). In Rio de Janeiro, newborn screening started in the 1980s, when the State Institute of Diabetes and Endocrinology (IEDE) and an independent philanthropic organization both put in practice programs for newborn screening of PKU and congenital hypothyroidism, which reached a coverage of 81% of the state’s live births in 2007 (Botler, Camacho, & Cruz, 2012). The incidence of PKU in Rio de Janeiro disclosed by this newborn screening program was 1 in 25,000 live births in the same year (Botler et al., 2012). Currently, IEDE is the state reference for the screening, diagnosis and follow-up of patients with PKU, and circa 150 patients are followed at the institution.

The diversity of mutations in the PAH gene found in the Brazilian population seems to be a consequence of genetic drift and founder effect upon a highly admixed population, resulting from five centuries of interaction between three large ethnic groups: Europeans, Africans, and Amerindians (Saloum de Neves Manta et al., 2013). However, the proportion of these three large ethnic groups is very variable in various Brazilian populations, considering that the historical processes of population formation occurred in very diverse ways in each of the regions of the country. To date, four reports have described the molecular basis of PKU in Southeast – São Paulo (Acosta et al., 2001) and Minas Gerais (Santos et al., 2008), and South Brazil (Perez et al., 1996; Santana da Silva et al., 2003). Unpublished material has described PKU mutation spectrum in Northeast Brazil (Boa Sorte, T.R.S.A. (2010) Estudo de bases moleculares de Fenilcetonúria no Nordeste do Brasil. (Thesis), Fundação Oswaldo Cruz, Centro...
de Pesquisas Gonçalo Moniz, Salvador]. The PKU muta-
tional profile in the state of Rio de Janeiro is unknown so
far. Rio de Janeiro’s population has a predominance of
European ancestry - especially Iberian, followed by African
and, to a lesser extent, Amerindian ancestry (Manta et al.,
2013). It is expected that most of the PKU pathogenic vari-
ants in this Southeast Brazil state have originated in the
Iberian Peninsula. However, it is likely that mutations that
are rare in Europe, of African origin or that are characteris-
tic of the admixed population of the state will also be
found. This study seeks to produce knowledge that will
contribute to settle these issues.

2 | MATERIALS AND METHODS

2.1 | Ethical compliance

This study was approved by the National Research Ethics
Commission of Brazil, and conducted in line with the
Guidelines and Standards for Research in Human Beings,
established by Resolution No. 466/2012 of the Brazilian
National Council of Health (Brazil’s Ministry of Health,
National Council of Health, 2012). Informed consent was
obtained from all patients and parents of minors engaged
in this study.

2.2 | Patients

One hundred two patients, including seven pairs of sib-
lings, were included in this study. The patients belonged to
95 unrelated families. The patients sample represents
approximately 68% (102/150) of the patients followed at
IEDE. The majority of the patients (95) were diagnosed by
newborn screening; seven patients had a late diagnosis. A
diagnosis of PKU was defined as a blood Phe level
≥10.0 mg/dl (600 µmol/L) in a confirmatory sample.
Patients with blood Phe levels persistently ≥6.0–9.9 mg/dl
were diagnosed as having MHP. The allocation of patients
to one of the following phenotypes was done according to
pretreatment Phe levels: classic PKU, ≥1,200 µmol/L
(≥20 mg/dl); moderate PKU, ≥900 µmol/L and
<1,200 µmol/L (≥15 mg/dl and <20 mg/dl); mild PKU,
≥600 µmol/L and <900 µmol/L (≥10 mg/dl and <15 mg/dl);
MHP, ≥360 µmol/L and <600 µmol/L (≥6 mg/dl and
<10 mg/dl). A total of 54 (52.9%) patients were classified
as classic PKU, 25 (24.5%) as moderate PKU, 20 (19.6%)
as mild PKU, and 3 (3.0%) as MHP.

2.3 | Molecular genetic analysis

Dried blood spot samples (DBS) were collected from
patients at IEDE in Rio de Janeiro, Brazil, and sent by air
transport to the Biochemical Genetics Unity, Dr Jacinto
Magalhães Medical Genetics Center, Porto, Portugal, where
all genetic analyses were performed. Genomic DNA was
isolated from DBS using the EZ1 DNA Tissue Kit in com-
bination with the BioRobot EZ1 workstation (Qiagen, Hil-
den, Germany). Sanger sequencing was used for PAH gene
variant identification. Primers for the 13 exons and intronic
boundaries of the PAH gene were designed employing the
NCBI Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/
tools/prime blast) (Table S1). These primers were tagged
with a M13 sequence for the later cycle sequencing reac-
tion. PCR was carried out using the EmeraldAmp MAX
PCR Master Mix (Takara Bio Inc., Kusatsu, Shiga, Japan).
PCR products were purified with ExoSAP-IT (Affymetrix,
Santa Clara, CA, USA), and subjected to a cycle sequenc-
ing reaction using BigDye Terminators v3.1 kit (Applied
Biosystems, Foster City, CA, USA), and M13 primers
[M13(21)F: 5’-TGTAAGACGCGCAGT-3’, M13R: 5’-CAGGAAACGCTATGACC-3’]. Sequencing products
were purified by gel filtration on a DyeEx 96-well plate
(Qiagen). The purified sequencing products were analyzed
in an automatic ABI Prism 3130xl genetic analyzer
(Applied Biosystems).

The sequences of all 13 exons and intron-exon bound-
daries were aligned and compared with the wild-type human
PAH Ensembl reference sequence (ENSG00000171759)
[http://www.ensembl.org/Homo_sapiens/Gene/Summary?g=
ENSG00000171759;r=12:102836885-102958410] using
SeqScape Software v2.5 (Applied Biosystems). The
observed variants were referred to the NCBI reference
sequence for human PAH gDNA, NG_008690.2, and tran-
script NM_000277.2.

2.4 | MLPA

After PAH gene sequencing, large deletions and duplica-
tions were screened by multiplex ligation-dependent probe
amplification (MLPA) in patients in whom only one
heterozygous pathogenic variant had been identified.
MLPA was performed according to the manufacturer’s pro-
tocol (MRC-Holland, Amsterdam, The Netherlands) using
the kit SALSA MLPA probemix P055 PAH (Lot # D1-
1015). This contains 20 probes for each of the 13 encoding
exons of the PAH gene (two probes for exons 1-7 and one
probe for exons 8-13). Between 20 and 50 ng of DNA was
used in a MLPA reaction, which was performed on a PCR
thermocycler with heated lid (Biometra, Göttingen, Ger-
many). The PCR products were analyzed on an ABI Prism
3100 genetic analyzer (Applied Biosystems) with
GeneMapper Software version 4.0 (Applied Biosystems),
using as internal standards GeneScan 500 LIZ Size Stan-
ards (Applied Biosystems). Data obtained were analyzed
by GeneMarker v2.6.0 (Softgenetics, State College, PA,
USA).
For normalization, relative probe signals were calculated by dividing each measured peak area by the sum of all peak areas of that sample. Four control samples of healthy individuals were used to calculate the ratio of each relative probe signal from the patient sample. An exon deletion and duplication was considered when the ratio was lower than 0.75 or higher than 1.30, respectively. All positive results were subjected to a second MLPA analysis. To test if a low signal obtained by MLPA was indeed due to a deletion of an exon and not due to a sequence variant in the probes’ target sequences, all the suspected exons were screened for polymorphisms.

2.5 | Variant pathogenicity criteria

The databases Ensembl Human (Homo sapiens) [http://www.ensembl.org/Homo_sapiens/Info/Index], HGMD (http://www.hgmd.cf.ac.uk/ac/index.php) and ClinVar (http://www.ncbi.nlm.nih.gov/clinvar/) were checked in to determine if variants found in our population, especially those in exons or splicing sites, had already been functionally evaluated. The in silico predictive tools PRO-VEAN, SIFT, PolyPhen-2, and Mutation Taster were employed to assess the potential biological effect of selected, ambiguously described, rare or new variants - missense, nonsense, and small deletions. Human Splicing Finder was the in silico tool to evaluate some splicing site variants.

2.6 | Haplotype analysis

Haplotypes were established after PCR of five RFLP biallelic polymorphisms BgIII, PvuIIa, PvuIIb, MspI, and XmnI and of the multiallelic VNTR system at HindIII site at the 3’ untranslated region of the PAH gene (Rivera, Leandro, Lichter-Konecki, Tavares de Almeida, & Lechner, 1998). Haplotype numbering was adapted from Eisensmith and Woo (1992), as EcoRI and EcoRV polymorphisms were not evaluated. To aid in the haplotype determination, selected silent SNPs (c.-71A>C, IVS2+19T>C, p.Q232Q, p.V245V, and p.L385L) were also examined (Lichter-Konecki, Schlotter, & Konecki, 1994). Some haplotypes were inferred both from the remaining RFLP and VNTR polymorphisms studied, and the mutation/haplotype association table available at PAHdb database (http://www.pahdb.mcgill.ca/?Topic=Search&Section=Main&Page=0).

2.7 | Homozygosity index (j) calculation

Homozygosity index (j) at the PAH locus in a given population is determined by

\[ j = \sum x_i^2 \]

where \( x_i^2 \) is the frequency of the allele (Aldamiz-Echevarria et al., 2016; Rivera et al., 2011).

3 | RESULTS

3.1 | Mutational spectrum

This study involved the molecular characterization of 204 alleles from 102 patients, including 14 (seven pairs of) siblings. It was possible to complete genotype all the patients. The second mutant allele, a large deletion of exon 5 found in three patients, was detected only by MLPA. Consequently, the diagnostic efficiency of Sanger sequencing plus MLPA reached 100%. The majority of the patients, 93 (91.2%), were compound heterozygous. Nine (8.8%) patients were homozygous - three cases of c.782G>A (p.R261Q), and one case each of c.1162G>A (p.V388M), c.1066-11G>A (IVS10-11G>A), c.503delA (p.Y168Sfs*27), c.498C>G (p.Y166*), c.473G>A (p.R158Q), and c.168+5G>C (IVS2+5G>C). Seven of the 9 homozygous patients had a family history of consanguinity.

The mutational spectrum included 37 causative mutations, which were distributed along almost the entire PAH gene sequence (Table 1). Most alleles (67.2%) conveyed mutations that were clustered in exons 7, 11, 10, 3, 5, and 9, with respective frequencies of 24.0%, 12.7%, 12.2%, 6.9%, 5.9%, and 5.4%. A significant number of alleles presented mutations in splicing sites of introns 10 (10.3%) and 2 (6.4%). Exons 12, 2, and 6 carried lower mutant allele frequencies (4.9%, 2.9%, and 2.5%, respectively). The remainder of the alleles displayed mutations in splicing sites of introns 12 (2.9%), 11 (2.0%), 4 (0.5%), and 7 (0.5%). No causative mutations were identified in exons 1, 4, 8, and 13. The 37 causative mutations were distributed in this manner: 22 in the catalytic domain (59.5%), seven in the regulatory domain (18.9%), two in the tetramerization domain (5.4%) and six in the intronic regions (16.2%). Figure 1 summarizes data on the distribution of the mutant alleles according to gene region and protein domain.

Most mutant alleles carried nucleotide substitutions, of which 130 were missense mutations (63.7%), 46 mutations at splicing sites (22.6%), and 6 nonsense mutations (2.9%). Large (three mutant alleles - 1.5%), and small deletions, inframe (11 mutant alleles - 5.4%) and with frameshift (eight mutant alleles - 3.9%), comprised the remainder.

Thirteen causative mutations were observed in only one patient or in two related individuals (private mutations), and six mutations were found in only two unrelated individuals, totaling 19 rare mutations (frequency <1.5%) (Table 1; Figure 2). The most prevalent pathogenic variant in our population was c.1162G>A (p.V388M), in exon 11, found in 26 mutant alleles (12.7%). Four pairs of siblings were compound heterozygotes for this variant; therefore, it was the second most frequent variant (11.6% - 22 in 190) among mutant alleles in unrelated individuals. The next two most frequent pathogenic variants were c.782G>A (p.R261Q), in
**TABLE 1** Mutational spectrum found in 102 phenylketonuria (PKU)/mild hyperphenylalaninemia (MHP) patients from Rio de Janeiro, Southeast Brazil

| DNA change | Protein effect or trivial name | Type | Gene region | Protein domain | PAHvdh | N   | RF (%) | N/RF-UI (%) |
|------------|--------------------------------|------|-------------|---------------|--------|-----|--------|-------------|
| c.1162G>A  | p.V388M | Missense | Exon 11 | Catalytic | Deleterious | 26 | 12.7 | 22/11.6 |
| c.782G>A   | p.R261Q | Missense | Exon 7 | Catalytic | Deleterious | 24 | 11.8 | 23/12.1 |
| c.1066-11G>A | p.Q355_Y356insGLQ | Splicing | Intron 10 | — | Not defined | 21 | 10.3 | 21/11.0 |
| c.168+5G>C | IVS2+5G>C | Splicing | Intron 2 | — | Not defined | 13 | 6.4 | 13/6.8 |
| c.1045T>C  | p.S349P | Missense | Exon 10 | Catalytic | Deleterious | 13 | 6.4 | 11/5.8 |
| c.752C>T   | p.R252W | Missense | Exon 7 | Catalytic | Deleterious | 11 | 5.4 | 10/5.3 |
| c.194T>C   | p.I65T | Missense | Exon 3 | Regulatory | Deleterious | 9 | 4.4 | 9/4.7 |
| c.967_969delACA | p.T323del | Inframe deletion | Exon 9 | Catalytic | Not defined | 9 | 4.4 | 8/4.2 |
| c.842C>T   | p.P281L | Missense | Exon 7 | Catalytic | Deleterious | 7 | 3.4 | 7/3.7 |
| c.1042G>A  | p.L348V | Missense | Exon 10 | Catalytic | Deleterious | 6 | 2.9 | 5/2.6 |
| c.473G>A   | p.R158Q | Missense | Exon 5 | Catalytic | Deleterious | 5 | 2.5 | 5/2.6 |
| c.1055delG | p.G352Vfs*48 | Frameshift deletion | Exon 10 | Catalytic | Not defined | 5 | 2.5 | 5/2.6 |
| c.1241A>G  | p.Y414C | Missense | Exon 12 | Oligomerization | Deleterious | 5 | 2.5 | 5/2.6 |
| c.745C>T   | p.L249F | Missense | Exon 7 | Catalytic | Deleterious | 4 | 2.0 | 4/2.1 |
| c.1199+17G>A | IVS11+17G>A | Splicing | Intron 11 | — | Not defined | 4 | 2.0 | 4/2.1 |
| c.250G>T   | p.D84Y | Missense | Exon 3 | Regulatory | Deleterious | 3 | 1.5 | 3/1.6 |
| c.442-?_509+?del | Unknown | Large deletion | Exon 5 | Catalytic | Not defined | 3 | 1.5 | 3/1.6 |
| c.116_118delTCT | p.F39del | Inframe deletion | Exon 2 | Regulatory | Not defined | 2^h | 1.0 | 1/0.5 |
| c.136G>A   | p.G46S | Missense | Exon 2 | Regulatory | Deleterious | 2 | 1.0 | 2/1.1 |
| c.204A>T   | p.R68S | Missense | Exon 3 | Regulatory | Deleterious | 2 | 1.0 | 2/1.1 |
| c.498C>G   | p.Y166* | Nonsense | Exon 5 | Catalytic | Not defined | 2^g | 1.0 | 2/1.1 |
| c.503delA  | p.Y168Sfs*27 | Frameshift deletion | Exon 5 | Catalytic | Not defined | 2^g | 1.0 | 2/1.1 |
| c.526C>T   | p.R176* | Nonsense | Exon 6 | Catalytic | Not defined | 2 | 1.0 | 2/1.1 |
| c.561G>C   | p.W187C | Missense | Exon 6 | Catalytic | Deleterious | 2^h | 1.0 | 1/0.5 |
| c.809G>A   | p.R270K | Missense | Exon 7 | Catalytic | Deleterious | 2 | 1.0 | 2/1.1 |
| c.934G>T   | p.G312C | Missense | Exon 9 | Catalytic | — | 2^h | 1.0 | 1/0.5 |
| c.1222C>T  | p.R408W | Missense | Exon 12 | Catalytic | Deleterious | 2 | 1.0 | 2/1.1 |
| c.1243G>A  | p.D415N | Missense | Exon 12 | Oligomerization | Tolerated | 2 | 1.0 | 2/1.1 |
| c.143T>C   | p.L48S | Missense | Exon 2 | Regulatory | Deleterious | 1 | 0.5 | 1/0.5 |
| c.165delT  | p.F55Lfs*6 | Frameshift deletion | Exon 2 | Regulatory | Not defined | 1 | 0.5 | 1/0.5 |
| c.441+5G>T | IVS4+5G>T | Splicing | Intron 4 | — | Not defined | 1 | 0.5 | 1/0.5 |
| c.618C>G   | p.Y206* | Nonsense | Exon 6 | Catalytic | Not defined | 1 | 0.5 | 1/0.5 |
| c.781C>T   | p.R261* | Nonsense | Exon 7 | Catalytic | Not defined | 1 | 0.5 | 1/0.5 |

(Continues)
exon 7, and c.1066-11G>\text{A} (IVS10-11G>\text{A}), in intron 10, which displayed a relative frequency of 11.8%, and 10.3% of the mutant alleles, respectively. The relative frequencies of these two variants in mutant alleles of unrelated patients were 12.1% (23 in 190 - highest frequency) and 11.0% (21 in 190), respectively (Table 1). The three most common pathogenic variants accounted for 34.8% of the mutant alleles. Six other major variants (present in \( \geq 3\% \) of the mutant alleles) were as follows: c.1042C>G (p.L348V) \( \left[ 13 \text{ alleles; 6.4\%} \right] \), c.1045T>C (p.R349P) \( \left[ 13; 6.4\% \right] \), c.754C>T (p.R252W) \( \left[ 11; 5.4\% \right] \), c.194T>C (p.I65T) \( \left[ 9; 4.4\% \right] \), c.967_969delACA (p.T323del) \( \left[ 9; 4.4\% \right] \), and c.842C>T (p.P281L) \( \left[ 7; 3.4\% \right] \) (Table 1; Figure 2). Eight minor variants (present in \( \geq 1.5\% - <3\% \) of the mutant alleles) were as follows: c.842+1G>A (IVS7+1G>A), c.994G>A p.G332R (IVS10-11G>\text{A}), c.1223G>A p.R408Q Missense Exon 12 Catalytic Deleterious 1 0.5 1/0.5

Total 204 100 190/100

One novel variant was detected: c.934G>T (p.G312C) \( \left[ \text{rs763115697, accession number PAH1068 in PAHvdb}, a missense variant in exon 9, affecting residue 312 in the protein catalytic domain (Figure 3). It was found out in two early-treated white sisters with a moderate/mild form of PKU, in compound heterozygous with IVS10-11G>A, a splicing variant in intron 12. The in silico analysis of this variant employing the tools SIFT (http://sift.jcvi.org/www/SIFT_enst_submit.html), Provean (http://provean.jcvi.org/seq_submit.php), and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) revealed scores of 0 (damaging), –8.734 (deleterious), and 1.00 (probably damaging), respectively. Mutation Taster (http://www.mutationtaster.org) predicted the variant to be disease causing with a probability >.9999. Using a computational algorithm to specific address this variant potential effect on splicing, Human Splicing Finder (http://www.umd.be/HSF3) revealed a potential alteration of splicing, an activation of an exonic cryptic donor site, and the creation of an exonic splicing silencer (ESS) site.

Another missense variant c.934G>A (p.G312S) is also described at dbSNP database at rs763115697 (https://www.ncbi.nlm.nih.gov/snp/rs763115697#gene_change_pr_22_tp),
however it is clearly distinct from c.934G>T, the novel variant that is being described in this paper. Moreover, c.934G>A, contrary to our novel variant (https://www.ncbi.nlm.nih.gov/clinvar/variation/446506/), has not been reported to ClinVar or PAHvdb (http://www.biopku.org/pah/search-start.asp). The variant c.934G>C (p.G312R) described from PKU families in India (Bashyam et al., 2014), and reported at PAHvdb, but not at dbSNP, although affecting the same transcript position as our novel mutation, has a completely different effect on amino acid sequence (p.G312R).

Applying the criteria for classifying a variant as pathogenic recommended by the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (Richards et al., 2015), we arrived at the conclusion that the new variant c.934G>T (p.G312C) is likely pathogenic. Two moderate pathogenic criteria were identified – PM1 (hot-spot region with 10 pathogenic variants out of 10 classified variants, 100.0%, which is >70.0%, using a hot-spot width of 36 base pairs), and PM2 (allele not found in Exome Sequencing Project, 1000 Genomes Project, and Exome Aggregation Consortium). In addition, three supporting criteria were also identified – PP1 (cosegregation with PKU in two sisters in gene PAH, definitively known to cause the disease), PP2 (missense variant in gene PAH that has 317 pathogenic missense variants versus 1 benign missense variant, ratio 317.0, which is greater than the threshold three, associated with PKU and mild hyperphenylalaninemia), and PP3 (five pathogenic predictions from SIFT, Provean, Poly-Phen-2, MutationTaster, and Human Splicing Finder vs. no benign predictions).

Homozygosity rate in Rio de Janeiro PKU population revealed a high genetic heterogeneity (j = 0.063), similar to that found in South Portugal (0.059) (Rivera et al., 2011), Galicia (0.054) (Couce et al., 2013), USA (nationwide index of 0.06) (Guldberg et al., 1996), and in California for the European descent subgroup (0.06) (Enns et al., 1999). Nevertheless, considering that the higher the value of j, the more homogeneous the population with respect to the PAH locus, our population was more homogeneous

FIGURE 2 Relative frequencies of PAH mutant alleles from 102 PKU/MHP patients from Rio de Janeiro, Brazil

FIGURE 3 Sanger sequencing electropherogram of a PKU patient sample showing the novel variant c.934G>T (p.G312C), a missense variant in the catalytic domain of exon 9, in the compound heterozygous state
than the PKU population of Spain (0.029) (Aldamiz-Echevarria et al., 2016), and the Hispanic population of California (0.04) (Enns et al., 1999).

3.2 | Haplotype characterization and association with mutant alleles

Of the 204 mutant alleles ascertained in this study, it was possible to resolve completely 188 (92.2%) of the associated haplotypes. VNTR was settled on for all mutant chromosomes. The lack of EcoRV and EcoRI RFLP evaluation did not permit to determine precisely some haplotypes linked with mutant alleles: 5.9/6.9 haplotypes associated with two c.116_118delTCT (p.F39del) mutant chromosomes, and one c.994G>A (p.G332R) mutant chromosome; 39.7/60.7 associated with one IVS10-11G>A mutant chromosome; 14.8/15.8 associated with one c.498C>T (p.Y166*) mutant chromosome, the novel c.934G>T (p.G312C) variant (two alleles in two siblings), and with two out of three mutant chromosomes bearing the large exon 5 deletion, c.442-?_509+?del, detected by MLPA (for the third chromosome, also on VNTR 8, we could not predict the haplotype based on RFLP results); 2.3/24.3 associated with three p.R261Q mutant chromosomes – the other 2.3 linkage disequilibria were settled on according to Rivera et al. (2011) and Zschocke and Hoffmann (1999); three other alleles associated each with VNTR3, VNTR7, and VNTR9 (Table 2). The analysis revealed that approximately 16 different haplotypes were associated with the mutant alleles (Table 2). Haplotype 1 was the most prevalent among mutant alleles with 16 different mutations associated with it, 14 with VNTR 8 (haplotype 1.8), and four with VNTR 7 (haplotype 1.7). The other completely resolved haplotypes in mutant chromosomes were associated with only one VNTR: 2.3, 3.8, 4.3, 5.9, 6.7, 7.8, 9.8, 11.8, 15.9, and 52.8. Among these, haplotypes 6.7, 11.8, 15.9, and 52.8 were associated with only one pathogenic variant, while the others were linked with 2-4 mutations. Eight of the nine most frequent mutations (>3.0% of the mutant alleles), p.V388M, p.R261Q, IVS10-11G>A, p.S349P, p.R252W, p.I65T, p.T323del, and p.P281L were in linkage disequilibrium to the haplotype usually associated with them in the Iberian Peninsula, especially Portugal: 1.7, 1.8, 6.7, 4.3, 1.8, 9.8, 1.8, and 2.3/1.8 respectively (Table 2). The pathogenic variant IVS2+5G>C, of non-Iberian origin, was associated to haplotype 5.9, as in other states of Brazil (Acosta et al., 2001; Santos et al., 2008), Germany and Turkey (Zschocke & Hoffmann, 1999). There were also important exceptions to the aforementioned linkages: the association of p.V388M to haplotype 1.8 in two patients, an occurrence previously observed in Minas Gerais, Brazil (Santos et al., 2008) and Spain (Perez, Desviat, & Ugarte, 1997); the linkage of p.R261Q with haplotype 2.3/24.3 in three patients, also formerly reported in Minas Gerais (Santos et al., 2008) and São Paulo, Brazil (Acosta et al., 2001); the finding of the rare haplotype 39.7/60.7 in a patient carrying the IVS10-11G>A pathogenic variant, previously reported for a homozygous patient in São Paulo (Acosta et al., 2001; Perez et al., 1996); and the association of p.R252W to haplotype 52.8 in one mutant allele, formerly found in two alleles in São Paulo (Acosta et al., 2001).

4 | DISCUSSION

The three most frequent mutations in our study, representing 34.8% of the alleles, were also the most common in Southeast Brazil (Acosta et al., 2001; Santos et al., 2008), Portugal (Rivera et al., 2011), and Spain (Aldamiz-Echevarria et al., 2016): p.V388M, p.R261Q, IVS10-11G>A. Nevertheless, in Spain they represented only 22.7% of the PKU alleles (p = 0.0004), and in Minas Gerais they responded for 52.5% of the alleles (p = 0.0008). These differences may reflect the higher heterogeneity of PKU alleles in Spain (j = 0.029), and the lower heterogeneity in Minas Gerais, Southeast Brazil (j = 0.110), in comparison to our population (Figure 4a,b). In South Brazil, IVS10-11G>A had a much lower frequency among PKU alleles than in Southeast Brazil (Figure 4a), the Iberian Peninsula (Figure 4b), and Hispanic America (Figure 4c). The low number of PKU alleles analyzed from South Brazil patients (41) (Santana da Silva et al., 2003) precludes any further conclusion concerning the observed differences.

We could not find in our population p.V388M PKU alleles on haplotype 4.3, an association of probable Amerindian inheritance found in Chile and Mexico (Desviat et al., 1995).

Two other frequent mutations of probable Iberian origin, p.S349P (6.4%) and p.T323del (4.4%), associated with haplotypes 4.3 and 1.8, respectively, have not been previously described in Brazil (Acosta et al., 2001; Santana da Silva et al., 2003; Santos et al., 2008). The missense pathogenic variant, p.S349P, has been observed in 3.4%, 1.3%, and 1.0% of PKU alleles in Spain (Aldamiz-Echevarria et al., 2016), South Portugal (Rivera et al., 2011), and Galicia (Couce et al., 2013), respectively. In South Portuguese alleles it was also found on haplotype 4.3; we are not aware of any haplotype linkage data for Spanish and Galician p.S349P alleles. In turn, the inframe deletion, c.967_969delACA (p.T323del), has been observed in 2.5% of South Portuguese alleles, on haplotypes 1.7 and 1.8. The variant p.S349P on haplotype 4.3 was firstly observed in PKU alleles in Jews from Morocco and Tunisia (Weinstein et al., 1993); c.967_969delACA (p.T323del) was originally described in one mutant allele from a Californian
### TABLE 2  PAH gene mutations and linked haplotypes in phenylketonuria (PKU)/ mild hyperphenylalaninemia (MHP) patients from Rio de Janeiro, Southeast Brazil

| DNA change | Protein effect or trivial name | N | Relative frequency (%) | Haplotype (N)b | Previous linkage description |
|------------|--------------------------------|---|------------------------|----------------|-------------------------------|
| c.1162G> A | p.V388M                       | 26 | 12.7                  | 1.7 (24)       | MG, SP, Ch, Cu, P, E         |
|            |                               |    |                       | 1.8 (2)        | MG, E                        |
| c.782G> A  | p.R261Q                       | 24 | 11.8                  | 1.8 (21)       | MG, SP, Ch, P, E             |
|            |                               |    |                       | X.3 (3)f       | MG, SP                       |
| c.1066-11G>A | p.Q355_Y356insGLQd | 21 | 10.3                | 6.7 (20)       | MG, SP, Ch, Cu, P, E         |
|            |                               |    |                       | X.7 (1)f       | SP                           |
| c.168+5G>C | IVS2+5G>C                     | 13 | 6.4                   | 5.9 (12)       | MG, SP                       |
|            |                               |    |                       | X.9 (1)b       | —                            |
| c.1045T>C  | p.S349P                       | 13 | 6.4                   | 4.3 (13)       | P                            |
| c.754C>T   | p.R252W                       | 11 | 5.4                   | 1.8 (9)        | MG, SP, P                    |
|            |                               |    |                       | 1.7 (1)        | —                            |
|            |                               |    |                       | 52.8 (1)       | SP                           |
| c.194T>C   | p.I65T                        | 9  | 4.4                   | 9.8 (9)        | MG, SP, Ch, P, E             |
| c.967_969delACA | p.T323del | 9  | 4.4                   | 1.8 (9)        | P                            |
| c.842C>T   | p.P281L                       | 7  | 3.4                   | 2.3 (4)f       | SP, P                        |
|            |                               |    |                       | 1.8 (3)        | MG, SP, P                    |
| c.1042C>G  | p.L348V                       | 6  | 2.9                   | 9.8 (6)        | MG, SP, P                    |
| c.1315+1G>A | IVS12+1G>A                  | 6  | 2.9                   | 3.8 (6)        | MG, SP, Ch                   |
| c.473G>A   | p.R158Q                       | 5  | 2.5                   | 4.3 (5)        | MG, SP, Cu, P                |
| c.1055delG | p.G352Vfs*48                  | 5  | 2.5                   | 2.3 (5)f       | MG, SP                       |
| c.1241A>G  | p.Y414C                       | 5  | 2.5                   | 4.3 (5)        | SP, Ch, P, E                 |
| c.745C>T   | p.L249F                       | 4  | 2.0                   | 1.7 (4)        | MG                           |
| c.1199+17G>A | IVS11+17G>A                  | 4  | 2.0                   | 7.8 (4)        | SP                           |
| c.250G>T   | p.D84Y                        | 3  | 1.5                   | 15.9 (2)       | —                            |
|            |                               |    |                       | X.3 (1)g       | —                            |
| c.442_-509del | Unknown                        | 3  | 1.5                   | X.8 (2)m       | —                            |
|            |                               |    |                       | X.8 (1)m       | —                            |
| c.116_118delTCT | p.F39del                  | 2  | 1.0                   | X.9 (2)i       | SP                           |
| c.136G>A   | p.G46S                        | 2  | 1.0                   | 5.9 (2)        | —                            |
|            |                               |    |                       | 1.8 (2)        | SP, P                        |
| c.204A>T   | p.R68S                        | 2  | 1.0                   | 1.8 (1)        | —                            |
| c.498C>G   | p.Y166*                       | 2  | 1.0                   | 1.8 (1)        | —                            |
|            |                               |    |                       | X.8 (1)f       | —                            |
| c.503delA  | p.Y168Sfs*27                  | 2  | 1.0                   | 3.8 (2)        | —                            |
| c.526C>T   | p.R176*                       | 2  | 1.0                   | 1.8 (1)        | MG, SP, P                    |
|            |                               |    |                       | 7.8 (1)        | —                            |
| c.561G>C   | p.W187C                       | 2  | 1.0                   | 1.8 (2)        | —                            |
| c.809G>A   | p.R270K                       | 2  | 1.0                   | 1.8 (2)        | SP, P                        |
| c.934G>Tc  | p.G312C                       | 2  | 1.0                   | X.8 (2)i       | —                            |
| c.1222C>T  | p.R408W                       | 2  | 1.0                   | 2.3 (2)f       | MG, SP, Ch, Cu               |
| c.1243G>A  | p.D415N                       | 2  | 1.0                   | 7.8 (2)        | E                            |
| c.143T>C   | p.L48S                        | 1  | 0.5                   | 4.3 (1)        | MG, SP, E                    |

(Continues)
TABLE 2 (Continued)

| DNA changea | Protein effect or trivial name | N   | Relative frequency (%) | Haplotype (N)b | Previous linkage description |
|-------------|--------------------------------|-----|------------------------|---------------|-----------------------------|
| c.165delT   | p.F55Lfs*6                      | 1   | 0.5                    | 11.8 (1)      | —                           |
| c.441+5G>T  | IVS4+5G>T                       | 1   | 0.5                    | 1.8 (1)       | —k                         |
| c.618C>G    | p.Y206*                         | 1   | 0.5                    | 1.8 (1)       | —k                         |
| c.781C>T    | p.R261*                         | 1   | 0.5                    | 1.8 (1)       | —k                         |
| c.842+1G>A  | IVS7+1G>A                       | 1   | 0.5                    | 1.8 (1)       | Ch, E                       |
| c.994G>A    | p.G332R                         | 1   | 0.5                    | X.9 (1)       | —k                         |
| c.1223G>A   | p.R408Q                         | 1   | 0.5                    | X.7 (1)       | —                           |
| Total       |                                | 204 | 100                    | —             | —                           |

*Stop codon.

Reference sequence: NM_000277.2.

Haplotype numbering corresponds to that of Eisensmith and Woo (1992), which includes RFLP polymorphisms and VNTR. An “X” denotes RFLP haplotype uncertainty due to the lack of EcoRI and EcoRV polymorphism assays.

Novel mutation, dbSNP: 763115697.

Trivial name: IVS10-11G-A.

Data were quoted from Santos et al. (2008), in Minas Gerais, Brazil (MG), Acosta et al. (2001, 2001), in Sao Paulo, Brazil (SP), Perez et al. (1999), in Chile (Ch), Desviat et al. (2001), in Cuba (Cu), Rivera et al., 2011; in Portugal (P), and Perez et al., 1997, in Spain (E).

Haplotype 2.3 cannot be distinguished from 24.3 without EcoRV RFLP assay; for p.P281L, p.G352V fs*48, and p.R408W settled on as 2.3 according to Rivera et al. (2011), for the first, and, and Zschocke and Hoffmann (1999), for the last two.

Haplotype 39.7 or 60.7: EcoRI RFLP assay capable of distinguishing them not done.

RFLP haplotype tentatively settled on as 77 (BglII +, PvuII(a) +, PvuII(b) +, MspI +, XmnI –).

Haplotype 5.9 or 6.9: EcoRV RFLP assay capable of distinguishing them not done; for p.G46S settled on as 5.9 according to Zschocke and Hoffmann (1999) and the mutation/haplotype association table available at PAHdb database (http://www.pahdb.mcgill.ca/?Topic=Search&Section=Main&Page=0).

Haplotype 14.8 or 15.8: EcoRV RFLP assay capable of distinguishing them not done.

Mutation previously described in Chile (Hamilton et al., 2017), Mexico (Vela-Amieva et al., 2015), Spain (Aldamiz-Echevarria et al., 2016; Trujillano et al., 2014) and/or Galicia (Couce et al., 2013) but haplotype not informed.

Mutation not previously described in Brazilian, Latin American, and Iberian Peninsula populations.

Undetermined haplotype, not 14.8 or 15.8.

RFLP haplotype tentatively settled on as 69 (BglII +, PvuII(a) --, PvuII(b) --, MspI +, XmnI --).

A total of 15 of the 48 mutant allele-linked haplotype combinations found in our PKU population, comprising 133 of the 204 mutant alleles (65.2%), was probably of Portuguese origin.

The most frequent pathogenic PAH variant of non-Iberian origin, IVS2+5G>C (6.4%), associated with haplotype 5.9, has been previously reported as a common variant in Southeast and South Brazil (Figure 4a). It has not been reported in Portugal, Spain, and Hispanic America (Desviat et al., 2001; Hamilton et al., 2017; Perez et al., 1999; Vela-Amieva et al., 2015) (Figure 4b,c). It is found in Middle Eastern and Central and Eastern European PKU patients (Biglari et al., 2015; Danecka et al., 2015; Kasnauksiene, Giannattasio, Lattanzio, Cimbalistiene, & Kucinskas, 2003; Zschocke & Hoffmann, 1999; Zschocke et al., 2003).

The second most frequent pathogenic variant of non-Iberian origin was c.1315+1G>A (IVS12+1G>A), on haplotype 3.8, a splicing pathogenic variant of North European origin. Rare in Portugal (0.6%) and Spain (1.0%), it reached a reasonable frequency in our population, 2.9% of PKU alleles (statistically higher than in Spain’s PKU population – p = .0327, but not Portugal’s – p = .1175).
An interesting contribution of non-Iberian European heritage to the PKU allele diversity in our population was the pathogenic variant c.136G>A (p.G46S). It was found in two PKU alleles of two compound heterozygous unrelated males (one of mixed, African and European, ethnicity), both linked with haplotype 5.9. The variant has been reported in 2.4% and 1.0% of Galician (Couce et al., 2013) and Spanish (Aldamiz-Echevarria et al., 2016) PKU alleles, respectively. But it is in Scandinavia that it reaches its peak, being present in 7.2% of Norwegian PKU alleles (Eiken et al., 1996) and in 4.3% of Swedish PKU alleles (Ohlsson et al., 2017), also linked with haplotype 5.9.

Some rare PAH pathogenic variants, found previously in geographical areas with no historical links to Rio de Janeiro, may represent recurrent mutational events. They have not been previously described in other Brazilian regions (Acosta et al., 2001; Santana da Silva et al., 2003; Santos et al., 2008), Portugal (Rivera et al., 2011) and Spain (Aldamiz-Echevarria et al., 2016; Couce et al., 2013; Perez et al., 1997; Trujillano et al., 2014). The nonsense pathogenic variant c.498C>G (p.Y166*) was observed in a homozygous early-treated black female, with no history of consanguinity, being each mutant allele linked with a distinct haplotype: 1.8 and 14.8/15.8. This variant was originally described in Southwestern China (Wang et al., 1999), and has been subsequently found in other parts of China (Song et al., 2005; Yu et al., 2014). The frameshift deletion c.503delA (p.Y168 fs*27) was detected in a homozygous early-treated white female, child of a consanguineous couple, linked with haplotype 3.8. It has been formerly reported in a heterozygous patient from Australia (Ho et al., 2014), and in two patients by a private reference laboratory in Georgia, USA, reported to ClinVar. Finally, the missense variant c.561G>C (p.W187C), observed in our

FIGURE 4 (a) Relative frequencies of prevalent PAH mutant alleles in different Brazilian regions. These data were quoted from the present study in Rio de Janeiro, Santos et al. (2008), in Minas Gerais, Acosta et al. (2001), in São Paulo, and Santana da Silva et al. (2003), in South Brazil (Rio Grande do Sul). (b) Relative frequencies of prevalent PAH mutant alleles in Rio de Janeiro, Brazil, and the Iberian Peninsula. These data were quoted from the present study in Rio de Janeiro, Rivera et al. (2011), in Portugal, and Aldamiz-Echevarria et al. (2016), in Spain. (c) Relative frequencies of prevalent PAH mutant alleles in Rio de Janeiro, Brazil, and Hispanic America. These data were quoted from the present study in Rio de Janeiro, Hamilton et al. (2017), in Chile, and Vela-Amieva et al. (2015), in Mexico
population in two early-treated white heterozygous siblings, was reported for the first time in a PKU patient in Japan (Okano et al., 1998), and, as far as we know, has not been described in other populations. In these siblings, it was linked with haplotype 1.8.

Other rare mutations might be of Spanish or Hispanic origin as, although not previously described in Brazil and Portugal, they have indeed been reported in Spain, Galicia or Hispanic America. The splicing variant c.441+5G>T (IVS4+5G>T) was found in a patient whose other allele carried a large deletion of exon 5 (c.442-509+?del). This pathogenic variant is frequent in Mexico and Chile, representing 7.3%, and 3.0% of the mutant alleles in PKU patients in those countries, respectively (Hamilton et al., 2017; Vela-Amieva et al., 2015). The nonsense pathogenic variant c.618C>G (p.Y206*) was originally described on haplotype 1 (VNTR not determined) in a patient of Spanish ethnicity in Belgium (Michiels, Francois, Raus, & Vandevyver, 1998), see also the PAHdb database (http://www.pahdb.mcgill.ca/?Topic=Search&Section=Main&Page=0). Not previously described in Brazil or Portugal, the variant occurs in 0.2% of Spanish alleles (Aldamiz-Echevarria et al., 2016). It was detected in a late diagnosed compound heterozygous white male on haplotype 1.8. The missense pathogenic variant c.994G>A (p.G332R) was firstly described in a compound heterozygous Spanish patient, with a large deletion of exon 5 on the other allele (Trujillo et al., 2014). It was detected in a early-treated compound heterozygous male, on haplotype X.9 (5.9 or 6.9), with c.1162G>A (p.V388M) on the other allele.

The geographic or ethnic origin of some rare mutant alleles was hard to resolve. The c.250G>T (p.D84Y) is an striking example of this uncertainty. Two of these alleles were found on haplotype 15.9. A third allele was found on VNTR 3, haplotype tentatively settled on as 69. These alleles were from three unrelated early-treated compound heterozygous males, two black and the third white. In the Czech Republic and in Germany, c.250G>T (p.D84Y)
alleles have been reported on 4.3 haplotype, according to the PAHdb database (http://www.pahdb.mcgill.ca/?Topic=Search&Section=Main&Page=0). In Minas Gerais, Brazil, two c.250G>T (p.D84Y) alleles were previously described on haplotypes 5.9 and 11.9 (Santos et al., 2008). Haplotype 15.9 differs from haplotypes 11.9 and 5.9 by one and two point mutations at RFLP sites, respectively. In turn, haplotypes 4.3 and 69.3 differ by four point mutations. Consequently, the c.250G>T (p.D84Y) allele on VNTR 3 found in a black male in our study probably does not share a common origin with Czech and German alleles, and the relationship among the Brazilian c.250G>T (p.D84Y) alleles on haplotypes 15.9, 11.9, and 5.9 remains to be settled.

The two c.526C>T (p.R176*) alleles found in our population might have different geographic origins. One was found on haplotype 7.8 in an early-treated compound heterozygous mestizo (black and white) male. Another allele, in an early-treated compound heterozygous white female, was detected on the usual 1.8 haplotype as formerly reported in São Paulo (Acosta et al., 2001), Minas Gerais (Santos et al., 2008), and Portugal (Rivera et al., 2011). Haplotypes 7.8 and 1.8 differ by five point mutations at RFLP sites, as a result, these two alleles probably do not share a common origin. Finishing the list of rare variants of uncertain origin, a c.165delT (p.F55Lfs*6) allele was observed on haplotype 11.8 in an early-treated compound heterozygous male. One c.165delT (p.F55Lfs*6) allele was formerly detected on haplotype 1.8 in Minas Gerais (Santos et al., 2008), the same haplotype on which alleles from the United Kingdom, Germany, Norway, and Italy were reported, according to the PAHdb database (http://www.pahdb.mcgill.ca/?Topic=Search&Section=Main&page=0). We can deduce that c.165delT (p.F55Lfs*6) 1.8 and c.165delT (p.F55Lfs*6) 11.8 do not share a common origin as haplotypes 11.8 and 1.8 differ by four point mutations at RFLP sites. The variant c.165delT (p.F55Lfs*6) on haplotype 1.8 might represent a recurrent mutational event.

It is worth turning our attention to the large deletion covering exon 5 (c.442-?_509+?del) found in three
mutant alleles exclusively by MLPA. Large deletions in PAH gene have not been previously detected among PKU alleles in Brazil (Acosta et al., 2001; Santana da Silva et al., 2003; Santos et al., 2008). However, this is probably not due to its absence in the Brazilian PKU population, but a result of the masking effect of the non-deleted allele that do not permit the identification of deletions encompassing one or more whole exons in a heterozygous state, when standard variant screening methods such as Sanger sequencing are exclusively employed (Birk Moller et al., 2007; Cali et al., 2010). Large deletions of exon 5 have been formerly reported, among other countries, in Chile (Hamilton et al., 2017), Spain (Desviat, Perez, & Ugarte, 2006; Trujillano et al., 2014), Italy (Cali et al., 2010), Denmark and Germany (Birk Moller et al., 2007), Czech Republic (Kozak et al., 2006), and Slovakia (Polak et al., 2013). Although a relatively high number of alleles with large deletions were supposedly a characteristic of Slavic (Czech, Polish, Slovak) PKU populations (Bik-Multanowski & Pietrzyk, 2008; Kozak et al., 2006; Polak et al., 2013), deletion of exon 5 reached a relative frequency of 14.9% in Chile (Hamilton et al., 2017). Moreover, the relative frequency reported by Kozak et al. (2006) in the Czech Republic (31 large deletions among 1042 mutant alleles) did not differ significantly from ours (three large deletions among 204 mutant alleles - \( p = .2313 \)), and both were significantly higher than the frequency in the Germanic population of Birk Moller et al. (2007) [four among 1,140 alleles - \( p < .0001 \) and \( p = .0362 \), respectively].

As MLPA was not used in the investigation of Mexican and Portuguese PKU alleles, the frequency of large deletions in these populations cannot be estimated from traditional DNA analysis results.

Kozak et al. (2006) observed, in the Czech Republic, that a 955 bp deletion covering exon 5 (from intron 4 to intron 5; g.50448_51402del955) was associated with haplotype 1.7 and that a longer 4232 bp deletion of exon 5, with an insertion of 268 bp in the breakpoint junction (g.47563_51794del4232 g.56161_56430ins268), was associated with haplotype 4.3. Cali et al., in Italy, confirmed that g.50448_51402del955 was associated with VNTR 7. Our three c.442?-?509+?del mutant alleles were associated with VNTR 8, two on haplotype 14/15, and the third on a non-determined haplotype. We plan to determine the breakpoints of these three mutant alleles in the future.

Concluding, the Iberian Peninsula, especially Portugal, is the major source of PKU alleles in Rio de Janeiro. This does not mean that our PKU patients are predominantly of European descent. Although ethnic origin was not thoroughly investigated, many patients were blacks or mestizos, some of which carried rare mutations, for example, c.498C>G (p.Y166*), or mutations on new haplotypes, for example, c.250G>T (p.D84Y) on 15.9, and c.526C>T (p.R176*) on 7.8. We could not find massive independent genetic incidents as the one that occurred in Mexico, where the c.60+5G>T variant, a very rare pathogenic variant in Spain, was identified in 20.8% of the alleles (Velas-Amieva et al., 2015) (Figure 4c). Nevertheless, the presence of a novel variant, c.934G>T (p.G312C), and of rare mutations previously described in geographical areas that had a very scanty contribution to Rio de Janeiro’s population gene pool, like China, prove that autochthonous events, besides genetic drift and founder effect, played important roles in shaping Rio de Janeiro’s PAH mutational spectrum.

ACKNOWLEDGMENTS

The authors thank Diego de Sousa Lima for the collection of dried blood samples, Tiago Silva Cabral Quirino and Mónica Miranda Nogueira for helping to obtain informed consent from patients and parents of minors, Prof. Isabel Rivera, Faculty of Pharmacy, University of Lisbon, Portugal, for providing details of PCR for RFLP and VNTR system analyses.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

Dr. Vieira Neto reports a public grant from Coordination for the Improvement of Higher Level Personnel (Capes) of the Ministry of Education, Brazil, and private grants from FBM Pharmaceutical Industry Ltd., Anápolis, Goiás, Brazil, and from Danone Ltd., São Paulo, Brazil, during the conduct of the study.

ORCID

Eduardo Vieira Neto http://orcid.org/0000-0002-0247-837X

REFERENCES

Acosta, A., Silva, W. Jr, Carvalho, T., Gomes, M., & Zago, M. (2001). Mutations of the phenylalanine hydroxylase (PAH) gene in Brazilian patients with phenylketonuria. Human Mutation, 17 (2), 122–130. https://doi.org/10.1002/humu.4300170222
Aldamiz-Echevarria, L., Llarena, M., Bueno, M. A., Dalmau, J., Vitoria, I., Fernandez-Marmiesse, A., . . . Couce, M. L. (2016). Molecular epidemiology, genotype-phenotype correlation and BH4 responsiveness in Spanish patients with phenylketonuria. Journal of Human Genetics, 61(8), 731–744. https://doi.org/10.1038/jhg.2016.38
Danecka, M. K., Woidy, M., Zschocke, J., Feillet, F., Muntau, A. C., de Carvalho, T. M., dos Santos, H. P., dos Santos, I. C., Vargas, P., Birk Moller, L., Nygren, A. O., Scott, P., Hougaard, P., Bieber Niel-Bik-Multanowski, M., & Pietrzyk, J. J. (2008). Single exon deletions promote phenylalanine hydroxylase (PAH) deficiency from Galicia region of Spain. Journal of Cellular Biochemistry, 115(3), 566–574. https://doi.org/10.1002/jcb.24692

Biglari, A., Safiari, F., Rashvand, Z., Alizadeh, S., Najafipour, R., & Sahmani, M. (2015). Mutations of the phenylalanine hydroxylase gene in Iranian patients with phenylketonuria. Springerplus, 4, 542. https://doi.org/10.1186/s40064-015-1309-8

Bik-Multanowski, M., & Pietrzyk, J. J. (2008). Single exon deletions in the PAH gene in Polish PKU-patients. Molecular Genetics and Metabolism, 94(2), 267. https://doi.org/10.1016/j.mgen.2008.02.007

Birk Moller, L., Nygren, A. O., Scott, P., Hougaard, P., Bieber, J., Hartmann, C., Romano, V. (2010). Exon deletions of the phenylalanine hydroxylase gene in Italian hyperphenylalaninemics. Experimental & Molecular Medicine, 42(2), 81–86. https://doi.org/10.3858/emm.2010.42.2.009

de Carvalho, T. M., dos Santos, H. P., dos Santos, I. C., Vargas, P. R., & Pedrosa, J. (2007). Newborn screening: A national public health issue for a Brazilian newborn screening program. Cadernos de Saúde Pública, 23(9), 1623–1631. https://doi.org/10.1590/S0102-311X2012000900002

Brazil’s Ministry of Health. National Council of Health. (2012). Resolution No. 466/2012 on guidelines and standards regulating researches involving human beings. Retrieved from http://consho.saude.gov.br/resolucoes/2012/466_english.pdf

Cali, F., Ruggeri, G., Vinci, M., Meli, C., Carducci, C., Leuzzi, V., ... Romano, V. (2010). Exon deletions of the phenylalanine hydroxylase gene in Italian hyperphenylalaninemics. Experimental & Molecular Medicine, 42(2), 81–86. https://doi.org/10.3858/emm.2010.42.2.009

Desviat, L. R., Perez, B., & Ugarte, M. (2006). Identification of exonic deletions in the PAH gene causing phenylketonuria by MLPA analysis. Clinica Chimica Acta, 373(1–2), 164–167. https://doi.org/10.1016/j.cca.2006.05.003

Donlon, J., Sarkissian, C., Levy, H., & Scriver, C. R. (2014). Hyperphenylalaninemia: Phenylalanine hydroxylase deficiency. In A. L. Beaudet, B. Vogelstein, K. W. Kinzler, S. E. Antonarakis, A. Balaban, K. M. Gibson & G. Mitchell (Eds.), The online metabolic and molecular bases of inherited disease. New York, NY: The McGraw-Hill Companies Inc.

Eiken, H. G., Knappskog, P. M., Bomun, H., Thune, K. S., Kaada, G., Motzfeldt, K., & Apold, J. (1996). Relative frequency, heterogeneity and geographic clustering of PKU mutations in Norway. European Journal of Human Genetics, 4(4), 205–213. https://doi.org/10.1000/s40064-01500472200

Eisensmith, R. C., & Woo, S. L. (1992). Updated listing of haplootypes at the human phenylalanine hydroxylase (PAH) locus. American Journal of Human Genetics, 51(6), 1445–1448.

Enns, G. M., Martinez, D. R., Kuzmin, A. I., Koch, R., Wakeem, C. K., Woo, S. L., ... Packman, S. (1999). Molecular correlations in phenylketonuria: Mutation patterns and corresponding biochemical and clinical phenotypes in a heterogeneous California population. Pediatric Research, 46(5), 594–602. https://doi.org/10.1203/00006450-199911000-00017

Guldberg, P., Levy, H. L., Hanley, W. B., Koch, R., Matalon, R., Rouse, B. M., ... Guttl, F. (1996). Phenylalanine hydroxylase gene mutations in the United States: Report from the Maternal PKU Collaborative Study. American Journal of Human Genetics, 59(1), 84–94.

Guldberg, P., Rey, F., Zschocke, J., Romano, V., Francois, B., Michiels, L., ... Guttl, F. (1998). A European multicenter study of phenylalanine hydroxylase deficiency: Classification of 105 mutations and a general system for genotype-based prediction of metabolic phenotype. American Journal of Human Genetics, 63(1), 71–79. https://doi.org/10.1086/301920

Guthrie, R., & Susi, A. (1963). A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants. Pediatrics, 32, 338–343.

Hamilton, V., Santa Maria, L., Fuenzalida, K., Morales, P., Desviat, L. R., Ugarte, M., ... Cornejo, V. (2017). Characterization of phenylalanine hydroxylase gene mutations in Chilean PKU patients. JIMD Reports, 2017 Dec 30 [Epub ahead of print]. https://doi.org/10.1007/8904_2017_85

Ho, G., Alexander, L., Bhattacharya, K., Dennison, B., Ellaway, C., Thompson, S., ... Christodoulou, J. (2014). The molecular bases of phenylketonuria (PKU) in New South Wales, Australia: Mutation profile and correlation with tetrahydrobiopterin (BH4) responsiveness. JIMD Reports, 14, 55–65. https://doi.org/10.1007/8904_2013_284

Kasanauskienė, J., Giannattasio, S., Lattanzio, P., Cimbalistiene, L., & Kucinskas, V. (2003). The molecular basis of phenylketonuria in Lithuania. Human Mutation, 21(4), 398. https://doi.org/10.1002/humu.9113

Kozak, L., Hrabincova, E., Kintr, J., Horky, O., Zapletalova, P., Blahakova, L., ... Prochazkova, D. (2006). Identification and characterization of large deletions in the phenylalanine hydroxylase (PAH) gene by MLPA: Evidence for both homologous and non-homologous mechanisms of rearrangement. Molecular Genetics and Metabolism, 89(4), 300–309. https://doi.org/10.1016/j.ymgme.2006.06.007
Lichter-Konecki, U., Schlotter, M., & Konecki, D. S. (1994). DNA sequence polymorphisms in exonic and intronic regions of the human phenylalanine hydroxylase gene aid in the identification of alleles. *Human Genetics*, 94(3), 307–310.

Manta, F. S., Pereira, R., Caiafa, A., Silva, D. A., Gusmao, L., & Carvalho, E. F. (2017). The spectrum of PAH mutations and increase of milder forms of phenylketonuria in Sweden during 1965–2014. *JIMD Reports*, 34, 19–26. https://doi.org/10.1007/8904_2016_4

Okano, Y., Asada, M., Kang, Y., Nishi, Y., Hase, Y., Oura, T., & Isshiki, G. (1998). Molecular characterization of phenylketonuria in Japanese patients. *Human Genetics*, 103(5), 613–618. https://doi.org/10.1007/s004390050877

Perez, B., Desviat, L. R., De Lucca, M., Cornejo, V., Raimann, E., & Ugarte, M. (1999). Mutation analysis of phenylalanine hydroxylase deficiency in Chile. Mutations in brief no. 243. https://doi.org/10.1002/humu.100796-7192(03)00032-5

Santana da Silva, L. C., Carvalho, T. S., da Silva, F. B., Morari, L., Fachel, A. A., Pires, R., ... Saraiva Pereira, M. L. (2003). Molecular characterization of phenylketonuria in South Brazil. *Molecular Genetics and Metabolism, 79*(1), 17–24. https://doi.org/10.1016/S1096-7192(03)00032-5

Ishikawa, G. (1998). Molecular characterization of phenylketonuria in Mexico. *Journal of Inherited Metabolic Disease*, 32(1), 19–22. https://doi.org/10.1016/s1096-7192(03)00032-5

Trujillo, D., Perez, B., Gonzalez, J., Tornador, C., Navarrete, R., Escaramis, G., ... Estivill, X. (2014). Accurate molecular diagnosis of phenylketonuria and tetrahydrobiopterin-deficient hyperphenylalaninemia using high-throughput targeted sequencing. *European Journal of Human Genetics*, 22(4), 528–534. https://doi.org/10.1038/ejhg.2013.175

Vela-Amieva, M., Abreu-Gonzalez, M., Gonzalez-del Angel, A., Ibarra-Gonzalez, I., Fernandez-Lainez, C., Barrientos-Rios, R., ... Alcantara-Ortigoza, M. A. (2015). Phenylalanine hydroxylase deficiency: Molecular epidemiology and predictable BH4-responsiveness in South Portugal PKU patients. *Molecular Genetics and Metabolism, 104*(Suppl), S86–S92. https://doi.org/10.1016/j.ymgme.2011.07.026

Saloum de Neves Manta, F., Pereira, R., Vianna, R., Rodolfo Beuttenmuller de Araujo, A., Leite Goes Gitai, D., Aparecida da Silva, D., ... Gusmao, L. (2013). Revisiting the genetic ancestry of Brazilians using autosomal AIM-Indels. *PLoS ONE, 8*(9), e75145. https://doi.org/10.1371/journal.pone.0075145

Santana da Silva, L. C., Carvalho, T. S., da Silva, F. B., Morari, L., Fachel, A. A., Pires, R., ... Saraiva Pereira, M. L. (2003). Molecular characterization of phenylketonuria in South Brazil. *Molecular Genetics and Metabolism, 79*(1), 17–24. https://doi.org/10.1016/S1096-7192(03)00032-5

Santos, L. L., Castro-Magalhaes, M., Fonseca, C. G., Starling, A. L., Januario, J. N., Aguia, M. J., & Carvalho, M. R. (2008). PKU in Minas Gerais State, Brazil: Mutation analysis. *Annals of Human Genetics, 72*(Pt 6), 774–779. https://doi.org/10.1111/j.1469-1809.2008.00476.x

Song, F., Qu, Y. J., Zhang, T., Jin, Y. W., Wang, H., & Zheng, X. Y. (2005). Phenylketonuria mutations in Northern China. *Molecular Genetics and Metabolism, 86*(Suppl 1), S107–S118. https://doi.org/10.1016/j.ymgme.2005.09.001

Trefz, F. K., Scheidle, D., Gotz, H., & Frauendienst-Egger, G. (2009). Significance of genotype in tetrahydrobiopterin-responsive phenylketonuria. *Journal of Inherited Metabolic Disease, 32*(1), 19–22. https://doi.org/10.1016/s1096-7192(03)00032-5

Trujillo, D., Perez, B., Gonzalez, J., Tornador, C., Navarrete, R., Escaramis, G., ... Estivill, X. (2014). Accurate molecular diagnosis of phenylketonuria and tetrahydrobiopterin-deficient hyperphenylalaninemia using high-throughput targeted sequencing. *European Journal of Human Genetics*, 22(4), 528–534. https://doi.org/10.1038/ejhg.2013.175

Vela-Amieva, M., Abreu-Gonzalez, M., Gonzalez-del Angel, A., Ibarra-Gonzalez, I., Fernandez-Lainez, C., Barrientos-Rios, R., ... Alcantara-Ortigoza, M. A. (2015). Phenylalanine hydroxylase deficiency: Molecular epidemiology and predictable BH4-responsiveness in South Portugal PKU patients. *Molecular Genetics and Metabolism, 104*(Suppl), S86–S92. https://doi.org/10.1016/j.ymgme.2011.07.026

Saloum de Neves Manta, F., Pereira, R., Vianna, R., Rodolfo Beuttenmuller de Araujo, A., Leite Goes Gitai, D., Aparecida da Silva, D., ... Gusmao, L. (2013). Revisiting the genetic ancestry of Brazilians using autosomal AIM-Indels. *PLoS ONE, 8*(9), e75145. https://doi.org/10.1371/journal.pone.0075145

Santana da Silva, L. C., Carvalho, T. S., da Silva, F. B., Morari, L., Fachel, A. A., Pires, R., ... Saraiva Pereira, M. L. (2003). Molecular characterization of phenylketonuria in South Brazil. *Molecular Genetics and Metabolism, 79*(1), 17–24. https://doi.org/10.1016/S1096-7192(03)00032-5

Santos, L. L., Castro-Magalhaes, M., Fonseca, C. G., Starling, A. L., Januario, J. N., Aguia, M. J., & Carvalho, M. R. (2008). PKU in Minas Gerais State, Brazil: Mutation analysis. *Annals of Human Genetics, 72*(Pt 6), 774–779. https://doi.org/10.1111/j.1469-1809.2008.00476.x

Song, F., Qu, Y. J., Zhang, T., Jin, Y. W., Wang, H., & Zheng, X. Y. (2005). Phenylketonuria mutations in Northern China. *Molecular Genetics and Metabolism, 86*(Suppl 1), S107–S118. https://doi.org/10.1016/j.ymgme.2005.09.001

Trefz, F. K., Scheidle, D., Gotz, H., & Frauendienst-Egger, G. (2009). Significance of genotype in tetrahydrobiopterin-responsive phenylketonuria. *Journal of Inherited Metabolic Disease, 32*(1), 19–22. https://doi.org/10.1016/s1096-7192(03)00032-5

Trujillo, D., Perez, B., Gonzalez, J., Tornador, C., Navarrete, R., Escaramis, G., ... Estivill, X. (2014). Accurate molecular diagnosis of phenylketonuria and tetrahydrobiopterin-deficient hyperphenylalaninemia using high-throughput targeted sequencing. *European Journal of Human Genetics*, 22(4), 528–534. https://doi.org/10.1038/ejhg.2013.175

Vela-Amieva, M., Abreu-Gonzalez, M., Gonzalez-del Angel, A., Ibarra-Gonzalez, I., Fernandez-Lainez, C., Barrientos-Rios, R., ... Alcantara-Ortigoza, M. A. (2015). Phenylalanine hydroxylase deficiency: Molecular epidemiology and predictable BH4-responsiveness in South Portugal PKU patients. *Molecular Genetics and Metabolism, 104*(Suppl), S86–S92. https://doi.org/10.1016/j.ymgme.2011.07.026
Zschocke, J., Preusse, A., Sarnavka, V., Fumic, K., Mardesic, D., Hoffmann, G. F., & Baric, I. (2003). The molecular basis of phenylalanine hydroxylase deficiency in Croatia. *Human Mutation, 21*(4), 399. https://doi.org/10.1002/humu.9115

**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**How to cite this article:** Vieira Neto E, Laranjeira F, Quelhas D, et al. Mutation analysis of the *PAH* gene in phenylketonuria patients from Rio de Janeiro, Southeast Brazil. *Mol Genet Genomic Med.* 2018;6:575–591. https://doi.org/10.1002/mgg3.408