REVIEW

Human Mutation

OFFICIAL JOURNAL
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Genetics of Phenylketonuria: Then and Now

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For the 25th Anniversary Commemorative Issue

Received 8 December 2015; accepted revised manuscript 12 February 2016.
Published online 26 February 2016 in Wiley Online Library (www.wiley.com/humanmutation). DOI: 10.1002/humu.22980

ABSTRACT: More than 950 phenylalanine hydroxylase (PAH) gene variants have been identified in people with phenylketonuria (PKU). These vary in their consequences for the residual level of PAH activity, from having little or no effect to abolishing PAH activity completely. Advances in genotyping technology and the availability of locus-specific and genotype databases have greatly expanded our understanding of the correlations between individual gene variant, residual PAH activity, tetrahydrobiopterin (BH_4) responsiveness, and the clinical PKU phenotype. Most patients (~76%) have compound heterozygous *PAH* gene variants and one mutated allele may markedly influence the activity of the second mutated allele, which in turn may influence either positively or negatively the activity of the biologically active heterotetrameric form of the PAH. While it is possible to predict the level of BH_4 responsiveness (~71%) and PKU severity (~78%) from the nature of the underlying gene variants, these relationships remain complex and incompletely understood. A greater understanding of these relationships may increase the potential for individualized management of PKU in future. Inherited deficiencies in BH_4 metabolism account for about 1%–2% of all hyperphenylalaninemias and are clinically more severe than PKU. Almost 90% of all patients are deficient in 6-pyruvoyltetrahydropterin synthase and dihydropteridine reductase.
Hum Mutat 37:508–515, 2016. © 2016 Wiley Periodicals, Inc.

KEY WORDS: phenylketonuria; PKU; phenylalanine hydroxylase; PAH; hyperphenylalaninaemia; tetrahydrobiopterin; sapropterin

Then: A Brief History of Phenylketonuria

Phenylketonuria (PKU; MIM #261600) is caused by variants on the gene for phenylalanine hydroxylase (PAH), with a resulting accumulation of phenylalanine (Phe) to neurotoxic levels [Blau et al., 2010; Scriver, 2007]. This condition occupies a unique place in the history of the study of metabolic disease, as not only the most common inborn error of amino acid metabolism to be identified, but also the first specific cause of mental retardation to be discovered, and the first serious genetic condition to be treated effectively, allowing its sufferers to lead a fulfilling life [Camp et al., 2014].

In 1929, Pearl S. Buck, winner of the Pulitzer in 1932 and the Nobel in 1938, travelled with her daughters Carol and Janice, from China back to the United States. In her novel "The Child Who Never Grew" [Buck, 1992], Buck wrote about her daughter Carol:

> "I remember when she was three months old that she lay in her little basket upon the sun deck of a ship. I had taken her there for the morning air as we travelled. The people who promenaded upon the deck stopped often to look at her, and my pride grew as they spoke of her unusual beauty and of the intelligence of her deep blue eyes."

Carol, who became severely retarded and was institutionalized in a special school in New Jersey, was diagnosed with PKU in the 1960s, far too late [Finger and Christ, 2004]. This was probably the first description of a child with PKU.

The actual story begins in 1934, when Asbjørn Følling, a physician studying metabolic diseases, identified an excess of phenypyruvic acid (a metabolite of Phe) as the cause of a strange, musty odor from the urine of two Norwegian children: this was the first demonstration of the underlying metabolic abnormality in a child with PKU [Fölling, 1934]. Further research in the 1930s, Penrose (1935) in the UK, led to the coining of the term, phenylketonuria, and identification of the autosomal-recessive nature of its genetic transmission. Although Penrose's attempts at dietary intervention failed, George Jervis (USA) and Horst Bickel (UK) laid the foundations for dietary intervention in PKU in the 1950s [Bickel et al., 1953], which is still the cornerstone of its management today [MacDonald et al., 2011]. The development of the first screening test for PKU in the early 1960s by Robert Guthrie made possible fast and inexpensive detection of PKU and other forms of hyperphenylalaninaemia (HPA) in all newborns [Guthrie and Susi, 1963].

Cloning of the gene for PAH in the 1980s set the scene for our current understanding of the genetics of PKU [Woo et al., 1983]. About 10 years later, both the cDNA sequence and the full-length genomic *PAH* sequence were obtained [Konecki et al., 1992] and deposited in the *PAHdb* knowledgebase [Scriver et al., 2003]; *PAHdb* was curated until 1999 and was a major source of information for the genetics of PKU [Scriver et al., 2000].

PKU always causes HPA, but not all HPA is PKU. Several cases described in the 1970s involved children with HPA unresponsive to dietary Phe restriction ("atypical" or "malignant" PKU), with developmental delay and neurological pathology [Bartholomé, 1974; Smith et al., 1975]. The author attributed these presentations to a deficiency of tetrahydrobiopterin (BH_4), a cofactor for PAH [Blau et al., 2001]. BH_4 is itself oxidized during the conversion of Phe to tyrosine by PAH and is regenerated by a separate biochemical pathway [Werner et al., 2011], the alterations of which can cause BH_4 deficiency [Thöny and Blau, 2006]. Introduction of the chemical cleavage of mismatch methods by Richard (Dick) Cotton and his

Additional Supporting Information may be found in the online version of this article.

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Contract grant sponsors: FP7-HEALTH-2012-INNOVATION-1 EU (grant no. 305444).

colleagues to screen for unknown mutations opened the potential to detect close to 100% of single base mutations [Forrest et al., 1991]. Many of these variants exhibit a high degree of association with specific restriction fragment-length polymorphism haplotypes at the *PAH* locus [Eisensmith and Woo, 1992] and for some of them in vivo and in vitro correlation with the phenotype was found [John et al., 1992; Waters et al., 1998; Pey et al., 2003].

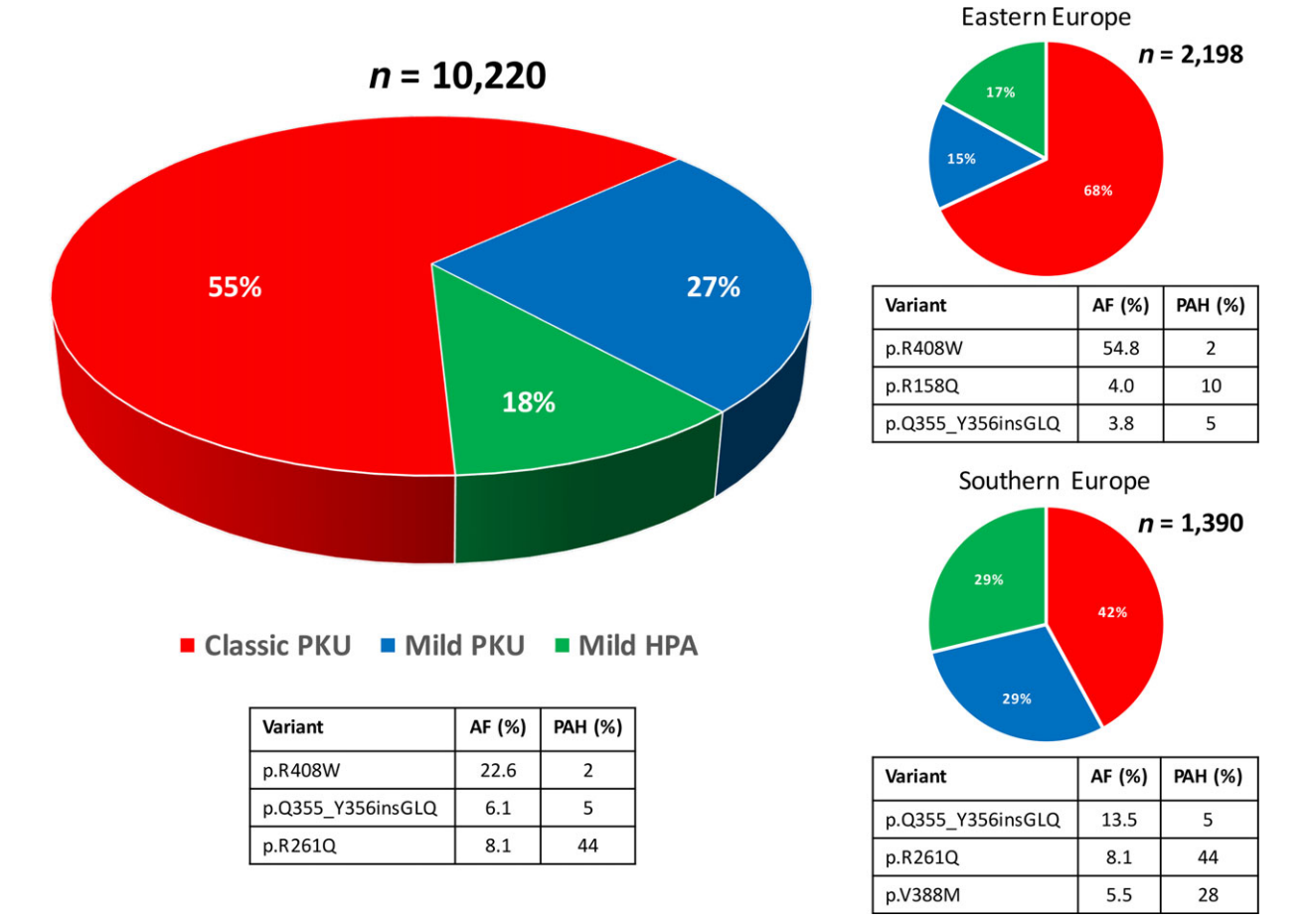
This concise review summarizes the progress we have made in understanding the genetics of PKU, building on the work of these pioneers of medicine.

Then and Now: Clinical Overview of PKU and BH_4 Deficiency

Presentation

Most countries detect PKU via routine neonatal screening to detect HPA [Blau et al., 2014]. The normal circulating level of blood Phe for newborns is up to 120 μmol/L (~2 mg/dl). PKU presents as a continuum of phenotypes from mild HPA that does not require treatment (120–360 μmol/L) to clinically defined PKU, with higher values of blood Phe [Camp et al., 2014]. Here also, there is a continuum of blood Phe levels within the population with PKU [Mitchell et al., 2011]. The most severe phenotype, often termed "classical PKU" is defined on the basis of untreated blood Phe concentrations of >1,200 μmol/L and is also the most common one worldwide [Blau et al., 2010]. The distribution of metabolic phenotypes varies with the frequency of regional genotypes and is different for different world regions (Fig. 1). While classic PKU is more common in the Eastern Europe, Mediterranean countries in Europe report milder phenotypes.

BH_4 acts as a cofactor for PAH in the hydroxylation of Phe to tyrosine, emerging from the reaction as 4a-hydroxy BH_4 and a recycling pathway involving two enzymes restores BH_4 via a qinonoid dihydrobiopterin intermediate [Kaufman, 1987; Blau et al., 2010; Werner, et al., 2011; Heintz et al., 2013]. BH_4 is also a cofactor for other amino acid hydroxylases, notably those present in the synthetic pathways of monoamine neurotransmitters [Werner et al., 2011]. Accordingly, BH_4 deficiency arising from a mutational defect



Variant	AF (%)	PAH (%)
p.R408W	22.6	2
p.Q355_Y356insGLQ	6.1	5
p.R261Q	8.1	44

Variant	AF (%)	PAH (%)
p.R408W	54.8	2
p.R158Q	4.0	10
p.Q355_Y356insGLQ	3.8	5

Variant	AF (%)	PAH (%)
p.Q355_Y356insGLQ	13.5	5
p.R261Q	8.1	44
p.V388M	5.5	28

Figure 1. Distribution of phenylketonuria (PKU) phenotypes worldwide and examples for two European regions. Phenotype frequency depends on the allele frequency of particular *PAH* variants and is specific for different world regions. There is, for example, a visible decreasing frequency of the severe classic PKU between the eastern to the southern Europe and the opposite (increasing) frequency of the mild hyperphenylalaninemia (HPA). Calculations based on 10,220 PKU/HPA patients from all over the world. Accordingly, mild PAH variant c.782G>A (p.R261Q) with a substantial in vitro residual PAH activity are more frequent in southern Europe, whereas the severe c.1222C>T (p.Arg408Trp) variant with almost no residual activity accounts for more than 50% of all mutations in Eastern Europe. Similar information can be retrieved for other world regions from the BIOPKU database (http://www.biopku.org/home/biopku.asp). In addition to the genotype information, BIOPKU includes information on the patient's phenotype, responsiveness to BH_4, blood Phe concentrations before treatment initiation, and tolerance to dietary Phe intake. All BIOPKU records are directly linked to the *PAH* locus-specific database. AF, allele frequency; PAH, in vitro enzyme activity (% of the wild-type activity).

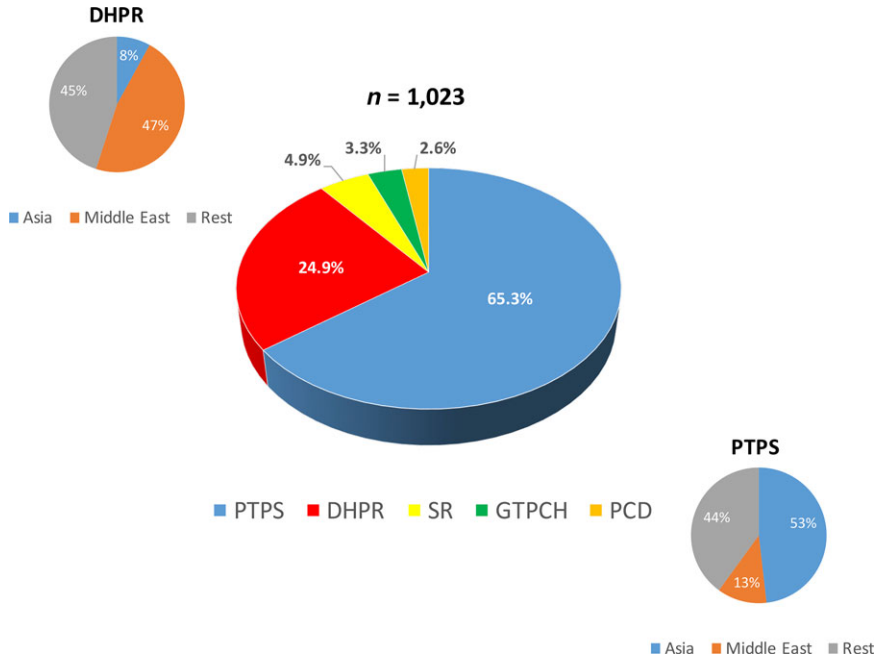


Figure 2. Frequency of different tetrahydrobiopterin (BH_4) deficiency defects. Data based on 1,023 patients tabulated in the BIODEF database (http://www.biopku.org/home/biodef.asp). Particular forms of BH_4 deficiency are common in certain world regions. The two small pies represent distribution of dihydropteridine reductase (DHPR) and 6-pyruvoyl-tetrahydropterin synthase (PTPS) deficiencies in Asia, Middle East (incl. Turkey), and the rest of the world, with DHPR deficiency being more common in the Middle East and Turkey and PTPS deficiency in Asia. BIODEF database tabulates the most common clinical and laboratory data related to hyperphenylalaninemia and BH_4 deficiencies. Additionally, there are data regarding treatment, outcome, and DNA analysis. SR, sepiapterin reductase; GTPCH, GTP cyclohydrolase I; PCD, pterin-4a-carbinolamine dehydratase.

in BH_4 synthesis or recycling may give rise to a heterogeneous range of, often severe, presentations associated with peripheral and/or central nervous dysfunction [Blau et al., 2001; Opladen et al., 2012]. Some, but not all, of these presentations include HPA, with the possibility of mental retardation, motor dysfunction, difficulty swallowing, seizures/convulsions, dystonias, dyskinesias, hyper-reflexia, or spasticity, among other symptoms, depending on the enzyme involved [Blau et al., 2001; Opladen, et al., 2012; Burlina and Blau, 2014]. BH_4 deficiencies are more rare than PKU (incidence about 1%–2% of all HPAs or 1:500,000 newborns), but quite common in the Turkey, Middle East, or Asia [Ye et al., 2013]. More than 1,000 patients with different forms of BH_4 deficiency are tabulated in the BIODEF database (http://www.biopku.org/home/biodef.asp) (Fig. 2). Neonates with any elevation of blood Phe should be screened for BH_4 disorders [Opladen et al., 2012]. Mutation analysis of such patients ensured that atypical mild presentations of BH_4 deficiency are not missed [Blau et al., 1992], but CSF investigations are essential here. Investigations of biogenic amines, BH_4 metabolites, and folates are particularly important for the differential diagnosis between mild and severe forms and for the treatment follow up [Opladen et al., 2012].

Current Management

A lifelong Phe-restricted diet, applied immediately on diagnosis, is the mainstay of the management of PKU, in which natural sources of protein are substituted with Phe-free "medical foods" [Camp et al., 2014]. People with PKU typically use special nutritional products [Belanger-Quintana et al., 2012], where foods are categorized according to their Phe content, to design their diet according to their daily Phe tolerance [MacDonald and Blau, 2013]. Good compliance with the diet protects the brain and allows patients to lead full and fulfilling lives. However, subtle neuropsychological defects remain, on average, relative to their non-PKU peers, and periodic evaluations of executive, emotional, behavioral, and other neuropsychological functions are recommended, in addition to nutritional status [Camp et al., 2014].

The PKU diet is onerous and compliance is often poor, particularly during the emotionally challenging teenage years [MacDonald et al., 2010]. Pharmacological doses of BH_4 increase the activity of PAH in the setting of certain PKU-causing mutations and a pharmaceutical preparation of BH_4 (sapropterin dihydrochloride; Kuvan®) is available for the management of PKU [Kure et al., 1999; Zurflüh et al., 2008; Heintz et al., 2013]. Responders to sapropterin benefit from increased Phe tolerance, permitting some (or even complete) relaxation of the Phe-restricted diet [Muntau et al., 2002; Trefz et al., 2009; Blau, 2010; Camp et al., 2014]. This treatment is only sufficiently effective in about one patient in five overall, however, and especially in milder PKU phenotypes [Fiege and Blau, 2007; Shintaku et al., 2008].

BH_4 deficiencies presenting with HPA may also be managed with BH_4 replacement, as first noted in 1975 [Danks et al., 1975], with application of the Phe-restricted diet if required [Opladen et al., 2012; Burlina and Blau, 2014]. Most patients require treatment with neurotransmitter precursors that enter the synthesis pathways of neurotransmitters distal to the BH_4-requiring step, such as L-dopa (for dopamine), or 5-hydroxytryptophan (for serotonin). Enzyme inhibitors (e.g., carbidopa, seligiline, entacapone) and agonists (e.g., bromocriptine, pramipexole) may be useful in a combination with the standard therapy [Longo, 2009; Porta et al., 2009; Burlina and Blau, 2014].

Dick Cotton's Contributions

The most severe form of BH_4 deficiency, dihydropteridine reductase (DHPR) deficiency, was one of the central points of Richard (Dick) Cotton's research between 1975 and 2000. Following the report of first Australian patients with BH_4 deficiency in 1975, intravenous therapy with synthetic BH_4 was proposed and introduced for the first time [Danks et al., 1975] and BH_4 loading test was suggested as the best method to identify cases with "malignant PKU" or BH_4 deficiency [Danks et al., 1979]. Development of an assay for DHPR in peripheral blood cells was basis for an accurate diagnosis at protein level [Firgaira et al., 1979], and radioimmunoassay, immunoprecipitation, affinity chromatography, and two-dimensional gel electrophoresis were used to test cultured cells from families with DHPR deficiency for a catalytically incompetent product of the gene variants, thus proving the heterogeneity of the disease [Firgaira et al., 1981a]. Furthermore, it has been shown that the same structural gene encodes for DHPR in human liver, fibroblasts, and lymphocytes [Firgaira et al., 1981b]. Using an inhibitor of BH_4 biosynthesis (diaminohydroxypyrimidine) in the diet, an animal model for BH_4 deficiency was created and subsequently rescued by BH_4, dihydrobiopterin, or sepiapterin administration [Cotton, 1986]. Dick's group also showed that metabolic response to BH_4 depends on the underlying *QDPR* variant and that some DHPR-deficient patients do not respond to the low-dosage administration of the cofactor BH_4 [Cotton et al., 1986a, 1986b]. Obviously, these patients needed higher dosage of BH_4 (20 mg/kg) for a full response [Ponzone et al., 1991]. With the isolation of a cDNA clone for human *QDPR* that spans the complete coding region, the nucleotide sequence and the predicted amino acid sequence were presented [Dahl et al., 1987]. This opened the era of molecular genetics for DHPR deficiency [Ponzone et al., 1988; Dahl et al., 1988; Howells et al., 1990; Blau et al., 1992; Dianzani et al., 1998; Smooker et al., 1999]. The main interests of Dick's research in the field of BH_4 is summarized in a recent review article [Heintz et al., 2013].

Now: A Closer Look at the Molecular Genetics of PKU

The *PAH* Gene and Databases

The *PAH* gene is 90 kb in length (about 171 kb if flanking regions are included) with 13 exons [Scriver, 2007]. Many variations of *PAH* have been described, over some 25 years of research [Scriver et al., 2000], with variations occurring in all exons, but most commonly in exons 3, 6, 7, and 11 (Supp. Fig. S1) [Blau et al., 2014]. PKU is inherited in an autosomal-recessive manner (as recognized by Følling and other early pioneers of PKU research); thus, two mutated copies of *PAH* are required for the PKU phenotype.

Locus-specific databases have been an important resource for understanding the nature, prevalence, and impact on PAH deficiency [Scriver, et al., 2003; Blau et al., 2014]. The open-access *PAH*vdb database (http://www.biopku.org/home/pah.asp) reports 957 variants of this gene (January 30, 2016) [Blau et al., 2014]. The reference accession number for the *PAH* sequence is ENSG00000171759; RefSeq NM_000277.1. An analysis of this database showed that 60% of *PAH* variants are missense mutations, with other common variants being splice variants and deletions (14% each) [Blau et al., 2014]. Genotypes and clinical phenotypes of more than 10,000 patients with PKU are tabulated in the BIOPKU database (January 30, 2016) (http://www.biopku.org/home/biopku.asp): 55% had the classical phenotype and 27% had a mild phenotype, with the remainder having non-PKU mild HPA (Fig. 1). Most patients with PKU (~76%) are compound heterozygotes [Scriver, 2007; Wettstein et al., 2015].

Severity of Variations and BH_4 Responsiveness

The most common variations of *PAH* in the BIOPKU database are c.1222C>T (p.Arg408Trp) and c.1066-11G>A (p.Gln355_Tyr356insGlyLeuGln) (23% and 6% of all mutations, respectively); these are severe mutations that essentially abolish PAH activity [DiLella et al., 1987; Gjetting et al., 2001]. A number of other mutations have varying effects on the activity of PAH: for example, alleles c.782G>A (p.Arg261Gln) and c.1241A>G (p.Tyr414Cys), also commonly occurring in these databases (5% and 3%, respectively), have been shown to have about 44% and 57% of the activity of wild-type PAH, respectively [Zurflüh et al., 2008; Wettstein et al., 2015]. Other genetic variants are effectively silent, with little or no effect on the activity of PAH, such as variants c.569T>C (p.Val190Ala) (~100% of the activity of wild-type PAH) or c.204A>T (p.Arg68Ser) (97% residual activity) [Wettstein et al., 2015].

Mild *PAH* mutations with a substantial residual enzyme activity are most likely to demonstrate increased activity in the presence of BH_4. This can be seen in Figure 3, where the residual PAH activity associated with various muted forms of the PAH protein in vitro has been plotted according to whether or not the mutation was considered to be BH_4 responsive (where known; this was determined in patients with these mutations treated with BH_4) [Zurflüh et al., 2008; Wettstein et al., 2015]. There is a clear separation between BH_4-responsive and nonresponsive mutations in terms of residual PAH activity.

Mutated forms of PAH are less structurally stable than wild-type PAH [Gamez et al., 2000]. BH_4 appears to be a molecular chaperone to PAH, in that it protects the protein from misfolding during synthesis or promotes reconstitution of the correct 3-dimensional structure in the cytosol. An experimental study of several known

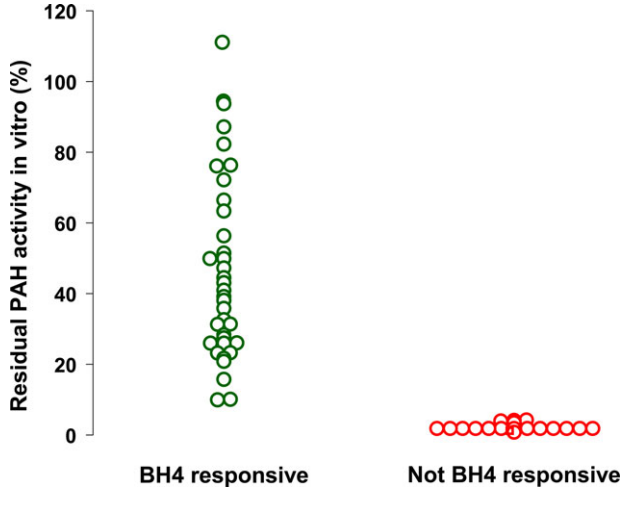


Figure 3. Tetrahydrobioperin (BH_4) responsiveness according to severity of the underlying variation in *PAH*. The activity of individual mutated PAH alleles for converting phenylalanine to tyrosine was studied in vitro. BH_4 responsiveness was determined by a BH_4 loading test in patients with PKU carrying these mutations. Drawn from data presented in Zurflüh et al. (2008).

BH_4-sensitive mutated forms of PAH demonstrated increased activity of variations known to be associated with misfolding defects, consistent with this hypothesis, but also preserved the activity of other mutated forms [Pey et al., 2004]. PAH assembles into homodimers or homotetramers in vivo, and mutations in the oligomeric binding domains, which may be involved in allosteric regulation of the protein [Jaffe et al., 2013], appear to be especially associated with BH_4 responsiveness [Wettstein et al., 2015]. The protection of PAH by BH_4 is therefore likely to be multifactorial in nature [Erlandsen et al., 2004; Gersting et al., 2008].

Genotype–Phenotype Correlations

Since the genotype determines the activity of PAH and thus the metabolic phenotype, there is growing evidence of genotype–phenotype correlation [Trefz et al., 1993; Kayaalp et al., 1997; Benit et al., 1999; Jennings et al., 2000; Kasnauskiene et al., 2003; Pey et al., 2003; Bercovich et al., 2008; Daniele et al., 2009; Bueno et al., 2013Polak et al., 2013; Reblova et al., 2013; Tao et al., 2015; Trunzo et al., 2015]. It has been known for about 20 years that PAH activity predicts the clinical phenotype (blood Phe) in PKU [Eisensmith and Woo, 1992]. More recent studies have confirmed a significant relationship between allelic phenotype, enzyme activity, and BH_4 responsiveness. The statistical and analytic power of large mutational databases has been used to explore the relationship between genotype and phenotype in PKU. The nature of the mutation, or the known effect of the mutation on PAH structure can be used to predict the enzyme activity in a number of cases, but not all [Erlandsen and Stevens, 1999]. Protein truncations, large deletions, active-site mutations, or production of fusion of proteins (missense mutations of splicing variants) are more likely to result in severe phenotypes than mutations in regulatory or oligomerization domains of PAH [Jennings et al., 2000].

A recent study used predictive algorithms for estimating the damage caused to PAH by various missense mutations [Wettstein et al., 2015]. Protein stability predicted enzyme activity and the allelic phenotype (mild, moderate, or severe PKU), and enzyme activity also predicted the allelic phenotype. Overall, BH_4 responsiveness was predicted correctly for 71% of patients. In other studies, the level of residual activity of PAH, and the concentration-dependent manner in which Phe and BH_4 regulate the activity of PAH (the "functional landscape" of PAH mutations), were strong predictors of BH_4 responsiveness [Staudigl et al., 2011; Danecka et al., 2015]. Accordingly, information on the genotype is likely to be more useful for identifying people for a BH_4 loading test, rather than accurate prediction of their BH_4 responsiveness based on the phenotype alone [Tao et al., 2015].

In principle, Phe catabolism is improved in humans if either of the patient's copies of *PAH* is BH_4 sensitive, which has important implications for therapy with BH_4 [Erlandsen et al., 2004]. However, studies involving coexpression of differently mutated PAH have shown that one mutated form can influence the other when assembled into a tetramer. This process, known as interallelic complementation [Leandro et al., 2006], can increase or decrease the BH_4 responsiveness of the resulting PAH subunits [Heintz et al., 2013; Shen et al., 2016]. Figure 4 shows the effects of coexpression of different mutated forms of PAH (one essentially null mutation with a second, milder mutation) on enzyme activity in vitro, and on BH_4 responsiveness and PKU phenotype in patients carrying them [Shen et al., 2016]. In general, the common c.1222C>T (p.Arg408Trp) variant together with c.473G>A (p.Arg158Gln) (another severe variant) resulted in little enzyme activity, little BH_4 responsiveness, and a mainly classic PKU phenotype. With very few emptions (e.g. c.1223G>A / p.Arg408Gln), combining other mutations result in predicted phenotype; coexpression of c.1222C>T (p.Arg408Trp) with c.1241A>G (p.Tyr414Cys) resulted in significant PAH activity, frequent BH_4 responsiveness, and a mild PKU phenotype.

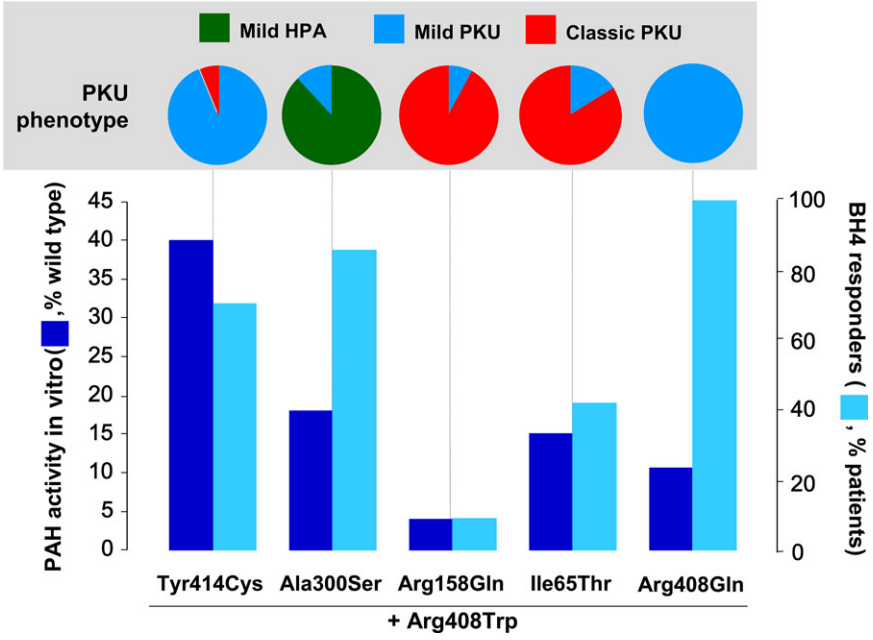


Figure 4. Graphical representation of the relationships between PAH activity of mutated forms of coexpressed in vitro and BH_4 responsiveness and PKU phenotype in carriers of each pair of mutations. Each pair of columns and the pie chart immediately above are for coexpression of (in vitro) or carriers of (in patients) the null c.1222C>T (p.Arg408Trp) variant of phenylalanine hydroxylase (PAH) together with other individual mutations shown. Drawn from data presented by Shen at al. (2016).

Then, Now, Tomorrow

Our understanding of the molecular basis of PKU has increased dramatically in recent years. This has been driven largely by the availability of ever-more powerful techniques for analyzing and visualizing the effects of mutations on proteins now, compared with when the properties of PAH and BH_4-regenerating proteins were first being studied using molecular techniques [Ratnam et al., 1989; Forrest et al., 1991; Blau et al., 2014]. In future, high-throughput automated sequencing techniques promise to revolutionize the molecular diagnosis of PKU and BH_4 disorders [Trujillano et al., 2014].

The concept of PAH landscapes promises to bring a new era of personalized medicine to the management of PKU, by addressing the complexity of the interactions between genotype and phenotype in the context of the compound heterozygous *PAH* mutations found in most people with PKU [Danecka et al., 2015]. New treatments under development to address the HPA of PKU include injecting (or perhaps administering orally) an alternative enzyme to PAH, phenylalanine ammonia lyase, that does not require a cofactor [Strisciuglio and Concolino, 2014; Blau and Longo, 2015], administering novel formulations of PAH, including encapsulation of PAH within erythrocytes [Yew et al., 2013; Rossi et al., 2014], or gene therapy approaches to replace the defective PAH protein itself [Viecelli et al., 2014]. Novel molecules to act as molecular chaperones for PAH, or to increase its stability, are also being investigated [Santos-Sierra et al., 2012; Underhaug et al., 2013; Muntau et al., 2014]. Fundamental basis for all above-mentioned projects is a full-length structure of human PAH, which is to date not published. There are, however, some very recent attempts to identify at least allosteric sites for Phe [Carluccio et al., 2015; Zhang and Fitzpatrick, 2016; Arturo et al., 2016] and dimerization mechanisms of regulatory domain [Zhang et al., 2015].

It is an exciting time to be working on the molecular pathology, diagnosis, and genetics of PKU and BH_4 deficiency. This is due in no small part to the dedication and skill of the scientists and physicians who have advanced this challenging field to its position today. Among these, Richard (Dick) Cotton, my colleague and friend for many years, left his own mark on the history of this important field of science and medicine and I am proud to have cited here some of the research we published together.

Acknowledgments

This work is part of the RD-CONNECT initiative. Dr. Mike Gwilt provided the editorial help.

Disclosure statement: Author of this article received educational grants and/or acted as an advisory board member to Merck Serono, Germany, Biomarin Pharmaceutical, USA and Codexis, USA.

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