Review Article

Phenylketonuria: translating research into novel therapies

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Abstract: Phenylketonuria (PKU) is an inborn error of metabolism of the amino acid phenylalanine. It is an autosomal recessive disorder with a rate of incidence of 1 in 10,000 in Caucasian populations. Mutations in the phenylalanine hydroxylase (*PAH*) gene are the major cause of PKU, due to the loss of the catalytic activity of the enzyme product PAH. Newborn screening for PKU allows early intervention, avoiding irreparable neurological damage and intellectual disability that would arise from untreated PKU. The current primary treatment of PKU is the limitation of dietary protein intake, which in the long term may be associated with poor compliance in some cases and other health problems due to malnutrition. The only alternative therapy currently approved is the supplementation of BH_4, the requisite co-factor of PAH, in the orally-available form of sapropterin dihydrochloride. This treatment is not universally available, and is only effective for a proportion (estimated 30%) of PKU patients. Research into novel therapies for PKU has taken many different approaches to address the lack of PAH activity at the core of this disorder: enzyme replacement via virus-mediated gene transfer, transplantation of donor liver and recombinant PAH protein, enzyme substitution using phenylalanine ammonia lyase (PAL) to provide an alternative pathway for the metabolism of phenylalanine, and restoration of native PAH activity using chemical chaperones and nonsense read-through agents. It is hoped that continuing efforts into these studies will translate into a significant improvement in the physical outcome, as well as quality of life, for patients with PKU.

Keywords: Phenylalanine hydroxylase (*PAH*); phenylketonuria (PKU); mutation; phenotype-genotype correlation; therapy; diet

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Phenylketonuria (PKU) and hyperphenylalaninaemia (HPA)

Phenylketonuria (PKU, OMIM 261600) is an inborn error of metabolism, predominantly caused by mutations in the phenylalanine hydroxylase (*PAH*) gene. The mode of inheritance is autosomal recessive. Mutations in *PAH* lead to impaired function of the hepatic enzyme PAH (EC 1.14.16.1), which catalyses the conversion of the essential amino acid L-phenylalanine (L-Phe) to L-tyrosine (L-Tyr), a precursor of the neurotransmitters dopamine, noradrenaline and adrenaline. The resulting elevated levels of L-Phe, a condition known as hyperphenylalaninaemia (HPA), which is the primary biochemical marker of PKU and the more benign form mild hyperphenylalaninaemia (MHP).

PKU was described by A. Følling in 1934 (1). The accumulation of L-Phe drives an alternate metabolic pathway, resulting in the detection of phenylketones (phenylpyruvate and phenylacetate) in the urine of PKU patients (2). These ketones are also excreted in sweat, creating a 'mousy' odour characteristic of the disease. The unravelling of the metabolic pathway from L-Phe to L-Tyr to subsequent products, and the subsequent development of a newborn screening strategy (3) were significant in the establishment of a treatment and in contributing to the understanding of metabolic diseases at that time. Prior to this, children with PKU failed to attain developmental milestones, ultimately exhibiting profound intellectual

Table 1 Incidence rates of PKU in different populations/countries

Ethnic group/ population	Incidence of PKU	Reference
Sicily	1:2,700	Guldberg *et al*. [1993] (12)
Turkey	1:4,200	Ozalp *et al*. [2001] (13)
Ireland	1:4,500	Zschocke *et al*. [1997] (14)
Catalonia	1:6,600	Mallolas *et al*. [1999] (15)
Israel	1:8,200 non-Jews; 1:12,500 Jews	Bercovich *et al*. [2008] (16)
Caucasian	1:10,000	Zschocke [2003] (11)
Northern China	1:11,000	Song *et al*. [2005] (17)
Cuba	1:20,000	Desviat *et al*. [2001] (18)
Korea	1:41,000	Lee *et al*. [2004] (19)
Taiwan	1:55,000	Chien *et al*. [2004] (20)
Japan	1:80,500	Kimura *et al*. [2001] (21)
Finland	1:100,000	Guldberg *et al*. [1995] (22)

PKU, phenylketonuria.

impairment. Hyperactivity and seizures also featured later in life, and many untreated individuals have a fairer complexion and hair colour than other family members (4). Most cases of PKU are caused by mutations in the *PAH* gene (1). The remaining cases are caused by mutations affecting either the synthesis or the regeneration of tetrahydrobiopterin (BH_4), the co-factor of PAH (5). The genes involved in the BH_4-deficiency type HPA include guanosine triphosphate cyclohydrolase I (*GTPCH*) (6), 6-pyruvoyl-tetrahydropeterin synthase (*PTPS*) (7), pterin-4a-carbinolamine dehydratase (*PCD*) (8), and quinoid dihydropteridine reductase (*DHPR*) (9). Sepiapterin reductase (*SR*) is also a genetic factor in BH_4-deficiency disorders, but mutations in *SR* do not lead to HPA (10). The remainder of this review focuses on issues more relevant to HPA caused by a primary deficiency of PAH.

PKU occurs in approximately 1 in 10,000 Caucasian births (11), equivalent to a carrier rate of 1 in 50. However, the incidence rate may vary widely amongst different countries or regions (*Table 1*). Finland and Japan have the lowest reported incidence of PKU, at 5 and 8 cases per million births respectively (21,23). Incidence rates are highest in Sicily with 1 in 2,700 births diagnosed as PKU, and 1 in 4,200 in Turkey (13,23). There is also a high level of clinical heterogeneity in PKU. Disease severity can be classified by blood Phe levels. In patients with classic PKU, the most severe form, blood Phe levels can rise to over 1,200 µmol/L, compared to 120 µmol/L in a normal healthy person. In moderate and mild PKU, the values range from 900 to 1,200 µmol/L and 600 to 900 µmol/L respectively, and in non-PKU MHP, between 120 and 600 µmol/L (4).

An additional complication is the effect of maternal PKU on foetal development. The features in the affected newborn (who in most cases does not have PKU) include microcephaly, congenital heart defects, dysmorphic facial features, intrauterine growth retardation, with often severe cognitive impairment becoming apparent in childhood (24,25). Treatment of women with PKU during pregnancy is vital for reducing the likelihood of the so-called maternal PKU syndrome in the offspring, and can be achieved by maintaining a Phe level of 120-360 µmol/L from the first trimester, and ideally even before conception (24).

Molecular bases of PKU

Mutations in the *PAH* gene accounts for 98% of cases of PKU (25). The human *PAH* gene maps to chromosome region 12q23.2, on the minus strand, spanning close to 80 kb (NCBI reference sequence NM_000277.1, *Figure 1*) (26,27). The gene consists of thirteen exons, forming a transcript with an open reading frame of 1,359 bases and encoding a polypeptide of 452 amino acids (NCBI reference sequence NP_000268.1).

The PAH polypeptide is highly homologous in mammals and retains a high level of homology even in more distant eukaryotes. The 52 kDa polypeptide is divided into three functional domains (28). The N-terminus is the regulatory domain (amino acid residues 1-142), followed by the catalytic domain (residues 143-410) and the C-terminal tetramerisation domain (residues 411-452). The hepatic PAH enzyme catalyses the conversion of L-Phe to L-Tyr, in the presence of the co-factor BH_4 [(6*R*)-L-*erythro*-5,6,7,8-tetrahydriobiopterin] and O_2 (29). The L-Tyr is then further converted to neurotransmitters dopamine, noradrenaline and adrenaline. In the absence of the PAH, an alternative metabolic pathway breaks down L-Phe to phenylketones (*Figure 2*). In plants and fungi, another enzyme, PAL is involved in the catabolism of L-Phe, and enzyme substitution therapy using PAL is currently being investigated (30).

There are currently over 560 *PAH* mutations reported in the *PAH*db (http://www.pahdb.mcgill.ca) (31). Mutations causing PKU have been identified in all three domains, but a mutation hotspot in exon 7 of PAH corresponds to the catalytic active site of the protein. The majority

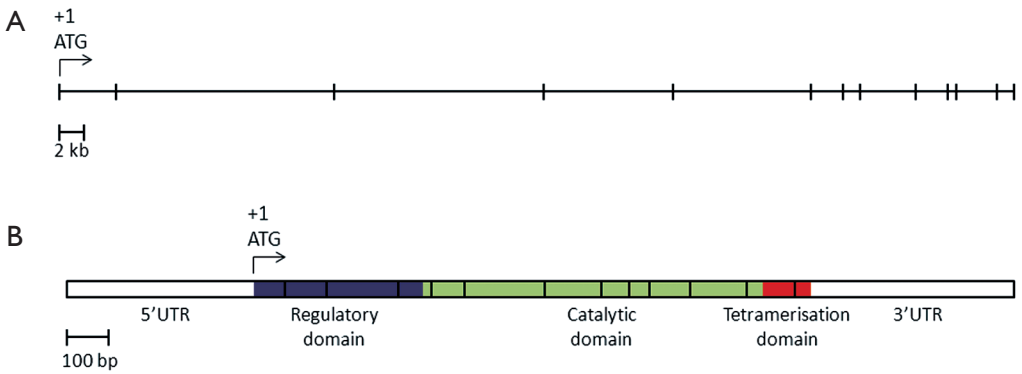

Figure 1 (A) Structure of the human *PAH* gene. The horizontal line represents the full length of the *PAH* gene, spanning 79.3 kb. Each vertical bar represents an exon. The location of the start codon ATG in exon 1 is indicated (+1); (B) Schematic representation of *PAH* mRNA. The vertical lines mark the boundaries between exons. The three functional domain of PAH are coloured purple for the regulatory domain, light green for the catalytic domain and orange for the tetramerisation domain. UTR, untranslated regions; PAH, phenylalanine hydroxylase.

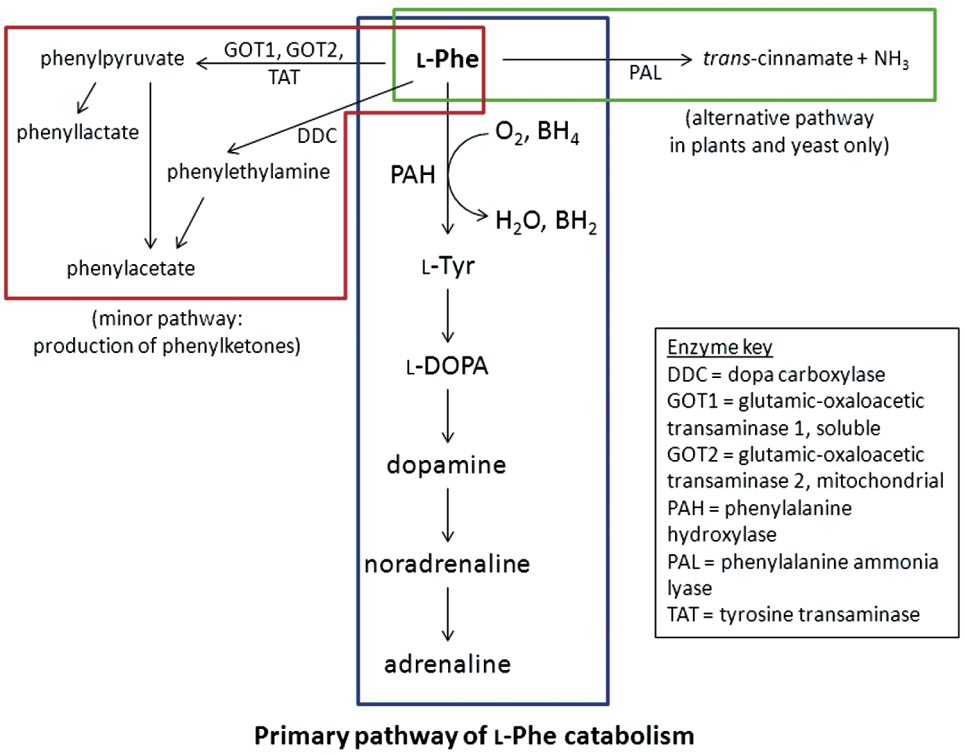

Figure 2 Metabolic pathway of L-Phe. The primary pathway (blue box) is the catalytic conversion of L-phe to L-Tyr by phenylalanine hydroxylase (PAH). In phenylketonuria (PKU), the deficiency of PAH enzyme leads to the production of phenylketones by an alternative pathway (red box). A third pathway (green box) can be found in plants and yeast involving the enzyme phenylalanine ammonia lyase (PAL).

of pathogenic mutations in *PAH* are missense mutations (65.4% of mutations reported in *PAH*db), followed by small insertions and/or deletions (both in-frame and frame-shift, 16.4%), mutations affecting splicing (12.2%), then nonsense mutations (5.2%). Large deletions and duplications, involving an entire exon or exons, have also been reported, but they are thought to account for less than 1% of disease alleles within a population (32-34).

As more *PAH* mutation data were made available, it became clear that there was a relationship between the mutations and the severity of the disorder, allowing classification of some *PAH* mutations as being likely to cause a particular phenotype (17,35-38). *In silico* prediction of phenotype (the severity of disease) from genotype may be useful in refining diagnosis and providing a baseline for treatment (36). However, the establishment of a system by which phenotype may be predicted from genotype is hampered by the large number of *PAH* mutations resulting in a larger number of possible genotype combinations.

The classification of a mutation as 'severe', 'moderate', 'mild' or 'MHP' is based upon individuals who are functionally hemizygous, whereby one of the alleles is shown or predicted to be a null allele (39). Null alleles (which are classed as 'severe' mutations) include nonsense mutations, frameshift mutations, splicing mutations affecting the canonical AG-GT dinucleotides at the exon-intron boundaries, and missense mutations that result in little residual enzymatic activity, such as p.Arg252Trp, p.Arg408Trp, p.Pro281Leu (36). Although most analyses have shown that this model is successful in correlating phenotype to genotype to a large degree, a significant number of discrepant findings have also been reported. This is most striking in patients who are homozygous for one mutation. For example, of the six patients homozygous for p.Leu48Ser reported in Bercovich *et al.*, (16) two were classified as having classic PKU, 2 with moderate PKU and the other 2 with mild PKU. Similarly, in another study, three patients who are homozygous for p.Ile65Thr had the three classifications PKU, variant and non-PKU MHP (35).

Phenotype classification may vary between different centres and misclassification may contribute to some of the incongruities (36). The method of phenotype classification (by plasma Phe levels or dietary Phe tolerance) is also a possible source of misclassification, with the former more closely aligned with genotype predictions than the latter (36). Different studies or centres may also use different cut-off for classifying the severity of phenotypes. These arbitrary cut-off points may not necessarily reflect the continuum in both the clinical phenotype and in the residual activity in the various mutations. In addition, it is highly probable that other genetic factors play a role in determining PKU severity. Modifier genes, such as the loci involved in the regeneration of BH_4, are postulated to have an effect on the phenotypic outcome (40). Similarly, the enzymes involved in the alternate catabolic pathway of L-Phe can modulate dietary Phe tolerance (41). Differences in the transport of L-Phe across the blood-brain barrier have also been suggested as a possible source of phenotypic variance (42).

In vitro studies have been valuable in demonstrating the nature of pathogenicity in many missense mutations and in elucidating the specific role of individual amino acids in the PAH peptide (43-45). In particular, analyses of the enzymatic activity of missense mutations have shown strong correlation to disease severity (46-48), and for rare missense mutations these studies are useful in predicting the metabolic phenotype. Missense *PAH* mutations may be grouped under broad headings: (I) null mutants with no detectable enzymatic activity; (II) V_{max} mutants with reduced maximum activity; (III) kinetic mutants with altered K_M for either substrate or co-factor and (IV) unstable mutants with decreased levels of PAH polypeptide (25).

Understanding the molecular defect of the missense mutations may also have therapeutic implications. For example, the aggregation of PAH protein carrying single amino acid changes such as p.Gly46Ser is thought to be caused by misfolding (49). Co-expression of bacterial chaperonins in *Escherichia coli* and other novel molecular chaperones increase both PAH protein levels and residual activity (50), giving rise to a new class of potential chemical compounds that may be of benefit for the treatment of PKU.

Treatment of PKU

The primary treatment of PKU is the restriction of dietary protein (and thus L-Phe). Due to early detection through newborn screening, treatment can be started in the first weeks of life. Early intervention and good dietary compliance are essential for cognitive outcome (measured as IQ) and for reducing the risk of neurological complications and behavioural problems in PKU patients (51). Whilst foods high in protein, including meat, dairy, nuts and legumes, should be eliminated or highly restricted, the intake of foods high in starch, e.g., potatoes, pasta, bread, needs to be monitored as well. Patients with the severe classic form of PKU must adhere most strictly to the dietary limitations and patients with non-PKU MHP may not require any dietary restrictions at all. Supplementary diet formulae exist for a variety of age ranges, and are critical to ensure the correct balance of the other essential amino acids, vitamins, minerals and trace nutrients are provided to reach recommended daily intake targets (4).

The target level of Phe concentration varies according to the age of the patient (52). In children under two years of age,

the target concentration is generally below 360 μmol/L, and increasing to up to 1,200 μmol/L in adults in countries such as Germany, Austria and France, whilst at our clinic the target for adolescents and young adults is 750 μmol/L. The stringent restrictions at an early age are due to the particular sensitivity of the developing brain in young children to the neurotoxic effects of the elevated Phe levels (52). Diet termination at eight years of age resulted in a decrease of intellectual function in children (53), although the effects may be subtle (54). Similar findings have been shown in adult patients, as well as higher risks of eczema, phobias, depression and neurological problems (55). Therefore, maintaining treatment for the duration of their lifespan may be beneficial for patients with PKU. At the same time, it is important to remember that early intervention was only made possible by the introduction of newborn screening for PKU in the 1960s (3), and that the long term effects of treatment in PKU patients are still to be fully determined (56).

Concerns over dietary treatment of PKU

There are three main issues relating to the current treatment regimen for PKU: (I) the level of adherence to Phe-restricted diets, especially in older children and adults; (II) the nutritional adequacy of the Phe-restricted diet; and (III) the effects of the diet and management on physical and psychosocial patient wellbeing, including their quality of life.

The level of diet adherence in patients with PKU is similar to that reported by the World Health Organization for patients with chronic disease complying with treatment recommendations (57). A study of children in Italy under the age of 18 with PKU found 56% of them to be adherent to the dietary prescriptions, based on food diaries (58). Across ten PKU management centres in Europe, blood Phe concentrations revealed a marked decrease in diet compliance with increasing age, with 88% of children under one year old meeting their target Phe range, compared to only 65% in adults (59). The main challenges to diet adherence are inconvenience of meal preparations, limitations on food choices, palatability of the diet, cost and availability of treatment. Additional barriers include poor social functioning, social and family support and relationships, illiteracy or language difficulties in the patient or carers, social stigma, knowledge and ability of the carer (59). Cotugno *et al.* also found that the education level of the mother to be a major determinant of diet adherence in their study (58). An overly restrictive PKU diet can lead to growth impairment in early childhood (60), likely due to malnutrition. These diets are also low in long-chain polyunsaturated fatty acids (LCPUFA) and docosahexaenoic acid (DHA), both of which are important in neurological development (61,62). Supplementation of pre-formed LCPUFA have been shown to improve neurological function in children with PKU (63,64). Micronutrient deficiency is far more common and poses a greater danger (60). In patients not taking dietary supplements, deficiencies of vitamins A, C and E, coenzyme Q10, iron, zinc, calcium, manganese and selenium have been reported (52,65). Vitamin B_{12} deficiency, which can lead to neurological impairment, is also very common in older patients, due to its main source being meat and seafood (66-68). The problem is further compounded by the level of serum vitamin B_{12} not correlating to the level of functional vitamin B_{12}, masking its deficiency in PKU patients (69). Children on amino acid-restrictive diets also have decreased bone mineral density, placing them at high risk of fractures and osteoporosis, despite receiving adequate levels of calcium, phosphorus and magnesium, and independent on the length of time on the diet (70,71). In addition, recent report has also suggested that patients on Phe-free formulae are at higher risk of developing chronic kidney disease (72).

Additional issues in the dietary treatment that require further addressing are related to the neurological or psychosocial outcomes and quality of life in patients with PKU (73). In spite of early intervention, children with PKU have lower intellectual functioning than their siblings and the general population (74,75), and poorer measures in other neurocognitive outcomes (76). Social and emotional difficulties have also been documented (77,78). Studies have also been carried out to determine how quality of life (QoL) in PKU patients is affected with a general perceived decreased in many aspects of QoL in both children and adults with PKU (79-83).

The discovery that a Phe-restricted diet was beneficial to PKU patients remains a prime example of nurture triumphing over nature (84). However, mounting evidence relating to diet adherence, nutritional deficiency and suboptimal neurological outcomes (and the interplay between the three factors) supports the need to find alternate treatment strategies for PKU.

Tetrahydrobiopterin (Sapropterin) treatment for PKU

Tetrahydrobiopterin (BH_4) is the natural co-factor of PAH, and is a requisite for its catalytic activity. In 1999, Kure *et al.* first demonstrated that pharmacological doses of BH_4 may

Table 2 Proportion of patients with MHP, mild or classical PKU who are BH_4-responsive after a BH_4-loading test

Reference	Phenotype[a]		
	MHP [%]	Mild/moderate [%]	Classical [%]
Muntau *et al*., [2002] (86)	10 of 10 [100]	17 of 21 [81]	0 of 7 [0]
Desviat *et al*., [2004] (98)	6 of 6 [100]	Mild 6 of 12 [50]; moderate 1 of 5 [20]	1 of 8 [13]
Pérez-Dueñas *et al*., [2004] (99)	10 of 10 [100]	9 of 12 [75]	9 of 42 [21]
Hennermann *et al*., [2005] (100)	11 of 12 [92]	4 of 5 [80]	3 of 23 [13]
Fiori *et al*., [2005] (101)	87 of 90 [97]	3 of 7 [43]	1 of 10 [10]
Matalon *et al*., [2005] (102)	2 of 3 [67]	8 of 11 [73]	12 of 24 [50]
Mitchell *et al*., [2005] (97)	5 of 6 [83]	Mild 8 of 9 [89]; moderate 3 of 7 [43]	1 of 15 [7]
Leuzzi *et al*., [2006] (103)	5 of 5 [100]	8 of 15 [53]	4 of 30 [13]
Bóveda *et al*., [2007] (104)	4 of 4 [100]	4 of 7 [57]	2 of 25 [8]
Burton *et al*., [2007][b] (95)	31 of 57 [54]	Mild 38 of 157 [24]; moderate 14 of 135 [10]	13 of 136 [10]
Fiege and Blau, [2007][c] (96)	54 of 64 [84]	Mild 46 of 64 [72]; moderate 24 of 55 [44]	11 of 110 [10]
Burlina and Blau, [2009] (105)	11 of 11[d] [100]	12 of 19[d] [63]	–
Anjema *et al*., [2011] (94)	34 of 37 [92]	33 of 47 [70]	18 of 84 [21]

[a], unless otherwise specified, phenotypes classed by the following baseline Phe levels: MHP, 300-600 μM; mild, 600-900 μM; moderate, 900-1,200 μM; classical, >1,200 μM. [b], Responsiveness was measured at day 8 with daily BH_4 dose (10 mg/kg), compared to 24 h and 20 mg/kg BH_4 in other studies; [c], Data taken for responsiveness (>30% reduction) at 24 h; MHP, <500 μM; mild, 500-900 μM; moderate, 900-1,300 μM; classical, >1,300 μM; [d], MHP, <450 μM, mild/moderate, 450-900 μM. PKU, phenylketonuria; MHP, mild hyperphenylalaninaemia.

lead to decreases in plasma Phe concentrations in patients with HPA caused by mutations in *PAH* (85). BH_4 supplementation has also been shown to lead to a decrease in blood Phe level, improved Phe tolerance, i.e., the amount of Phe allowed in diet, resulting in relaxation of the Phe-restricted diets in many patients (86-88). Measures of Phe:Tyr and stability of Phe levels, which are hypothesised to be determinants of long-term neurological outcomes, have also been found to improve upon BH_4 treatment (89,90). Other benefits reported include improvement of depression and panic attacks in an adult female patient (91).

BH_4 supplementation is beneficial only in a proportion of PKU patients (so-called BH_4-responsive). BH_4-responsiveness can be assessed by a BH_4 loading test, in which patients are given a single oral dose of 20 mg/kg. A reduction of Phe levels after 24 h of greater than 30% is defined as responsive (92). However, other variations in loading levels, time points at which measurements are taken, and Phe starting levels, have also been reported (93-95), and these may yield different response classifications, as some patients may show an initial Phe decrease (at 8 h) but return to baseline Phe levels at 24 h (96,97). On the other hand, some patients may return a positive response only at 48 h (94). Studies involving larger cohorts of patients revealed that BH_4 supplementation is more effective in decreasing Phe levels in patients with milder forms of PKU, compared to those with the severe classical form (*Table 2*) (86,93). Overall, the percentage of PKU patients for whom BH_4 doses may be of benefit is estimated to be 30% to 50% (106).

Certain *PAH* mutations are frequently identified in BH_4-responsive patients, suggesting that genotype may be an important factor in BH_4-responsiveness (85). An allele is usually only considered to be BH_4-responsive if it is identified in a homozygous patient or a patient compound heterozygous with a second null allele (107). However, there is also evidence that genotype is not the sole determinant of BH_4-response. Whilst some mutations (e.g., p.Leu48Ser, p.Ile65Thr, p.Arg261Gln) can be found in BH_4-responsive patients at a high frequency, some of these mutations may also be found in patients who are non-

BH_4-responsive (101,108,109). These discrepancies may be incidental, relating to the methods of ascertaining and interpreting BH4-responsiveness, but there are also possible differences specific to each individual patient, such as BH_4-absorption, protein catabolic rate, and Phe intake during the test (94,96,97). The improvement of enzymatic activity in certain mutant PAH proteins expressed *in vitro* in BH_4 supplemented media strongly supports the notion that genotype may play a role in determining BH_4-response (110). However, the BH_4-loading test is still the best determinant for BH_4-responsiveness in PKU patients.

Although the treatment with BH_4 offers a safe and effective alternative for many patients with PKU, its appropriateness is limited to primarily patients with a milder biochemical phenotype. Most classical PKU patients, in whom the disease is more difficult to manage and the consequences of dietary non-compliance are greater, are unlikely to benefit from this treatment, and further research into other therapies is required to provide alternative solutions for this group of patients.

Alternate treatments for PKU

New dietary approaches

Research on new dietary supplements for the treatment of PKU is ongoing, including more palatable medical formulae, medical foods using glycomacropeptide (GMP), a naturally low-Phe protein, and supplementation of large neutral amino acids (LNAA). Supplementation of LNAA has been shown to stabilise the concentration of cerebral Phe, despite an increase observed in the plasma level, suggesting that the influx of LNAA may block the transport of Phe across the blood brain barrier (111). Other clinical trials have also found decreases in blood Phe concentrations with the added LNAA intake, although brain Phe levels were not measured in some of these studies, and it has been suggested that these LNAAs may also exert their effect by competing with Phe for active transport across the intestinal mucosa (112-115). This treatment is currently only recommended for adult patients not complying with a low-Phe diet, as those on diets would generally be supplemented with a medical formulae encompassing these amino acids (116).

GMP is a by-product of cheese production, and is a 64 amino peptide with no Phe residues, which makes food made from GMP a good alternative source of protein for patients with PKU (117). The use of these products in the murine model of PKU has shown promising results regarding growth rates and bone mineral density (118,119), and a number of these products are now commercially available (http://www.cambrookefoods.com).

Gene therapy

Research into novel treatments for PKU involves different methods for overcoming the deficiency of the host PAH by gene therapy or enzyme substitution/replacement.

Recombinant adeno-associated virus (AAV) vectors have been used to deliver *PAH* gene to the liver in a murine PKU model, allowing correction of HPA of up to one year (120-122). The loss of PAH activity over time is due to the continual regeneration of hepatocytes and the loss of the AAV vector. Antibody-mediated immune responses also reduced the efficacy of any reinjections of the same vector. There was also an apparent gender-bias in the efficacy of transduction, with female mice showing a poorer improvement in blood Phe levels (123). Using an AAV8-pseudotype vector with a self-complementary AAV genome, Yagi *et al.* (124) were able to achieve high levels of liver transduction and expression of PAH with complete phenotypic correction and normal blood Phe for over one year, with equivalent levels of improvement in male and female mice.

Skeletal muscle has been considered a more promising target for gene therapy, as it is easily accessible compared to the liver and the cells are longer-lived (125). On the other hand, the enzymes required for the biosynthesis of BH_4, the co-factor of PAH, are not expressed in this tissue. Co-expression of PAH with two of these enzymes, delivered via AAV2 pseudotype 1, led to long-term and stable reduction of blood Phe in the Pah^{enu2} mouse model (126). Development in viral vectors is likely to continue to drive improvement in both liver- and muscle-directed gene therapies.

Enzyme replacement and substitution therapies

Enzyme replacement via introduction of wild-type, functional PAH protein has been hampered by the instability of the protein produced *in vitro* (127), rendering large-scale production and purification of the protein costly and inefficient. Although storage of the protein in high glycerol concentrations or mannitol provides some protection against protein denaturation and aggregation, the loss in enzymatic activity after a period of storage is significant (128). Therapeutic liver repopulation, whereby wild-type hepatocytes would be transplanted onto the livers

of patients with PKU, has also been suggested as a potential therapy (129). This concept has been proven in the PKU mouse model, in which the introduction of hepatocytes from wild-type and heterozygous (but phenotypically normal) mice to the livers of homozygous affected mice resulted in normal blood Phe levels if there is a high enough level (greater than 10%) of repopulation of wild-type (or heterozygous) cells (130).

An alternative to enzyme replacement is substitution with phenylalanine ammonia lyase (PAL, EC 4.3.1.5). PAL, an enzyme normally found in plants and fungi, catalyses the deamination of phenylalanine to ammonia and *trans*-cinnamic acid, the latter of which is then quickly converted into hippurate and excreted in urine (131). The use of recombinant PAL (with polyethylene glycol polymers covalently linked to lysine residues, so-called PEGylation) avoids the immune-mediated degradation of the enzyme (132). Weekly subcutaneous injections of PEGylated-PAL enzyme in a murine PKU model can sustain correction of blood Phe for up to one year (133). A phase II clinical trial using this formulation has since commenced. Given the constant requirement of therapy, a less invasive method of delivery would ensure greater compliance to treatment in patients, especially as they enter adulthood. Although initial work on orally-administered PAL enzyme in mice was unsuccessful due to proteolytic degradation in the gut (134,135), there has also been renewed interest in this form of enzyme administration (136). Again, PEGylation of the PAL enzyme is useful in protecting the enzyme from protease degradation in the gut, and oral-administration to mice with HPA significantly reduced (but not completely corrected) blood Phe levels (137).

Chaperone therapy

Another group of novel treatments examine how native PAH in patients can be restored to sufficient catalytic activity such that normal range blood Phe levels can be achieved. Indeed, BH_4 (sapropterin) treatment is likely to act via this method, although the exact mechanism is uncertain. During *in vitro* expression of mutant PAH protein, the presence of BH_4 leads to increased protein levels and thus catalytic activity, supporting a chaperone-like role of the BH_4, increasing the half-life of the mutant PAH protein and protecting it from targeted degradation in the ubiquitin-dependent proteolytic pathway (138-141). Similar screening of chemical libraries found other candidate chaperones for the p.Val106Ala variant, which is the mutation carried in one of the PKU mouse models, *Pah*^{enu1}. These compounds not only normalised blood Phe concentrations *in vivo*, but also showed higher efficacy than BH_4 (142). It remains to be seen how these compounds might perform with a wider range of mutations and whether a larger proportion of PKU patients would benefit from treatment with these compounds.

As indicated above, most mutations in the *PAH* gene cause single amino acid (missense) substitutions. A subset of these mutants are known to cause aggregation of the PAH protein, hypothesised to be a result of misfolding of the mutant polypeptide (49). In other genetic diseases, it has been shown that such misfolding and aggregation would lead to a greater rate of targeted degradation (143,144). By expressing the mutant PAH in an *in vitro* expression system in the presence of a chemical chaperone, both PAH protein levels and residual activity can be restored, suggesting that such molecular chaperones are good candidates as therapeutic agents for PKU (145). Due to its readiness to form high molecular weight aggregates, the missense mutation p.Gly46Ser (p.G46S) has been used for testing the ability of a range of chaperones to restore proper folding and enzymatic activity (50). Several compounds have shown to inhibit formation of p.Gly46Ser aggregates, including glycerol and Compound III (146), identified as a potential chaperone by Pey *et al.* (145) for other missense *PAH* mutations. Other anti-aggregation agents (such as trehalose and sodium 4-phenylbutyrate) may also be good candidate drugs to test *in vitro* for similar missense mutations causing protein aggregation (147-149).

Nonsense read-through therapy

Another novel type of therapy of genetic disorders is the use of the nonsense read-through agents, such as the aminoglycoside antibiotic gentamicin, for treating individuals with nonsense mutations, e.g., in cystic fibrosis, Duchenne muscular dystrophy (150,151). The development of other novel chemical compounds to overcome toxicity problems associated with traditional aminoglycosides has made nonsense read-through compounds a potentially viable long-term treatment option (152-154). *In vitro* testing of two aminoglycosides against four *PAH* nonsense mutations have demonstrated their ability to restore PAH enzyme activity (155). In some populations, the proportion of patients with a nonsense mutation can be as high as 22% (19). This mode of therapy therefore merits further study, in particular in a clinical setting, as to allow evaluation of the extent of the

restoration of enzyme activity on regulation of Phe levels and of the short- and long-term effects on the patients.

Conclusions

Different modes of therapy for PKU are now under development. These therapies illustrates the range of solutions that can be deployed to address the root problem of PKU, the loss of catalytic activity of the PAH enzyme. Although many of these have been high efficacious in the murine model of PKU, they all require greater research efforts and clinical testing to ensure the safety and longevity of treatment, with the goal of eliminating the rigorous dietary restrictions in many, if not all, patients with PKU. These treatment solutions may also be highly applicable to other genetic disorders of metabolism, and research into PKU therapies will have far-reaching consequences in the field of genetic medicine in the next few decades.

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Footnote

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