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 BH₄, 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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impaired. 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 (BH4), the co-factor of PAH (5). The 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 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 domain (amino acid residues 1-142), followed by the catalytic domain (amino acid residues 143-410) and the C-terminal 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 BH4 [(6R)-l-erythro-5, 6,7,8-tetrahydrobiopterin] and O2 (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 PAHdb (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

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

PKU, phenylketonuria.
of pathogenic mutations in PAH are missense mutations (65.4% of mutations reported in PAHdb), 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 BH4, 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 chaperons 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). Cottugno 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 B12 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 B12 not correlating to the level of functional vitamin B12, 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 (BH4) 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 BH4 may
lead to decreases in plasma Phe concentrations in patients with HPA caused by mutations in PAH (85). BH₄ 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 BH₄ treatment (89,90). Other benefits reported include improvement of depression and panic attacks in an adult female patient (91).

BH₄ supplementation is beneficial only in a proportion of PKU patients (so-called BH₄-responsive). BH₄-responsiveness can be assessed by a BH₄ 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 BH₄ 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 BH₄ doses may be of benefit is estimated to be 30% to 50% (106).

Certain PAH mutations are frequently identified in BH₄-responsive patients, suggesting that genotype may be an important factor in BH₄-responsiveness (85). An allele is usually only considered to be BH₄-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 BH₄-response. Whilst some mutations (e.g., p.Leu48Ser, p.Ile65Thr, p.Arg261Gln) can be found in BH₄-responsive patients at a high frequency, some of these mutations may also be found in patients who are non-

Table 2 Proportion of patients with MHP, mild or classical PKU who are BH₄-responsive after a BH₄-loading test

<table>
<thead>
<tr>
<th>Reference</th>
<th>MHP [%]</th>
<th>Mild/moderate [%]</th>
<th>Classical [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muntau et al., [2002] (86)</td>
<td>10 of 10 [100]</td>
<td>17 of 21 [81]</td>
<td>0 of 7 [0]</td>
</tr>
<tr>
<td>Fiori et al., [2005] (101)</td>
<td>87 of 90 [97]</td>
<td>3 of 7 [43]</td>
<td>1 of 10 [10]</td>
</tr>
<tr>
<td>Matalon et al., [2005] (102)</td>
<td>2 of 3 [67]</td>
<td>8 of 11 [73]</td>
<td>12 of 24 [50]</td>
</tr>
<tr>
<td>Mitchell et al., [2005] (97)</td>
<td>5 of 6 [83]</td>
<td>Mild 8 of 9 [89]; moderate 3 of 7 [43]</td>
<td></td>
</tr>
<tr>
<td>Leuzzi et al., [2006] (103)</td>
<td>5 of 5 [100]</td>
<td>8 of 15 [53]</td>
<td>4 of 30 [13]</td>
</tr>
<tr>
<td>Bóveda et al., [2007] (104)</td>
<td>4 of 4 [100]</td>
<td>4 of 7 [57]</td>
<td>2 of 25 [8]</td>
</tr>
<tr>
<td>Fiege and Blau, [2007] (96)</td>
<td>54 of 64 [84]</td>
<td>Mild 46 of 64 [72]; moderate 24 of 55 [44]</td>
<td></td>
</tr>
<tr>
<td>Anjema et al., [2011] (94)</td>
<td>34 of 37 [92]</td>
<td>33 of 47 [70]</td>
<td>18 of 84 [21]</td>
</tr>
</tbody>
</table>

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 BH₄ dose (10 mg/kg), compared to 24 h and 20 mg/kg BH₄ 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.
BH4-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 BH4-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 BH4 supplemented media strongly supports the notion that genotype may play a role in determining BH4-response (110). However, the BH4-loading test is still the best determinant for BH4-responsiveness in PKU patients.

Although the treatment with BH4 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 LNAA’s 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 BH4, 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 Pahenu2 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, BH₄ (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 BH₄ leads to increased protein levels and thus catalytic activity, supporting a chaperone-like role of the BH₄, 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*<sup>enu1</sup>. These compounds not only normalised blood Phe concentrations *in vivo*, but also showed higher efficacy than BH₄ (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 illustrate 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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None.

**Footnote**

*Conflicts of Interest:* The authors have no conflicts of interest to declare.

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