Università degli Studi di Palermo

AREA RICERCA E TRASFERIMENTO TECNOLOGICO
SETTORE DOTTORATI E CONTRATTI PER LA RICERCA
U. O. DOTTORATI DI RICERCA

D070- Dottorato di Ricerca "Medicina Molecolare e Clinica"
Dipartimento di Promozione della Salute, Materno-Infantile, di Medicina Interna e Specialistica di Eccellenza "G. D'Alessandro" (PROMISE)
06/MEDS-05- MEDICINA INTERNA

IMPLEMENTATION OF A CLINICAL, NUTRITIONAL AND GENETIC-MOLECULAR PATHWAY FOR THE TREATMENT OF PATIENTS WITH PHENYLKETONURIA

IL DOTTORE
DOTT.SSA MARINA LANZA

IL COORDINATORE
PROF. ANTONINO TUTTOLOMONDO

IL TUTOR
PROF. ANGELO BALDASSARE CEFALU'

CICLO XXXVIII
ANNO CONSEGUIMENTO TITOLO 2025/2026

SUMMARY

1. INTRODUCTION

Phenylketonuria (PKU) is an autosomal recessive disorder related to phenylalanine metabolism caused by defect of phenylalanine hydroxylase (PAH); the enzyme responsible for hydroxylation of phenylalanine (Phe) to tyrosine (Tyr) (1). This enzyme is expressed predominantly in the liver but also kidney and pancreas and requires the co-substrate tetrahydrobiopterin (BH4), molecular oxygen and iron. BH4 can also act as a chaperone to facilitate the correct folding of the PAH monomer, together with DNAJC12. For these reasons, in a small number of cases the hyperphenylalaninemia (HPA) is caused by defect in BH4 metabolism or the presence of pathogenetic variants in DNAJC12 (2,3). The incidence of BH4 deficiency is very rare, it is 1-2% of all HPA cases or 1:500.000 newborns (4). Defects in the PAH gene cause accumulations of Phe in the blood and brain, and if left untreated, the condition is characterized by intellectual disability, microcephaly, motor deficits, eczematous rash, autism, seizures, development problems, aberrant behaviour and psychiatric symptoms (5).

The frequency is of 1:10.000 lives births in Europe, with including Italy, but the prevalence changes significantly by based on geographic regions of origin and ethnic groups. From data dating back to 2018, from bibliographic data collected in 64 countries, the number of subjects with PKU was 360,466. Prevalence is higher in European and Middle Eastern populations. Italy and Ireland, of 1:4,000 and 1:4,545 respectively, had a higher prevalence than some Central European countries, such as Germany (1:5,360), the Czech Republic (1:5,521), Austria (1:5,764) and Slovakia (1:5,753). Pathology is also less frequent in some Western European countries, for example France (1:9,091), the United Kingdom (1:10,000), Belgium (1:11,000) or the Netherlands (1:11,546), and in Southern Europe, for example in Spain (1:10,115) or Portugal (1:12,500). While Northern Europe had the lowest rates of PKU in Europe, for example Norway (1:11,457), Sweden (1:12,681), Denmark (1:13,434) or only 1:112,000 in Finland. To date, of 3400 PAH gene variants have been identified in people with PKU, thus causing different effects on the gene and observable clinical phenotypes (4,6).

The classification of the pathology is based both on tolerance to Phe before the age of 5 and on blood Phe values in the blood. In the first case, the classification proposed by Guldberg provides:

- Classic PKU with a tolerance <250-350 mg of Phe per day.
- Moderate PKU with a tolerance of 350–400 mg of Phe per day.
- Mild PKU with a tolerance of 400–600 mg of Phe per day.
- Mild HPA with plasma Phe values below 600 μmol/L and a normal diet (7).

As regards plasma Phe concentrations, the classification includes mild-moderate PKU with values between 360 and 1200 μmol/L and >1200 μmol/L defined as classic PKU (8). According to recent European guidelines, the previous classification can no longer be applied because, thanks to prenatal screening, patients with PKU begin treatment before reaching maximum Phe concentrations in the bloodstream. Therefore, patients can be classified as: patients who do not require treatment (Phe<360 μmol/L), patients who require cofactor-responsive treatment, and patients who require non-cofactor-responsive treatment (9).

According to a study conducted in 2020, analyzing data available worldwide in 2018, substitutions are the most frequent variants (80.5%), followed by deletions (12.9%) and duplications (2.1%). On exon 6 are most of the variants identified (14.1%), followed by exon 7 (12.2%) (6).

Følling first discovered and described PKU, observing the case of twins of Norwegian origin, later, in 1953 Bickel formulated the first treatment involving dietary control, and 10 years later, thanks to Guthrie and Susi, newborn screening for the condition was introduced, using dried blood spot (DBS) to measure Phe concentration in blood (3). The test has been made mandatory in many countries, such as Italy. If the result is positive, the test will be repeated after ten days. Some newborns may have immature enzyme systems linked to amino acid metabolism, resulting in temporary increases in amino acids (10). The main treatment is to follow a diet avoiding foods with moderate or high phenylalanine intake. The diet must be followed for life; therefore, some patients have difficulty maintaining this stringent dietary regimen. For this reason, pharmacological treatments have been developed that would facilitate adherence to dietary treatment (11).

1.1 HISTORY OF THE PATHOLOGY

The first clinical case described dates to 1934, by a Norwegian doctor named Asbjørn Følling, who examined two brothers with mental retardation. He performed a urine test on the two subjects, where he detected the presence of ketone bodies, specifically phenylpyruvic acid. His intuition was that the substance derived from dietary phenylalanine (12).

In 1953, Bickel treated the case of a 2-year-old girl unable to stand, walk or talk. Aware at the time that a typical feature of the condition was the accumulation of Phe in the blood and cerebrospinal fluid, he decided to give the patient a specifically prepared diet low in Phe. The child was treated in the hospital, to ensure careful observation, for about four weeks, at the end of which no significant changes in her condition had been recorded, except for loss of weight and characteristic musty odor and normalization of Phe levels in plasma and urine. Phenyl pyruvic acid excretion had ceased, and the ferric chloride reaction was negative. Subsequently, a return of biochemical abnormalities was recorded, and the diet was then reformulated by adding small amounts of Phe about 0.3-0.5g in whole milk. With these changes, weight gain and improvement in the patient's biochemical condition could be observed. Gradually, there were also improvements in the health status of the child who was able to crawl and sit up. This demonstrated how the controlled introduction of Phe into the diet of subjects with the condition resulted in improvements in the patient's health status (13). Thus, it became necessary to develop tests that would allow early identification of individuals with the condition to benefit from dietary treatment.

In 1959, Joseph Wortis mailed several families a paper test that allowed them to detect the presence of phenyl pyruvic acid in their urine. The test consisted of dipping this paper into a sample of fresh urine, and the presence of phenyl pyruvic acid would produce a change in the colour of the paper from its original yellowish white colour to blue. This was not a specific test for phenylketonuria because the change in the colour of the paper from the original to other colours other than blue was an indicator of the presence of other substances (14). The first test for identifying hyperphenylalaninemia was developed by Robert Guthrie in the 1960s. It is a test that uses the bacterium Bacillus subtilis, which grows only in the presence of phenylalanine, exploiting the inhibition process. A plate contains the

bacterial inhibitor beta-2-thienylalanine, which physiologically prevents the growth of the bacterium. The test uses filter paper discs onto which a drop of blood is placed and allowed to dry. The disc with the dried blood is then placed inside the plate with the inhibitor. If high amounts of phenylalanine are present, the latter counteracts the action of the inhibitor, promoting bacterial growth, which suggests that the subject may be a carrier of the disease (15).

1.2 PHENYLALANINE HYDROXYLASE

The gene encoding PAH is located on chromosome 12 (region q22–24.1) consisting of 13 exons and 12 introns, covering a total of 100 kb of genetic data (5). Under physiological conditions, the PAH gene produces the PAH protein consisting of 452 amino acids (7). Phenylalanine hydroxylase is an enzyme belonging to the family of aromatic amino acid hydroxylases, which also includes tyrosine hydroxylase (TH) and two tryptophan hydroxylases (TH1 and TH2). These enzymes are found mainly in the liver of mammals, and to a lesser extent in the kidneys. In the specific case of phenylalanine hydroxylase, their function is to prevent the neurotoxic effects caused by the accumulation of phenylalanine and, at the same time, to prevent it from being completely catabolised, as it is an essential proteinogenic amino acid. For this reason, this family of amino acids has evolved structures that allow it to perform this dual function. The reaction consists of the conversion of L-phenylalanine (L-Phe) into L-tyrosine (L-Tyr) through a process of para-hydroxylation of the aromatic side chain. In mammals, this process is highly dependent on the presence of BH4, oxygen and iron (16). In mammals, phenylalanine hydroxylase is composed of 52 kDa subunits that assemble into a tetrameric form. Each subunit consists of an N-terminal regulatory domain, a catalytic domain, and a C-terminal oligomerisation domain responsible for dimerization and tetramerization (17). The regulatory domain possesses ACT domains. These are regulatory domains first observed in proteins involved in the amino acid synthesis process and, in most cases, they are also allosteric enzymes with complex regulation due to ligand binding (18). The regulatory domain allows the enzyme's activity to be regulated; in fact, it is activated in the presence of the substrate, L-Phe, and inhibited by tetrahydrobiopterin (BH4). Experiments were conducted on the rat enzyme, which has a sequence homology with the human

enzyme of 92%. In one experiment, it was observed that, in the absence of pretreatment with phenylalanine, enzyme activity was reduced by approximately 5% compared to the enzyme previously incubated with the substrate (19). This cooperative enzyme-substrate mechanism is believed to have important physiological significance in the homeostasis of L-Phe in the blood; in fact, the presence of the substrate induces conformational changes in the enzyme (20). The catalytic domain contains the binding sites for iron, the cofactor and the substrate. Iron binds to two histidines, His285 and His290, and to a glutamate, Glu330. The oligomerisation domain has an antiparallel sheet, responsible for dimerization, and a helix that allows tetramerization (16). The enzyme has two states: an inactive state in which the N-terminal regulatory domain covers the active site, which also results in a lower affinity for the substrate, and an active state through the binding of L-Phe (21).

1.3 CATALYTIC MECHANISM

As described above, phenylalanine hydroxylase is an enzyme that belongs to the non-haem iron monooxygenase family. These enzymes introduce an oxygen atom into a substrate in the presence of molecular oxygen and a reducing agent. The iron is defined as non-haem because, unlike that contained in haemoglobin, it is not bound to a haem group but to two histidines and a glutamate present in the catalytic domain of the enzyme (22). Experiments were conducted on a truncated form of the enzyme, PheHΔ117, deprived of the regulatory domain, to study in detail the kinetics of phenylalanine hydroxylase, using interrupted flow absorbance spectroscopy. The study showed that the first bond to form is between the enzyme and the BH4 substrate, followed by a slower bond with phenylalanine. Once this ternary complex has formed, oxygen binds rapidly. The bond with oxygen causes variations in absorbance (first an increase at 248 nm and then a reduction at 340 nm), indicating that an intermediate is formed first, followed by the formation of 4a-hydroxypterin and Fe(IV)O (23). The above reaction leads to the formation of a transient Fe (II)-O-O-BH4 complex, followed by the cleavage of the O-O bond, thus generating the Fe(IV)=O intermediate, known as oxyferryl, together with the formation of the BH4 intermediate, i.e. 4a-hydroxypterin, which will subsequently lose a water molecule to form quinonoid-BH2 and hydrogen peroxide (24). Fe(IV)O is the oxidising agent involved in the hydroxylation of Phe, through the

transfer of an oxygen atom to the aromatic ring of the amino acid (25). 4a-hydroxypterin must be reduced to reform the BH4 cofactor. This process involves two enzymes: pterin carbinolamine dehydratase (PCD) and dihydropterin reductase (DHPR) (26). 4a-hydroxypterin is converted to qBH2 by the removal of a water molecule in a process catalysed by PCD. Subsequently, qBH2 is regenerated to BH4 in a reaction involving NAD(P)H as an electron donor (27).

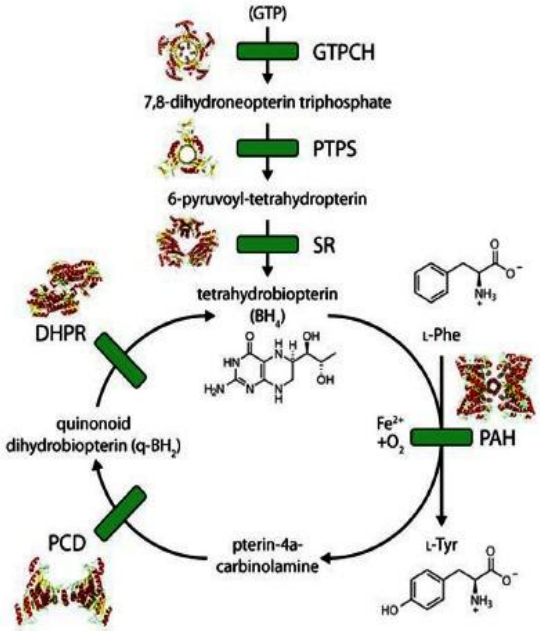


Figure 1: The image schematically shows the process of BH4 synthesis by the enzymes GTPCH, PTPS and SR. Once produced, BH4 enters a cycle that determines the para-hydroxylation of L-Phe to L-Tyr and the subsequent regeneration of the cofactor through the involvement of the enzymes PCD and DHPR. (*J. Underhaug et al. Phenylalanine Hydroxylase Misfolding and Pharmacological Chaperones†. Curr Top Med Chem. 2012 Nov;12(22):2534–2545).*

1.4 PATHOPHYSIOLOGICAL MECHANISMS

Brain toxicity in hyperphenylalaninaemia is multifactorial. Several hypotheses have been put forward to explain the mechanism, including competitive inhibition of a neutral amino acid transporter (LNAA) present in the blood-brain barrier, a reduction in cholesterol synthesis with a consequent reduction in myelin production, inhibition of tyrosine hydroxylase and tryptophan hydroxylase, and oxidative stress (28). In the first case, phenylalanine, together with other amino

acids (valine, isoleucine, leucine, methionine, threonine, tryptophan, tyrosine and histidine) called LNAAs, bind to the large neutral amino acid transporter type 1 (LAT1) to cross the blood-brain barrier. This is a competitive transport process, whereby high concentrations of Phe in the blood inhibit the transport of other amino acids. This results in a poor supply of LNAAs across the barrier and can cause brain deficiencies, compromising the synthesis of neurotransmitters or brain proteins. As a result, individuals may develop cognitive abnormalities (29). It has also been observed that some patients experienced degeneration or reduction of myelin and poor neuronal maturation (30). One of the direct consequences of phenylalanine hydroxylase deficiency, in addition to increase Phe levels, is a reduction in tyrosine concentration. Tyrosine is a neutral amino acid involved in the production of:

- Dopamine, norepinephrine and epinephrine, important brain neurotransmitters.
- Melanin, the pigment responsible for skin and hair colour.

Alterations in this enzyme cause brain dysfunction, and in some individuals with this condition, reduced skin pigmentation has also been observed (31). In vitro studies have shown that the condition may be related to increased oxidative stress. In fact, high concentrations of Phe can be associated with damage to DNA, proteins and lipids. The causes may be related to metabolites produced from Phe (phenylpyruvate, phenyl lactate and phenyl acetate) that interfere with the action of antioxidant enzymes (catalase, superoxide dismutase and glutathione peroxidase), thus leading to an increase in the production of reactive oxygen species (32).

1.5 BH4 DEFICIENCY

The BH4 cofactor is also responsible for the development of the disease, although there are fewer cases of individuals affected by HPA with pathogenic variants in the enzymes involved in the synthesis and regeneration of BH4. Synthesis and regeneration are processes that occur in several steps and are catalysed by five enzymes: guanosine triphosphate cyclohydrolase I (GTPCH), 6-pyruvoyltetrahydropterin synthase (6-PTPS), sepiapterin reductase (SR), pterin 4-alpha-carbinolamine dehydratase (PCD) and q-dihydropterin reductase (DHPR). The first three enzymes are responsible for the cofactor synthesis process and the

other two are involved in regeneration (28). The enzyme GTP cyclohydrolase I inhibit synthesis in the event of excess tetrahydrobiopterin and stimulates synthesis in the event of an increase in phenylalanine concentration. 6-PTPS removes a triphosphate from 7,8-dihydroneopterin triphosphate and performs a redox reaction that generates the precursor of BH4, 6-pyruvoyltetrahydrobiopterin, which is then converted into its final form by the enzyme SR. Sepiapterin is not the only enzyme capable of catalysing this reaction. In fact, in the event of SR deficiency, enough BH4 can be synthesised in the liver thanks to aldose reductase (AR), carbonyl reductase (CR) and dihydrofolate reductase, but not in the brain. This explains why patients with SR deficiency only present neurological dysfunction and not hyperphenylalaninaemia. Individuals with mutations in the genes involved in the synthesis and regeneration of tetrahydrobiopterin have been identified in patients with phenylketonuria who did not respond to dietary treatment. In these patients, mutations affect the 6-PTS gene in 60% of cases and the DHPR gene in 30% of cases, while in a smaller number of cases they affect GPTCH and PCD in 5% of cases (33).

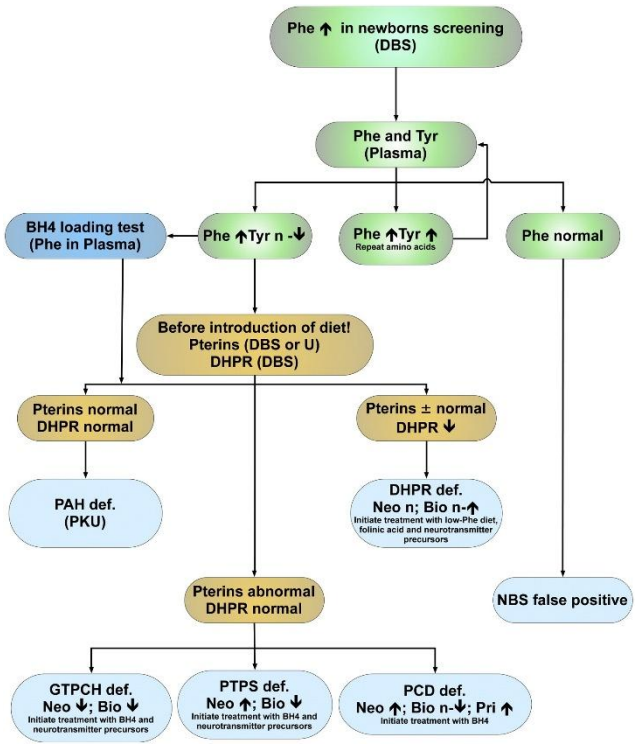


Figure 2: The image shows the diagnostic flowchart to be followed for the diagnosis of PKU and BH4 deficiencies (*N. Blau et al. Diagnosis, classification, and genetics of phenylketonuria and tetrahydrobiopterin (BH4) deficiencies.*

Molecular Genetics and Metabolism. Volume 104, Supplement, 2011, Pages S2-S9).

As described above, DBS (dried blood spots) are used for diagnosis, as they are useful for testing the enzymatic activity of subjects. In some cases, the test may generate false positives. In addition to DBS, further tests may be carried out, such as urine tests, which measure the amount of excreted pterins, which is directly proportional to the amount of Phe present in the blood. However, some patients, such as those with DHPR deficiency, show normal levels of neopterin and biopterin in their blood or urine. Therefore, measuring DHPR activity is useful in diagnosis. Following the diagnostic scheme shown in the image, based on the levels of neopterin (Neo), biopterin (Bio) and primapterin (Pri) and DHPR activity, it is possible to establish the source of the BH4 deficiency:

- Low or undetectable neopterin and biopterin deficiency due to GTP cyclohydrolase I (GTPCH).
- High neopterin and low or undetectable biopterin deficiency due to 6-pyruvoyl-tetrahydropterin synthase (PTS).
- Normal neopterin, normal or elevated biopterin, and no DHPR activity deficiency due to dihydropterin reductase (DHPR).
- Elevated neopterin, low or normal biopterin, and elevated primapterin deficiency due to pterin-4a-carbinolamine dehydratase (PCD) (34).

The prevalence of BH4 deficiency is unknown, but it is estimated to account for approximately 1–2% of known HPA cases (28). Patients with tetrabiopterin deficiency show normal plasma phenylalanine levels after oral administration of BH4. It has also been shown that some patients respond to BH4 administration with a reduction in plasma Phe levels. For this reason, patients can be divided into three categories: non-responsive PAH deficiency, responsive PAH deficiency and BH4 deficiency. A small percentage of patients with BH4 deficiency show a more severe phenotype with low phenylalanine tolerance and are therefore subjected to dietary therapy (35). Treatment for individuals with BH4 deficiency is different. A low-Phe diet is not effective, while the administration of drugs such as saprobiopterin dihydrochloride guarantees a good outcome (34). The recommended daily dose is 20 mg/kg and should be taken with food or a formula to improve absorption. A reduction in Phe levels equal to or greater than 30% of

the concentration present in the blood is considered an optimal response. Studies conducted in Europe and Japan have shown that patients who respond to sapropterin also experience an increase in tolerance to the dietary regimen (36).

1.6 DNAJC12 AND HYPERPHENYLALANINEMIA

The PAH gene and the genes involved in the synthesis and regeneration of BH4 are not solely responsible for the development of HPA. The DNAJC12 gene, together with Heat Shock 70KDa Proteins (HSP70), is involved in the folding process of the PAH protein. From a phenotypic point of view, clinical manifestations are wide-ranging and include intellectual disability and severe neurological symptoms or mild symptoms or even subjects with a normal picture (37, 38). In 2017, the case of two brothers aged 6 and 17, both born at term from uncomplicated pregnancies, was studied. The younger brother was diagnosed with mild PHA at birth. Until the age of six, he followed a normal diet and was then given sapropterin. Sequencing of the PAH gene did not reveal any pathogenic mutations. The older brother, on the other hand, tested negative in neonatal screening but, following his younger brother's diagnosis, underwent an amino acid analysis which revealed increased Phe levels consistent with a diagnosis of mild HPA. The investigation was carried out because both brothers had cognitive dysfunction and had been diagnosed with autism spectrum disorder (ASD) during childhood. It was decided to subject the older brother to exome analysis, which revealed a homozygous c.58_59delGG p.Gly20Metfs*2 mutation in the DNAJC12 gene. The analysis was then extended to family members, showing that the parents were heterozygous for the variant and the brother was also homozygous (39). Clinically, DNAJC12 deficiency is like BH4 deficiency, therefore drug treatment involves the administration of sapropterin. Analysis of this gene could be considered in cases of individuals with mild neurological symptoms of unknown origin (9). This and other studies suggest the involvement of the DNAJC12 gene in the development of HPA, particularly in patients who test negative for mutations in the PAH gene or other genes involved in the synthesis and regeneration of BH4. DNAJC12 mutations are associated with marked clinical and symptomatic heterogeneity, which is why, to date, there is no single diagnostic parameter available that allows for the immediate identification of a deficiency in this gene.

1.7 PREGNANCY AND PHENYLKETONURIA

Untreated maternal PKU and HPA can lead to complications during pregnancy. One of the most common consequences is the development of microcephaly, coronary heart disease, intestinal disorders and low birth weight. For this reason, strategies are needed to prevent the development of maternal phenylketonuria syndrome (40). Diet remains the cornerstone of treatment. This involves a low-protein vegetarian diet supplemented with vitamins and minerals. Check-ups must be more frequent, with visits twice a week, to keep Phe blood levels under control, which must be lower than the normal acceptable levels for the condition (41). A study conducted on 47 subjects, children of 24 women with PKU, aged between 1 month and 26 years, investigated the long-term effects of the condition on offspring and the correlation between the diet followed during pregnancy and the outcome. It was found that mothers who receive adequate treatment develop a higher IQ and are also less likely to develop anxiety and depression. Another important finding of this study is that problems associated with maternal phenylketonuria syndrome often arise later in life. In fact, no abnormalities were observed in infants, while in children over the age of 5, 25% had learning difficulties, 31% had attention deficit hyperactivity disorder (ADHD) and 34% had been diagnosed with anxiety and/or depression. For this reason, assessment during childhood and adolescence is also essential (42).

1.8 THERAPY

Diet is the main therapeutic strategy for controlling blood phenylalanine levels in patients with PKU. To prevent neurological damage caused by excessive Phe accumulation, dietary treatment must begin at birth and continue throughout life. This is a low-protein diet, so foods such as meat, fish, eggs and dairy products must be severely restricted. One of the complications arises from maintaining this dietary regime throughout life. Natural foods that are low in protein and high in starch, such as potatoes and vegetables, can be consumed but always in limited quantities. Although the diet can reduce the complications associated with the condition, there are limitations. The first is due to the difficulty for patients to maintain this strict regime throughout their lives, as well as the cost of medicinal

foods, and the second is related to nutritional deficiencies (43). To date, strategies such as protein substitutes such as large neutral amino acids (LNAA) and glycomacropeptide or pharmacological treatments such as sapropterin dihydrochloride (BH4) and enzyme replacement therapy are used. Protein substitutes make up approximately 52-80% of the total protein required in the diet and essential amino acids. Studies have shown that dietary restriction without an adequate intake of protein substitutes is associated with multiple deficiencies, such as vitamins (B6 and B12), folate, magnesium and protein, which can also lead to poor growth (44). LNAAs are capsules containing a Phe-free amino acid mixture. They contain tyrosine, tryptophan, threonine, methionine, valine, leucine, isoleucine and histidine. Except for tyrosine, these are essential amino acids (45). Phe can cross the blood-brain barrier and competes for transport with other LNAAs. The latter use a family of proteins called the L system for transport, which contain LAT1, the catalytic subunit and the type II glycoprotein subunit (4F2hc). The glycoprotein subunit is selective for LNAAs and facilitates their transport across the blood-brain barrier (46). LNAA supplementation in individuals with PKU has resulted in significant improvements, such as reduced Phe concentrations in both the blood and brain, increased synthesis of brain neurotransmitters, and increased brain concentrations of non-Phe LNAAs (45). Glycomacropeptide, or caseinomacropeptide, is a glycophosphopeptide isolated from cheese whey with prebiotic properties (47). It contains higher concentrations of threonine and isoleucine. As it is a protein that is naturally produced incompletely, it is used as an ingredient for various applications. In the treatment of PKU, it is supplemented with essential amino acids and tyrosine to be used as a substitute for daily proteins. It has been shown that the use of medicinal foods containing CGMP improves satiety, increases brain control and, thanks to their prebiotic properties, contributes to the taste and acceptance of food in patients (48). One of the pharmacological treatments used to treat the condition is saprobiopterin. This drug has been available in the United States and Europe since 2008 and is known to have positive effects in controlling Phe levels and increasing dietary tolerance (49). It is a synthetic version of the 6R isomer of tetrahydrobiopterin (BH4) and is administered orally. Responsive subjects are identified by performing a BH4 loading test. The efficacy of this treatment has been demonstrated in numerous studies conducted on patients of various ages

(50,51). In newborns, the loading test is performed after a positive HPA test and after ensuring that there are no other conditions that could cause false positives. During the test, the newborn must follow a normal diet. First, a blood sample is taken to measure the Phe level, and then 20mg/kg of sapropterin, dissolved in breast milk or formula, is administered. Blood samples are taken 4, 6, 8, 12, 16 and 24 hours after administration, and the entire test must be performed within 24 hours (51).

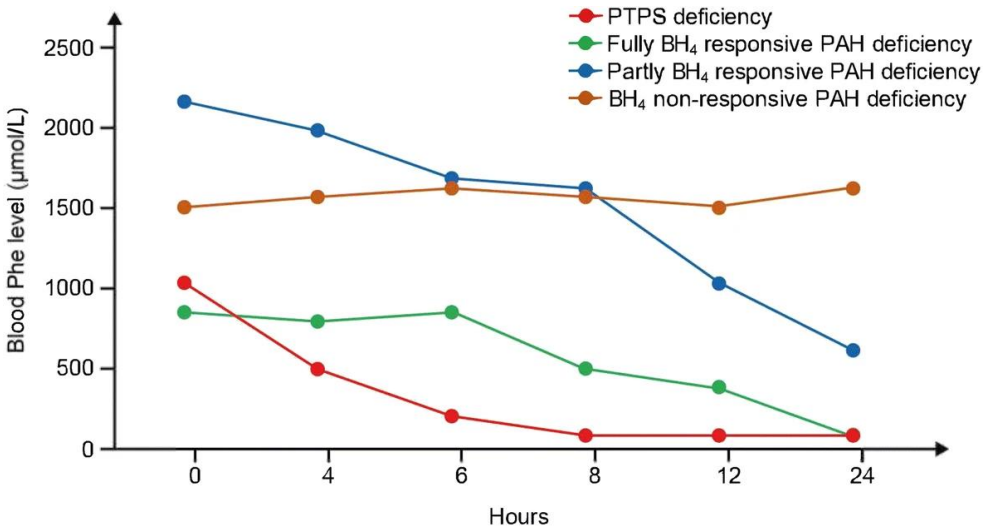


Figure 3: The image describes the possible responses observed following the administration of sapropterin (*Muntau, A.C., du Moulin, M. & Feillet, F. Diagnostic and therapeutic recommendations for the treatment of hyperphenylalaninemia in patients 0–4 years of age. Orphanet J Rare Dis 13, 173 (2018)).*

In the graph above, the green and blue lines describe the condition of subjects who are totally or partially responsive to treatment with sapropterin, respectively. In general, a subject is defined as responsive when blood Phe levels are reduced by approximately 30%. However, in partially responsive subjects, long-term trials are often necessary to understand whether they can respond satisfactorily to therapy. The red line, on the other hand, describes a specific condition of deficiency, a rapid reduction in levels 2-6 hours after administration, which is linked to a deficiency of PTPS or DHPR. In this case, further tests, such as pterin tests, will be necessary. The orange line describes a situation of non-responsiveness (51). Hypotheses have also been formulated on the correlation between genotype and possible response to

treatment. In fact, it is known that patients with null mutations do not respond to therapy. Therefore, through the residual enzymatic activity in mutated subjects, it is possible to distinguish between patients who respond and those who do not respond to treatment. The BH4 loading test depends on many methodological factors, and what has been observed is that, in addition to some patients who showed a response to therapy 24 hours after administration but no long-term response, there are also subjects who show no immediate response to a single dose, but a reduction occurs after several days. For this reason, there is a need to study new approaches which, in addition to the loading test, allow the identification of subjects who respond to therapy (52). Studies have shown that heterozygous patients with two null mutations are associated with severe cases of PKU and have low or no residual enzyme activity. Consequently, sapropterin cannot be administered to these patients, as it is a drug treatment that requires a certain amount of residual activity. Patients with severe forms (Phe> 1200 μmol/L) but with at least one partially active allele have been found to respond to therapy. Patients with mild mutations, in which both mutations result in partial residual activity, may respond to BH4. Other cases in which compound heterozygotes with a severe mutation and a mild mutation are present are still being studied to understand whether these individuals are responsive to BH4 and, above all, at what doses the treatment can be effective (53). The latest treatment is enzyme replacement therapy, the drug pegvaliase (Palynziq), a PEGylated form of phenylalanine ammonia-lyase, produced through a process of recombination from the cyanobacterium Anabaena variabilis, which has the function of converting Phe into ammonia and trans-cinnamic acid, which are then metabolised by the liver and excreted in the urine. It is administered subcutaneously and is independent of residual enzyme activity (54,55). This therapy is characterised by a higher frequency of adverse events, which are mainly related to the patient's immune response. Basically, these are hypersensitivity reactions (56). Many of the treated subjects developed anti-PAL and anti-PEG antibodies. A different antibody response could also be observed at different times. From the start of treatment up to 6 months, a higher number of anti-PEG (IgM and IgG) and anti-PAL (IgM) antibodies were observed, while after 6 months there was a higher number of anti-PAL IgG antibodies, with specific antibodies against the drug (IgG4). The antibodies bind to the drug, thus creating circulating immune complexes (CICs)

(57). This drug was approved by the FDA in 2018. The first study conducted in humans was a phase 1 study, which included patients over the age of 18 who had been diagnosed with classic PKU and had poor diet adherence. Twenty-five patients were recruited in the United States and divided into five groups, each receiving different doses of the drug (0.001, 0.003, 0.10, 0.03 and 0.1 mg/kg). Each patient was followed for six weeks; 80% of subjects reported an adverse event during the study, which were more frequent at higher doses of the drug. Treatment reduced Phe concentrations 6 days after drug administration, and it was observed that the reduction in Phe levels was related to the concentration of the drug, highlighting a dose-dependent effect (58,59).

1.9 NEW THERAPEUTIC FRONTIERS

To date, studies are underway on mouse models to investigate new therapies based on viral vectors, mRNA or genome editing. The aim of these therapies is to introduce copies of unmutated PAH mRNA into human hepatocytes to enable their integration into liver cells and the expression of unmutated, functioning proteins. For example, it is possible to introduce wild-type copies of mRNA using adenoviruses. One of the main side effects of this therapy is due to the immune response, which, shortly after the introduction of the adenovirus into murine liver cells, attacks and destroys the infected cells. Other side effects observed in these models are the development of tumour cells, again due to the insertion of viral vectors. Another study concerns the introduction of WT mRNA in order to restore PAH activity in hepatocytes. The mRNA bound to a lipid nanoparticle (LNP) can be administered intravenously and, once in circulation, by binding to ApoE, it will be absorbed into the liver by endocytosis. These and many other gene therapies are still being studied and clinical trials on humans have not yet begun, as there are still critical issues that need to be overcome. The goal of gene therapies compared to those currently in use is to enable the re-expression of wylde-type PAH mRNA in human liver cells and thus the expression of a functioning protein, overcoming the limitations imposed by current therapies on the market (60).

2. RESEARCH PROJECT OBJECTIVES

The research project is based on four fundamental objectives:

1) Recruitment of subjects with hyperphenylalaninaemia

2) Genetic-molecular analysis of the PAH gene to identify pathogenic variants responsible for the clinical phenotype in subjects with PKU and implementation, through new sequencing technologies, of custom panels for the study of new genes related to phenylalanine metabolism.

3) Classification of pathogenic variants identified using bioinformatic software (PKU Database, HGMD, GnoMAD).

4) Correlation of the different clinical phenotypes of patients with PKU with their genotypes, to identify individuals eligible for sapropterin therapy.

The aim of this project is to study the correlation between genotype and phenotype in subjects who have already been diagnosed with hyperphenylalaninaemia or phenylketonuria at birth. The subjects followed at our facility, the A.O.U.P. “Paolo Giaccone” in Palermo, and the samples collected were processed at the “Laura Notarbartolo” Laboratory of Biochemistry, Biology and Molecular Genetics of Lipids. Specifically, the PAH gene was sequenced with the aim of identifying pathogenic variants in patients and understanding the correlation between mutations and residual enzyme activity to identify individuals eligible for treatment with sapropterin dihydrochloride (KUVAN). Thanks to a collaboration with the University Medical Centre Hamburg-Eppendorf, in Germany, it was possible to analyse in detail the residual enzyme activity of each genotype that had not been previously analysed, using HPLC methods. Treatment with saprobiopterin is important as it would allow specific diets to be formulated for each patient, with the possibility of reducing dietary restrictions and improving the patient's lifestyle and psychological well-being.

3. MATERIALS AND METHODS

3.1 PATIENT RECRUITMENT

At the Laura Notarbartolo Laboratory of Biochemistry, Biology and Molecular Genetics of Lipids, a total of 22 patients with suspected hyperphenylalaninemia (HPA) or prenatally diagnosed with classic phenylketonuria (PKU) were enrolled in the study. Most patients had been receiving a specific dietary treatment since birth. Consequently, classification based solely on plasma phenylalanine (Phe) concentration was deemed unreliable, as these values are affected by early dietary management and do not reflect the patients' initial metabolic status prior to nutritional intervention. All participants underwent a comprehensive plasma amino acid analysis prior to admission to our facility. Based on the measured Phe concentrations, patients were classified as follows: three with suspected mild HPA, seven with HPA type III or mild hyperphenylalaninemia (Phe levels between 2–10 mg/dL or 120–600 μM), eight with HPA type II or mild PKU (10–20 mg/dL or 600–1200 μM), and four with classic PKU (Phe levels >20 mg/dL or >1200 μM). Genetic analysis revealed that one patient did not carry any pathogenic variants in the PAH gene and was therefore excluded from the study. Among the remaining 21 patients, the majority were found to harbor pathogenic PAH variants either in homozygosity or in compound heterozygosity. In contrast, five patients were found to carry only a single heterozygous variant. Specifically, among the 16 genotyped patients, 7 carried variants in homozygosity and 9 in compound heterozygosity. The variants were classified using different bioinformatic software: BioPKU, GnomAD, and HGMD.

3.2 GENOMIC DNA EXTRACTION

Genomic DNA was extracted from circulating leukocytes obtained through whole blood sampling. A commercial purification kit (Wizard® Genomic DNA purification Kit, PROMEGA) was used for extraction, consisting of four steps: red blood cell lysis, white blood cell and nucleus lysis, protein precipitation, DNA concentration and washing. To isolate DNA from leukocytes, the first step is to lyse erythrocytes using 900 μL of Cell Lysis Solution, transferred to a sterile 1.5 mL Eppendorf tube, to which 300 μL of blood will be added. The Eppendorf tube

will be gently inverted and incubated at room temperature for 10-15 minutes. At the end of incubation, centrifugation at 14,000 x g for 4 minutes at room temperature will follow. After centrifugation, the pellet will be visible at the bottom of the tube. Using a disposable pipette, the supernatant is removed, leaving the pellet at the bottom. To lyse the white blood cells, 300 µL of Nuclei Lysis Solution is added to the pellet. Using a pipette, move up and down to obtain a viscous solution free of cell aggregates. For protein precipitation, 100 µL of Protein Precipitation Solution is added to the cell lysate, and the mixture is then vortexed vigorously for about 10-20 seconds. The sample was then centrifuged at 14,000 x g for 3 minutes at room temperature; at the end of centrifugation, a protein pellet, recognisable by its dark brown colour, can be observed at the bottom of the Eppendorf tube. In the last step, the supernatant obtained from the previous centrifugation is transferred to a clean 1.5 mL Eppendorf tube containing 300 µL of 100% isopropanol. The solution is inverted until the white DNA strand is visible. The sample is then centrifuged at 14,000 x g for 1 minute, after which a white DNA pellet can be observed. The supernatant is removed, 300 µL of 70% ethanol is added to the Eppendorf tube, and the tube is gently inverted to wash the pellet and walls. The tube is centrifuged at 14,000 x g for 1 minute, after which the supernatant is removed and, once inverted, placed on absorbent paper to allow the ethanol to evaporate completely. Finally, 100 µL of DNA Rehydration Solution is added to rehydrate the DNA sample overnight at room temperature. The DNA is then stored at 4°C for subsequent analysis.

3.3 DESIGN OF PRIMERS

For each of the 13 exons of the PAH gene, pairs of forward and reverse primers were designed to amplify the exon and some intronic portions upstream and downstream of the coding sequences. The primers were designed using NCBI (National Centre for Biotechnology Information).

1	**F**	5’-CAAGGCAGTGTGCTTAGAGG-3’
	R	5’-ACCAGCAGTCTTCGGATCTC-3’
2	**F**	5’-ATAGCTCATTGTATTGCAAGATT-3’

	R	5’-AAGGTCACACAGTTGGTTAATG-3’
3	**F**	5’-TTCCTGTTCTGGTTCTGCAT-3’
	R	5’-AACAGTCTTCCAAGGCATTATT-3’
4	**F**	5’-GCCAAGATCACATGCAACTG-3’
	R	5’-ACATTGCTCCAAGTAGAGAAGG-3’
5	**F**	5’-GCACATTGGAATCCACAGC-3’
	R	5’-AACACATGCACACACAGAAGG-3’
6	**F**	5’-GATGGCAGCTCACAGGTTCT-3’
	R	5’-CCTCTCCTCTGCCTCAATCC-3’
7	**F**	5’-TGAAGCCAAGTCTGCCTAGC-3’
	R	5’-ACCTCATTCTTGCAGCAGGA-3’
8	**F**	5’-TCTGCATATCACTTATTCCTGG-3’
	R	5’-GGATGAGTGATTCACCAACC-3’
9	**F**	5’-GCTGGAGTCAAGAGAAGAAGC-3’
	R	5’-GCCATAGCCTATAGCACTCCA-3’
10	**F**	5’-GTCCACTGACTCACATGCCA-3’
	R	5’-GCATAGGTCAGCCTTGGAAT-3’
11	**F**	5’-GCTGTGATGTAGAAGGAATCG-3’
	R	5’-CAACCTTCCAACAGAGAACG-3’
12	**F**	5’-ATACACACATACAGAGGCTTCC-3’
	R	5’-TTGTCAAGTTCTTCTCCATCA-3’
13	**F**	5’-CCATAATGAGGCTGTAAGCTCC-3’
	R	5’-TCTCCATCAACAGATTCACAGC-3’

3.4 GENOMIC DNA AMPLIFICATION

Amplification involves:

- Polymerase activation phase at 95°C for 5 minutes.
- Denaturation phase at 95°C for 1 minute, at the end of the process the single-stranded targets will be obtained.

- Annealing phase, there are different temperatures for the primers used and the reaction lasts one minute. During this phase, the primers can pair with the target portions.
- Extension phase at 72°C for 1 minute. The polymerase recognises the 3'-OH region of the primer and begins the polymerisation phase.

These steps are repeated for 35 cycles, followed by a final phase called the extension phase at 72°C for 7 minutes to complete all amplification products. At the end of all the previous phases, the samples are kept at 4°C for an indefinite period of time. The PCR reaction was performed in a final volume of 30 µL containing dNTPs (at a final concentration of 2.5 µM each), 3 µL of 10X Buffer, 1.5 mM MgCl2, 20 pmol of each primer, 2U of Taq polymerase (Taq Invitrogen) and 100 ng of genomic DNA. Each amplification product was evaluated in a standard 1.5% agarose gel using Sybr Safe, a non-specific double-stranded DNA intercalating dye.

3.5 PURIFICATION OF PCR PRODUCTS

The fragments obtained through the amplification reaction were purified by electrophoresis on 1% Low Melting Point agarose gel in 1X TAE (0.04 M Tris acetate, 0.001 M EDTA) and eluted through column purification (Wizard® PCR Preps DNA Purification System- Promega). The samples were resuspended in MQ H2O. The regions of genomic DNA suspected of being involved in the alteration of the pathogenic picture were subjected to PCR amplification. After purification, the PCR products were sequenced using the Sanger method (named after its inventor), based on the selective incorporation of chain terminator dideoxynucleotide triphosphate (ddNTP). A ddNTP is very similar to its dNTP counterpart but lacks the hydroxyl group at the 3' carbon position.

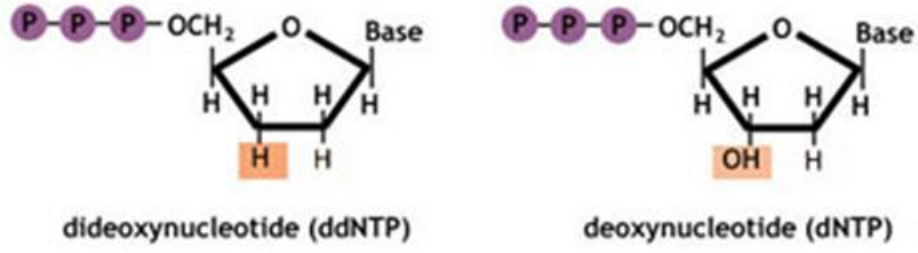


Figure 4: Comparison between the structure of a dideoxynucleotide and a deoxynucleotide

For each cycle, DNA fragments will be synthesised which can be interrupted if they have incorporated a ddNTP, as the 3'-OH group is not available for the addition of further nucleotides, and which can be separated based on their length. The substrate for sequencing is DNA produced by PCR amplification, which is converted into single-stranded form and used as a template for sequencing. The four ddNTPs can be distinguished as they are labelled with different fluorochromes that emit fluorescence at different wavelengths. The fluorescent DNA fragments are analysed using an automatic sequencer based on capillary electrophoresis, the 3500 Genetic Analyser (Applied Biosystems). Electrophoresis takes place inside eight capillaries containing a polymer such as polyacrylamide gel (POP7, which allows fragments of varying lengths to be separated). A laser beam strikes the capillary, exciting the fluorochromes marking the fragments of different lengths. The fluorescence emission is read by a CCD (Charge-Coupled Device) camera. The fluorescence emitted by the excited molecules is collected as a band of a particular wavelength and stored as a digital signal. The raw data is analysed using a specific programme capable of determining which base corresponds to a given fluorescence intensity (Sequencing Analysis 6). The result is a graph with coloured peaks, where each peak corresponds to a specific base; this graph is the electropherogram. The amplified, purified and quantised products, read by spectrophotometry, are used as a template for the direct sequencing reaction. Approximately 10 ng of template DNA per 100 bp to be sequenced is used in the presence of 3.2 pM of one of the primers specific for the exon in question (usually the forward primer is used) and 1 µl of BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem) containing deoxynucleotides, labelled dideoxynucleotides, reaction buffer and specific Taq in a final volume of 10 µl. Specific Taq is used because it has a point mutation in the N-terminal domain that eliminates 5' → 3' nuclease activity.

The sequencing reaction involves:

- A first step at 96°C for 10 seconds in which the DNA denatures.
- A second step at 50°C for 5 seconds to allow primer annealing.

- A third step at 60°C for 4 minutes in which Taq polymerase extends the chain until it randomly encounters a ddNTP.

These steps are repeated for 25 cycles with a temperature increase of 1°C per second. These reactions were purified on a plate using the BigDye XTerminator® Purification Kit (Applied Biosystems) by adding only two reagents:

- XTerminator™ Solution, which removes what has not been incorporated and free salts after the sequencing reaction.
- SAM™ Solution, which improves the performance of the first reagent and also stabilises post-purification reactions.

The purified reaction is subjected to capillary electrophoresis for 56 minutes (10 minutes to fill the capillary with the polymer, 10 minutes of pre-run at 6-10 mA constant current and 36 minutes for electrophoresis, again at 6-10 mA). The raw data is analysed using the Sequencing Analysis 6 programme; the subsequent comparison with the sequences in the database is performed using the SeqScape 3 programme.

3.6 MLPA (MULTIPLEX LIGATION-DEPENDENT PROBE AMPLIFICATION)

Patients carrying a single heterozygous variant were sequenced using MLPA (Multiplex Ligation-Dependent Probe Amplification) to highlight the possible presence of deletions or duplications. The SALSA MLPA Probemix P055 PAH kit was used. The DNA must have a concentration between 50-250ng; therefore, each sample was quantified using Qubit 2.0 Fluorometer (Invitrogen, Thermo Fisher Scientific, Italy) and checked on a standard 0.7% agarose gel in 1X TAE (0.04 M Tris acetate, 0.001M EDTA). The test involves several steps:

- Denaturation phase: 5 µL of sample are placed in the thermal cycler and heated first to 98°C and then cooled to 25°C.
- Hybridisation phase: a mixture is prepared using 1.5 µL of MLPA Buffer and 1.5 µL of MLPA probemix, and 3 µL of the mixture is added to each sample, followed by incubation at 98°C for one minute and hybridisation at 60°C for 16-20 hours.
- Ligation phase: prepare a mixture using 25 µL of ultrapure water, 3 µL of Buffer A, 3 µL of Buffer B and 1 µL of Ligase-65. After cooling the samples to 54°C, add

the mixture and incubate at the same temperature for 15 minutes. Then, raise the temperature to 98°C for a further 5 minutes of incubation in order to inactivate the ligase, and cool to 20°C.

- PCR phase: prepare a mix using 7.5 µL of ultrapure water, 2 µL of primer mixture and 0.5 µL of polymerase. Add 20 µL of mix to each sample, pipetting carefully, and perform a new reaction consisting of 35 PCR cycles at 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 60 seconds, 1 cycle at 72°C for 20 minutes and a pause at 15°C.

  The samples are then prepared on a plate for sequencing using an automatic sequencer based on capillary electrophoresis, 3500 Genetic Analyser (Applied Biosystems). Nine µL of Hidi formamide, 0.7 µL of PCR products and 0.2 µL of (LIZ) GS500 are added to the plate. The plate is sealed, heated for 3 minutes at 98°C and cooled for 2 minutes at 4°C. The results are then analysed using Coffalyser.Net™ software.

3.7 VARIANT FUNCTIONAL STUDIES

The variants were cloned using Site-Directed Mutagenesis (SDM) and cloned into the pEF-DEST51 vector (Invitrogen, USA). For each genotype, 4 µg of DNA were used and transfected into 4 million COS-7 cells by electroporation (Amaxa Nucleofector II, Lonza, Switzerland) and cultured for 26-30 hours. The cells were then lysed (20 mM HEPES (Sigma-Aldrich, USA), 200 mM NaCl (Chemsolute, Germany), complete mini-EDTA-free protease inhibitor (Roche, Switzerland) through cycles of freezing and thawing. The products of the previous lysis were pipetted into 384-well plates automatically (FreedomEvo150, Tecan, Switzerland), allowing the analysis of 8 genotypes per run (each run always included a WT control sample analysed in duplicate and a negative sample without DNA). Each genotype was analysed in triplicate. For the analysis, 10 µL of lysate was combined with a buffer consisting of 22.35 mM NaHEPES (Sigma-Aldrich, USA) (pH 7.3), 1 U/µl catalase (Sigma-Aldrich, USA) and 10 µM ferrous ammonium sulphate (Sigma-Aldrich, USA) and L-phenylalanine. After a pre-incubation phase, the reaction was started by adding BH4 and stopped after 15 minutes by adding 2µL of 9 N HCl (Sigma-Aldrich, USA). For each plate, the concentration of Phe varied incrementally along the column (0 to 2500 µM) while the concentration of

BH4 varied along the rows (0-250 μM). Protein content in lysates was measured by Bradford assay (Bio-Rad, USA; CLARIOstar, BMG LABTECH, Germany). The tyrosine produced by the reaction was quantified by high-performance liquid chromatography on a Hypersil ODS-2 column column (Dionex UltiMate 3000, Thermo Fisher Scientific, USA) with a running buffer (pH 4.6) containing 1.57% ammonium hydroxide (Sigma-Aldrich, USA) and 2% acetic acid (AppliChem, Germany) at 2 mL/min (61).

4. RESULTS

The twenty-one genotyped patients were found to be carriers of homozygous or biallelic heterozygous variants. The genotypes identified are shown in the table below.

cDNA	Allele 1	cDNA	Allele 2
c.194T>C	p. Ile65Thr	c.194T>C	p. Ile65Thr
c.194T>C	p. Ile65Thr	c.473 G>A	p.Arg158Gln
c.284_286delTCA	p.Ile95del	c.284_286delTCA	p.Ile95del
c.556del	p.Thr186HisfsTer9	c.1171_1172del	p.Ser391PhefsTer2
c.601C>T	p.His201Tyr	c.782G>A	p.Arg261Gln
c.638T>C	p.Leu213Pro	c.638T>C	p.Leu213Pro
c.781C>T	p.Arg261Ter	c.1066-11G>A	p.Gln355_Tyr356insGlyLeuGln
c.782G>A	p.Arg261Gln	c.782G>A	p.Arg261Gln
c.782G>A	p.Arg261Gln	c.1066-11G>A	p.Gln355_Tyr356insGlyLeuGln
c.842C>T	p.Pro281Leu	c.1066-11G>A	p.Gln355_Tyr356insGlyLeuGln
c.898G>T	p.Ala300Ser	c.1066-11G>A	p.Gln355_Tyr356insGlyLeuGln
c.898G>T	p.Ala300Ser	c.1208C>T	p.Ala403Val

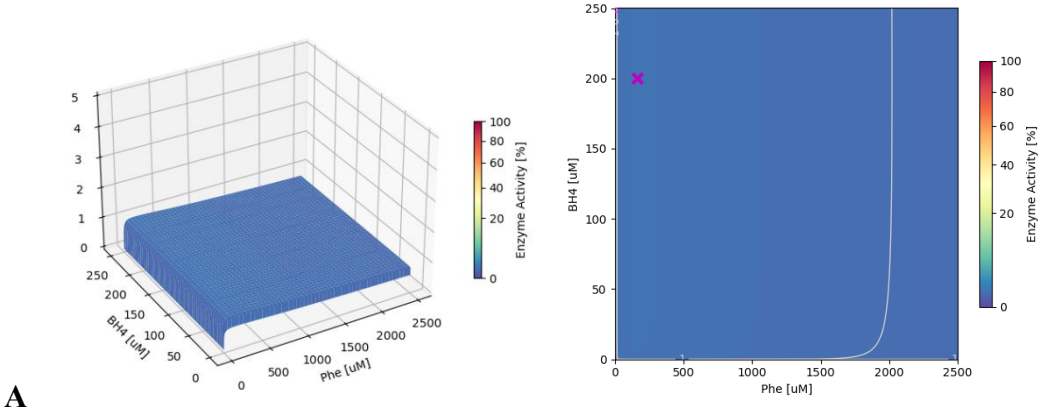
The electropherograms for each genotype are shown in 6. Supplementary Material.

Missense variants represent the most frequent class, followed by frameshift variants and a single intronic variant.

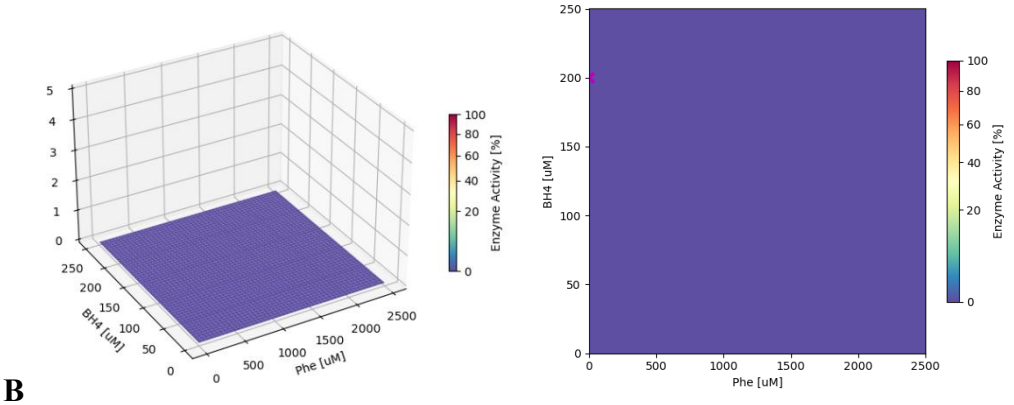
In five patients, single heterozygous variants were also found, including: c.121C>T p.Leu41Phe, c.1208G>A p.Ala403Val and c.782delG p.Arg261fs*80. These patients underwent MLPA (Multiplex Ligation-dependent Probe Amplification) analysis to rule out the presence of duplications or deletions not detectable by Sanger sequencing. The outcome of these analyses was negative.

Thanks to a collaboration with the University Medical Centre Hamburg-Eppendorf, it was possible to evaluate the residual enzymatic activity of some previously unanalysed genotypes in vitro. In cases of compound heterozygosity, the analysis of individual homozygous variants was also conducted in order to understand the specific contribution of each allele.

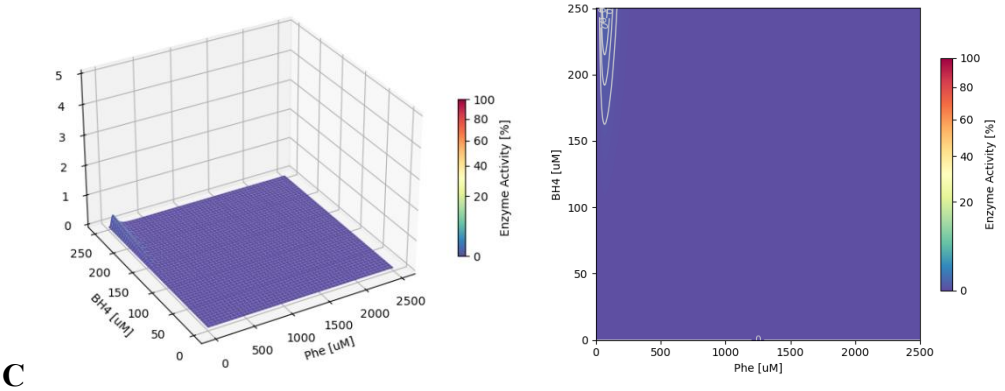
The genotypes p.(Thr186Hisfs9);(Ser391Phefs2), p.(Leu213Pro);(Leu213Pro) and p.(Ile95del);(Ile95del) were found to be completely devoid of residual enzyme activity. Therefore, patients carrying these genotype combinations are not eligible for BH4 (tetrahydrobiopterin) therapy and must necessarily follow a dietary restriction, possibly combined — in some cases — with enzyme replacement therapy.



A



B

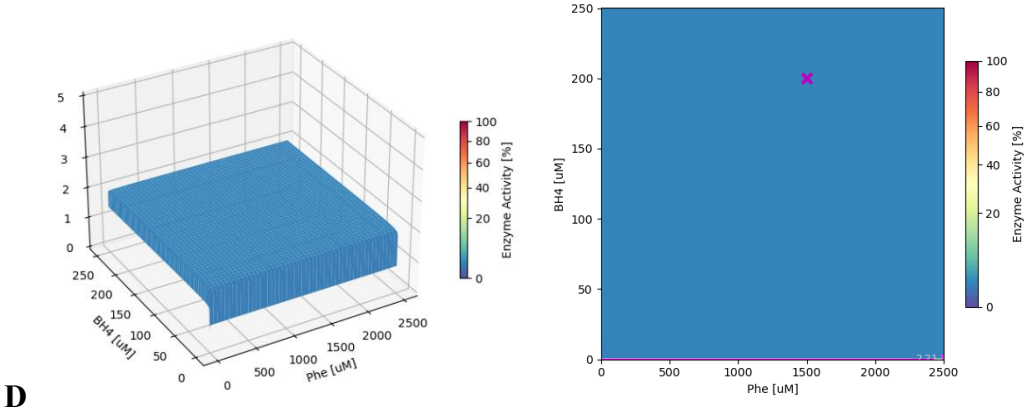


C

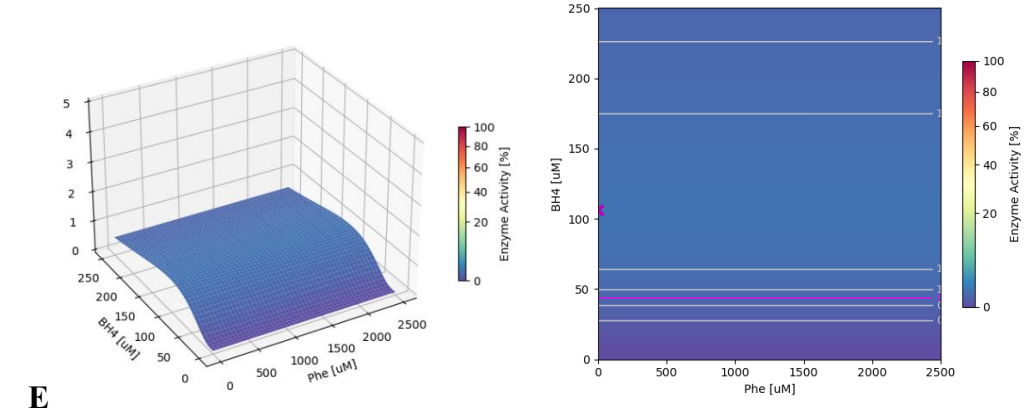
The figure shows graphs relating to the genotypes mentioned above (A) p.(Thr186HisfsTer9);(Ser391PhefsTer2) (B) p.(Leu213Pro);(Leu213Pro) (C) p.(Ile95del);(Ile95del). The 3D (left) and 2D (right) graphs show residual enzyme activity based on a colorimetric system: blue-violet shades indicate zero or very low levels of activity (0–1%).

This evidence is also reflected in clinical practice. The two patients homozygous for the p.(Leu213Pro) variant are currently being treated with Palynziq 20 mg, which has led to a significant reduction in plasma phenylalanine (Phe) levels and the reintroduction of a normal diet. The other patients, carriers of the remaining variants with total loss of function, are following dietary therapy exclusively.

The p.(Thr186Hisfs*9) and p.(Ser391Phefs*2) variants were also analysed in homozygosity. Although already known in the literature as pathogenic, no experimental data on their enzymatic activity were available. As expected, since these are frameshifts that cause the formation of premature stop codons, the enzyme is completely inactive.



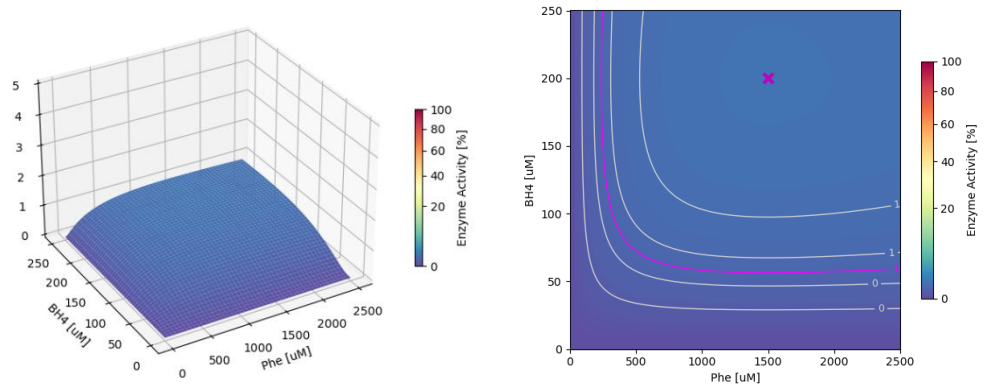
D



E

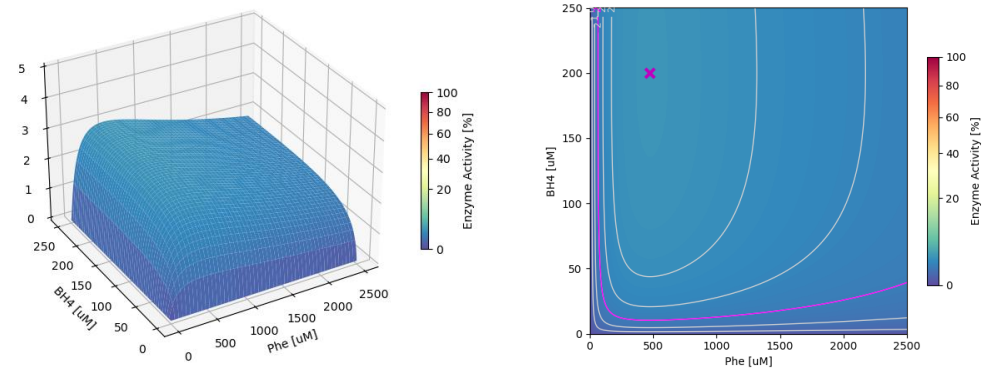
The relevant graphs are shown in (D) p.(Ser391PhefsTer2);(Ser391PhefsTer2) and in (E) (Thr186HisfsTer9); (Thr186HisfsTer9)

An analysis was also conducted on the c.782delG p.(Arg261fs*80) variant, identified as heterozygous in one patient. In the absence of a second identified variant, enzyme activity was assessed considering the homozygous genotype. The result confirms an activity of less than 1%, in line with what is expected for a frameshift with a premature stop codon. The results are shown in figure (F).



F

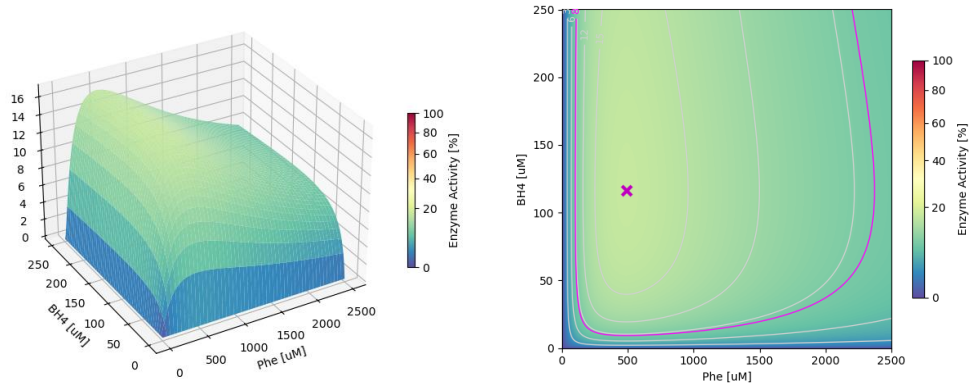
Some genotypes, on the other hand, showed appreciable residual enzymatic activity. In particular:



G

The images in (G) show the graphs relating to the variant (Ile65Thr);(Arg158Gln). In the specific case of these graphs, the x indicates the optimal values of BH4 and the concentrations of Phe for which maximum enzymatic activity is obtained. As can be seen, this is achieved for Phe values of 475 µM and BH4 values of 200 µM. The pink line delimits the area within which 50% of the enzyme's activity can be achieved, i.e. the optimum functioning. In the specific case of this mutation, 50%

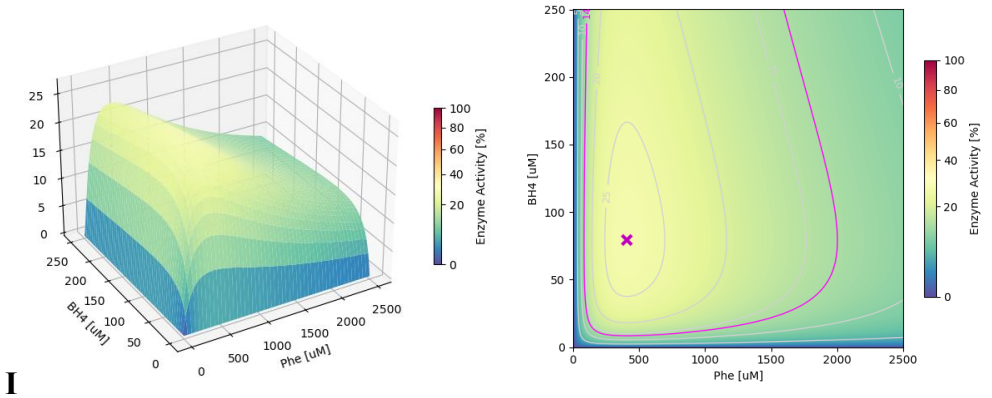
of enzyme activity is achieved for [Phe] values between 65-3466 µM and BH4 levels between 10-3841 µM. In the 3D model, it can be seen that the enzyme maintains a residual activity of 3%, with a peak activity corresponding to the values shown above. Generally, patients are considered likely to respond to BH4 treatment but, considering the low residual activity and evaluating how the peak activity corresponds to low [Phe] but high BH4 levels, in order to obtain benefits from KUVAN therapy, it would be advisable to increase the initial doses of the drug.



H

The variant p.(His201Tyr);(Arg261Gln), whose graphs are shown in (H), shows a residual activity of 17%. The maximum peak activity is recorded for a concentration of Phe of 490 µM and BH4 of 116 µM. The presence of a high peak activity is consistent with subjects who may have a response to BH4. It is necessary to adjust the test taking into account the Phe values, as the therapy may only have positive effects in the case of higher amino acid concentrations. Fifty per cent of enzyme activity is achieved for [Phe] 101-2370 µM and BH4 9-1454 µM.

In vitro analysis of the p.(His201Tyr);(His201Tyr), variant was also performed; image (I), which was not present in their databases, and it was found that the enzyme has a residual enzymatic activity of more than 20% with a peak activity for Phe concentrations of 410 µM and BH4 concentrations of 79 µM. Like the previous genotype, this genotype could also respond favourably to treatment when higher concentrations of Phe are considered.



I

The other variants had already been analysed by their research group, and the results relating to the genotype landscape are available on the website http://pah-activitylandscapes.org/.

Specifically, the data reported indicate that the variants p.(Pro281Leu);(Gln355_Tyr356insGlyLeuGln) and p.(Arg261Ter);(Gln355_Tyr356insGlyLeuGln) are classified as variants that do not respond to treatment with BH4. In fact, their enzymatic activity is considered to be zero. The three patients are not undergoing any enzyme replacement therapy but are following diet therapy exclusively.

The variants p.(Ala300Ser);(Gln355_Tyr356insGlyLeuGln), p.(Ala300Ser);(Ala403Val) p.(Arg261Gln);(Gln355_Tyr356insGlyLeuGln), p.(Arg261Gln); (Arg261Gln) and p.(Ile65Thr);(Ile65Thr) maintain some residual activity and are therefore classified as potentially responsive. Among those listed above, the only one that determines a mild phenotype is p.(Ala300Ser);(Ala403Val), which is the one that could give the most effective response to treatment with BH4. The other responsive variants could give a positive response only by considering the elevated [Phe] for the start of administration or a higher BH4 value for the loading test.

Overall, in vitro analysis has identified seven genotypes that are potentially responsive to treatment with sapropterin dihydrochloride. Currently, only one patient is being treated with KUVAN 300 mg, in combination with dietary therapy and supplements (Afenil Micro 3H, Neutrafenil Micro R). This patient is homozygous for p.(Arg261Gln), which has a residual enzyme activity of 7%.

The other potentially eligible patients underwent responsiveness testing but showed intolerance to the drug, making it necessary to discontinue treatment. In some cases, dietary therapy was supplemented with amino acid supplements to compensate for any nutritional deficiencies.

5. CONCLUSIONS

In phenylketonuria (PKU), dietary restriction of phenylalanine remains the cornerstone of treatment, effectively preventing the neurological complications associated with the accumulation of this amino acid in the brain. Early diagnosis and timely initiation of therapy are therefore critical to achieving a favorable clinical outcome. However, long-term adherence to the restrictive diet poses substantial challenges, particularly during adolescence and adulthood, potentially compromising metabolic control and quality of life.

In this context, alternative or adjunctive therapeutic strategies have become increasingly important. These include specific nutritional supplements (such as large neutral amino acids, LNAA, or glycomacropeptide), pharmacological treatment with sapropterin dihydrochloride (Kuvan), and enzyme substitution therapy with pegvaliase (Palynziq). Among these, sapropterin has shown clinical efficacy only in individuals whose phenylalanine hydroxylase (PAH) enzyme retains sufficient residual activity to be stimulated by tetrahydrobiopterin (BH4).

Functional studies on homozygous or compound heterozygous PAH variants are thus essential to predict the potential effectiveness of pharmacological treatment. The findings of this study allowed the identification of seven genotypes potentially responsive to BH4. Among these, the p.(Ala300Ser);(Ala403Val) genotype—associated with a mild phenotype—emerged as the most promising candidate for a favorable therapeutic response.

Of the genotyped patients, only one individual carrying the p.(Arg261Gln);(Arg261Gln) genotype demonstrated a clinically significant response to sapropterin, as confirmed by the BH4 loading test. Other genotypes, although theoretically responsive, did not show positive outcomes, possibly due to pharmacokinetic or metabolic factors. It is also worth noting that therapy data are unavailable for some patients who have since been transferred to other clinical centers. Conversely, two patients homozygous for the p.(Leu213Pro) variant successfully tolerated Palynziq therapy, achieving a marked reduction in phenylalanine levels and enabling a transition to an unrestricted diet.

A major limitation of this study lies in the small sample size, which reduces the ability to extrapolate findings to broader populations. A larger cohort would

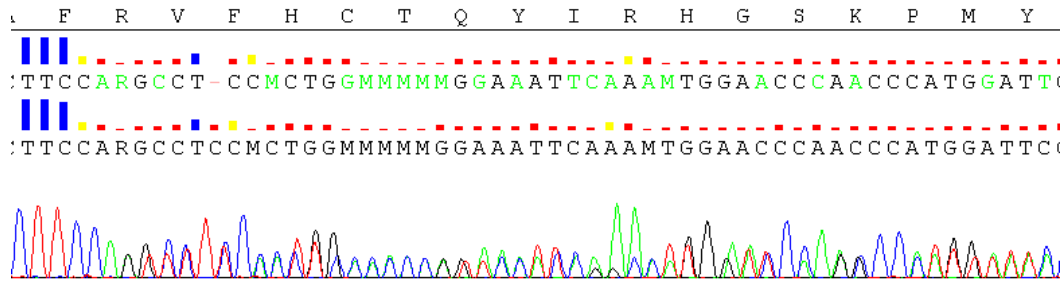
increase the likelihood of identifying a wider spectrum of genotypes and their correlations with phenotypic expression.

Overall, this work highlights the predictive value of genetic characterization in the context of personalized medicine for PKU. However, genotyping alone is not sufficient. A comprehensive understanding of how individual variants affect PAH protein function is equally essential. The enzyme's residual activity must also be evaluated in relation to substrate (Phe) and cofactor (BH4) concentrations, as responsiveness may only manifest under specific biochemical conditions. Indeed, genotypes classified as potentially responsive may exhibit positive responses only at elevated phenylalanine concentrations or with higher initial doses of BH4.

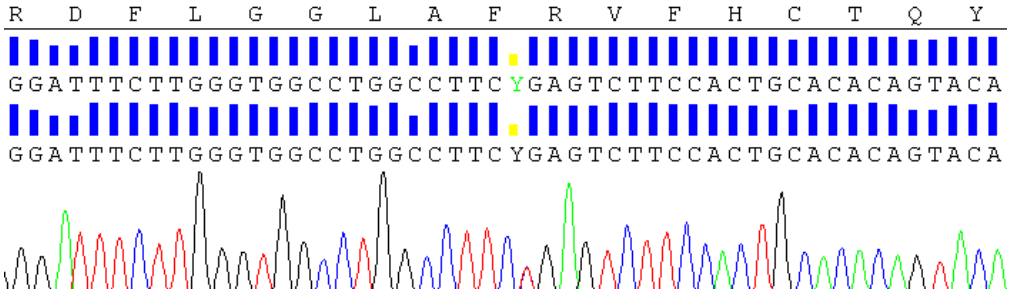
In vitro characterization of the molecular mechanisms leading to preserved or abolished PAH activity is thus a crucial step toward optimizing therapeutic strategies. Nonetheless, it is also necessary to account for interindividual variability, which can influence both treatment response and adherence.

Despite being a rare disease, PKU presents with significant clinical and molecular heterogeneity. While dietary management remains a reliable therapeutic baseline, the expanding availability of pharmacological and biotechnological options calls for continuous refinement of treatment protocols. In this context, the present work contributes to improving our understanding of the disease and supports a shift toward a more personalized and targeted treatment model, ultimately aimed at enhancing the quality of life for individuals living with PKU.

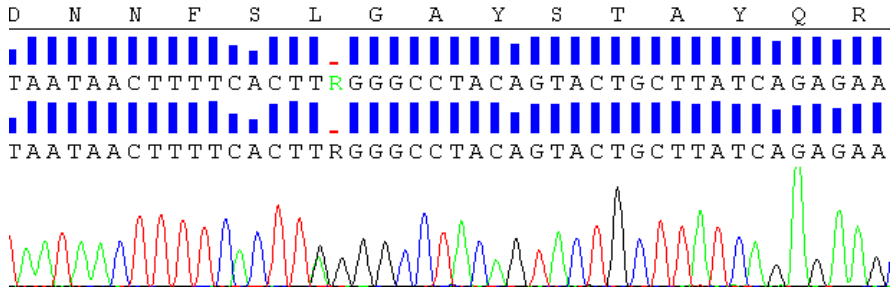
6. SUPPLEMENTARY MATERIAL



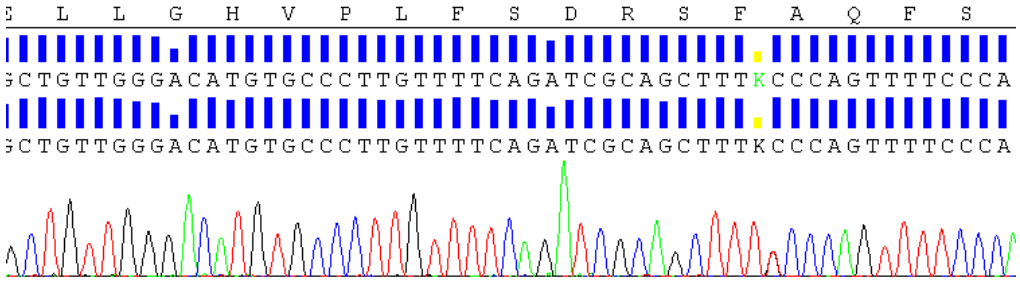
PAH: c.782delG p.Arg261fs80Ter in heterozygosity (exon 7).



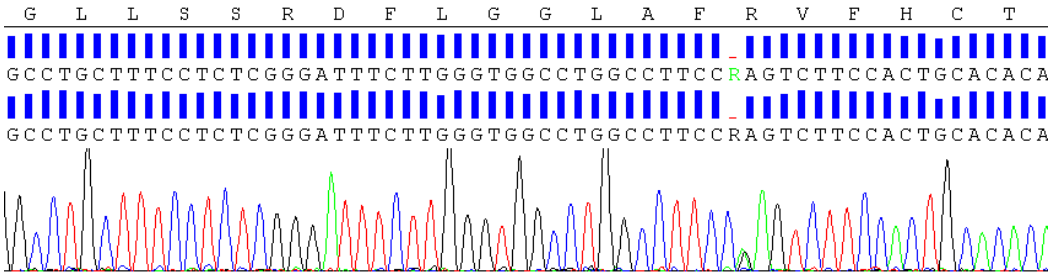
PAH: c.781C>T p.Arg261Ter in heterozygosity (exon 7).



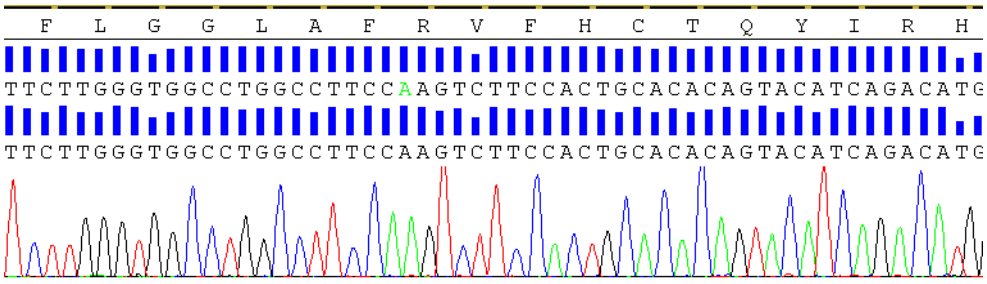
PAH: c.1066-11G>A p.Gln355_Tyr356insGlyLeuGln in heterozygosity (IVS10).



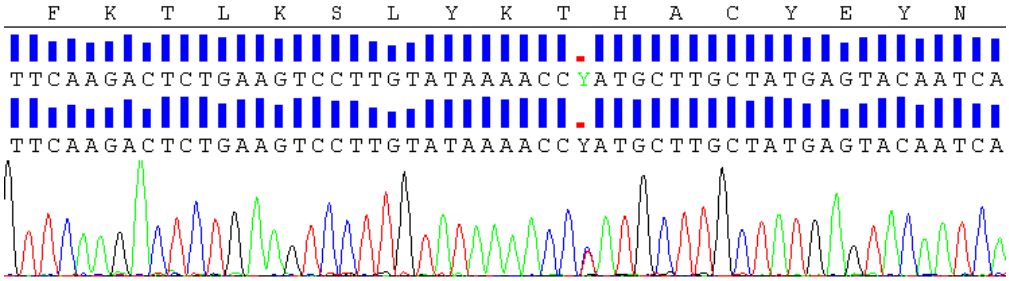
PAH: c.898G>T p.Ala300Ser in heterozygosity (exon 8).



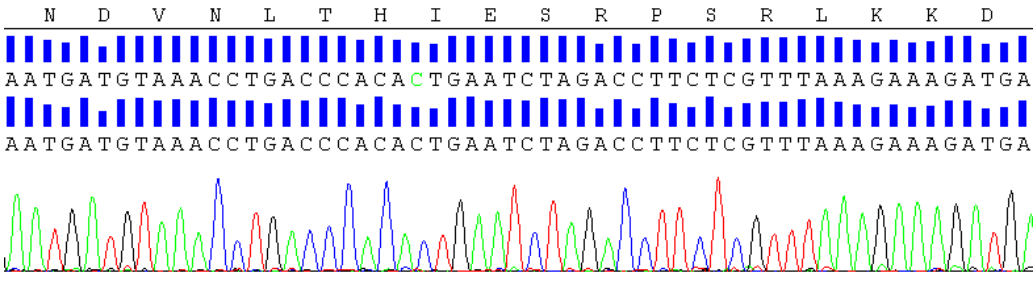
PAH: c.782G>A p.Arg261Gln in heterozygosity (exon 7).



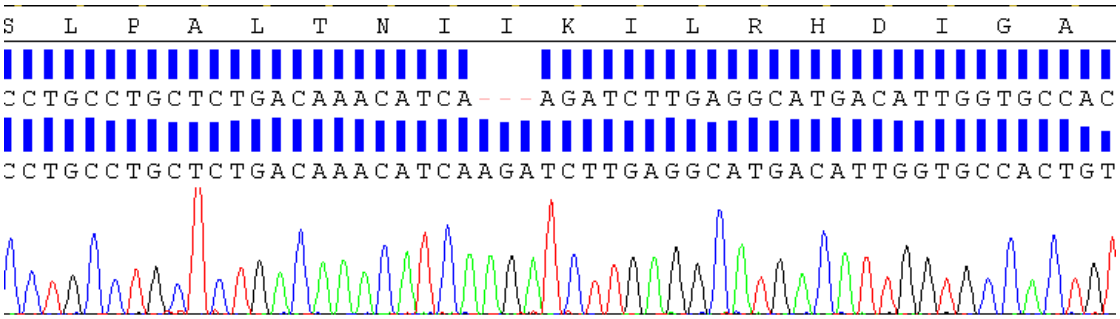
PAH: c.782G>A p.Arg261Gln in homozygosity (exon 7).



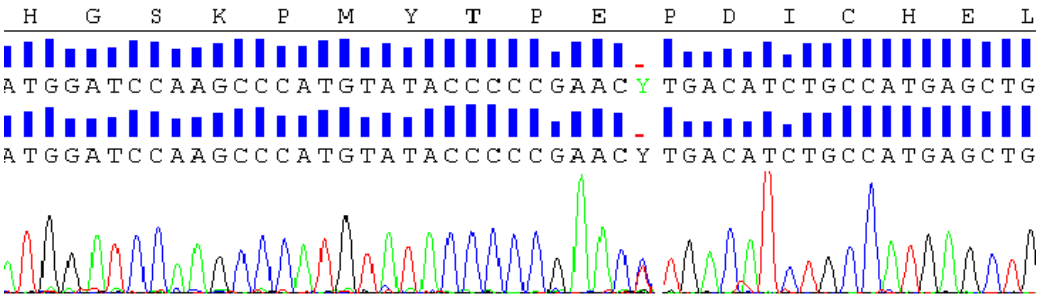
PAH: c.601C>T p.His201Tyr in heterozygosity (exon 6).



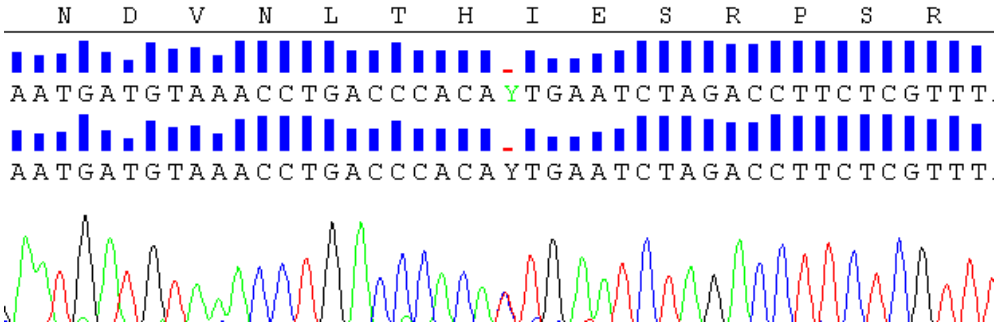
PAH: c.194T>C p.Ile65Thr in homozygosity (exon 3).



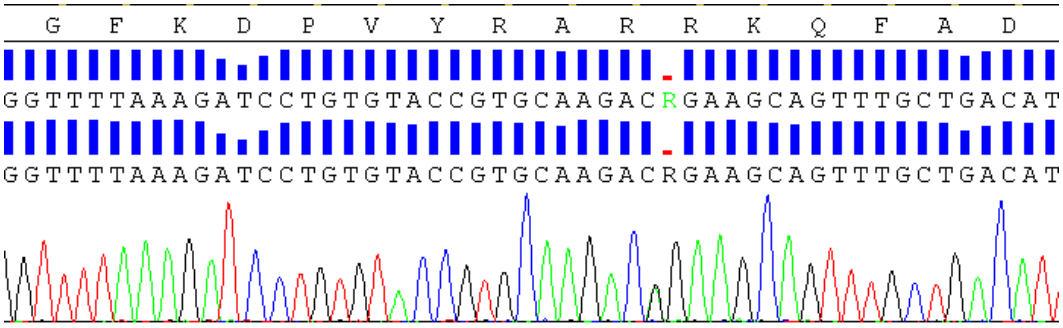
PAH: c.284_286delTCA p.Ile95del in homozygosity (exon 3).



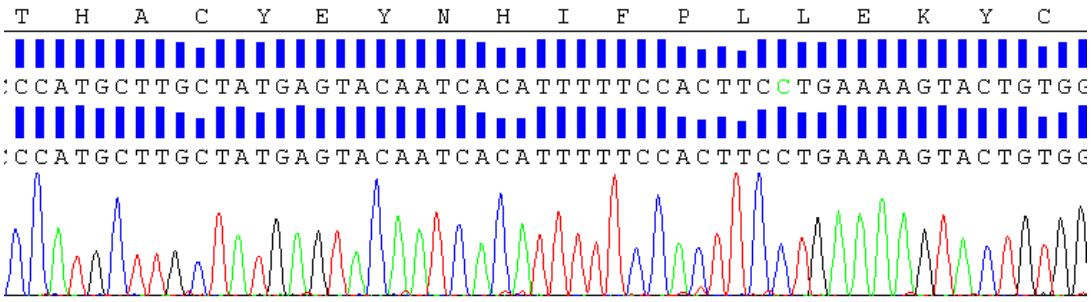
PAH: c.842C>T p.Pro281Leu in heterozygosity (exon 7).



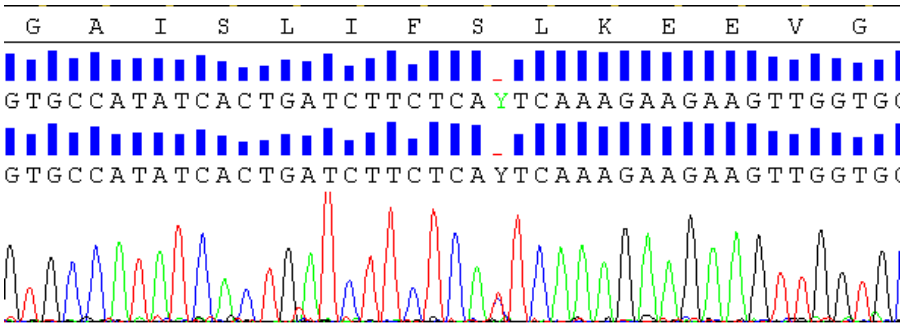
PAH: c.194T>C p.Ile65Thr in heterozygosity (exon 3).



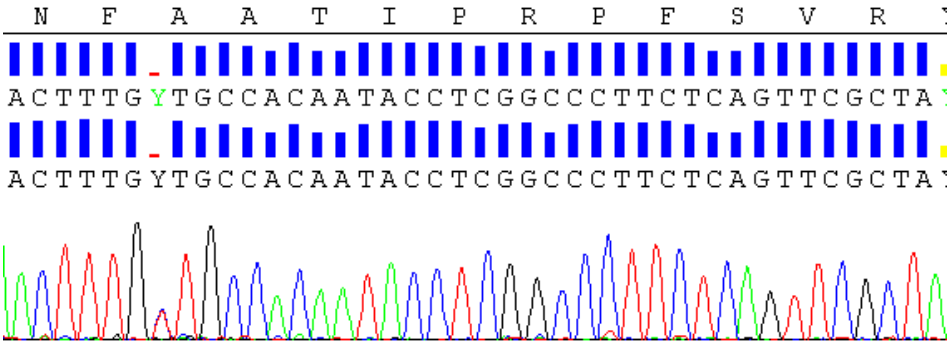
PAH: c.473G>A p.Arg158Gln in heterozygosity (exon 5).



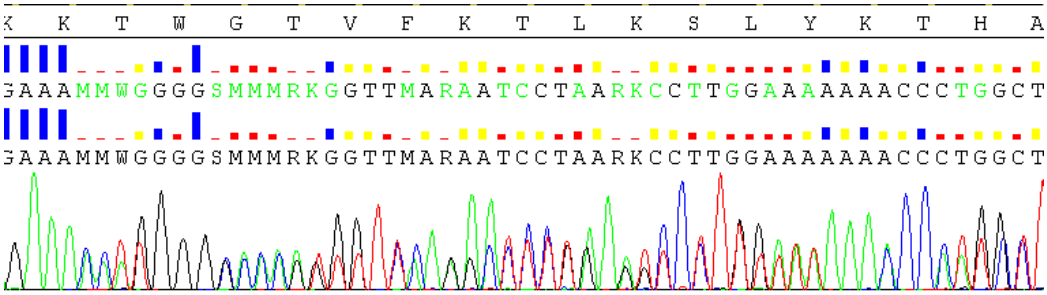
PAH: c.638T>C p.Leu213Pro in homozygosity (exon 6).



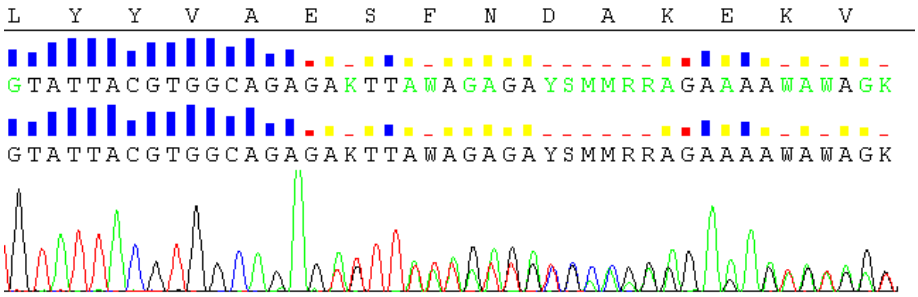
PAH: c.121C>T p.Leu41Phe in heterozygosity (exon 2).



PAH: c. 1208C>T p.Ala403Val in heterozygosity (exon 12).



PAH: c.556del p.Thr186HisfsTer9 in heterozygosity (exon 6).



PAH: c.1171_1172del p.Ser391PhefsTer2 in heterozygosity (exon 11).

7. BIBLIOGRAPHY

(1) J. Thomas et al. Pegvaliase for the treatment of phenylketonuria: Results of a long-term phase 3 clinical trial program (PRISM); Molecular Genetics and Metabolism 124 (2018) 27–38.

(2) N.A. Elhawary et al. Genetic etiology and clinical challenges of phenylketonuria. Human Genomics (2022) 16:22.

(3) F.J. van Spronsen et al. Phenylketonuria. Nat Rev Dis Primers; 7(1): 36.

(4) N. Blau. Genetics of Phenylketonuria: Then and Now. Hum Mutat. 2016;37(6):508–15.

(5) A.M.J. van Wegberg et al. The complete European guidelines on phenylketonuria: diagnosis and treatment. Orphanet Journal of Rare Diseases (2017) 12:162.

(6) A. Hillert et al. The Genetic Landscape and Epidemiology of Phenylketonuria. Am J Hum Genet. 2020 Jul 14;107(2):234–250.

(7) J.J. Mitchell et al. Phenylalanine hydroxylase deficiency. Genetics in medicine 2011 Aug;13(8):697-707.

(8) A. Regnault et al. Development and psychometric validation of measures to assess the impact of phenylketonuria and its dietary treatment on patients' and parents' quality of life: the phenylketonuria – quality of life (PKU-QOL) questionnaires. Orphanet J Rare Dis . 10 (1): 59.

(9) A.M.J. van Wegberg et al. European guidelines on diagnosis and treatment of phenylketonuria: First revision. Molecular Genetics and Metabolism. Volume 145, Issue 2, June 2025, 109125.

(10) R. Trunzo et al. Mutation analysis in hyperphenylalaninemia patients from South Italy. Clinical Biochemistry. Volume 46, Issue 18, December 2013, Pages 1896-1898.

(11) T. Remmington, S. Smith. Tyrosine supplementation for phenylketonuria (Review). Cochrane Database of Systematic Reviews 2021, Issue 1. Art. No.: CD001507.

(12) L.I. Woolf and J. Adams. The Early History of PKU. Int J Neonatal Screen. 2020 Jul 29;6(3):59.

(13) Bickel H. Gerrard J & Hickmans EM Influence of phenylalanine intake on phenylketonuria. Lancet 265, 812–813 (1953).

(14) Wortis J & Giancotti AM. A new simple test paper for mass detection of phenylketonuria. Am. J. Public Health Nations Health 49, 463–464 (1959).

(15) R. Wallace et al. Phenylketonuria—The Guthrie Screening Test—A Method of Quantitation, Observations on Reliability and Suggestions for Improvement. Calif Med. 1966 Dec;105(6):429–434.

(16) F.I. Marte, M. Aurora. Phenylalanine hydroxylase: Function, structure, and regulation. IUBMB LifeVolume 65, Issue 4 p. 341-349.

(17) M.I. Flydal et al. Structure of full-length human phenylalanine hydroxylase in complex with tetrahydrobiopterin. Proc Natl Acad Sci U S A. 2019 May 22;116(23):11229–11234.

(18) J. S. Liberles et al. Allosteric mechanisms in ACT domain containing enzym es involved in amino acid metabolism. Amino Acids (2005) 28: 1–12.

(19) P.F. Fitzpatrick. Allosteric Regulation of Phenylalanine Hydroxylase. Arch Biochem Biophys. 2011 Oct 7;519(2):194–201.

(20) M. Thórólfsson et al. Activation of Phenylalanine Hydroxylase: Effect of Substitutions at Arg68 and Cys237†. Biochemistry. 2003 Apr 1;42(12):3419-28.

(21) R.R. Lopes et al. Modulation of Human Phenylalanine Hydroxylase by 3-Hydroxyquinolin-2(1H)-One Derivatives. Biomolecules. 2021 Mar 19;11(3):462.

(22) J.A. Pavon and P.F. Fitzpatrick. Insights into the Catalytic Mechanisms of Phenylalanine and Tryptophan Hydroxylase from Kinetic Isotope Effects on Aromatic Hydroxylation†. Biochemistry. 2006 Sep 12;45(36):11030–11037.

(23) H. M. Roberts et al. The Kinetic Mechanism of Phenylalanine Hydroxylase: Intrinsic Binding and Rate Constants from Single Turnover Experiments†. Biochemistry. 2013 Jan 29;52(6):1062–1073.

(24) E. Olsson et al. Formation of the Iron–Oxo Hydroxylating Species in the Catalytic Cycle of Aromatic Amino Acid Hydroxylases. Chemistry – A European Journal, 2011 vol. 17, issue 13, pp. 3746-3758.

(25) P.F. Fitzpatrick. Mechanism of aromatic amino acid hydroxylation. Biochemistry. 2003 Dec 9;42(48):14083–14091.

(26) J. Underhaug et al. Phenylalanine Hydroxylase Misfolding and Pharmacological Chaperones†. Curr Top Med Chem. 2012 Nov;12(22):2534–2545.

(27) H. Fanet et al. Tetrahydrobioterin (BH4) Pathway: From Metabolism to Neuropsychiatry. Curr Neuropharmacol. 2021 Apr 29;19(5):591–609.

(28) T. Opladen et al. Consensus guideline for the diagnosis and treatment of tetrahydrobiopterin (BH4) deficiencies. Orphanet J Rare Dis. 2020 May 26; 15:126.

(29) M.J. de Groot et al. Pathogenesis of cognitive dysfunction in phenylketonuria: Review of hypotheses. Molecular Genetics and Metabolism. Volume 99, Supplement, 2010, Pages S86-S89.

(30) M. Hoeksma et al. Phenylketonuria: High plasma phenylalanine decreases cerebral protein synthesis. Molecular Genetics and Metabolism. Volume 96, Issue 4, April 2009, Pages 177-182.

(31) R.A. Williams et al. Phenylketonuria: An Inborn Error of Phenylalanine Metabolism. Clin Biochem Rev. 2008 Feb;29(1):31–41.

(32) P. F. Schuck et al. Phenylketonuria Pathophysiology: on the Role of Metabolic Alterations. Aging Dis. 2015 Oct 1;6(5):390–399.

(33) N. Longo. Disorders of biopterin metabolism. Symposium on Neurotransmitter Disorders. Volume 32, pages 333–342, (2009).

(34) N. Blau et al. Diagnosis, classification, and genetics of phenylketonuria and tetrahydrobiopterin (BH4) deficiencies. Molecular Genetics and Metabolism. Volume 104, Supplement, 2011, Pages S2-S9.

(35) L. Fiori et al. Incidence of BH4-responsiveness in phenylalanine-hydroxylase-deficient Italian patients. Molecular Genetics and Metabolism Volume 86, Supplement, December 2005, Pages 67-74.

(36) H. Levy et al. Recommendations for evaluation of responsiveness to tetrahydrobiopterin (BH4) in phenylketonuria and its use in treatment. Molecular Genetics and Metabolism. Volume 92, Issue 4, December 2007, Pages 287-291.

(37) S. Wang et al. Impaired cognitive function and decreased monoamine neurotransmitters in the DNAJC12 gene knockout mouse model. Orphanet Journal of Rare Diseases volume 20, Article number: 60 (2025).

(38) E. Fino et al. DNAJC12 deficiency: Mild hyperphenylalaninemia and neurological impairment in two siblings. Molecular Genetics and Metabolism Reports. Volume 37, December 2023, 101008.

(39) D. Veenma et al. DNAJC12-associated developmental delay, movement disorder, and mild hyperphenylalaninemia identified by whole-exome sequencing re-analysis. Eur J Hum Genet. 2018 Aug 23;26(12):1867–1870.

(40) B. W. Prick, Wim CJ Hop, J. J. Duvekot. Maternal phenylketonuria and hyperphenylalaninemia in pregnancy: pregnancy complications and neonatal sequelae in untreated and treated pregnancies. The American Journal of Clinical Nutrition. Volume 95, Issue 2, 2012, Pages 374-382.

(41) R.M. Roberts et al. Promoting psychological well-being in women with phenylketonuria: Pregnancy-related stresses, coping strategies and supports. Mol Genet Metab Rep. 2014 Apr 17; 1:148–157.

(42) S.E. Waisbren et al. Maternal Phenylketonuria: Long-term Outcomes in Offspring and Post-pregnancy Maternal Characteristics. JIMD Rep. 2015 Feb 25; 21:23–33.

(43) N.A. Hafid and J. Christodoulou. Phenylketonuria: a review of current and future treatments. Transl Pediatr. 2015 Oct;4(4):304–317.

(44) S.H.L. Yi and R.H. Singh. Protein substitute for children and adults with phenylketonuria. Cochrane Database Syst Rev. 2015 Feb 27;2015(2):CD004731.

(45) F.J. van Spronsen et al. Large neutral amino acids in the treatment of PKU: from theory to practice. J Inherit Metab Dis. 2010 Oct 26;33(6):671–676.

(46) A.P. Burlina et al. Large Neutral Amino Acid Therapy Increases Tyrosine Levels in Adult Patients with Phenylketonuria: A Long-Term Study. Nutrients. 2019 Oct 21;11(10):2541.

(47) D.M. Ney et al. Glycomacropeptide for nutritional management of phenylketonuria: a randomized, controlled, crossover trial. Am J Clin Nutr. 2016 Jul 13;104(2):334–345.

(48) F.N. Keskin et al. Protein substitutions as new generation pharmanutrition approach to managing phenylketonuria. Clin Exp Pediatr. 2022 Nov 1;66(8):320–331.

(49) M.L. Couce et al. Long-Term Pharmacological Management of Phenylketonuria, Including Patients Below the Age of 4 Years. JIMD Rep. 2011 Sep 6; 2:91–96.

(50) U. R. Somaraju and M. Merrin. Sapropterin dihydrochloride for phenylketonuria. Cochrane Database Syst Rev. 2015 Mar 27;2015(3):CD008005.

(51) Muntau, A.C., du Moulin, M. & Feillet, F. Diagnostic and therapeutic recommendations for the treatment of hyperphenylalaninemia in patients 0–4 years of age. Orphanet J Rare Dis 13, 173 (2018).

(52) I. Karačić et al. Genotype-predicted tetrahydrobiopterin (BH4)-responsiveness and molecular genetics in Croatian patients with phenylalanine hydroxylase (PAH) deficiency. Molecular Genetics and Metabolism. Volume 97, Issue 3, July 2009, Pages 165-171.

(53) N. Blau and H. Erlandsen. The metabolic and molecular bases of tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency. Molecular Genetics and Metabolism. Volume 82, Issue 2, June 2004, Pages 101-111.

(54) J. Thomas et al. Pegvaliase for the treatment of phenylketonuria: Results of a long-term phase 3 clinical trial program (PRISM). Molecular Genetics and Metabolism. Volume 124, Issue 1, May 2018, Pages 27-38.

(55) I. Scala et al. Pegvaliase therapy for phenylketonuria: Real-world case series and clinical insights. Molecular Genetics and Metabolism. Volume 142, Issue 1, May 2024, 108151.

(56) N. Longo et al. Evidence- and consensus-based recommendations for the use of pegvaliase in adults with phenylketonuria. Genet Med. 2018 Dec 14;21(8):1851–1867.

(57) O. Hausmann et al. Pegvaliase: Immunological profile and recommendations for the clinical management of hypersensitivity reactions in patients with phenylketonuria treated with this enzyme substitution therapy. Molecular Genetics and Metabolism. Volume 128, Issues 1–2, September–October 2019, Pages 84-91.

(58) T. Hydery and V. Azzopardi Coppenrath. A Comprehensive Review of Pegvaliase, an Enzyme Substitution Therapy for the Treatment of Phenylketonuria. Drug Target Insights. 2019 Jun 21; 13:1177392819857089.

(59) N. Longo et al. Phase 1 Trial of Subcutaneous rAvPAL-PEG in Subjects with Phenylketonuria. Lancet. 2014 Apr 14;384(9937):37–44.

(60) M. Martinez et al. State-of-the-Art 2023 on Gene Therapy for Phenylketonuria. J Inherit Metab Dis. 2023 Aug 3;47(1):80–92.

(61) Gundorova P. et al. Personalized Genotype-Based Approach for Treatment of Phenylketonuria. Journal of Inherited Metabolic Disease, 2025; 48: e70067.

(62) F. J van Spronsen et al. Key European guidelines for the diagnosis and management of patients with phenylketonuria. Lancet Diabetes Endocrinol 2017;5: 743–56.

(63) P. Guldberg et al. A European Multicenter Study of Phenylalanine Hydroxylase Deficiency: Classification of 105 Mutations and a General System for Genotype-Based Prediction of Metabolic Phenotype. Am. J. Hum. Genet. 63:71–79, 1998.hawary

(64) L.L. Moldawer et al. The contribution of phenylalanine to tyrosine metabolism in vivo. Studies in the post-absorptive and phenylalanine-loaded rat. Biochem J. 1983 Mar 15;210(3):811–817.

(65) R. Shiman et al. A Simple Purification of Phenylalanine Hydroxylase by Substrateinduced Hydrophobic Chromatography. The Journal of Biological Chemistry Vol. 254, No 22, Issue of November 25, pp. 11300-11306, 1979.

(66) K.A. Konovalov et al. Conformational selection turns on phenylalanine hydroxylase. J Biol Chem. 2018 Dec 21;293(51):19544–19545.

(67) M.K. Danecka, M. Woidy, J. Zschocke et al. Mapping the functional landscape of frequent phenylalanine hydroxylase (PAH) genotypes promotes personalised medicine in phenylketonuria. J Med Genet 2015;52:175–185.