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MOLECULAR ANALYSIS AND GENOTYPE-PHENOTYPE CORRELATION IN PATIENTS AFFECTED BY HYPERPHENYLALANINEMIAS IN SOUTHERN ITALY

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To my angel and my family,
for love and patience
during these years

There is no difficulty in holding current views, and we are all clever in thinking the fashionable thoughts. But to create a new idea, and foresee the development before the time is ripe, that is insight.
To see further than the obvious, and to put things into a wider context, is insight.

Asbjörn Fölling, described by his son

Sommario

L'Iperfenilalaninemia (HPA), una malattia genetica autosomica recessiva, è il risultato di un difetto dell'attività dell'enzima Fenilalanina Idrossilasi (PAH), con conseguente accumulo plasmatico di Fenilalanina. Nella maggior parte dei casi (circa il 98%) l'espressione fenotipica della patologia è il risultato della presenza di mutazioni a carico del gene PAH, codificante per l'enzima fenilalanina idrossilasi, con locus 12q24.1 sul cromosoma 12. La patologia ha una frequenza nella popolazione caucasica di circa 1 ogni 10.000, corrispondente ad una frequenza di portatori di circa 1 a 50; la maggior parte degli individui affetti sono identificati nel periodo neonatale mediante programmi di screening (obbligatori in diversi Paesi, quali l'Italia) sulla popolazione generale.

La HPA è espressa con una significativa variabilità di espressione fenotipica e con differenti gradi di gravità. La terapia alimentare, basata su una ristretta assunzione di fenilalanina, previene i danni neurologici.

I fenotipi dell'Iperfenilalaninemia sono definiti in base ai livelli di fenilalanina nel sangue alla nascita: Fenilchetonuria classica (PKU), con valori di fenilalanina nel plasma >1200 μmol/L (>20mg/dl); forma lieve di PKU con livelli di fenilalaninemia compresi tra 600 and 1200 μmol/L (10-20 mg/dl); Iperfenilalaninemia non-PKU (HPA- non PKU o MHP) quando i valori di fenilalaninemia sono al di sotto di 600 μmol/L (<10 mg/dl). Ad oggi, tramite l'analisi molecolare del gene PAH, sono state individuate circa 600 mutazioni (la maggior parte di tipo missenso) e oltre il 50% in condizione di eterozigosi composita. Il numero considerevole di mutazioni individuate rappresenta una consistente evidenza di eterogeneità allelica alla base della patologia che ne giustifica l'espressività variabile (anche nell'ambito di una stessa famiglia).

Il progetto ha avuto, innanzitutto, l'obiettivo di incrementare le conoscenze sui meccanismi molecolari e biochimici, ancora oggi non del tutto noti, che sono alla base dell'eziopatogenesi ed espressività variabile dell'HPA basandoci su

un'attenta analisi delle mutazioni responsabili della patologia in pazienti provenienti dal Sud Italia.

La caratterizzazione genotipica dei pazienti con elevati livelli di Phe individuati con lo screening neonatale viene spesso eseguita per completare la diagnosi. Da questi dati, abbiamo anche correlato i genotipi con le attività enzimatiche residue (PRA) ottenute in esperimenti di espressione in vitro ed elencate nel database PAHdb (*http://www.pahdb.mcgill.ca/*), eseguendo una correlazione genotipo-fenotipo.

Nella sezione finale della tesi, si è puntato l'attenzione sulle implicazioni della PKU a livello metabolico, con particolare attenzione alla risposta al BH_4: la quantificazione dell'attività dell'enzima PAH, espresso in cellule in coltura, è stata eseguita tramite la tecnica della spettrometria di massa tandem presso la sezione di malattie metaboliche della clinica universitaria di Heidelberg (Germania). La spettrometria di massa consente l'uso di isotopi stabili per la quantificazione della fenilalanina e della tirosina e la misura dell'attività dell'enzima PAH delle mutazioni PKU, consentendo di testare e predire la loro risposta al BH_4 in un sistema cellulare di mammifero.

In sintesi, i dati ottenuti in questo studio sulla frequenza e la distribuzione delle mutazioni del gene PAH rafforzano l'idea della notevole eterogeneità delle mutazioni nei pazienti HPA, con particolare riferimento al Sud Italia. Questo lavoro ha portato anche alla conclusione che il genotipo è il principale determinante del fenotipo biochimico nella maggior parte dei pazienti con deficit di PAH ed ha un grande valore nel determinare la risposta al cofattore. Inoltre, il calcolo dell'attività residua enzimatica proveniente dalle informazioni ottenute dai nostri esperimenti in vitro e da quelle disponibili nel database potrebbe essere utile per la previsione e/o l'esclusione di potenziali candidati per la terapia con BH_4.

I risultati qui presentati forniscono quindi una delucidazione sui genotipi PKU, sui fenotipi, e sulla risposta al BH_4 come riferimento per i medici, operatori sanitari e ricercatori per la diagnosi e la definizione di un trattamento su misura

dei pazienti. Un numero significativo di pazienti affetti da PKU potrebbero trarre beneficio dalla terapia col BH_4 che, combinata con una dieta meno rigida, o usata in casi particolari come monoterapia, potrebbe ridurre al minimo le carenze nutrizionali e le disfunzioni neurologiche e psicologiche, contribuendo ad una migliore qualità di vita di questi pazienti.

Parole chiavi: fenilalanina idrossilasi umana, fenilchetonuria, mutazioni, espressione *in vitro*.

Abstract

The hyperphenylalaninemia (HPA), an autosomal recessive genetic disorder, is the result of a defect of enzyme phenylalanine hydroxylase (PAH), resulting in the accumulation of phenylalanine (Phe) in the blood. In most cases (about 98%), the phenotypic expression of disease is the result of the presence of mutations in the PAH gene, coding for the enzyme PAH, with 12q24.1 locus on chromosome 12. The disease has a frequency in the Caucasian population of about 1 in 10,000 live births, corresponding to a carrier frequency of about 1 to 50; the majority of affected individuals are identified in the neonatal period by screening programs (mandatory in several countries, such as Italy) on the general population.

HPA is expressed with a significant variability of phenotypic expression and with different degrees of severity. Food therapy, based on a limited intake of phenylalanine, prevents neurological damage. Phenotypes of hyperphenylalaninemia are defined based on the levels of phenylalanine in the blood at birth: classical phenylketonuria (PKU), with values of Phe in the plasma >1200 μmol/L (>20 mg/dl); mild form of PKU with phenylalaninemia levels comprised between 600 and 1200 μmol/L (10-20 mg/dl); Non-PKU hyperphenylalaninemia (HPA-non PKU or MHP) when the values of phenylalaninemia are below 600 μmol/L (<10 mg/dl). To date, through the molecular analysis of PAH gene, about 600 mutations have been identified (the majority are missense) and over 50% of patients are composite heterozygous. The considerable number of identified mutations is a substantial evidence of allelic heterogeneity of the underlying pathology that justifies the variable expressivity (even within the same family).

The project aims first to increase the knowledge about the molecular and biochemical mechanisms of the disease, still not fully known, which are the basis of the etiology and variable expressivity of HPA, relying on the analysis

of gene mutations responsible of the disease in patients from Southern Italy. Genotyping of patients with elevated Phe levels detected in newborn screening is often performed to complete diagnosis. From these data, we also correlated genotypes with predicted residual activities (PRA) from in vitro expression experiments tabulated in PAHdb (*http://www.pahdb.mcgill.ca/*), performing a genotype–phenotype correlation.

The molecular bases of PKU and their implications at the metabolic level with focus on BH_4 responsiveness were addressed in the final section of thesis. The quantification of PAH activity expressed in cultured cells was performed by a tandem mass spectrometry assay at the section of Dietmar-Hopp-Metabolic Center of Universitatsklinikum of Heidelberg (Germany). Mass spectrometry allows the use of stable isotopes for Phe and Tyr quantification and PAH activity measurement of PKU mutations.

In summary, the data obtained in this study on the frequency and distribution of mutations in the PAH gene reinforce the idea of considerable heterogeneity of mutations in patients HPA, with particular reference to Southern Italy. This work has also led to the conclusion that the genotype is the main determinant of the biochemical phenotype in most patients with PAH deficiency and has greater value in estimation of BH_4-responsiveness. In addition, calculating the residual PAH activity from the information obtained from our in vitro experiments and those available in the database may be useful for predicting and/or exclusion of potential candidates for BH_4 therapy.

The results presented herein provide then a clarification on PKU genotypes, on phenotypes, and response to BH_4 as a reference available for clinicians, health care professionals and researchers for diagnosis and establishment of tailored treatment of patients. A significant number of PKU patients is likely to benefit from BH_4 treatment which, combined with a less strict diet, or in some cases as monotherapy, may reduce nutritional deficiencies and neurological and

psychological dysfunctions, contributing to a better quality of life of these patients.

Keywords: human phenylalanine hydroxylase, phenylketonuria, mutations, *in vitro* expression.

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Contributions to the publications

This thesis is based on the following papers.

I. **Mutation analysis in Hyperphenylalaninemia patients from South Italy.**

   Trunzo R, Santacroce R, D'Andrea G, Longo V, De Girolamo G, Dimatteo C, Leccese A, Lillo V, Papadia F, Margaglione M.
   Clin Biochem. 2013 Dec;46(18):1896-8.

II. **Intra-familiar discordant PKU phenotype explained by mutation analysis in three pedigrees.**

   Trunzo R, Santacroce R, D'Andrea G, Longo V, De Girolamo G, Dimatteo C, Leccese A, Lillo V, Papadia F, Margaglione M.
   Clin Biochem. 2014 Feb;47(3):233-5

III. **Phenylalanine hydroxylase deficiency in South Italy: Genotype–phenotype correlations, identification of a novel mutant PAH allele and prediction of BH_4 responsiveness.**

   Trunzo R, Santacroce R, D'Andrea G, Longo V, De Girolamo G, Dimatteo C, Leccese A, Bafunno V, Lillo V, Papadia F, Margaglione M.
   Clin Chim Acta. 2015 Oct 23;450:51-5.

Abbreviations

BBB	Blood-Brain Barrier
BH_2	7,8-Dihydrobiopterin
BH_4	(6R)-L-erythro-5,6,7,8-tetrahydrobiopterin ((6R)-BH_4)
bp	Base pair
CBR	Cofactor Binding Region
cDNA	Complementary Deoxyribonucleic Acid
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
DBS	Dried Blood Spots
DHFR	Dihydrofolate Reductase
DHPR	Dihydropteridine Reductase
DNA	Deoxyribonucleic Acid
GTPCH	Guanosine Triphosphate Cyclohydrolase
HMG-CoA Reductase	3-hydroxy-3-methyl-glutaryl-CoA Reductase
HPA	Hyperphenylalaninemia
HPLC	High-performance liquid chromatography Liquid
LC-ESI-MSMS	Chromatography Electrospray Ionization Tandem Mass Spectrometry

LNAA	Large Neutral Amino Acids
MHP	Mild Hyperphenylalaninemia
mRNA	Messenger Ribonucleic Acid
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
PAH	Phenylalanine Hydroxylase
PAHdb	Phenylalanine Hydroxylase Locus Knowledgebase
PAL	Phenylalanine Ammonia Lyase
PCR	Polymerase Chain Reaction
Phe	L-Phenylalanine
PKU	Phenylketonuria
PTP	6-pyruvoyl-5,6,7,8-tetrahydropterin
PTPS	6-pyruvoyl-tetrahydropterin Synthase
qBH_2	Quinoid-dihydrobiopterin
RNA	Ribonucleic Acid
SR	Sepiapterin Reductase
TH	Tyrosine Hydroxylase
TMS	Tandem Mass Spectrometry
TPH	Tryptophan Hydroxylase

Trp	L-Tryptophan
Tyr	L-Tyrosine

CHAPTER 1 –INTRODUCTION

1.1 PHENYLKETONURIA AND HYPERPHENYLALANINEMIA

1.1.1 HISTORY OF PHENYLKETONURIA (PKU)

PKU was first described by Asbjørn Følling, one of the first Norwegian physicians to apply chemical methods to the study of medicine. In 1934, the mother of two intellectually impaired children approached Følling to ascertain whether the strange musty odour of her children's urine might be related to their intellectual impairment. The abnormally excreted substance in urine of these children was identified as phenylpyruvic acid, a transamination product from Phenylalanine (Phe). In this way, in 1934, Følling described a biochemical disorder associated with intellectual disability, the first such description. Further investigations lead to the conclusion that the defect is due to elevated concentrations of Phe in the blood. The name, "phenylketonuria", was given to this disorder by Lionel Penrose, the first to recognize the importance of Følling's discovery and to identify the autosomal recessive nature of inheritance of PKU.

Phe is an essential amino acid for humans taken up with nutrition. The evidence soon emerged that Phe-low diet could prevent the metabolic phenotype and thus mental retardation. The knowledge that PKU can be treated successfully greatly improved patients' and families' lives. This was also the urge of the bacteriologist Robert Guthrie, successfully trying to develop a more accurate test for PKU and promoting the need to test all newborns. He developed the first practical screening test for PKU in the early 1960s. The Guthrie test is a bacterial inhibition assay using a drop of blood from a heel prick spotted on filter paper. The filter paper is applied to an agar gel containing Bacillus subtilis, which requires Phe for growth. Bacterial growth only appears in samples with elevated Phe levels and the size of the bacterial colony gives a rough estimate of the Phe concentration in the sample. This test was cheap, reliable and robust. Today, neonatal screening and the use of Phe-restricted

diets is accepted as clinically effective and cost effective, and is universally applied across most of the world.

PKU emerged as the prototype for treatable human inherited genetic diseases by early diagnosis and showed that a simple Mendelian phenotype can also be a complex disorder. The understanding of the links between gene, mutations, enzyme function, metabolism and clinical phenotype provide opportunities to better understand the pathophysiology of disease.

1.1.2 EPIDEMIOLOGY OF PKU

Today, almost all cases of PKU are identified through national neonatal screening programs. The prevalence of phenylketonuria varies widely around the world. It is highest in Caucasian or east Asian subjects, at about 1 case per 10 000-15 000 live births [1,2].

Persistent hyperphenylalaninemia is detected in about one in every 4 000 births in Turkey because of high consanguinity within the population, and in Northern Ireland. Finland has the lowest prevalence in Europe with one case per 100 000. In the USA the prevalence is one case per 15 000. In Latin America it varies from about 1 per 50 000 to 1 per 25 000 births, with a generally lower prevalence in the north. Estimates of prevalence rates in Asia vary from about 1 per 15 000 to 1 per 100 500 births in regions of China [3,4], less than one per 200 000 in Thailand [5], and about one per 70 000 in Japan. Spain differs from other European countries, having a relatively high incidence of relatively mild elevations of blood Phe arising from partial activation of PAH [6].

The prevalence of PKU in subjects of African or South Asian descent may be lower than the prevalence in Caucasian populations.

1.1.3 Diagnosis and Classification of Hyperphenylalaninemias

Hyperphenylalaninemia (HPA) and phenylketonuria (PKU; OMIM #261600) are a heterogeneous group of autosomal recessive disorders [7] with an incidence of 1/10 000 in Caucasians and a variable frequency in other populations. It is caused by a deficiency of phenylalanine hydroxylase (PAH, EC 1.14.16.1), a hepatic enzyme which catalyzes the conversion of phenylalanine (Phe) to tyrosine, using tetrahydrobiopterin (BH_4) as its coenzyme. The deficiency of PAH enzyme, which determines an accumulation of L-Phe in body fluids and the conversion to other metabolites toxic for the central nervous system, is caused by mutations in the PAH gene (GenBank accession no. U49897.1). Based on blood phenylalanine (Phe) levels, we can distinguish between mild hyperphenylalaninemia (MHP) with blood Phe levels less than 600 µmol/L (< 10mg/dl), mild PKU showing Phe levels between 600 and 1200 µmol/L (10-20 mg/dl), and classical PKU with Phe levels above 1200 µmol/L (> 20mg/dl) (Table 1). All infants are screened for PKU between the ages of 2 and 7 days, in order to allow timely dietary intervention to protect children with PKU from neurological damage. The newborn screening test involves the measurement of blood L-Phe levels by Guthrie test, a bacterial inhibition assay or by Tandem Mass Spectrometry (TMS). Normally, blood L-Phe levels are below 120µmol/L (2mg/dl). Some infants, particularly those born prematurely, may demonstrate immaturity of enzyme systems involved in amino acid metabolism, resulting in a transient elevation of blood Phe to a level sufficient to test positive in a PKU screening test. A second test is required for confirmation of HPA and to eliminate the possibility of transient HPA. The results of early PKU screening should also be interpreted with caution in sick neonates, due to interference with a Guthrie test by antibiotics [8]. If not diagnosed early, untreated or late-treated, classical PKU patients can develop severe intellectual disability, seizures, ataxia, motor deficits, and behavioural

problems, and in many cases, features of autism. Furthermore, this biochemical defect can result in a variety of cutaneous abnormalities, including diffuse hypo-pigmentation, eczema and photosensitivity.

	Blood Phe (µmol/L)
Mild HPA (no treatment required)	**120 - 600**
Mild PKU	**600 - 1200**
Classic PKU	**> 1200**
BH_4-responsive HPA/PKU	**> 360**

Table 1: *Classification of hyperphenylalaninemias according to blood Phe levels. Normal blood Phe levels are below 120 µmol/L.*

PAH requires BH_4 as a cofactor. About 1–2% of cases of hyperphenylalaninemia are due to mutations in genes coding for enzymes involved in BH_4 biosynthesis or regeneration pathways lead to disturbed Phe metabolism. The differentiation of a PAH defect and a defect in BH_4 metabolism is done by analysis of urinary or blood pterins and determination of dihydropteridine reductase (DHPR).

1.1.4 Pathophysiology of Phenylketonuria and outcome

The major effect of hyperphenylalaninemia (HPA) in PKU patients is on the brain function and development. Symptoms of untreated or late-diagnosed PKU patients are generally summarized as mental retardation, but the clinical effects of HPA/PKU are highly variable (several intellectual disability, seizures, ataxia, motor deficits, behavioural problems, and, in many cases, features of autism). In

adults, anxiety disorders and depression have been reported [9,10,11]. Microcephaly and impaired growth were revealed in adult non-treated PKU patients. A characteristic of classic PKU already known from 1930s is a mousy odor that results from the excretion of phenylketone bodies in the urine. Hypopigmentation in PKU patients is a result of deficient melanin synthesis inhibited by the elevated Phe levels [12]. Many of these symptoms are only rarely observed nowadays because of the introduction of neonatal screening. Children with a late diagnosis and start of treatment can still markedly benefit from dietary treatment. Cognitive performances may improve in children as well as intellectual functioning, behavior and improved quality of life but they depend on the age at which the treatment is started. Nevertheless, intellectual disabilities remain and chronic neurotoxic consequences from high Phe levels are irreversible.

All these symptoms led to the conclusion that a PAH deficiency mainly affects the central nervous system and leads to impaired brain development and function.

The pathogenesis of brain dysfunction is not completely known. However, there are several hypotheses addressing possible causes of the neurotoxicity. It is highly likely that the neurocognitive damage in PKU is caused by different processes, all occurring together, discussed briefly below:

- Impared LNAA uptake into brain and dopamine and serotonin synthesis

The most important neurotoxic pathophysiological mechanisms are thought to be the direct effects of high Phe levels in combination with reduced levels of brain large neutral amino acids (LNAAs). Phe’s entry into the brain is mediated by the large neutral aminoacid carrier L-aminoacid transporter 1 (LAT1). The other LNAA, valine, leucine, isoleucine, methionine, threonine, histidine, tryptophan and tyrosine, are also using this route in a competitive manner [13]. In addition, for each LNAA taken into the brain, the LAT1 transporter excretes one LNAA. Moreover, the LAT1 transporter shows highest affinities for Phe among all the LNAA’s. High concentrations of Phe in the blood can inhibit LAT1 and other large neutral amino acids, including tyrosine (Tyr) and tryptophan (Trp), from

entering the brain, increasing the potential for neurotransmitter dysfunction and their availability for protein synthesis. The low levels of Tyr and Trp might contribute to the depletion of the neurotransmitters dopamine and serotonin. The dopamine depletion in patients with PKU, either due to a deficiency of its precursor Tyr or secondary to hypomyelinisation, might play a prominent role in the development of the neurocognitive impairments. Since the prefrontal cortex is highly susceptible for changes in Tyr levels, the deficits established in PKU patients are frequently specific to the cognitive functioning of the frontal lobes of the brain, which is rich in nerve endings from dopamine-containing neurons, although not all studies support this hypothesis. In addition, cerebral serotonin deficiency may explain the increased occurrence of anxiety and depression disorders in PKU patients. Synthesis of serotonin occurs by hydroxylation of tryptophan by tryptophan hydroxylase. Like dopamine, tryptophan competes with Phe across the BBB. At elevated plasma Phe concentrations, brain tryptophan concentrations, and consequently brain serotonin levels, might be reduced. Although dopamine and serotonin are involved in postnatal brain development and maturation, these findings do not explain the severe mental retardation of PKU patients, but they are likely to explain certain cognitive deficiencies.

- Altered myelin metabolism (white matter abnormalities)

Myelin is a cerebral protein often found abnormal in PKU and associated with white matter abnormalities detected in PKU patients. High concentrations of Phe and/or reduced availability of other LNAA may inhibit the development of myelin formation in untreated patients, and to oedema within myelin in early-treated patients with PKU. These lesions may be reversible over a period of months following the achievement of improved Phe control. White matter damage on magnetic resonance imaging has been correlated with cognitive impairment in some patients with PKU. However, such structural impairments usually do not correlate with functional neuropsychological status, and impaired

myelin function may not completely account for the principal pathophysiological defect in PKU.

- Other mechanisms

Other possible mechanisms for hyperphenylalaninaemia-induced damage to the brain include reduced activity of pyruvate kinase, disturbed glutamatergic neurotransmission, reduced activity of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), and the function of monoamine oxidase B as a modifying gene (Figure 1). HMG-CoA reductase is a rate-limiting enzyme in the metabolic synthesis pathway of cholesterol.

As protein and cholesterol are essential parts of myelin, this finding is in line with observations of hypomyelination and gliosis in brain cells of PAHenu2 and wild type mice and in brains of deceased PKU patients [14].

It is however not clear, whether impaired cholesterol synthesis leads to reduced myelination in PKU or whether synthesis of HMG-CoA reductase is reduced, suggesting that reduced cerebral protein synthesis may affect enzymes in myelin formation. Finally, DNA damage and oxidative stress may also play a role on PKU pathogenesis [15,16]. The accumulation of toxic metabolites may lead to the induction of free radical production, increasing the risk of oxidative stress of tissue damage in PKU.

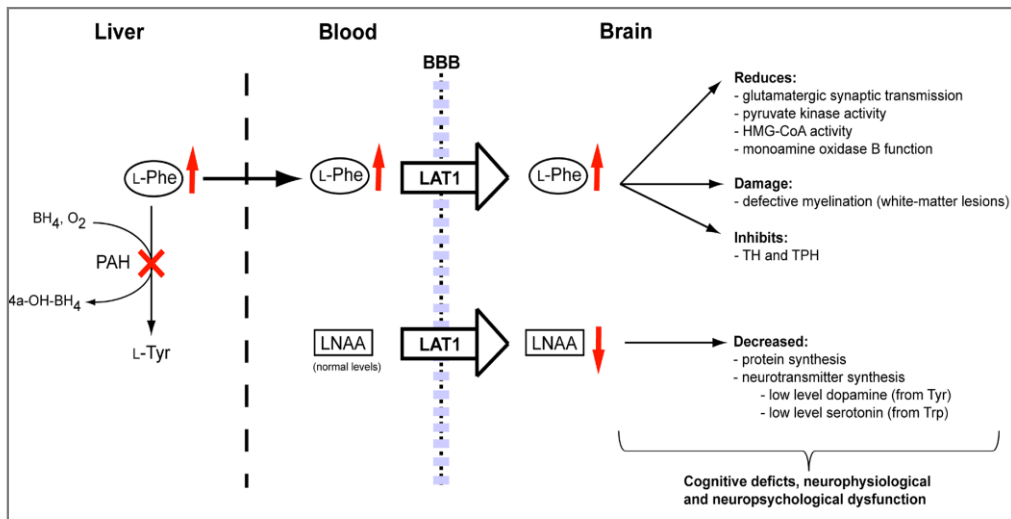


Figure 1. *Potential mechanisms of neurocognitive impairment by hyperphenylalaninemia (HPA). (Figure adapted from Feillet et al. 2010.).*

Patients with non-phenylketonuric hyperphenylalaninaemia have a lower risk of neuropsychological dysfunction than do those with phenylketonuria, although compared with healthy controls, some might have decreased executive functioning. Despite most development of the brain occurring in the early years of life, it seems that discontinuation of dietary management of phenylketonuria during adolescence leads to subtle but measurable deficits in neuropsychological functioning during adult life. The universal experience of those caring for individuals with phenylketonuria is that the dietary treatment results in a pronounced improvement in cognitive outcome but imposes a social burden.

1.1.5 PKU MATERNAL SYNDROME

Another important health concern related to PKU pathogenesis is the relation between prenatal L-Phe exposure and the offspring outcome. During pregnancy, Phe crosses the placenta by active transport, resulting in 70% to 80% increased fetal concentration of Phe compared with maternal concentration. An elevated Phe concentration is toxic and teratogenic to a developing fetus.

Untreated maternal phenylketonuria or hyperphenylalaninemia during pregnancy may lead to maternal phenylketonuria syndrome in the neonate. This syndrome consists of low birth weight, microcephaly, congenital heart disease, intellectual or developmental disability and facial dysmorphisms. In this respect, women of childbearing age with all forms of phenylketonuria, including mild variants such as mild hyperphenylalaninemia, are advised to use a strict diet that starts before conception to prevent teratogenic effects in the fetus. Normal pregnancy and neonatal outcome have been achieved in women with PKU who have blood Phe concentrations between 120 and 360 μmol/L before conception or by 8 weeks of gestation at the latest. Achieving this degree of control requires a major commitment by the woman and support by her treating professionals, continually throughout pregnancy, is important, as cognitive outcome in these offspring is better than in children whose mothers began or resumed dietary phenylalanine restriction after conception. Offspring born to women who are fed with a normal diet, unless, as in rare cases, also have phenylketonuria. Mothers with maternal phenylketonuria can breastfeed their non-phenylketonuria infants without restriction. These infants carry a mutant gene for phenylketonuria but their residual PAH activity is sufficient to adequately metabolize phenylalanine, even the additional amount they receive from their mother´s breast milk [7].

1.1.6 PKU ACTUAL TREATMENT: THE LOW PHE DIET

The aim of PKU treatment is the reduction of blood Phe to a level allowing normal brain development. An individual's blood Phe depends upon dietary intake of Phe and the residual activity of Phe hydroxylase. As stated in 1.1.3 and 1.1.4 sections, PKU related symptoms are severe, therefore it is necessary to treat this disease since birth. At present, PKU is treated with a lifelong dietary protein restriction, in which many common foods, such as milk and dairy products, meat, eggs, wheat, beans, corn, peanuts, lentils, and other grains, are prohibited to the patients (see also Figure 2).

Figure 2. Low Phe diet: foods that are allowed are in the inner part of the diagram.

A semi-synthetic diet is used which comprises:

- foods of low phe content in unlimited amounts such as many fruits and vegetables;

- weighed amounts of foods containing medium amounts of Phe (e.g. broccoli, potato). The amount of Phe ingested is often calculated using an exchange system. In the UK system 1 ‘exchange’= 50 mg Phe which is approximately 1 g protein;
- Phe-free amino acid mixtures to provide normal total protein intake;
- vitamins, minerals and trace elements.

The diet should be strictly followed with these food groups evenly distributed throughout the day. Aspartame should be avoided as it contains large amounts of Phe. Infant formulae feeds which are Phe-free are available; many contain added essential fatty acids. These are used in conjunction with a small amount of standard infant formulae. It is possible to continue breast feeding even in severe PKU by giving a measured amount of Phe-free formula prior to a breast feed. All PKU diets should be administered with the advice of a specialist dietician [17].

1.1.7 Alternatives approaches to treat PKU

As mentioned in 1.1.6 section, PKU related symptoms can be prevented by a strict dietary restriction from early infancy. Although it is recognized that dietary treatment initiated early in life is successful in avoiding the severe mental retardation of PKU, this treatment presents many limitations, since PKU patients must adhere to an unpalatable, expensive and lifelong diet that is frequently stopped prematurely, leading to an unsatisfactory clinical outcome.

As long-term dietary compliance is difficult, there is a need for alternative modes of treatments:

- Treatment with large neutral amino acids (LNAAs)

Because phenylalanine competes with other LNAAs for transport (LAT 1) across the blood–brain barrier, supplementation with these aminoacids other than Phe, could provide a potential treatment approach.

In fact, LNAA administration was shown to reduce toxic Phe levels in the brain of patients despite constantly elevated serum concentrations [18,19,20].

In this way, other amino acids with high affinity for the BBB transport system keep high plasma Phe concentrations from entering the brain and it seems that this treatment has a beneficial effect on executive functioning [7]. It may not replace Phe-low diet, at least in childhood or in pregnant women with PKU, but may help to relax dietary restriction of treated individuals or help in management of untreated adults with classic PKU. However, further clinical trials and data are required to examine safety and efficacy of LNAA therapy still in development.

- Glycomacropeptide

Another strategy is the supplementation of glycomacropeptide (GMP). Glycomacropeptide is a natural protein from cheese whey, that is rich in specific essential amino acids but contains no Phe, Tyr, or tryptophan (Trp).

It is being investigated as a useful adjunct to dietary treatment for PKU by allowing a greater intake of 'natural' protein in the diet, thus, allowing a decrease in artificial amino acid mixture. It has been shown in the mouse model to improve bone density [17] and some studies have given promising results also in terms of palatability, safety and improved compliance, but further evidence is still required [21,22,23].

- Enzyme substitution therapy

Enzyme therapy can be addressed in two different ways, either by replacing PAH or by substitution with a foreign protein capable of metabolizing Phe. Replacement of PAH is highly challenging, as the whole, intact multi-enzyme complex for PAH catalysis including BH_4-cofactor is required. The enzyme of choice for substituting PAH is phenylalanine ammonia lyase (PAL, EC 4.3.1.5), derived from plants and compared to PAH, sufficiently more stable as oral formulation. PAL targets the intestinal system and is also involved in Phe metabolism, capable of lowering blood Phe in humans. This exogenous enzyme converts Phe to transcinnaminic acid and negligible amounts of ammonia without the need of a cofactor, making it catalytically less complex than PAH. In the mouse model of phenylketonuria, blood and brain concentrations of Phe were reduced during 90 days of treatment with injections of PAL. The PEGylation (attachment of polyethylene glycol polymers to lysine side chains) diminished immune-mediated detection and elimination of the injected enzyme [24]. Clinical trials have been initiated in PKU patients and preliminary results showed a significant decrease in Phe blood levels after a single injection. A phase II clinical trial is in progress in addition to development of an orally administrable PAL form [25]. PAL would probably be used in addition to a less stringent Phe diet.

- BH_4 therapy

Some mutations are associated with a BH_4-sensitive phenotype of phenylketonuria, in which giving pharmacological doses of exogenous BH_4 results in an increase in the activity of PAH that is sufficient to reduce circulating Phe with a therapeutically relevant effect. These mutations usually present with substantial residual activity when expressed in eukaryotic cell systems and are located in all regions of PAH. However, the relation between

genotype and BH_4-responsiveness is complex. Thus, although genotyping is useful in excluding non-responders (classic phenylketonuria), it is insufficient for a reliable prediction of those who are BH_4 responsive. Mechanisms of BH_4-responsiveness are multifactorial, but the main mechanism seems to be stabilisation of the PAH tetramer by preventing misfolding, subunit aggregation, proteolytic degradation, and thermal inactivation [7].

Since 2008, sapropterin dihydrochloride (Kuvan® or Biopten®, BioMarin Pharmaceutical Inc), the synthetic analogue of BH_4, has been market approved for treatment and is commercially available in the US, Japan and Europe.

It acts as a pharmacological chaperone by stabilizing PAH. In BH_4-responsive patients, BH_4 decreases the blood Phe concentration and/or increases the dietary Phe tolerance. Correct and efficient identification of BH_4-responsive patients is important, both to improve the fast assessment, as well as to avoid false expectations and unnecessary costs. Unfortunately, there is still no golden standard on how to assess BH_4 responsiveness most efficiently. Three methods have been proposed for the prediction of BH_4 responsiveness: the 7–28 days BH_4 challenge, the 48-hour BH_4 loading test and, the START (Sapropterin Therapy Actual Response Test) BH_4 challenge and genotyping protocol. Genotype was frequently reported to be useful in predicting or excluding BH_4 responsiveness [26]. The proportion of BH_4-sensitive patients increases as the severity of the phenotype decreases, which is due to the mechanistic action of administered BH_4, requiring sufficient active hepatic PAH protein (residual enzyme activity). The online BIOPKU database (*www.biopku.org*) tabulates available data on almost 800 genotypes, phenotypes and BH_4-response in patients previously tested and is used as a reference tool in consulting whether to challenge a patient based on previous similar genotype results.

- Pharmacological Chaperons

The use of pharmacological chaperones to stabilize or promote correct folding of mutant proteins represents a promising new approach in the treatment of many genetic diseases causing protein misfolding. Proteins and small molecules in addition to tetrahydrobiopterin may act as chaperones to assist in the folding of PAH [27].

- Gene therapy

Gene therapy for the treatment of PKU has been the focus of multiple research groups over the last two decades. In a mouse model of PKU, important progress has been made by the use of an adenovirus related gene directed into the liver [28].

However, the vector's genome is gradually eliminated as it is not integrated into the hepatocyte's DNA and re-injection was not effective due to immunological responses. Studies of PKU mouse models have also shown that gene therapy can be successfully delivered to non-hepatic tissues such as the muscle. It is easily accessible and does not undergo cell division. In fact, the insertion into the muscle cells of vectors containing the necessary genes for PAH and tetrahydrobiopterin synthesis results in a system that could convert phenylalanine into tyrosine, mimicking the role of hepatic phenylalanine metabolism [29].

- Liver Transplantation

Hepatocyte transplantation has been performed in preclinical studies using various animal models, as well in humans with metabolic disorders, such as urea cycle defects or glycogen storage disorders. This cellular approach could be possible for the permanent treatment of PKU if a selective growth advantage

could be achieved for donor hepatocytes. This treatment has been reported to be successful in an animal model with a selective advantage for the donor cells. However, cell based therapies using stem cells or more differentiated progenitor cells may represent the future of cell transplantation for treatment of metabolic liver diseases such as PKU [27].

1.2 THE PHENYLALANINE HYDROXYLASE SYSTEM

1.2.1 STRUCTURAL BASIS AND REGULATION OF PHENYLALANINE HYDROXYLASE

PAH is an hepatic monooxygenase that catalyzes the conversion of Phe to Tyrosine (Figure 3), using 6R-lerythro-5,6,7,8-tetrahydrobiopterin (BH_4) as a coenzyme. Molecular oxygen and iron are essential for the hydroxylation of Phe to Tyrosine.

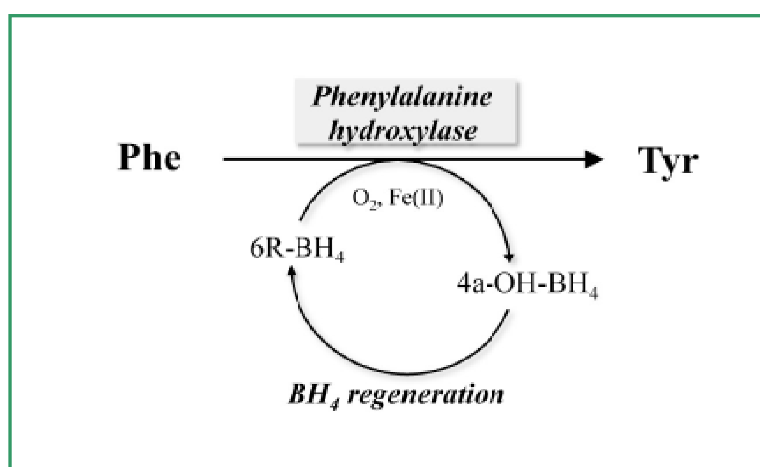


Figure 3. *Overview of the reaction catalyzed by phenylalanine hydroxylase.*

The structure of PAH is similar to the other principal aromatic amino acid hydroxylases, tyrosine hydroxylase (TH) and tryptophan hydroxylases (TPH) 1 and 2. The size of an individual subunit of PAH is about 50kDa, and the active enzyme exists as dimer or (mainly) tetramer of these subunits, with these forms

existing in equilibrium, according to cytosolic pH. Each subunit of PAH consists of three structural and functional domains:

- ❖ N-terminal regulatory domain (residues 1-142);
- ❖ Large catalytic domain (residues 143-410), which contains the active site with the iron center and the binding sites for Phe and BH_4;
- ❖ Small C-terminal tetramerization domain (residues 411-452), which mediates the association of subunits into tetramers (Figure 4.).

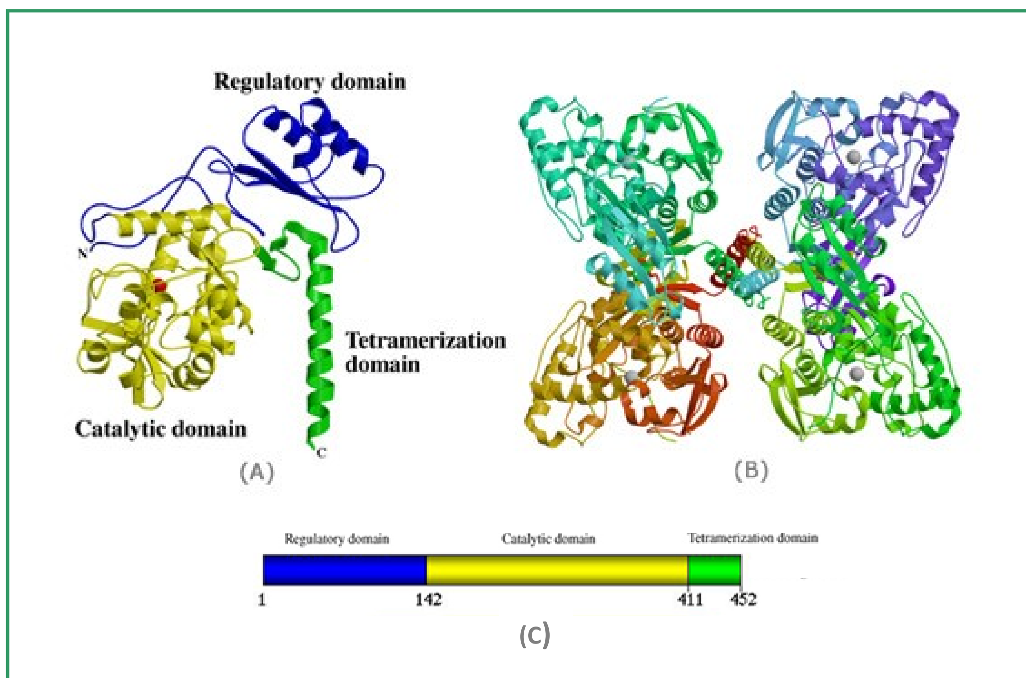


Figure 4. *3D crystal structure of the human PAH. A: Interactive site, the iron atom is shown in red. The N-terminus starting over the active site, as well as the rest of the regulatory domain is highlighted in blue; catalytic domain in yellow; and tetramerization domain is in green. B: The native tetramer form of the enzyme. C: Structure of a monomer of human Phenylalanine Hydroxylase full-length composite model. In figure are shown the regulatory domain (residues 19–142), the catalytic domain (residues 143–410) and the tetramerization domain.*

1.2.2 Catabolic Pathway of Phenylalanine

The L-Phe amino acid is hydroxylated by the enzyme PAH to form Tyr. PAH is an iron-containing enzyme depending on 6(R)-L-erythro-tetrahydrobiopterin (BH_4) as cofactor and molecular oxygen for efficient catalysis. During the reaction, BH_4 is oxidized to 4a-hydroxypterin and needs to be regenerated for continuous catalysis. The regenerating enzymes pterin-4a-carbinolamine (PCD) and dihydropteridine reductase (DHPR) complete the phenylalanine hydroxylating system (Figure 5).

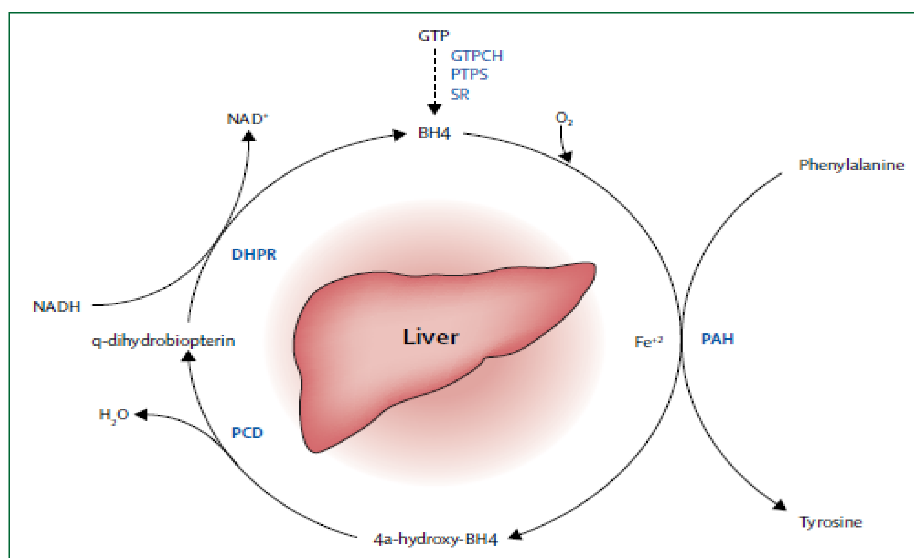


Figure 5. *Phenylalanine hydroxylating system: during the hydroxylation of phenylalanine by phenylalanine hydroxylase (PAH), and when molecular oxygen (O_2) and iron (Fe+2) are present, tetrahydrobiopterin (BH_4) is oxidised to a 4a-hydroxy-BH_4 intermediate, which is subsequently regenerated back to BH_4 via quinonoid (q) dihydrobiopterin by the enzymes carbinolamie-4adehydratase (PCD) and by the NADH-dependent dihydropteridine reductase (DHPR). BH_4 is synthesised from guanosine triphosphate (GTP) by three additional enzymes GTP cyclohydrolase I (GTPCH), 6-pyruvoyl-tetrahydropterin synthase (PTPS), and sepiapterin reductase (SR). Mutations in genes coding for PCD, DHPR, GTPCH, PTPS, and SR result in BH_4 deficiency.*

Tyr, via secondary biosynthetic pathways, is then converted in several products:

catecholamines (dopamine, adrenaline and noradrenaline), melanin and thyroid hormones.

Therefore, the biosynthesis of the neurotransmitter monoamines dopamine, norepinephrine and serotonin is dependent upon the availability of the precursor amino acids Tyr and Trp within the brain and the presence of a normal Phe concentration. A defect in PAH system, leading to excess Phe and a relative lack of Tyr and Trp in the brain, has consequences for the subsequent enzymatic reactions.

To maintain Phe homeostasis in vivo, PAH is highly sensitive to changes in substrate concentrations and its activity is tightly regulated and requires the binding of all three of its substrates- Phe, molecular oxygen and the cofactor BH_4– prior to any reaction occurs. The first 30 residues of PAH act as an autoregulatory sequence that includes the Ser16, a substrate for cAMP-dependent protein kinase and is termed autoregulatory, as it sterically limits substrate access unless Phe binding to the regulatory domain activates the enzyme.

The activation by the Phe substrate results in a significant increase in the initial rate of Tyr formation [30]. Activation is probably related to a conformational change induced in the PAH protein upon binding which is transmitted throughout the enzyme, displacing the autoregulatory sequence and leading to the propagation of the activating process to the adjacent subunit in the dimer and finally to the other dimer through the oligomerization domain. While Phe activates the enzyme, at the same time BH_4 acts as an inhibitor in addition to its role as a cofactor, keeping the enzyme in a low activity state, and blocking the substrate-activating conformational change [31]. BH_4 interacts with the N-terminal autoregulatory sequence and leads to a dead-end PAH–BH_4 complex, closing the entrance to the active site and leaving the enzyme in a latent, low-activity state [32,33,34]. Phe binds with lower affinity to PAH than the cofactor BH_4. The high affinity binding of BH_4 and the inhibitory regulatory effect in non-Phe activated PAH are associated with specific interactions of the BH_4 side

chain with residues from the catalytic and regulatory domain. The iron atom is also essential for catalytic activity. It is coordinated to two histidine residues (H285 and H290), as well as to one oxygen from E330. The rest of the coordination sites of iron are occupied by water molecules, which are all displaced upon substrate and cofactor binding. This leaves an open coordination site for the reaction with O_2 and generates an activated intermediate in the hydroxylation of the Phe and BH_4.

Therefore, the composite model of full-length PAH provided an important basis for analysis of the numerous mutations resulting in deficient PAH activity.

1.3 The Cofactor Tetrahydrobiopterin (BH_4)

1.3.1 Cofactor Biosynthesis and Functions

BH_4 is the short name of its correct chemical name 2-amino-4-hydroxyl-6-[L-erythro-1’,2’-dihydroxypropyl]-tetrahydrobiopterin. BH_4 is essential for many diverse processes and ubiquitously present in all tissues of higher organisms. Consequently, BH_4 plays a key role in a number of biological processes and pathological states associated with monoamine neurotransmitter formation, cardiovascular and endothelial dysfunction, the immune response and pain sensitivity. BH_4 is formed de novo from GTP via a sequence of three enzymatic steps carried out by GTP cyclohydrolase I (GTPCH), 6-pyruvoyltetrahydropterin synthase (PTPS) and sepiapterin reductase (SR). Detailed reviews of the biosynthesis of BH_4 have been provided in Figure 6.

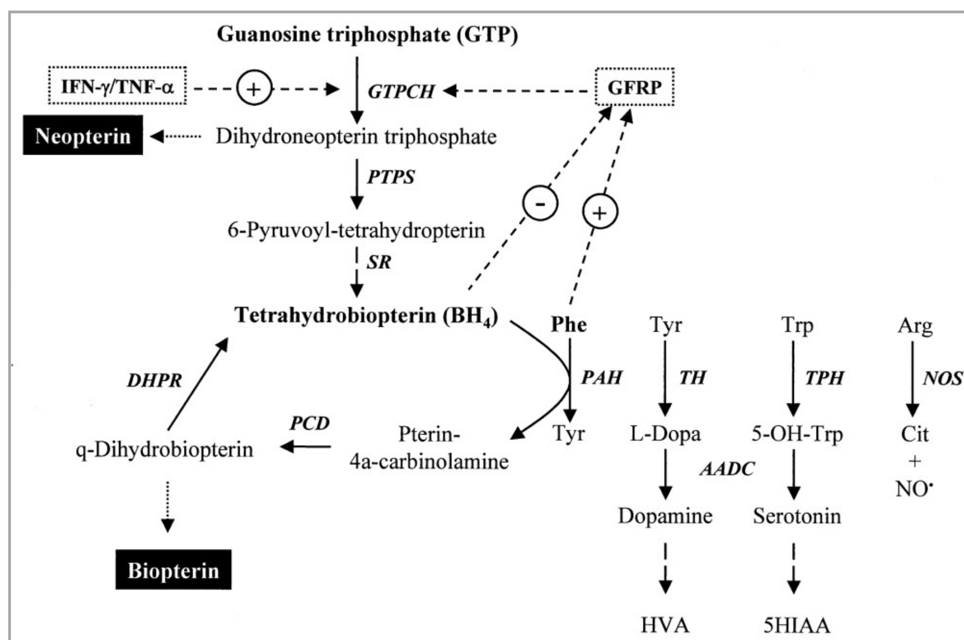


Figure 6. *Overview of the biosynthesis of tetrahydrobiopterin (BH_4) and a summary of its actions as a cofactor in enzymatic reactions.*

In the last two steps of the novo biosynthetic pathway, 6-pyruvoyl-tetrahydropterin is thought to be converted to tetrahydropterin derivatives with a different succession of side chain reductions involving SR but potentially also alternative carbonyl and aldose reductases.

BH_4 itself is hydroxylated during the aromatic amino acid reactions of PAH, TH and TPH. It is therefore essential that the cofactor be regenerated to ensure a continuous supply of reduced cofactor and to prevent accumulation of harmful metabolites. BH_4 is oxidized to BH_4-4a-carbinolamine and via two further catalytic reactions and quinoid dihydrobiopterin (qBH_2) intermediate reduced to BH_4. The enzymes involved are PCD and DHPR.

BH_4 acts not only as a cofactor of the aromatic amino acid monooxygenases as described above. Additional functions on the cellular level were found upon the discovery that BH_4 is essential for all three isoforms of nitric oxide synthase (NOS) [35]. Nitric oxide synthases convert L-arginine to nitric oxide and L-

citrulline in two-step reactions [36]. The role of BH_4 in these reactions is different from aromatic amino acid monooxygenases. BH_4 is not involved in oxygen activation, donates only one electron, and is regenerated without the need for external enzymes. Werner E. et al. recently compared the mechanisms involving BH_4 in hydroxylases and NOSs [37]. The synthases are ubiquitously involved in vascular and cardiac functions, establishing a role for BH_4 in diseases like hypertension, diabetes, atherosclerosis, cardiac hypertrophy and failure, but also Parkinson's and Alzheimer's disease.

1.3.2 ROLE OF BH_4 AS A MOLECULAR CHAPERONE

BH_4 supplementation is known to result in increased enzyme activity. Besides this non-chaperon stimulatory effect of BH_4 supplementation, the binding of BH_4 to PAH may also increase the stability of protein and prevent its degradation, a phenomenon described as a "chaperone" effect.

The term *molecular chaperones* identifies the large molecular machines that in an energy-dependent manner ensure the correct folding of intracellular proteins. Together with the ubiquitin-proteasome and the selective autophagy systems, molecular chaperones constitute the quality control system acting at different subcellular localizations, working in concert to reduce the accumulation of misfolded proteins by either refolding or destroying them (Figure 7).

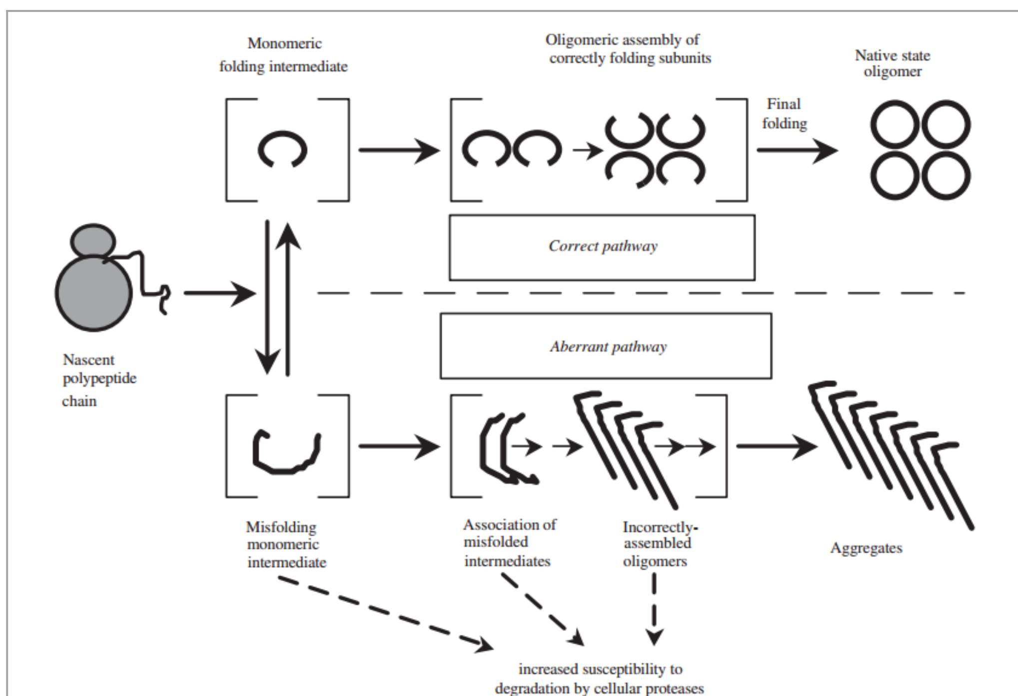


Figure 7. *Model of competing pathways for correct vs. aberrant protein folding and assembly (Figure based on ref. [38]).*

Many of the point mutations on the PAH sequence leading to missense in translation were found to affect protein folding and assembly leading to secondary effects on enzyme function. They can lead to increased aggregation and destabilized native conformation, causing loss-of-function pathologies (or eventually gain-of-function). The aggregates or nonfunctional conformations are no longer removed by the cells quality control systems like proteases. The protein architecture of PAH is very sensitive to single point mutations throughout the protein sequence. It results in a greater proportion of protein entering the aberrant pathway, where it is more susceptible to proteolytic degradation in human cells than protein proceeding through the pathway of correct folding and assembly.

However, BH_4 was shown to favour the formation of tetrameric PAH where specific mutations may have impaired the association of PAH dimers into

tetramers, and thus increased the stability of the PAH protein and slowed its inactivation in vitro [39,40].

These data suggest that molecular chaperones, like BH_4, can be an efficient treatment option for PKU, although further development regarding mechanism and action of clinical effects is required before application in patients is possible.

1.3.3 Inborn errors in Tetrahydrobiopterin Metabolism

About 2% all Phe level elevations detected by the newborn screening (higher in some countries where frequent consanguinity tends to maintain the presence of genetic disorders within families, e.g. Turkey or Saudi Arabia) are due to disorders in BH_4 metabolism, highlighting the importance of always considering the differential diagnosis for every even slightly elevated blood Phe level. BH_4 deficiencies are more severe than PKU with regard to their response to therapy and treatment is substantially different. Low-Phe diet is not effective and early substitution with dopamine and serotonin precursors, as well as with the synthetic BH_4 is crucial for a good outcome. Analysis of dried blood spot (DBS) or urine for neopterin and biopterin and measurement of DHPR activity in the DBS is essential for the exact diagnosis and should be performed as early as possible. A BH_4 loading test and measurement of neurotransmitter metabolites, pterins, and folates in cerebrospinal fluid add further important information about the severity of the disease. Some patients with DHPR deficiency show a normal blood or urinary neopterin and biopterin profile. Therefore, DHPR activity measurement is essential in all patients with HPA, regardless of pterin measurements [41].

BH_4 deficiencies arise from mutations in the genes coding for the enzymes in BH_4 biosynthesis and regeneration pathways. Due to the secondary disturbance in aromatic amino acid hydroxylase metabolism, they also affect central

nervous system neurotransmitter biosynthesis and generally lead to severe and heterogeneous phenotypes. BH_4 deficiencies are treatable diseases although with variable outcomes. The clinical signs and symptoms of a BH_4 deficiency largely arise from neurotransmitter depletion and compromised nitric oxide synthesis [42]. The few patients that present with a mild phenotype generally show normal brain neurotransmitter metabolism and require only BH_4 monotherapy. Symptoms of BH_4 deficiency may develop only after a few weeks or months of life. Therefore, most patients are initially maintained on a low-Phe diet until final diagnosis. All BH_4 deficiencies associated with elevated Phe levels are inherited in an autosomal recessive manner and often share common clinical symptoms. Clinical features manifested with GTPCH, PTPS and DHPR deficiencies are abnormal movements together with impaired tone and posture, convulsions, seizures, mental retardation, as well as light pigmentation and microcephaly.

1.4 Molecular Genetics

1.4.1 The PAH Gene

The human phenylalanine hydroxylase gene (PAH) is located on chromosome region 12q22 - 12q24.2 containing the nucleotide sequence coding for the hepatic enzyme phenylalanine hydroxylase (PAH, phenylalanine 4-monooxygenase, EC 1.14.16.1). The PAH gene is 90 kb long and consists of 13 exons and their required introns, adding up to 171 kb including the flanking regions, encoding a 51.9 kDa polypeptide sequence with 452 amino acids. The exonic sequences in PAH take up less than 1% of the genomic sequence. The complete sequences have been catalogued in GenBank under NM_000277 (mRNA, 2680 bp), U49897.1 (cDNA, 1359 bp), AF404777 (gDNA, 171 kb)

and NP_000268.1 (protein, 51.9 kDa) or in Ensembl under the reference number ENSG00000171759.

PKU arises from mutations on this gene and is inherited as an autosomal recessive disorder. Different possibilities of a child to develop PKU, according to the PAH status of its parents is depicted in Figure 8.

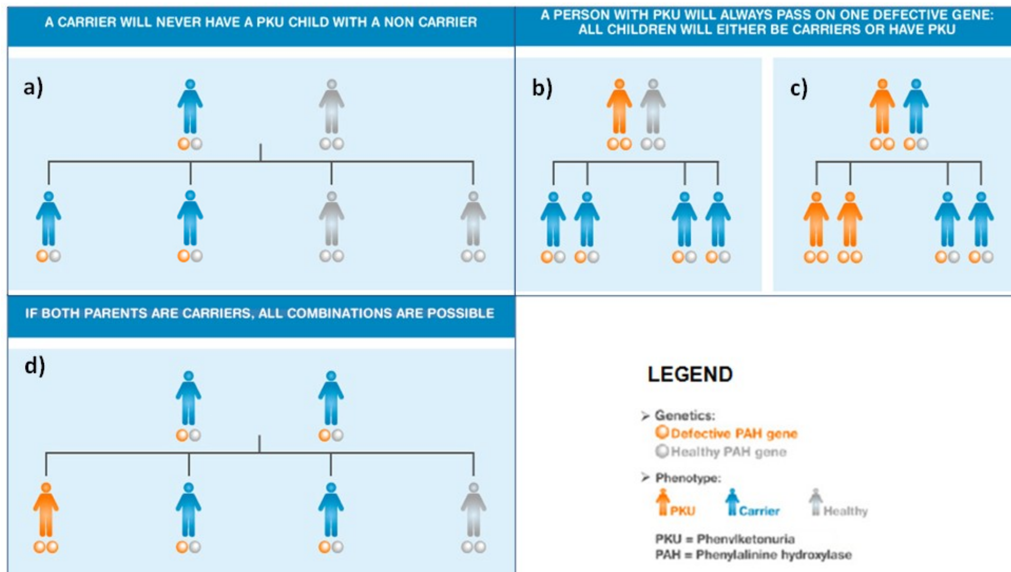


Figure 8. *Overview of the mode of inheritance of PKU (Figure adapted from website http://www.pku.com/en/What_is_PKU/Who_gets_PKU/who_gets_pku.html).*

Thus, if only one parent has a defective copy of the PAH gene, offsprings have a 50% chance of being a carrier themselves, with no possibility of inheriting clinical PKU (Figure 8. panel a). All children of a parent with PKU will have at least one defective copy of PAH, but at risk of PKU only if the other parent carries a PAH mutation that affects its activity (Figure 8. panels b and c). An offspring of parents who each have one defective copy of the PAH gene has a 25% chance of having PKU, a 50% chance of being a carrier of a PAH mutation, a 25% chance of inheriting two fully functional copies of the PAH gene (Figure 8. panel d). Finally, all children of two parents with PKU will inherit the disease.

1.4.2 Causative Mutations

The PAH gene is the only gene associated with PAH deficiency, which results from mutations in both alleles disturbing enzyme function. Isolation and sequencing of PAH cDNA opened new fields for intense exploration of the molecular causes of PKU. The Human PAH Mutation Knowledgebase (hPAHdb), a database of naturally occurring mutations in the human PAH gene, was summarised in 2009 and includes a total of 567 separate mutations. Since the latest updates of this database roughly 61% of all mutations listed are missense mutations, followed by small deletions (13%), splice mutations (11%), silent and nonsense mutations (5 - 6%), and small insertions (2%).

The position and nature of the mutation dictates its effect on the activity of the PAH enzyme, which determines the hyperphenylalaninemic phenotype of the patient. Little or no enzyme activity (for example p.R408W, the most common mutation) results in the classic phenylketonuria phenotype. Other mutation, such as p.E390G, p.Y414C and p.A300S only partly inhibit enzyme activity so that dietary Phe tolerance is higher, giving rise to mild phenylketonuria or mild hyperphenylalaninemia. The majority of mutations are scattered over the entire gene length but with a different frequency in distinct populations and geographic areas and a number of them have been analyzed and characterized. Thanks to different studies, it is now known that most of PAH missense mutations result in a misfolding of the protein which increases its turnover both in vitro and probably in vivo, pointing to a decreased conformational stability as the major molecular mechanism for the loss of PAH function in PKU [43].

1.4.3 Genotype-Phenotype correlations

The compilation of all information about PAH mutations and establishment of genotype-phenotype correlations is highly useful in predicting the course of disease for a patient’s diagnosis. The ability to predict the

phenotype already in a newborn with PAH deficiency not only enables the design and early implementation of an optimal dietary regimen, it also greatly improves counseling of the patient's family.

The BIOPKU database (*www.biopku.org*) was created at the University of Zürich and it is based on the genotype and phenotype data of over 9700 PAH-deficient patients from all over the world. More than 900 different PAH gene variants are tabulated in the database and these result in almost 2000 different genotypes. More than 75% of all PKU patients are compound heterozygotes bearing two different variants. Some of the mutation combinations of patients listed are not always associated with the same phenotype. In general, genotype-phenotype correlation is possible, but there are always exceptions. Mutations for which inconsistencies regarding genotype/phenotype correlation are reported in this database as well as in the literature, are the R261Q mutation in the homozygous state or in combination with the R158Q mutation, the L48S mutation in the homozygous state or in combination with the R158Q mutation, and the Y414C mutation in combination with the R408W mutation [41].

As already mentioned, a substantial number of PKU patients are compound heterozygotes expressing two different PAH alleles. Thus, the genotype is determined by the type of PAH gene variation, which may result in a totally inactive enzyme or a protein with substantial residual activity (up to 100% of the wild-type PAH). However, the interallelic complementation (IC) phenomena may occur, leading to a milder (positive IC) or a more severe phenotype (negative IC) than expected in the homozygous constellation [44]. Interallelic complementation effects arise from the combination of PAH monomer variants yielding a heterotetrameric PAH protein with functional and/or structural properties different from the wild-type homotetrameric protein. The interallelic complementation of variant protein monomers and effect of possible protein modifiers may complicate the interpretation and lead to inconsistencies between the same genotype and different phenotypes as demonstrated by Trefz et al. [45]. Lichter-Konecki et al. [46] correlated

genotypes and residual *in vitro* enzyme activity with the phenotypes of PKU patients. As expected, patients with classic PKU harbored more severe mutations with less or no residual PAH activity [47].

General truths about genotype/phenotype correlations in PKU that have emerged from the data cataloged in the databases, especially in the BIOPKU data base, and in literature are:

1. Mutation combinations that allow for <15% in vitro enzyme activity cause classic PKU and do not respond to BH_4. Mutation combinations that allow for >20% residual activity responds to BH_4. Responders have moderate to mild phenotypes.
2. Splice site mutations may cause classic or mild PKU depending on ‘read through’ (i.e. normal splicing may sometimes occur despite the mutation), and the fact that they have different phenotype associations is listed in the available databases.
3. Specific mild mutation/classic mutation combinations with identical predicted residual enzyme activity may have different phenotype associations (due to negative intra-allelic complementation) but the phenotype associations of different mutation combinations can be found in the available data bases making prediction unnecessary.
4. The BH_4 responsiveness of many mutation combinations (complete genotypes) has been well established several times. Patients that have those genotypes may not need to undergo BH_4 responsiveness testing [41].

Although many factors affect phenotypes, the specific PAH genotype is the main determinant of metabolic phenotype in most cases.

1.4.4 Responsiveness to BH_4

Some mutations have been associated with a BH_4- sensitive PKU phenotype, where the administration of pharmacological doses of exogenous BH_4 results in an increase in the activity of PAH sufficient to reduce circulating phenylalanine levels to a clinically significant extent and daily tolerance for phenylalanine.

These mutations usually give rise to milder forms of PKU, with substantial residual PAH activity. Some analyses have identified mutations which facilitate stabilization of the structure of the PAH protein by BH_4, which is consistent with the BH_4 responsive phenotype [48,49].

It was emphasized from these studies that a significant residual PAH activity is a prerequisite for BH_4 responsiveness. This was further characterized on biochemical, molecular and physiological levels and several possible mechanisms for BH_4 responsiveness were proposed based upon results of *in vitro* expression studies and structural implications of the mutations [50].

Many mutations and genotypes were found associated with responsiveness and are listed in the BIOPKU database (*www.biopku.org*).

These mutations with substantial residual activity are located in all regions of PAH (Figure 9).

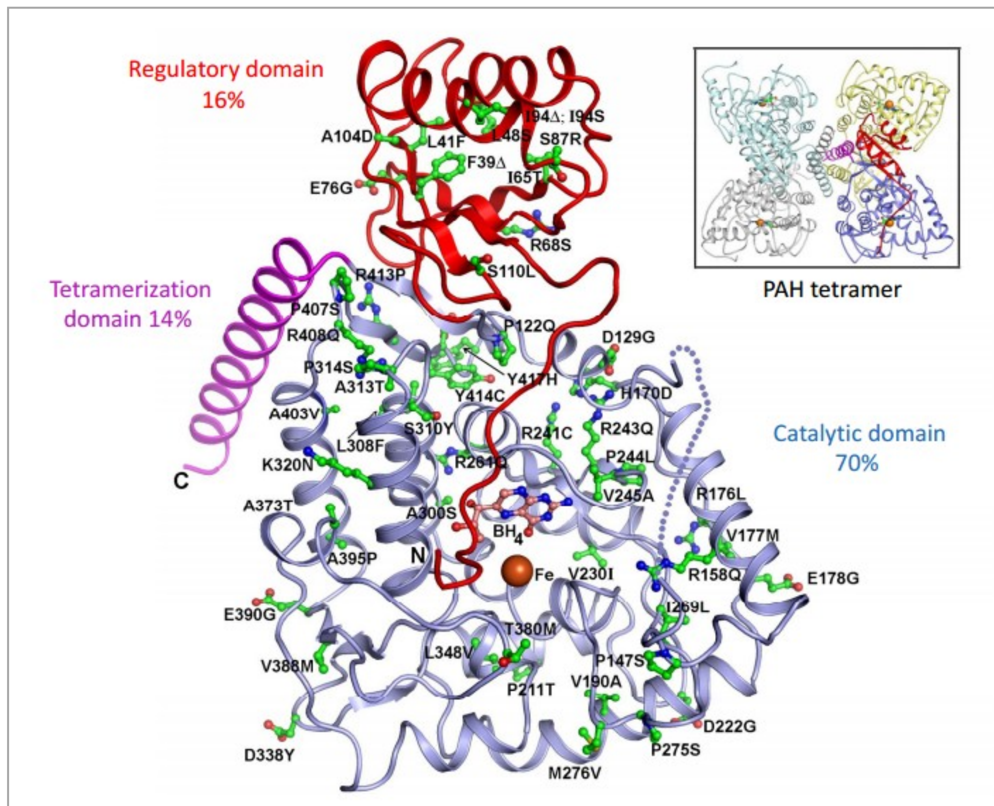


Figure 9.: *Three-dimensional crystal structure of the human phenylalanine hydroxylase monomer with most common BH_4-responsive mutations The most mutations (70%) were positioned in the catalytic domain (blue), 16% of mutations are in the regulatory domain (red), and 14% are in the tetramerization domain (lilac). (source BIOPKU database; http://www.bh4.org/biopku.html)*

About 70% of all mutations are located in the catalytic domain of PAH, 16% in the regulatory domain, 14% are located in the tetramerization domain.

Based on the presently detected genotypes of BH_4 -responsive patients, it would appear that the allelic PAH mutation-combination is the most important indicator of BH_4-responsiveness. Two severe mutations found on the two alleles for PAH will very likely result in severe PKU, and in turn little or no PAH enzymatic activity, and very likely no BH_4-responsive. It would be very difficult to propose a possible mechanism for BH_4-responsiveness in patients with homozygosity for severe null mutations. Nevertheless, a few severe/classical PKU patients (with blood Phe levels >1200 µmol/L) have been

found to be BH_4-responsive, all of them with at least one partially active allele (as determined by enzymatic activity assays performed on expressed protein *in vitro*). Similarly, patients with two mild mutations that show relatively high residual activity (i.e., for example >30% activity as compared to wild-type PAH) will likely display HPA or mild PKU, and possibly be BH_4-responsive. The combination of one mild mutation with one severe mutation will questionably be BH_4-responsive, and based on the currently known genotypes that are BH_4-responsive, this will depend upon the combination of the mutations present in the genotype. Thus, most of the genotypes found currently to be BH_4-responsive consist of one mild mutation and one severe mutation, or two relatively mild mutations, and they also display high residual enzymatic activity [51]. However, the relation between genotype and BH_4-responsiveness is complex. A study in Croatian patients with phenylketonuria showed that the prevalence of BH_4-responsive PKU was lower than would have been expected from genotyping alone [52]. This study also clearly documented that only the full genotype may predict the phenotype and BH_4–responsiveness. Overall, genotyping alone is currently insufficient for prediction of the BH_4 –sensitive PKU phenotype, but it is helpful in excluding non-responders to BH_4 therapy (classic PKU).

CHAPTER 2 –AIM OF THE THESIS

2. AIM OF THE THESIS

Ever since its discovery in 1934, it has been customary to consider PKU as a typical Mendelian trait with autosomal recessive inheritance. However, within the first two decades of its discovery, it became apparent that the disorder is also "multifactorial" with inherited (genetic) and acquired (dietary) components, both of which are necessary to establish the variant metabolic phenotype (HPA).

It became apparent that HPA also reflects locus heterogeneity [53]; although the vast majority of mutations responsible for HPA map to the PAH locus, some occur at loci controlling the synthesis and recycling of tetrahydrobiopterin, the essential cofactor for catalytic activity of phenylalanine hydroxylase enzyme. Furthermore, in the era of molecular genetics, expectations for a consistently close correlation between the mutant genotype and variant phenotype have been somewhat disappointing. In fact significant inconsistencies, both between in vitro and in vivo phenotypes and between different individuals with similar PAH genotypes, reveal that the HPA-phenotype is more complex than that predicted by Mendelian inheritance of alleles at the PAH locus.

Figure 10 summarizes the factors adding to the complexity of monogenic PKU resulting in challenging genotype-phenotype correlations. As the figure shows, the underlying genetic heterogeneity is the basis of the biochemical and clinical diversity of hyperphenylalaninemias. The prediction of the ‘monogenic’ phenotype from the PAH genotype is severely challenged by the fact that patients, even siblings, with identical PAH genotypes could have greatly different cognitive and metabolic phenotypes. Most probably, this results from other genetic contributions to the phenotype (e.g., modifier genes), which are complex and sparsely investigated in PKU. Many of the features mentioned in

Figure 10 also greatly influence the prediction of BH_4 responsiveness and often lead to inconsistent results.

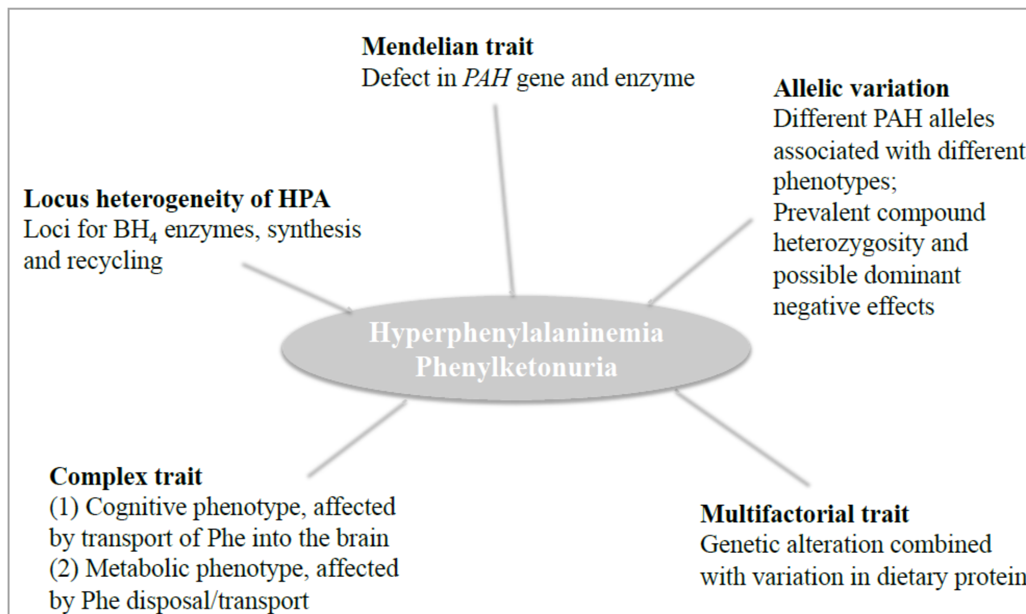


Figure 10.: *Factors influencing phenotype in phenylketonuria (Figure adapted from [54])*

Based on these evidences, in the first part of the project a molecular analysis in a group of unrelated HPA patients from Southern Italy has been performed. Genotyping of patients with elevated Phe levels detected in newborn screening is often useful to complete diagnosis of a HPA patient. From these data, we also planned to correlate genotypes with predicted residual activities (PRA) from in vitro expression tabulated in PAHdb, performing a genotype–phenotype correlation. The main goal of this part of the study was to summarize the mutation spectrum of the PAH gene in PKU population from Southern Italy and to provide more information on the predictive value of BH_4-responsive PAH mutations. The prediction facilitates the selection of potential PKU candidates for pharmacological therapy with BH_4. Null mutations for example are generally not associated with response to BH_4.

In the second part of this work we described the quantification of PAH activities expressed in cultured cells by a novel tandem mass spectrometry assay. Mass spectrometry allows the use of stable isotopes for amino acid quantification with increased specificity. In vitro expression analysis of PAH mutations has three major applications: 1) to confirm that a disease-associated mutation is genuinely pathogenic, 2) to assess the severity of a mutation's impact, 3) to examine how a mutation exerts its deleterious effects on the PAH enzyme, that is, to elucidate the molecular mechanisms involved, and 4) to predict the response to BH_4.

The thesis project outlined the molecular, in vitro analysis of PKU mutations and testing of their response to BH_4 in a mammalian cell system for better prediction of BH_4 responsiveness contributing to elucidate specific aspects of HPA and PKU.

CHAPTER 3 – MATERIAL AND METHODS

3.1 Mutation analysis of PAH gene

3.1.1 Patients

We analyzed 145 HPA patients (age ranged 1 month–26 years: mean 11 years), recruited in this study from the Clinic of Inborn Errors of Metabolism in the “Giovanni XXIII” Pediatric Hospital (Bari, Italy). All the patients studied with transient HPA were excluded. None of the patients with HPA was affected by defects in the synthesis or recycling of the cofactor BH_4. Phenotypes were classified on the basis of blood phenylalanine level at diagnosis and Phe dietary tolerance in: classic PKU (Phe ≥1200 µmol/L, low-Phe diet, with reduced natural protein intake, and supplemented with essential amino acids), mild PKU (Phe between 600 and 1200 µmol/L), and mild HPA (Phe <600 µmol/L, free diet) [55]. Thus, from the 145 HPA patients detected, 108 showed mild HPA, 37 classic PKU and mild PKU. Parents and/or siblings, if available (data on parents and siblings are included in the work only in the case of confirmation of HPA diagnosis), were investigated to confirm their carrier status. Moreover, Phe levels were regularly monitored through Guthrie cards with the dried blood spots and amino acids levels were quantified then through tandem mass spectrometry (TMS).

The study was performed according to the standards set by the Declaration of Helsinki. The experiments were undertaken with the understanding and written consent of all subjects or their guardians.

3.1.2 DNA extraction and quantification

Genomic DNA was extracted from buccal swab for the infants and from peripheral blood in adults and parents using the QIAamp DNA Mini-Kit (Qiagen, USA), according to the manufacturer's instructions. For each sample, the extracted DNA was quantified by spectrophotometric reading at 260nm. The quality of the extraction was evaluated by checking the relationship between the reading at 260nm and 280nm (optimal ratio ≥1.8).

3.1.3 PCR AND DIRECT SEQUENCING

Primers spanning the exons, including the splice sites in the intronic regions, were designed using PRIMER3 software (*http://frodo.wi.mit.edu/cgi-bin/primer3/primer3*) in order to amplify the various 13 exons by PCR and are listed in table 2.

Exon[a]	Primer Forward	Primer Reverse
1	CGTGCTGTTTGCAAACCTGC	TGGAGGCCCAAATTCCCCTAACTG
2	TGATCATTTAATTGCCCTGGA	GCCTGTTCCAGATCCTGTGT
3	GCCTGCGTTAGTTCCTGTGA	CTTATGTTGCAAAATTCCTC
4	GCCATGTTCTGCCAATCTGT	ATCTCATCCTACGGGCCAT
5	TCATGGCTTTAGAGCCCCCA	AGGCTAGGGGTGTGTTTTTC
6	CCGACTCCCTCTGCTAACCT	CAATCCTCCCCCAACTTTCT
7	TAGCGTCAAAGCCTATGTCC	AAACCTCATTCTTGCAGCAG
8	TGGCTTAAACCTCCTCCCCT	CTGGGCTCAACTCATTTGAG
9	ATGGCCAAGTACTAGGTTGG	GAGGGCCATAGACTATAGCA
10	ACACACCCCAAAATAATGCT	GAGTTCCCAGGTTGCATATC
11	TGAGAGAAGGGGCACAAATG	CCACCCACAGATGAGTGGCA
12	TTCTCCAAATGGTGCCCTTC	ACTGAGAAACCGAGTGGCCT
13F	GACACTTGAAGAGTTTTTGC	TTTTCGGACTTTTTCTGAT

Table 2. *Primers used for PCR-amplification and sequencing of all 13 exons plus flanking intronic regions of the human PAH gene.*
F, forward; R, reverse.

The PCR products were monitored by electrophoretic analysis on agarose gel at 2% and subsequently analyzed by direct sequencing.
Direct sequencing of all 13 exons of the PAH gene and their flanking introns was performed on both strands using an ABI Prism BigDye Terminator Cycle Sequencing Kit, version 3.1 (Applied Biosystems, USA) on an ABI Prism 3100 sequencer. All PCR fragments were sequenced using the same primers as used for PCR amplification. All identified mutations were confirmed using a new

PCR product of the abnormal fragment (forward and reverse). Novel missense mutations and previously described mutations were also analyzed in a control panel consisting of DNA from 140 healthy Caucasian individuals.

The sequences were compared to the wild-type sequence of the PAH gene (GenBank access no. NG_008690.1).

Two databanks were accessed: the PAH database (*http://www.pahdb.mcgill.ca*), and HGMD (*http://www. hgmd.cf.ac.uk/ac/index.php*) to check for mutations and polymorphisms in the PAH gene. The nomenclature of mutations was implemented according to the current HGVS recommendations (*http://www.hgvs.org/mutnomen/*).

3.1.4 MLPA Analysis

MLPA was carried out for patients who lacked PAH mutations on one or both alleles, based on previous sequencing analyses. The SALSA PO55 PAH MLPA kit (MRC Holland, Amsterdam, The Netherlands) contains 25 sets of probes: 13 PAH specific sets, and the remainder comprises control standard probes from other human genes. The assay was carried out in 200µl tubes in a thermal cycler according to the manufacturer's instructions. PCR products were identified and quantified using capillary electrophoresis on an ABI 3100 genetic analyser with the Gene Mapper software from Applied Biosystems (Foster City, CA). The obtained data were analyzed using the Coffalyser.Net software in combination with the appropriate lot-specific MLPA Coffalyser sheet.

3.2 IN VITRO EXPRESSION OF PAH MUTATIONS

3.2.1 MUTAGENESIS AND EXPRESSION IN E. COLI OF WILD TYPE AND MUTANT FORMS OF PAH

The expression plasmids pCMV-FLAG-PAH and pCMV-Myc-PAH wild-type were provided by L. R. Desviat, Madrid, Spain [56]. PAH wild type cDNA had been cloned into pFLAG-CMV-2 vector (Sigma) using SalI and NotI restriction sites. The pFLAG-CMV-2 vector is a high-copy plasmid with CMV promoter, leading to high levels of expression in mammalian cells. The wild-type and mutant human PAH proteins were fused to different peptide tags (FLAG and Myc) at the N-terminus in order to confirm and compare PAH activity of identical mutations and expression levels of each protein. Plasmid maps of the human PAH expression vectors are provided in Figure 11.

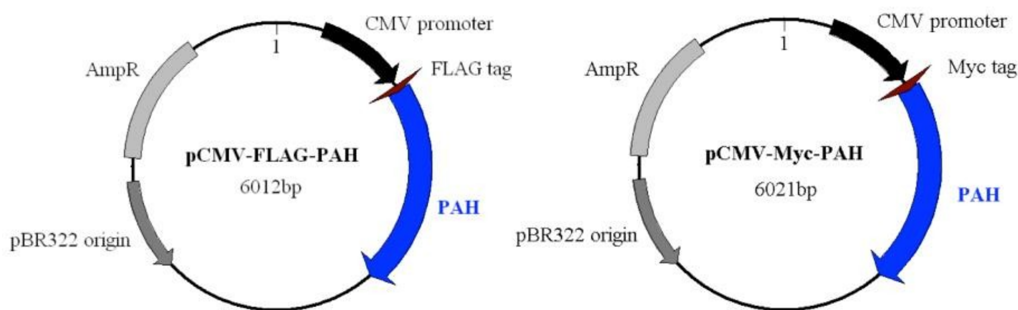


Figure 11. *Plasmid maps of human PAH expression vectors.*

Gene mutations were investigated whether no data exist or diverging reports on clinical phenotype and BH_4 responsiveness are reported, according to data in the BIOPKU database (*www.biopku.org*).

Mutations in the human PAH cDNA sequence were introduced by site-directed mutagenesis using a QuikChange II XL site-directed mutagenesis kit from Agilent Technologies (Santa Clara, CA, USA). Table 3 lists the mutagenic oligonucleotide primer sequences.

Primer name	Mutagenic primers (5'-3')[a]
PAH p.Y204C fwd	GGAAAAATGTGATTGTACTCACAGCAAGCATGGGTTTTATACAAG
PAH p.Y204C rev	CTTGTATAAAACCCATGCTTGCTGTGAGTACAATCACATTTTTCC
PAH p.L212P fwd	GAAGCCACAGTACTTTTCAAGAGGTGGAAAAATGTGATTGTACTC
PAH p.L212P rev	GAGTACAATCACATTTTTCCACCTCTTGAAAAGTACTGTGGCTTC
PAH p.L249P fwd	ATCCCGAGAGGAAGGCAGGCCAGCCAC
PAH p.L249P rev	GTGGCTGGCCTGCCTTCCTCTCGGGAT
PAH p.L249F fwd	ATCCCGAGAGGAAAACAGGCCAGCCACAG
PAH p.L249F rev	CTGTGGCTGGCCTGTTTTCCTCTCGGGAT
PAH p.R270K fwd	TTGGATCCATGTTTGATGTACTGTGTGCAGTGGAAG
PAH p.R270K rev	CTTCCACTGCACACAGTACATCAAACATGGATCCAA
PAH p.R261P fwd	GCAGTGGAAGACTGGGAAGGCCAGGCC
PAH p.R261P rev	GGCCTGGCCTTCCCAGTCTTCCACTGC
PAH p.S196Y fwd	CAAGCATGGGTTTTATACAAGTACTTCAGAGTCTTGAACACTG
PAH p.S196Y rev	CAGTGTTCAAGACTCTGAAGTACTTGTATAAAACCCATGCTTG
PAH p.T380M fwd	GGCTGGAACTCCATGACAGTGTAATTTTGGATGGCT
PAH p.T380M rev	AGCCATCCAAAATTACACTGTCATGGAGTTCCAGCC

Table 3. *Primer sequences, used for site-directed mutagenesis generating PAH mutants for in vitro expression.*
[a] *Corresponding nucleotide changes are underlined.*

The obtained constructs were sequenced to confirm the introduction of the desired modifications and were used to transform competent XL10-Gold cells. Competent XL10-Gold cells were thawed on ice for 3 minutes, mixed by gentle tapping and briefly centrifuged at 3000rpm in a tabletop centrifuge. They were then resuspended by gentle pipetting, added to pre-chilled DNA plasmid and mixed either by tapping or gentle pipetting. A 30 minute incubation on ice after that was followed by a 45 second heat shock at 42°C with gentle tapping once after 45 sec. Cells were then put directly on ice for 2 minutes. One ml of LB

medium was added and the transformation reaction was kept in a shaker (600rpm) at 37°C for 45 minutes. Following that, the volume was reduced by centrifugation and discarding of the supernatant to 100µl. Cells were then plated with a sterile glass spreader on pre-warmed LB-agar plates containing ampicillin antibiotic. Plates were dried for 10 minutes with an open lid at 37°C and then incubated overnight.
The resulting clones were extracted using the QIAGEN Plasmid mini kit and sequenced to verify the introduction of each single mutation.

3.2.2 TRANSFECTION EXPERIMENTS

The COS-7 cell line (monkey kidney cells) was cultured in DMEM supplemented with 10% fetal calf serum and 1% Penicillin-Streptomycin at 37°C under 5% CO_2. One day before transfection, COS-7 cells were plated in 100-mm culture dishes at 2×10^5 cells/ml. The cell density should be 50-70% confluent on the day of transfection. Fugene HD (Promega, Madison, USA) reagent was used in the transfection experiments and the experiments were performed according to the manufacturer's instructions. Specifically, 13 µg of the pCMV-FLAG-PAH plasmid and 13 µg of the pCMV-Myc-PAH (either wild type or mutant) were co-transfected with 2 µg of pSV-β-Galactosidase Control Vector (Promega, Madison, USA), using 45 µl of Fugene HD reagent in antibiotics-free media. Transfection experiments were performed in triplicate and always included expression of wild type PAH, several mutant PAH constructs, two already studied mutant PAH constructs of which we knew the PAH activity and the comparison to non-transfected COS-7 cells. One 100-mm cell plate was only transfected with 15 µg pSV-β-Galactosidase control vector as a control. Transfected cells were harvested after 48 h for the immediate determination of PAH activity or for flash freezing in liquid N_2 for storage at -80°C.

Transfection efficiency in transfected cell pellets was verified by determining β-galactosidase activity in 5 µl lysates in PAH assay cell lysis buffer (1×PBS pH 7.4, 0.25 M sucrose, complete protease inhibitors cocktail; Roche, Switzerland) using the β-Galactosidase Enzyme Assay System (Promega, Madison, USA).
PAH activities of wild type and mutants were normalized using the absorbance results at 420 nm of o-nitrophenol (yellow color development at 37°C with a linear range between OD420 0.1 and 0.9) as measure of transfection efficiency and protein concentration.

3.2.3 Sample preparation and PAH activity assay

Cell lysates were prepared and enzyme activity was determined according to previously described methods [57,58].
Free amino acids and contaminants of low molecular weight were previously removed from the extracts using Zebra Desalt Spin columns (Pierce Biotechnology). Under these conditions, PAH activity was linear to the amount of protein in the extracts.
In brief, 5 µl (containing 50-100 µg of total protein) of cell homogenate was mixed with 0.1 M Na-HEPES buffer, 2 µg catalase and 1 mM L-Phe. The cell mixture was incubated for 5 min. After 4 minutes preincubation at 25° C, 100 µM ferrous ammonium sulfate (Fe $(NH_4)^2(SO_4)^2$) was added and the reaction triggered after 1 minute by adding 200 µM BH_4 and 5mM DTT. After 15 min, the reaction was stopped by adding 50 µl of 2% (w/v) acetic acid in ethanol.
Samples were prepared for liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) according to the Phenomenex EZ: faast™ kit manual: 20 µl of each internal standard solution containing 100 µM Phe-d5 and 20 µM Tyr-d4 (in 50 mM HCl) was added to 20 µl of PAH assay samples. This kit consists of a solid phase extraction step followed by derivatization and liquid-liquid extraction, and the amount of derivatized tyrosine produced was determined by LC-ESI-MS/MS. Electrospray and ion

source conditions were optimized for the amino acid measurements using phenylalanine and tyrosine as internal standards. Specific PAH activities were expressed in mU per mg total protein to account for differences in total protein amount and with mU equal to nmol L-Tyr produced per minute. The mean PAH activities were calculated from the three sets of transfection data. The residual activities of mutant PAH enzymes were expressed as a percentage of wild-type enzyme activity and normalized to transfection efficiencies based on replicate β-galactosidase activities.

3.2.4 PHE AND TYR DETERMINATION BY LC-ESI-MSMS

We quantified Phe and Tyr in cell homogenates by LC-ESI-MSMS using a Quattro Ultima triple quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray ion source and a Micromass MassLynx data system. Electrospray and ion source conditions were optimized for the amino acid measurements using phe and tyr as internal standards and an eight-point calibration curve. Labeled internal standard stock solutions were prepared in 50 mM/L HCl, stored at -20°C, and freshly diluted to working concentrations (see section 3.2.3). Stock solutions of non-labeled Phe (50 mM/L) and Tyr (8 mM/L) were prepared for calibration curves in 50 mM/L HCl and stored at -20°C. Working solutions for calibration curves were freshly prepared from the non-labeled stock solutions in H2O from 0 to 600 µM for Phe and from 0 to 120 µM for tyrosine.

Specific PAH activity was expressed in mU/mg total protein, with mU equal to nmol tyrosine produced.

3.2.5 WESTERN BLOT

Western blot analyses for testing PAH protein expression were performed on cell lysates (25 µg in each lane) from the activity assay. After electrophoretic

separation (SDS-PAGE, 10% acrylamide), the proteins were blotted onto nitrocellulose filters (GE Healthcare) by a semidry transfer.

The blot was blocked with 5% non-fat milk in TBST and then incubated with primary antibody solution: anti-PAH (1:6000 dilution, Merck Millipore, MA, USA), anti-Flag (1:2000 dilution, Sigma-Aldrich, MO, USA), anti-Myc-tag (1:2000 dilution, Cell Signaling, MA, USA) and anti-β-actin (1:1000 dilution, Santa Cruz Biotechnology, CA, USA) overnight at 4°C. After washing with TBST, the membrane was incubated with goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, CA, USA) for 1 hr at room temperature. Antibody bindings were detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, MA, USA) as described by the manufacturer's instructions.

CHAPTER 4 – RESULTS

4.1 MOLECULAR DIAGNOSIS OF PAH MUTATIONS

We sequenced all the exons and adjacent intron regions of the PAH gene in 145 unrelated HPA patients from the South of Italy.

Patients are mainly from the Clinic of Inborn Errors of Metabolism in the "Giovanni XXIII" Pediatric Hospital (Bari, Italy) and, based on the L-Phe concentrations found in the blood at diagnosis, were divided into two groups: 37 (25.5%) were classified as PKU and mild PKU, and 108 (74.5%) as MHP.

When no mutation or one mutation was detected, MLPA analysis for detection of large gene deletions/duplications was performed but in all cases the results were negative. To determine the inheritance phase of sequence variants when >2 mutations were observed, specimens from parents and other first-degree relatives were recruited.

In our cohort of Italian HPA patients, we identified 60 different types of disease-causing mutations, of which 56 can be found in mutation databases PAH and HGMD (*http://www.pahdb.mcgill.ca/*, *http://www.hgmd.cf.ac.uk/ac/index.php*) and 4 were new and not described thus far. From these, 41 types were missense (68.3%), 4 nonsense (6.66%), 8 splicing (13.3%) and 7 deletion (11.6%). The results were divided into two sections considering phenotypic classes (see below).

4.1.1 PAH MUTATION SPECTRUM IN MHP PATIENTS

In 108 subjects presenting mild HPA, a gene mutation was identified in 151 out of 216 PAH alleles investigated, representing a mutation detection rate of 69.9%. Additionally, our genetic analysis established mild HPA diagnosis (blood Phe levels between 150 and 360 µmol/L) for three fathers, a mother and two sisters of five unrelated patients. These subjects were not aware of their

mild HPA genotypes and the presence of two mutations did not appear to lead to any pathological effect over time.

Overall, 42 different PAH gene mutations were identified in our patient cohort of which, 39 were previously described in the HGMD database (Table 4).

Mutation (protein level)	**Mutation (DNA level)**	**Alleles**	**Frequency**
p.A403V	c.1208C>T	42	18.42
p.A300S	c.898G>T	20	8.77
IVS10-11G>A	c.1066-11G>A	17	7.45
p.R261Q	c.782G>A	9	3.94
p.V245A	c.734T>C	8	3.5
p.F39del.	c.115_118del.	6	2.63
IVS4-5C>G	c.442-5C>G	6	2.63
p.T380M	c.1139C>T	5	2.19
p.P281L	c.842C>T	5	2.19
p.L48S	c.143T>C	4	1.75
p.R408W	c.1222C>T	3	1.31
p.R158Q	c.473G>A	3	1.31
p.R252W	c.754C>T	3	1.31
p.H201Y	c.601C>T	2	0.87
p.V230I	c.688G>A	1	0.43
p.R176Q	c.527G>A	1	0.43
p.I306V	c.916A>G	1	0.43
IVS7+3G>C	c.842+3G>C	1	0.43
IVS4+5G>T	c.441+5G>T	1	0.43
p.P407L	c.1220C>T	1	0.43

p.I65V	c.193A>G	1	0.43
p.L358F	c.1074A>T	1	0.43
p.L287V	c.859C>G	1	0.43
p.C217G	c.649T>G	1	0.43
p.E182G	c.545A>G	1	0.43
IVS4+1G>A	c.441+1G>A	1	0.43
p.Q134*	c.400C>T	1	0.43
p.A322G	c.965C>G	1	0.43
p.R241H	c.722G>A	1	0.43
p.V177L	c.529G>A	1	0.43
p.E178Q	c.533A>G	1	0.43
p.R243*	c.727C>T	1	0.43
p.I174V	c.781C>T	1	0.43
p.R261*	c.781C>T	1	0.43
p.E390G	c.1169A>G	1	0.43
p.F55del.	c.163_165del.	1	0.43
p.Y198Cfs*136	c.593_614del	1	0.43
p.Y168Sfs*27	c.503del	1	0.43
p.Q352Vfs*48	c.1055delG	1	0.43

Table 4. *Mutations identified in Italian MHP patients, alleles and their relative frequencies.*

Three mutations found in this group were new and not described in the PAH mutation and HGMD databases thus far. For the prediction of the causality of missense mutations, especially of novel mutations, we used the bioinformatic tool PolyPhen-2 (*genetics.bwh.harvard.edu/pph2/*). The functional impact of the novel missense mutations using PolyPhen were reported in table 5.

The alterations c.361 T>C (p.Phe121Leu), c.587 C>A (p.Ser196Tyr), and c.854 A>G (p. His285Arg) have been reported neither in the PAH database nor in the "1000 genome project" (*http://browser. 1000genomes.org/index.html*) database. These alterations and the other identified mutations were verified by additional sequencing of a new PCR product to eliminate the possibility that observed changes were artifacts of the sequencing method. Finally, to exclude the possibility that the novel variations would be a polymorphism, 280 alleles from randomly selected healthy individuals of Caucasian origin were analyzed.

Trivial name (Protein effect)	Systemic name (DNA effect)	Exon	Predicted effect (Polyphen-2)
p.F121L	c.361 T>C	4	probably damaging (score of 1.000)
p.S196Y	c.587 C>A	6	probably benign (score of 1.000)
p.H285R	c.854 A>G	8	probably damaging (score of 1.000)

Table 5. *Prediction of impact of new missense mutations using Polyphen-2.*

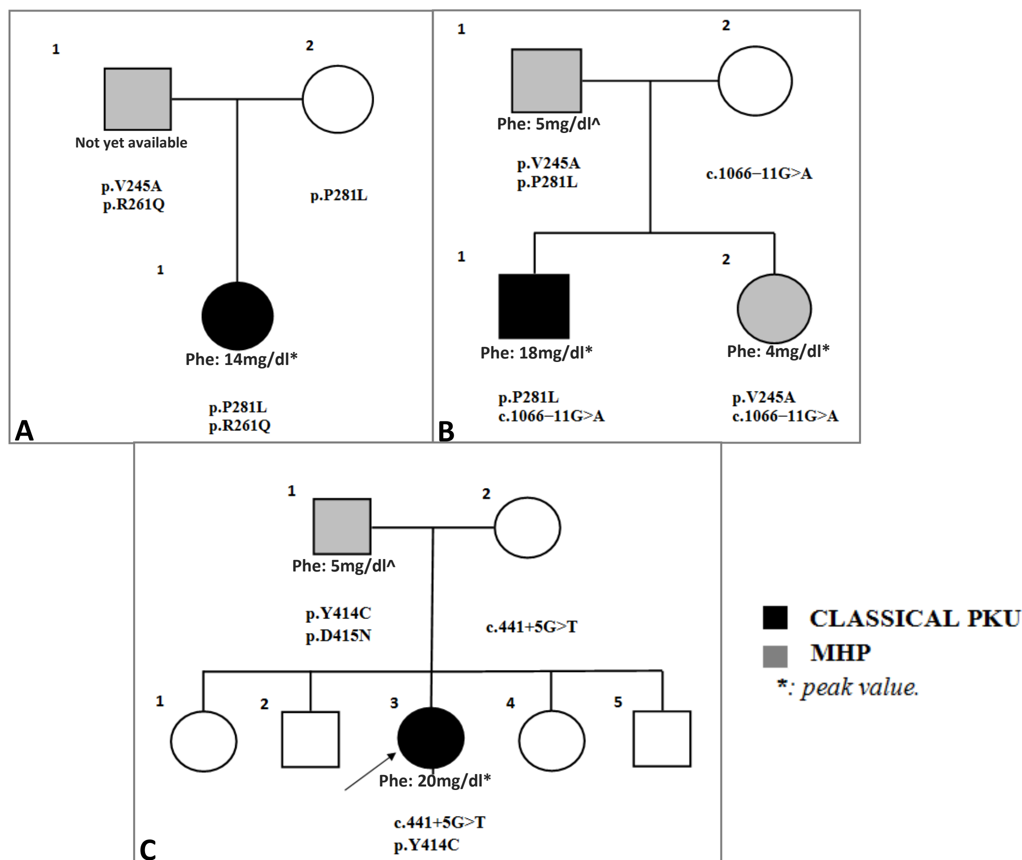
The p.Phe121Leu variant was predicted to be probably damaging (score of 1.000). The coexistence with the mutation p.Val230Ile, associated with HPA phenotype, was in agreement with the mild HPA phenotype recorded in the patient. The p.Ser196Tyr variant was predicted to be probably benign (score of 1.000) and found in association with the c.842+3G>C mutation, associated with a PKU phenotype. This compound heterozygosity well explained the mild HPA phenotype observed. The p.His285Arg mutation was found within a single allele in a patient with value of Phe around 160 µmol/L and it was predicted to be probably damaging.

Fifty-two subjects were compound heterozygotes and 5 homozygotes, whereas a mutation within a single allele was found in 38 patients. In the remaining 13

infants, no mutation was detectable. Interestingly, after reassessing blood phenylalanine concentrations, they were not confirmed as carrying mild HPA. In these subjects, the initial positivity was due to an unsuitable pick, performed at birth and that repeated later was persistently negative, or to prematurity, a condition associated with a physiological transient hyperphenylalaninemia for an incomplete development of the liver.

Furthermore, as already mentioned earlier, we established mild HPA diagnosis (blood Phe levels between 150 and 360 µmol/L) for six relatives of five unrelated patients. In particular, three of them were fathers who belonged to unusual families, in which three different alleles in the PAH gene were identified, including two associated with PKU and one associated with MHP (Figure 12A,B and C). Thus, three mutations in different combinations had given rise to different degrees of hyperphenylalaninemia. The unexpected outcome of discordant phenotypes within the families described was explained by previously unrecognized parental MHP. All three fathers were not aware of their HPA status until molecular analysis since they were phenotypically normal and showed no overt sign of impaired intellectual development or neurophysiological dysfunction.

After the results of the molecular PAH analysis, they were informed on clinical consequences and were advised to measure Phe concentrations. During periodic checks on amino acid levels, two of them had values of Phe in the blood around 5 mg/dl (300 µmol/L).



Figures 12A,B and C. *Different combinations of three mutations segregating within the families (Figures adapted from [59]).*

4.1.1 Genotype-phenotype correlation in MHP patients

A genotype–phenotype correlation was performed in the MHP patients group. From the analysis of the genotype-phenotype data, it resulted that p.A403V (18.42% of the total mutant alleles) and p.A300S (8.77% of the total mutant alleles) mutations, the most common ones in this group, were more related to mild HPA phenotype since they present with substantial residual activity (31-66% of the wild type PAH) when expressed in eukaryotic cell system. In fact, for some PAH gene missense mutations, the “in vitro” PRA is

known. Mutations that cause a severe phenotype are those in which the PRA is less than 10% [60].

In compound heterozygotes, it has been observed that the less severe mutation is the one that determines the phenotype [61]. Thus, when one of the mutations has a severe effect and the second one allows for at least a partially functioning PAH allele, the HPA metabolic phenotype will be less severe. Whereas, when the two mutations are in a compound heterozygote state and have the same predicted effect (mild effect), the resulting phenotype will be less severe [62]. For instance, when p.Ala403Val (PRA=66%) or p.Ala300Ser (PRA=31%) coexisted with a severe mutation, they will confer a mild PKU phenotype, and when they co-segregated, they will give rise to a mild HPA phenotype. Data from the present cohort revealed a good genotype/phenotype correlation, except in one case, where only a mutation (c.441+5 G>T) in the PAH gene was identified and MLPA analysis resulted negative. It was conceivable that it did not cause the recorded HPA phenotype in the patient (values >3 mg/dl): another mutation existed, which had gone undetected, probably in regions not screened (5′ UTR, deep intronic, 3′ UTR).

4.1.2 PAH MUTATION SPECTRUM IN PKU PATIENTS

Using the PAH nucleotide sequence analysis, among 74 independent alleles, we identified thirty previously described mutations (detection rate 98.6%) and one novel mutation, p.H290Q. These included six splice site mutations (19.4%), five frameshift (16.2%), seventeen missense (54.8%) and three nonsense mutations (9.6%). Of the 37 patients studied, thirty-one (83.78%) were compound heterozygotes, only four (10.81%) were in the homozygous form, a mutation within a single allele and a case with in cis compound mutations were found in two patients respectively.

IVS10−11 G>A was the most prevalent mutation in our PKU patients with a frequency of nearly 12.16%. In addition, three other mutations, p.R261Q, p.P281L and p.Y198Cfs*136, were found at relatively high frequencies (9.45%, 6.75% and 6.75% respectively). Together, 4 mutations, IVS10−11 G>A, p.R261Q, p.P281L and p.Y198Cfs*136 accounted for around 35% of mutations in our sample.

The mutations were distributed throughout the PAH gene but the exon 7 seemed to be a hot spot for mutations. It exhibits the greatest number of different mutations (5) and is affected in 25.6% (19/74) of the total mutant alleles. Therefore, the majority of mutations (n= 16) were distributed along the catalytic domain (51.6%), 8 mutations (25.8%) belonged to the regulatory domain, 1 (3.2%) to the tetramerization domain and 6 (19.3%) are intronic (Figure 13).

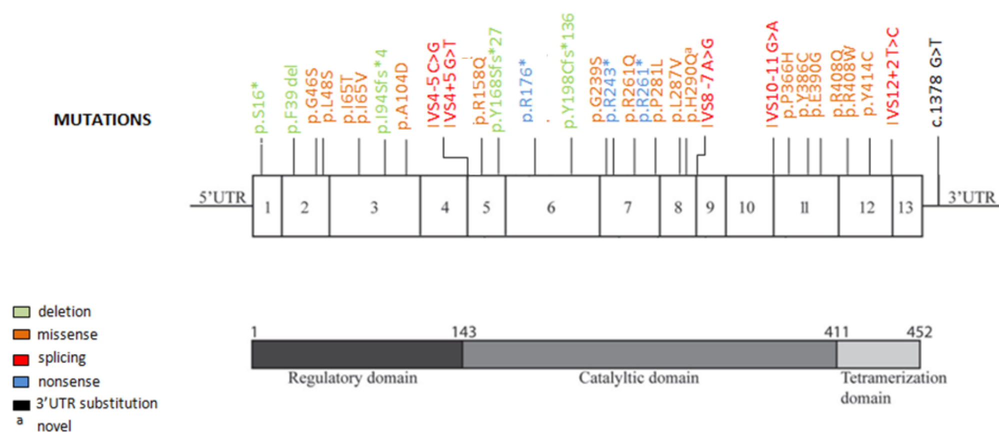


Figure 13. *Schematic representation of the PAH gene with the location of the 31 mutations detected in the Italian PKU population (Figure adapted from [63]).*

The alteration c.870 T>G (p.His290Gln) in exon 8, has not been described in the PAH mutation and HGMD databases. This conserved residue coordinates with other two amino acids the catalytic iron. Accordingly, the change is predicted to be probably damaging with a score of 1.000 using PolyPhen-2 (*http://genetics.bwh.harvard.edu/pph2/*), score values ranging from 0 (neutral)

to 1 (damaging). Furthermore, the analysis of 200 normal alleles from randomly selected healthy Caucasian individuals for this variant did not detect the alteration, confirming that it may be disease-causing.

4.1.3 GENOTYPE-PHENOTYPE CORRELATION AND PREDICTION OF BH_4 RESPONSIVENESS IN PKU PATIENTS

Mutation analysis revealed a spectrum of thirty-two different genotypes. Five identical genotypes were detected in at least two families.

A genotype–phenotype correlation was performed on patients and we found the expected correlation in most of the patients, but there were some inconsistencies when we compared phenotype and PRA in four mutations: E390G, R261Q, L48S and R158Q. Moreover, the genotype-based predictions of BH_4-responsiveness were assessed.

Number of Patients	Allele 1	Allele 2	Predicted Phenotype	Observed Phenotype	Prediction of BH_4 responsiveness*	Domain
Identical genotype reported on BIOPKUdb						
1	p.Y414C	IVS4+5G>T	Mild	Mild	Yes	Tetra/-
2	p.P281L	IVS10-11G>A	Classic	Classic	No	Cat/-
1	p.R261Q	p.P281L	Mild-Classic	Classic	No	Cat/Cat
1	p.R158Q	p.A104D	Mild	Classic[b]	Yes	Cat/Reg
2	IVS4-5C>G	IVS10-11G>A	Mild	Classic[b]	Yes	-/-
2	p.R408W	p.L48S	Mild-Classic	Classic	No	Cat/Reg
1	IVS10-11G>A	p.R243*	Classic	Classic	No	-/Cat
1	p.R408W	p.R408W	Classic	Classic	No	Cat/Cat
1	p.I65T	p.R158Q	Mild-Classic	Classic	No	Reg/ Cat
1	p.P281L	p.I94Sfs*4	Mild-Classic	Classic	No	Cat/Reg
1	p.R261Q	IVS10-11G>A	Classic	Classic	No	Cat/-
2	p.R261Q	p.R261Q	Mild	Classic[b]	Yes	Cat/Cat
1	IVS4+5G>T	IVS4+5G>T	Classic	Classic	No	-/-
At least one allele has been reported as BH_4 responsive						
1	p.I65V	p.Y386C	Mild-Classic	Classic	No/Yes	Reg/Cat
1	p.Y198Cfs*136	p.Y414C	Mild-Classic	Classic	No/Yes	Cat/Tetra
1	p.R408Q	p.R243*	Mild-Classic	Classic	Yes/No	Cat/Cat
1	p.R408Q IVS13+19 G>T	p.P281L	NR	Classic	Yes/NR/No	Cat/-/Cat
1	IVS10-11G>A	p.G46S	Classic	Classic	No/Yes	-/Reg
1	**p.H290Q**	p.R261Q	NR	Mild	NEW/Yes	Cat/Cat
1	p.F39del.	p.P366H	NR	Classic	Yes/NR	Reg/Cat
1	p.R243*	p.F39del	Classic	Classic	No/Yes	Cat/Reg

2	p.Y198Cfs*136	p.E390G	NR	Mild	No/Yes	Cat/Cat
1	IVS8-7A>G	p.F39del	Classic	Classic	No/Yes	-/Reg
Both alleles have been reported as BH_4 non-responders						
1	p.G239S	p.I65V	Mild-Classic	Classic	No/No	Cat/Reg
1	p.R176*	IVS4+5G>T	Classic	Classic	No/No	Cat/-
1	IVS8-7A>G	IVS10-11G>A	Classic	Classic	No/No	-/-
1	p.Y168Sfs*27	p.R261*	Classic	Classic	No/No	Cat/Cat
1	p.S16*	IVS10-11G>A	Classic	Classic	No/No	Reg/-
1	p.Y198Cfs*136	p.R261*	Classic	Classic	No/No	Cat/Cat
BH_4 response not determined						
1	p.L287V	p.R261*	NR	Classic	NR/No	Cat/Cat
1	p.Y198Cfs*136	IVS12+2T>C	NR	Classic	No/NR	Cat/-
1	-	p.L48S	NR	Classic	NR /Yes	-/Reg

NR: not reported;

[a]New variants are shown in bold type.

[b]Discordant genotype-phenotype correlation.

*reported on BIOPKUdb or literature.

Table 6. *Genotype-phenotype correlation and genotype-based prediction of BH_4 responsiveness in Italian PKU patients (Figure adapted from [63]).*

Four different genotypes were predicted to be BH_4-responsive, nine genotypes (eleven patients) were predicted to be BH_4-nonresponsive, ten genotypes (eleven patients) had at least one allele reported as BH_4-responsive, six genotypes (six patients) had both alleles reported as BH_4-non responders. The prediction of BH_4-responsiveness remained unknown for three genotypes found in three unrelated PKU patients because insufficient data was available (Table 6). Notably, less than half (F39del, G46S, L48S, I65T, I94Sfs*4, A104D, R261Q, Y386C, E390G, R408Q, Y414C, IVS4−5 C>G) of the 31 mutations

identified among Italian PKU patients belong to the group of more than 70 mutations already identified in BH_4-responsive patients, according to BIOPKU database, and interestingly, in contrast with other studies [64,65], the majority lies in the regulatory domain of the protein. The analysis of the allelic data allowed to predict that about 40% of the fully genotyped patients are potential responders to BH_4 treatment (Table 6).

4.2 *IN VITRO* RESIDUAL PHENYLALANINE HYDROXYLASE ACTIVITY

4.2.1 ENZYMATIC ACTIVITY ANALYSIS OF WILD TYPE AND MUTANT FORMS OF PAH

We measured the enzymatic activity of eight variant forms of PAH to investigate the effect of the gene variations on catalytic activity. Of these 8 variations, seven were associated with different phenotypic groups in HPA patients according to the data reported in the BIOPKU database (www.biopku.org/biopku/): p.S196Y (a rare novel variant found in our previous study [18]) and p.T380M are mild PKU variations; p.L249F is a mild-moderate PKU variation; p.R261P is a moderate PKU variation; p.L212P, p.L249P, p.R270K are severe PKU variations. According to Ellingsen et al. [19], for the p.Y204C variation, there was a change in the type of variation from a missense variation to a splice variation (Ex6-96 A>G). The information about PKU phenotype referred to the splice variation and not for the missense one. All variations are located in the catalytic domain: The Leu249 residue (exon7) is a pterin-binding residue in PAH. The Arg270 residue (exon7) is not an active-site residue, but is located on one side of the channel that leads to the active site. The Thr380 residue (exon11) maps to a similar region of the active site crevice that is opposite to the iron-binding site where the pterin-binding site is located. The Arg261 (exon7) substitution affects a residue that, based on the crystal structure, appears to be essential for maintaining the protein structure All

mutations occurred in the catalytic domain regions. L249 is a pterin-binding residue in PAH. The R270 is not an active-site residue, but is located on one side of the channel that leads to the active site. The T380 residue maps to a similar region of the active site crevice, opposite to the iron binding site where the pterin binding site is located. The R261P substitution affects a residue which appears from the crystal structure, to be essential for maintaining the protein structure and the substitution may be predicted to destabilize the secondary structure in the active site, as it disrupts hydrogen bonds (Figure 14).

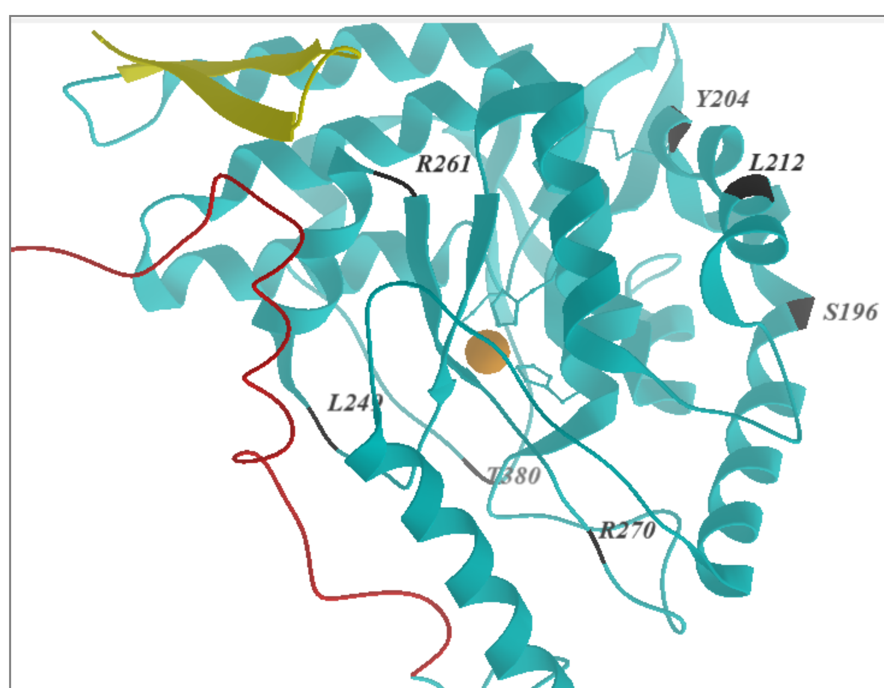


Figure 14. *The localization of the eight mutations is represented in the composite monomeric model of PAH (PDB code 1PAH). The catalytic domain, the regulatory domain, and the tetramerization domain are shown in blue, red, and yellow, respectively. Fe ion is drawn as a grey sphere. The figure was generated with the program Molsoft.*

In three independent experiments, we studied the transient expression of WT-PAH, the variants p.Y204C, p.L212P, p.L249P, p.L249F, p.R270K, p.R261P, p.S196Y, and p.T380M as well as the two variants p.R408W and p.P384S, which were used as controls, in COS-7 cells for 48 hours. Cells were harvested,

and steady-state PAH activity was measured in soluble cell extracts. The PAH activities of identical variations with either FLAG- or Myc-tagged proteins were compared. No significant differences in activity due to a different epitope tag was detected. The mean PAH activities were calculated from the three sets of transfection data. The residual activities of mutant PAH enzymes were expressed as a percentage of the wild-type enzyme activity. All mutant PAH activities were normalized to β-galactosidase, which was co-transfected into COS-7 cells. There was no enzyme activity in the untransfected cells.

As shown in Figure 15, the residual activities of the different PAH enzymes, expressed as a percentage of the wild-type enzyme activity, were: 94±6% (p.Y204C), 17.0±7.0% (p.L212P), 7.0±0.1% (p.L249P), 51.0±9.0% (p.L249F), 11.0±5.0% (p.R270K), 10.0±0.2 (p.R261P), 21.0±4.0% (p.S196Y), 28.0±6.0% (p.T380M). The residual activities of the variant PAH enzymes used as mild and severe variation controls, expressed as percentage of the wild-type activity, were 66.0±15.0 (p.P384S) and 8.0±4.0 (p.R408W), respectively.

Except for the p.Y204C variant, which was associated with near normal levels of residual PAH activity (94±6%), all other variant proteins showed reduced activities compared to the wild-type PAH protein. Three variations (p.L249F, p.S196Y, and p.T380M) were associated with low-intermediate PAH activity (21–51%), while variations p.L212P, p.L249P, p.R270K, and p.R261P showed very low residual activity (7-17%).

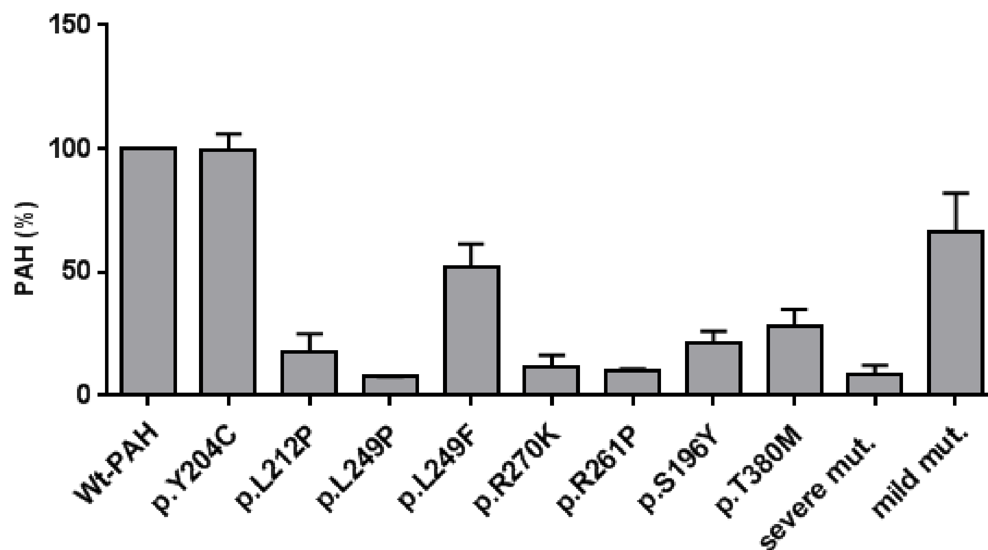


Figure 15. *PAH activities of PAH mutant proteins expressed in COS-7 cells and quantified by LC-ESI-MSMS. Activities are displayed as % wt activity. All PAH activities are normalized with β-galactosidase activity for correction of transfection efficiency.*

When comparing the residual in vitro enzyme activities and the metabolic phenotypes reported in the BIOPKU database, the obtained results were in full accordance (Table 1). The only exception was the p.L249F variation, which was associated with a mild-moderate PKU phenotype, but had substantial in vitro activity (51±9%).

Nucleotide aberration	Effect on protein	Gene location	Protein domain	SIFT score[a]	PolyPhen score[b]	FoldX value[c]	SNPs3D score[d]	PAH (%)	Associate phenotype[e]	Associate BH4 responsiveness[f]	APV[g]
c.587C>A	p.S196Y	Exon 6	catalytic	0.010	0.161	-	-0.20	21	1 MHP	-	-
c.611A>G	p.Y204C	Exon 6	catalytic	0.000	0.632	0.77	-0.70	94	10 mPKU, 56 cPKU	0/14	1
c.635T>C	p.L212P	Exon 6	catalytic	0.000	1.000	0.27	-1.25	17	2 cPKU	0/2	1
c.745C>T	p.L249F	Exon 7	catalytic	0.098	1.000	2.69	-2.04	51	4 mPKU, 1 cPKU	0/2	4
c.746T>C	p.L249P	Exon 7	catalytic	0.000	1.000	1.41	-2.73	7	9 cPKU	0/8	1
c.782G>C	p.R261P	Exon 7	catalytic	0.000	0.999	4.43	-2.32	10	11 mPKU, 11 cPKU	7/23	2
c.809G>A	p.R270K	Exon 7	catalytic	0.000	1.000	3.68	-2.40	11	2 mPKU, 18 cPKU	1/4	1
c.1139C>T	p.T380M	Exon11	catalytic	0.000	1.000	0.46	-1.55	28	28 MHP, 1 mPKU	5/6	8

[a]The SIFT Blink (http://sift.jcvi.org) algorithm estimates the functional impact of missense variant based solely on multiple sequence analysis. It predicts it to be either deleterious (<0.060) or tolerated (≥0.050).

[b]PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2) estimates the damaging effect of missense variants based on multiple sequence analysis. Continuous output ranges from 0 (benign) to 1.0 (probably damaging) with an intermediary category of possibly damaging.

[c]FoldX (http://foldx.crg.es) is a computer algorithm predicting the impact of variants on protein stability. Variations leading to a change of DDG41kcal/mol were considered destabilizing, those where the change in DDGo_1 kcal/mol counted as stabilizing, while variants in the range in between were considered neutral.

[d]The SNPs3D (http://www.snps3d.org) module assesses the effect of non-synonymous SNPs on protein stability and function. A negative SVM Score classifies the substitution as destabilizing to the folded state, while positive scores indicate non-deleterious substitutions.

[e]Phenotypes (number of patients) associated with homozygous or functional hemizygous genotypes in patients from the BIOPKU database (www.biopku.org).

[f]BH4 responsiveness (number of patients, of those tested) associated with homozygous or functional hemizygous genotypes in patients from the BIOPKU database.

[g]Allelic phenotype value (1 = classical PKU; 2 = moderate PKU; 4 = mild to moderate PKU; 8 = mild hyperphenylalaninemia) according to Guldberg et al. [61].

Table 7. *The comparison of the residual in vitro enzyme activities and the metabolic phenotypes reported in the BIOPKU database and our obtained results.*

4.2.2 Western Blot

Figure 16 illustrates the different PAH mutant proteins by western blotting. PAH expression was normalized to β-actin expression in COS-7 cells. The results showed variable amounts of proteins depending on the variation, and in some cases correlating with the level of residual PAH activity.

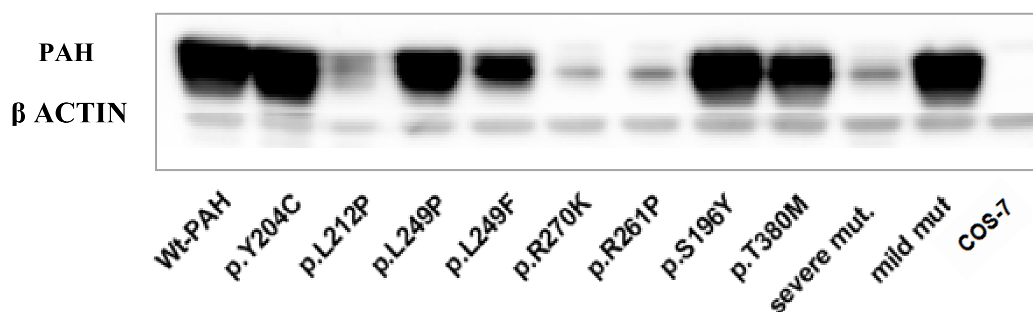


Figure 16. *Western blot analysis of wild type PAH and mutations tested for PAH activity. The same amount of total protein (25 µg) was analyzed per lane. PAH expression in all samples was normalized with β -actin expression. Non transfected COS-7 cells do not show PAH protein.*

All of the PAH mutant proteins with rather low activity (p.L212P, p.R270K, p.R261P) also exhibited reduced amounts of PAH protein, compared to wild type (Figure 16). Thus, these results were well in agreementwith the enzyme activity measurements.

In contrast, p.L249P, p.S196Y and p.T380M exhibited low activity and showed substantial amounts of protein. In particular, p.L249P showed a clear contrast between strong reduction of activity (7±0,1%) and high protein expression. Finally, the p.Y204C and p.L249F variants with rather high enzymatic activity yielded a relatively normal amount of PAH protein; in particular, for p.Y204C, the amount of protein was almost comparable with the levels of protein expressed by the wild-type enzyme.

CHAPTER 5 – DISCUSSION

5. DISCUSSION

5.1 PAH MUTATION ANALYSIS

Our study cohort summarizes the complete data from mutational analysis of 37 unrelated PKU and mild PKU patients and 108 MHP patients. Totally, the mutational spectrum involves 60 mutations, four of which have not previously been identified.

The present thesis work enlarged the molecular epidemiology of PAH mutations, in particular, with respect to Southern Italy. By scanning the PAH gene using direct sequencing, we detected a mutation in 98.6% of chromosomes from patients affected by PKU and 69,9% of chromosomes from patients affected by MHP.

The study confirmed the wide heterogeneity of PAH mutations in HPA patients also in our region. Given the wide heterogeneity of PAH mutations, it is necessary to scan the whole coding region of the gene for molecular analysis.

The PAH mutations were differently distributed in our HPA groups of patients, confirming that several mutations were more frequently associated with the PKU phenotype and others (mainly missense). In particular, we confirmed that p.R261Q, p.L48S and c.1066-11 G>A were more frequently associated with the PKU and mild PKU phenotypes, whereas p.A403V and p.A300S were peculiar to the milder phenotypes (MHP). Furthermore, in our PKU patients, we showed that for p.R261Q, p.L48S, p.E390G and p.R158Q mutations, there was a discrepancy between genotype and phenotype. Interestingly, indeed, we found that the mutation p.L48S was associated with a severe phenotype in our study when combined with mutation p.R408W, and it is inconsistent with previously reported studies for this mutation in Europe [66,67].

The intermediate degree of severity was observed also for the mutation p.R261Q, which has been associated with severe or moderate phenotypes in our group of PKU patients and this finding was in concordance with previous

European studies. In fact, the “mild” p.R261Q mutation in combination with the putative null mutations, p.P281L and IVS10−11 G>A, was associated with classical PKU; a curious inconsistency was observed in one homozygous patient for this mutation who was classified as cPKU. On the other hand, a mild PKU phenotype was observed in one patient with the p.[E390G]+[R261Q] genotype, in which both mutations display residual enzymatic activity >25%. In addition, two cases of genotype–phenotype inconsistencies involved the p.R158Q mutation which has 10% residual enzymatic activity, and which conferred a severe phenotype in two patients bearing, on the other allele, the missense p.A104D and the p.I65T, respectively. These controversial results have been experienced in many previous phenotype associations or in vitro expression studies.

Several reasons can be attributed to this discrepancy: incorrect phenotypic classifications; combination of different subunits encoded by mutant alleles in the homotetramer protein; the action of modifier genes or many other environmental or genetic factors.

Our data also revealed encouraging results about the evaluation of responsiveness to BH_4 treatment in our cohort of PKU patients: following the assumption that there are certain mutations associated with BH_4 responsiveness and previously classified as leading to a mild phenotype, and that patients with at least one BH_4-responsive mutation would be responsive to BH_4, about 46% of patients in our cohort could benefit from this treatment. Although calculating residual PAH activity from information available in *in vitro* experiments (PRA) may be useful in predicting potential candidates for BH_4 therapy, complete genotype has greater value in estimation of BH_4-responsiveness and is a useful tool in identifying those patients who could take advantage of this simple and inexpensive treatment. But, at the moment, the only precise method of determining patients' response to the drug is the BH_4-loading test.

In this thesis work, we also identified four novel missense mutations. Moreover, one of these mutations is located in regulatory domain (p.F121L), and the other

three in the catalytic domain (p.S196Y, p.H285R and p.H290Q). Two of these mutations (p.F121L and p.S196Y) could be considered “mild”, because they were found in patients bearing a “severe” mutation on the second allele (*http://www.pahdb.mcgill.ca*) and showed a mild biochemical phenotype. On the contrary, mutation p.H290Q can be considered a “severe” mutation because the patient bore a “mild” mutation on the second allele (i.e. p.R261Q) and shows a mild PKU phenotype. The p.H285R mutation was detected in a patient with only one mutant allele and values of Phe around 2mg/dl. The histidine 285 is an important residue which forms a hydrogen bond with residue serine 349 participating directly in the structural maintenance of the iron binding site. The maintenance of the iron binding site, coordinated by also residue H290 and E330, is crucial for the structural integrity of the monomer allowing correct folding/oligomerization of the protein.

In cases in which only one mutant PAH allele was detected (1 PKU patient and 38 MHP patients), these samples were further assessed by MLPA analysis, but negative results were recorded. A second mutation leading to an HPA phenotype may be deeply buried in an intron, which is not assessed in conventional genotyping methods.

Furthermore, we further supported the marked heterogeneity of hyperphenylalaninemia even within the same family due to the different combinations of mutations. We analyzed three families in which classical PKU, MHP and a normal phenotype occurred within each family due to the different combinations of three mutations segregating within the family. Indeed, sequence PAH analysis revealed three different alleles segregating in each family.

Thus, these results demonstrate that there are multiple and distinct mutations in the phenylalanine hydroxylase gene, with different levels of severity, and that various combinations of the mutant alleles in a family can result in different phenotypes of Phe metabolic disorders. These findings are important in subjects who are carriers of mutations which are associated with a severe phenotype

(classical PKU) or with mild phenotypes (MHP) because the MHP phenotype often results from the combination of a “mild” mutation and a “severe” mutation and therefore this individual is at risk of having a child with classical PKU.

This report also highlights that it might make an erroneous assumption that two siblings will have the same genotype and phenotype and an unnecessary dietary regime might be administered.

5.2 *IN VITRO* ANALYSIS

In this work, we expressed eight variant proteins to investigate the *in vitro* PAH activity. We reproduced *in vitro* the eight mutant enzymes and measured the PAH catalytic activity. The assays revealed reduced PAH activity ranging from 7% to 51% of the activity of wild-type PAH protein, with the exception of p.Y204C, which revealed no significant impact on PAH enzyme function. Furthermore, western blot analysis showed that five variants (p.Y204C, p.L249P, p.L249F, p.S196Y and p.T380M) yielded a relatively normal amounts of PAH protein.. This indicated that these mutations do not induce changes that lead to increased degradation of the mutants. The much lower amounts of protein and the low residual activities of the p.L212P, p.R270K and p.R261P variants, instead, could reflect both the decreased activity and the folding and stability defects.

As mentioned above, in our system, all variations resulted in significant decreases in enzyme activity, with the notable exception of p.Y204C. This variant, which resulted in a tyrosine to cysteine substitution, had previously been studied by Wang et al. [68] and was identified as a silent variation, with only a small reduction in enzymatic activity (> 80% residual activity in three different systems). *In vitro* assessment of the variant enzyme did not demonstrate significantly reduced activity and did not correspond to the severe

phenotype in PKU patients. It was suggested that *in vitro* expression systems produce p.Y204C PAH; however, *in vivo*, this variation (c.611 A>G) actually affects RNA splicing. In fact, Ellingsen et al. investigated the role of the c.611 A>G variation in mRNA processing because in vitro assessment of the mutant enzyme did not demonstrate significantly reduced activity and correspond to the severe phenotype in PKU patients [69]. Analysis of the *PAH* mRNA in a patient lymphoblast cell line demonstrated that the c.611A>G variation masquerades as a missense variation but actually creates a new 5' splice site resulting in a 96 nt deletion at the 3' end of exon 6 of *PAH*. The variation was thereafter renamed Ex6-96 A>G [69]. Therefore, the study of the p.Y204C variation represents a strong example of the importance of mRNA processing studies. The p.T380M variant is one of the most frequent variations in Italian patients with the MHP phenotype. The Thr380 cluster in the Cα12/Cβ6 is located in the region that is the most likely region for the Phe binding sites [70]. The expression of the p.T380M variant protein showed reduced activity compared to the wild-type PAH protein (28±4%); patients with this variant exhibit a mild phenotype, which is in accordance with residual PAH activity of >25%. The novel variant p.S196Y, which was found in one of our MHP patients in association with the c.842+3G>C mutation (associated with a PKU phenotype) [71], and results in reduction of the enzyme activity (21±4%). The results obtained were compatible with the metabolic phenotype of the affected patient (blood Phe concentrations between 120 and 240 µmol/L). The results of both the enzymatic activity and western blotting were also in accordance also for the p.L212P, p.R261P and p.R270K variants. In fact, the observed decreased enzymatic activity, 17±7% for p.L212P, 10±0.2% for p.R261P and 11±5% for p.R270K, and the low amounts of protein expression correlated well with severe phenotypes reported in the BIOPKU database. These results suggested that these variations affected the catalytic function of the enzyme and that they could simultaneously be responsible for structural changes that could also affect the stability of the protein. These findings could be useful for predicting the

responsiveness to BH_4, because residual PAH activity is likely the main prerequisite for BH_4-responsiveness, in the sense that at least one of the alleles in a responsive patient should harbor a partially active protein and severe functionally null variations do not contribute to the response [72]. Discordant results were observed for the p.L249P and p.L249F variants. The Leu249 residue is one of the pterin-binding residues located in the loop Cα6/Cβ1. It has been found in PKU patients, and the change to proline affects the formation of the secondary structure of the protein and does not allow for the formation of α helices or β sheets. Thus, it resulted in severely decreased activity but, interestingly, exhibited normal protein expression, suggesting that the rapid protein degradation of misfolded protein could be the reason for the loss of function. In contrast, p.L249F affects the same residue, but the change is from an aliphatic leucine to an aromatic phenylalanine. Our studies indicated that the p.L249F variant protein, resulted in a normal protein expression levels and partial enzymatic activity (51±9%), although it was reported in the BIOPKU database as associated with mild-moderate PKU phenotypes. These results might suggest that the mutation has a reduced affinity for the cofactor BH_4. Finally, the present study has also demonstrated that the type of amino acid substitution in a selected codon could have a differential effect on catalytic activity. In fact, as for the Leu249 residue, similar findings were observed upon analyzing variants at Arg261 residue. The Arg261 residue is located in the loop between Cα6 and Cβ2, forming hydrogen bonds to Gln304 and Thr238. Previous *in vitro* studies [56,58,73] indicated that the p.R261Q substitution resulted in decreased protein levels and partial activity (44%), suggesting that folding and stability were affected. In contrast, the p.R261P variant in the same codon, associated with a moderate PKU phenotype, exhibited only 10% of residual activity in our experiments. Also, in this case, the substitution from an arginine to a proline residue might destabilize the catalytic domain structure.

CHAPTER 6 – CONCLUSION

6. CONCLUSION

We have undertaken PAH mutation screening in a large clinic-based cohort of HPA patients from Southern Italy and have examined phenotype–genotype correlations, including potential BH_4 responsiveness. Mutation analysis of these patients revealed a wide spectrum of mutations present similar to what has been reported in other Caucasian populations. The decreasing costs of direct sequencing or the use of next generation sequencing technology will facilitate widespread genotyping of all HPA patients, as well as carrier testing for family members. Mutation screening may inform decisions relating to disease severity and management, especially with regard to the detection of alleles associated with BH_4 responsiveness and the identification of patients who may benefit from being given access to this treatment and other novel therapies.

In fact, HPA is responsive to BH_4 supplementation in vivo in more than 70% HPA phenotypes. Infants undergoing BH_4 treatment may be fed a less rigid restricted diet, with consequent positive effects on weight gain and quality of life. Moreover, this treatment improves intellectual development especially in patients who have difficulty in adhering to a semisynthetic Phe-restricted diet. As the BH_4 positive response requires some residual PAH activity, BH_4 is believed to activate the PAH enzyme. Indeed, classification of BH_4-responsive patients, based on genotype and biochemical phenotype, and elucidation of the molecular mechanism underlying BH_4-responsiveness are crucial for HPA treatment. Various mechanisms have been implicated in BH_4-responsiveness: increased enzyme activity, correction or compensation of the BH_4 decreased affinity, protection towards catalytic inactivation and chaperone-like activity that, by stabilizing the protein, protects it from proteolytic degradation. In all cases, BH_4 responsiveness is dependent on the genotype, even though in some cases the genotype is not a reliable predictor of phenotype. Correlations of the genotype and prediction of BH_4 responsiveness remain complex as long as

there is no gold standard test for assessing sensitivity to BH_4 and inconsistent results persist.

In addition, the molecular bases of HPA and their implications at the metabolic level were addressed in the second part of this present thesis. Results from initial patient-based studies were investigated in the laboratory by *in vitro* analysis and characterization of HPA mutations, using a novel method tandem mass spectrometry method for assessing *in vitro* PAH activity.

Detailed enzymatic analysis of mutant PAH enzymes and measurement of PAH protein in cell culture provided important information on the structure and function of wild-type PAH and a range of mutant forms. However, even *in vitro* studies performed on PAH do not reliably reflect in vivo conditions and may be associated with certain ambiguities. Although HPA caused by PAH deficiency is well established as a monogenic trait, it has been shown that other genes involved in phenylalanine metabolism can cause hyperphenylalaninemia, including those encoding enzymes responsible for the synthesis and regeneration of BH_4 and pterin-4a-carbinolamine dehydratase (PCD). Inherited dysfunction of any of these factors may modify the phenotype associated with a particular PAH mutation genotype and result in complex traits leading to unexpected phenotypes [74].

Further expression, structural and mechanistic studies will help to predict the likely effects of unclassified mutations associated with PKU, as well as new mutations that may be discovered.

Finally, we should bear in mind that the results of the expression analysis represent only another piece in the genotype–phenotype puzzle which should be complemented with the three dimensional structure predictions, the patients' data, and further additional studies, such as in vivo response to BH_4. This will help unravel the final consequences of a particular mutation on the structure-function relationship of the PAH enzyme and thus allow the implementation of an optimal and individually tailored treatment.

References

[1] Williams R, Mamotte C, Burnett J, Phenylketonuria: An Inborn Error of Phenylalanine Metabolism, Clin Biochem Rev. 2008 Feb; 29(1): 31–41.

[2] Loeber JG, Neonatal screening in Europe; the situation in 2004, J Inherit Metab Dis. 2007 Aug;30(4):430-8.

[3] Zhan JY, Qin YF, Zhao ZY. Neonatal screening for congenital hypothyroidism and phenylketonuria in China. World J Pediatr 2009; 5: 136–9.

[4] Jiang J, Ma X, Huang X, et al. A survey for the incidence of phenylketonuria in Guangdong, China. Southeast Asian J Trop Med Public Health 2003; 34 Suppl 3:185.

[5] Pangkanon S, Charoensiriwatana W, Janejai N, et al. Detection of phenylketonuria by the newborn screening program in Thailand. Southeast Asian J Trop Med Public Health 2009 May;40(3):525-9.

[6] Desviat LR, Perez B, Gamez A, et al. Genetic and phenotypic aspects of phenylalanine hydroxylase deficiency in Spain: molecular survey by regions. 1999 Apr; 7(3):386-92

[7] Blau N, Van Spronsen FJ, Levy HL, Phenylketonuria. Lancet 376 (2010) 1417-1427.

[8] Blau N, Burton B, Levy HL et al. Phenylketonuria and BH_4 deficiencies. 2th edn – Bremen: UNI – MED, 2013.

[9] Pietz J, Fatkenheuer B, et al. Psychiatric disorders in adult patients with early-treated phenylketonuria. Pediatrics. 1997 Mar;99(3):345-50.

[10] Weglage J, Funders, B, et al. Psychological and social findings in adolescents with phenylketonuria. Eur J Pediatr.1992 Jul;151(7):522-5.

[11] Waisbren SE and Zaff J. Personality disorder in young women with treated phenylketonuria. J Inherit Metab Dis.1994;17(5):584-92.

[12] Mazur A, Jarochowicz S, et al. Evaluation of somatic development in adult patients with previously undiagnosed and/or untreated phenylketonuria. Med Princ Pract. 2010;19(1):46-50.

[13] Smith QR, Momma S, et al. Kinetics of neutral amino acid transport across the blood-brain barrier. J Neurochem. 1987 Nov;49(5):1651-8.

[14] Bauman ML, Kemper TL. Morphologic and histoanatomic observations of the brain in untreated human phenylketonuria. Acta Neuropathol. 1982;58(1):55-63.

[15] Sitta A, Barschak AG, et al. Effect of short- and long-term exposition to high phenylalanine blood levels on oxidative damage in phenylketonuric patients. Int J Dev Neurosci. 2009 May;27(3):243-7.

[16] Sitta A, Barschak AG, Deon M, et al. Investigation of oxidative stress parameters in treated phenylketonuric patients. Metab Brain Dis. 2006 Dec;21(4):287-96.

[17] Cleary MA. Phenylketonuria. Paediatrics and Child Health 2014.

[18] Pietz J, Kreis R, et al. Large neutral amino acids block phenylalanine transport into brain tissue in patients with phenylketonuria. J Clin Invest. 1999 Apr 15; 103(8): 1169–1178.

[19] Koch R, Moseley KD, et al. Large neutral amino acid therapy and phenylketonuria: a promising approach to treatment. Mol Genet Metab. 2003 Jun;79(2):110-3.

[20] Moats RA, Moseley KD, et al. Brain phenylalanine concentrations in phenylketonuria: research and treatment of adults. Pediatrics. 2003 Dec;112(6 Pt 2):1575-9.

[21] Lim K, van Calcar SC, et al. Acceptable low-phenylalanine foods and beverages can be made with glycomacropeptide from cheese whey for individuals with PKU. Mol Genet Metab. 2007 Sep-Oct;92(1-2):176-8.

[22] Ney DM, Gleason ST, et al. Nutritional management of PKU with glycomacropeptide from cheese whey. J Inherit Metab Dis. 2009 Feb;32(1):32-9.

[23] Van Calcar SC, MacLeod EL, et al. Improved nutritional management of phenylketonuria by using a diet containing glycomacropeptide compared with amino acids. Am J Clin Nutr. 2009 Apr;89(4):1068-77.

[24] Gamez A, Wang L, et al. Structure-based epitope and PEGylation sites mapping of phenylalanine ammonia-lyase for enzyme substitution treatment of phenylketonuria. Mol Genet Metab. 2007 Aug;91(4):325-34.

[25] Kang TS, Wang L, et al. Converting an injectable protein therapeutic into an oral form: phenylalanine ammonia lyase for phenylketonuria. Mol Genet Metab. 2010 Jan;99(1):4-9.

[26] Anjema K, van Rijn M, Hofstede FC, et al. Tetrahydrobiopterin responsiveness in phenylketonuria: prediction with the 48-hour loading test and genotype. Orphanet J Rare Dis. 2013 Jul 10;8:103.

[27] Strisciuglio P, Concolino D. New Strategies for the Treatment of Phenylketonuria (PKU). Metabolites. 2014 Nov 4;4(4):1007-17.

[28] Ding Z, Georgiev P, Thöny B. Administration-route and gender independent long-term therapeutic correction of phenylketonuria (PKU) in a mouse model by recombinant adeno-associated virus 8 pseudotyped vector-mediated gene transfer. Gene Ther. 2006 Apr;13(7):587-93.

[29] Ding Z, Harding CO, Rebuffat A et al. Correction of murine PKU following AAVmediated intramuscular expression of a complete phenylalanine hydroxylating system. Mol Ther. 2008 Apr;16(4):673-81.

[30] Doskeland AP, Martinez A, Knappskog PM, Flatmark T. Phosphorylation of recombinant human phenylalanine hydroxylase: effect on catalytic activity, substrate activation and protection against non-specific cleavage of the fusion protein by restriction protease. Biochem J. 1996 Jan 15;313 (Pt 2):409-14.

[31] Kaufman S. New tetrahydrobiopterin-dependent systems Annu Rev Nutr. 1993;13:261-86.

[32] Mitnaul LJ, Shiman R. Coordinate regulation of tetrahydrobiopterin turnover and phenylalanine hydroxylase activity in rat liver cells. Proc Natl Acad Sci U S A.1995 Jan 31;92(3):885-9.

[33] Pey AL, Thorolfsson M, Teigen K, et al. Thermodynamic characterization of the binding of tetrahydropterins to phenylalanine hydroxylase. J Am Chem Soc. 2004 Oct 27;126(42):13670-8.

[34] Teigen K, Martinez A. Probing cofactor specificity in phenylalanine hydroxylase by molecular dynamics simulations. J Biomol Struct Dyn. 2003 Jun;20(6):733-40.

[35] Tayeh MA, Marletta MA. Macrophage oxidation of L-arginine to nitric oxide, nitrite, and nitrate. Tetrahydrobiopterin is required as a cofactor. J Biol Chem. 1989 Nov 25;264(33):19654-8.

[36] Gorren AC, Mayer B. Tetrahydrobiopterin in nitric oxide synthesis: a novel biological role for pteridines. Curr Drug Metab. 2002 Apr;3(2):133-57.

[37] Werner ER, Blau N, Thony B. Tetrahydrobiopterin: biochemistry and pathophysiology. Biochem J. 2011 Sep 15;438(3):397-414.

[38] Waters PJ. How PAH gene mutations cause hyper-phenylalaninemia and why mechanism matters: insights from in vitro expression. Hum Mutat. 2003 Apr;21(4):357-69.

[39] Gersting SW, Kemter KF, Staudigl M, et al. Loss of function in phenylketonuria is caused by impaired molecular motions and conformational instability. Am J Hum Genet. 2008 Jul;83(1):5-17.

[40] Erlandsen H, Pey AL, Gámez A, et al. Correction of kinetic and stability defects by tetrahydrobiopterin in phenylketonuria patients with certain phenylalanine hydroxylase mutations. Proc Natl Acad Sci U S A. 2004 Nov 30;101(48):16903-8.

[41] Blau N, Hennermann JB, Langenbeck U, et al. Diagnosis, classification, and genetics of phenylketonuria and tetrahydrobiopterin (BH_4) deficiencies. Mol Genet Metab. 2011;104 Suppl:S2-9.

[42] Zorzi G, Thony B, Blau N. Reduced nitric oxide metabolites in CSF of patients with tetrahydrobiopterin deficiency. J Neurochem. 2002 Jan;80(2):362-4.

[43] Pey AL, Stricher F, Serrano L, et al. Predicted effects of missense mutations on native-state stability account for phenotypic outcome in phenylketonuria, a paradigm of misfolding diseases. Am J Hum Genet. 2007 Nov;81(5):1006-24.

[44] Siddiqi O. Interallelic complementation in vivo and in vitro. Br Med Bull. 1965 Sep;21(3):249-53.

[45] Trefz FK, Scheible D, Gotz H, et al. Significance of genotype in tetrahydrobiopterin-responsive phenylketonuria. J Inherit Metab Dis. 2009 Feb;32(1):22-6.

[46] Lichter-Konecki U, Rupp A, Konecki DS, et al. Relation between phenylalanine hydroxylase genotypes and phenotypic parameters of diagnosis and treatment of hyperphenylalaninaemic disorders. German Collaborative Study of PKU. J Inherit Metab Dis. 1994;17(3):362-5.

[47] Heintz C, Cotton RG, Blau N. Tetrahydrobiopterin, its mode of action on phenylalanine hydroxylase, and importance of genotypes for pharmacological therapy of phenylketonuria. Hum Mutat. 2013 Jul;34(7):927-36.

[48] Kim SW, Jung J, Oh HJ, et al. Structural and functional analyses of mutations of the human phenylalanine hydroxylase gene. Clin Chim Acta. 2006 Mar;365(1-2):279-87.

[49] Pey AL, Pérez B, Desviat LR, et al. Mechanisms underlying responsiveness to tetrahydrobiopterin in mild phenylketonuria mutations. Hum Mutat. 2004 Nov;24(5):388-99.

[50] Erlandsen H, Patch MG, et al. Structural studies on phenylalanine hydroxylase and implications toward understanding and treating phenylketonuria. Pediatrics. 2003 Dec;112(6 Pt 2):1557-65. Review.

[51] Blau N, Erlandsen H. The metabolic and molecular bases of tetrahydrobiopterin-responsive. Mol Genet Metab. 2004 Jun;82(2):101-11.

[52] Karacić I, Meili D, Sarnavka V, et al. Genotype-predicted tetrahydrobiopterin (BH_4)-responsiveness and molecular genetics in Croatian patients with phenylalanine hydroxylase (PAH) deficiency. Mol Genet Metab. 2009 Jul;97(3):165-71.

[53] Scriver CR. Whatever happened to PKU? Clin Biochem. 1995 Apr;28(2):137-44.

[54] Scriver CR, Waters PJ. Monogenic traits are not simple: lessons from phenylketonuria. Trends Genet. 1999 Jul;15(7):267-72.

[55] Pérez-Dueñas B, Vilaseca MA, Mas A, et al. Tetrahydrobiopterin responsiveness in patients with phenylketonuria. Clin Biochem.2004 Dec;37(12):1083-90.

[56] Pey AL, Desviat LR, et al. Phenylketonuria: genotype-phenotype correlations based on expression analysis of structural and functional mutations in PAH. Hum Mutat. 2003 Apr;21(4):370-8.

[57] Pey AL, Ying M, Cremades N, et al. Identification of pharmacological chaperones as potential therapeutic agents to treat phenylketonuria, J Clin Invest. 2008 Aug;118(8):2858-67.

[58] Heintz C, Troxler H, Martinez A, et al. Quantification of phenylalanine hydroxylase activity by isotope-dilution liquid chromatography-electrospray

ionization tandem mass spectrometry. Mol Genet Metab. 2012 Apr;105(4):559-65.

[59] Trunzo R, Santacroce R, D'Andrea G, et al. Intra-familiar discordant PKU phenotype explained by mutation analysis in three pedigrees. Clin Biochem. 2014 Feb;47(3):233-5.

[60] Avigad S, Cohen BE, Bauer S, et al. A single origin of phenylketonuria in Yemenite Jews. Nature 1990;8(344):168–70.

[61] Guldberg P, Rey F, Zschocke J, 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 1998;63:71–9.

[62] Bercovich D, Elimelech A, Zlotogora J, et al. Genotype– phenotype correlations analysis of mutations in the phenylalanine hydroxylase (PAH) gene. J Hum Genet 2008;53:407–18.

[63] Trunzo R, Santacroce R, D'Andrea G, et al. Phenylalanine hydroxylase deficiency in south Italy: Genotype-phenotype correlations, identification of a novel mutant PAH allele and prediction of BH_4 responsiveness. Clin Chim Acta. 2015 Oct 23;450:51-5.

[64] Zurfluh MR, Zschocke J, Lindner M, et al. Molecular genetics of tetrahydrobiopterin responsive phenylalanine hydroxylase deficiency. Hum. Mutat. 29 (2008) 167–175.

[65] Rivera I, Mendes D, Afonso A, et al. Phenylalanine hydroxylase deficiency: molecular epidemiology and predictable BH_4-responsiveness in South Portugal PKU patients. Mol. Genet. Metab. 104 (2011) S86–S92.

[66] Konecki DS, Lichter-Konecki U, The phenylketonuria locus: current knowledge about alleles and mutations of the phenylalanine hydroxylase gene in various populations. Hum Genet. 1991 Aug;87(4):377-88.

[67] Tyfield LA, Zschocke J, Stephenson A, et al. Discordant phenylketonuria phenotypes in one family: the relationship between genotype and clinical outcome is a function of multiple effects. J Med Genet. 1995 Nov;32(11):867-70.

[68] Wang T, Okano Y, Eisensmith RC, et al. Missense mutations prevalent in orientals with phenylketonuria: Molecular characterization and clinical implications. Genomics.1991 Jun;10(2):449-56.

[69] Ellingsen S, Knappskog PM, Eiken HG. Phenylketonuria Splice Mutation (EXON6nt-96A>G) Masquerading as Missense Mutation (Y204C). Hum Mutat. 1997;9(1):88-90.

[70] Arturo EC, Gupta K, Heroux A, et al. First structure of full-length mammalian phenylalanine hydroxylase reveals the architecture of an autoinhibited tetramer. Proc Natl Acad Sci U S A. 2016.

[71] Trunzo R, Santacroce R, D'Andrea G, Longo V, De Girolamo G, Dimatteo C, Leccese A, Lillo V, Papadia F, Margaglione M. Mutation analysis in hyperphenylalaninemia patients from South Italy. Clin Biochem. 2013 Dec;46(18):1896-8.

[72] Perez B, Desviat LR, Gomez-Puertas P, et al. Kinetic and stability analysis of PKU mutations identified in BH_4-responsive patients. Mol Genet Metab. 2005 Dec;86 Suppl 1:S11-6.

[73] Gjetting T, Petersen M, Guldberg P, Güttler F. In vitro expression of 34 naturally occurring mutant variants of phenylalanine hydroxylase: correlation with metabolic phenotypes and susceptibility toward protein aggregation. Mol Genet Metab. 2001 Feb;72(2):132-43.

[74] Vockley J, Andersson HC, Antshel KM, et al. Phenylalanine hydroxylase deficiency: diagnosis and management guideline. Genet Med. 2014 Feb;16(2):188-200.

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