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Minireview

Diagnosis, classification, and genetics of phenylketonuria and tetrahydrobiopterin (BH4) deficiencies

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ABSTRACT

This article summarizes the present knowledge, recent developments, and common pitfalls in the diagnosis, classification, and genetics of hyperphenylalaninemia, including tetrahydrobiopterin (BH4) deficiency. It is a product of the recent workshop organized by the European Phenylketonuria Group in March 2011 in Lisbon, Portugal. Results of the workshop demonstrate that following newborn screening for phenylketonuria (PKU), using tandem mass-spectrometry, every newborn with even slightly elevated blood phenylalanine (Phe) levels needs to be screened for BH4 deficiency. Dried blood spots are the best sample for the simultaneous measurement of amino acids (phenylalanine and tyrosine), pterins (neopterin and biopterin), and dihydropteridine reductase activity from a single specimen. Following diagnosis, the patient's phenotype and individually tailored treatment should be established as soon as possible. Not only blood Phe levels, but also daily tolerance for dietary Phe and potential responsiveness to BH4 are part of the investigations. Efficiency testing with synthetic BH4 (sapropterin dihydrochloride) over several weeks should follow the initial 24–48-hour screening test with 20 mg/kg/day BH4. The specific genotype, i.e. the combination of both *PAH* alleles of the patient, helps or facilitates to determine both the biochemical phenotype (severity of PKU) and the responsiveness to BH4. The rate of Phe metabolic disposal after Phe challenge may be an additional useful tool in the interpretation of phenotype–genotype correlation.

Contents

Abbreviations: BH4, tetrahydrobiopterin; CNS, central nervous system; DHPR, dihydropteridine reductase; DBS, dried blood spot; HPA, hyperphenylalaninemia; KOUT, 1st order rate of metabolic disposal; MHP, mild HPA; PAH, phenylalanine hydroxylase; PBW, percent body weight; PKU, phenylketonuria; PROT, net protein synthesis; TMS, tandem mass-spectrometry.

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1. Diagnosis

1.1. Newborn screening

Phenylketonuria (PKU) is identified through national newborn screening programs [1]. The first efficient test for hyperphenylalaninemia (HPA) was a bacterial inhibition assay developed by Robert Guthrie [2]. The test was based on *Bacillus subtilis*, which requires phenylalanine (Phe) for growth. The Guthrie test was very useful for mass screening as the dried blood spot (DBS) can be obtained in the hospital or a doctor's office using a standardized filter paper ("Guthrie card") and mailed to reference laboratories in an envelope. Tandem mass-spectrometry (TMS) was developed as a fast method for achieving reliable and quantitative determination of concentrations of amino acids in small volumes of blood or plasma [3]. This method produces a lower rate of false positive results, by measuring levels of both Phe and Tyr and providing the Phe/Tyr ratio, and thus requires fewer resources to follow up such cases. In addition, other inborn errors of metabolism can be identified simultaneously.

All infants should be screened for PKU within the first days of life, in order to allow timely dietary intervention to protect children with PKU from neurological damage. Where screening is carried out in maternity wards, the blood sample is usually obtained between days 2 and 5; in general, however, screening is carried out mostly between the ages of 2 and 7 days [4]. In the U.S., samples are typically obtained at 24–48 h. A commonly used Phe cut-off level for diagnosis of PKU is 120–130 μmol/L (with a Phe/Tyr ratio >2), with TMS employed [5]. Concern has been expressed that screening too early, associated with a shift towards earlier discharge from maternity wards, can provide a false negative result, as there will have been insufficient opportunity for Phe from the diet to build up to diagnostic (and toxic) levels. It is currently accepted that the sensitivity of screening in a healthy neonate is adequate before 24 h of life, especially where the screening test involves measurement of Phe/Tyr ratios to increase sensitivity relative to Phe measurement alone [3,6]. However, as the pretreatment level is often used as a diagnostic parameter for the classification of the PKU phenotype new cut-offs have to be determined for classic and mild PKU and mild HPA when measuring the Phe level so early.

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. The results of early PKU screening should also be interpreted with caution in sick neonates or in neonates under parenteral nutrition or blood transfusion, and a second screening test should be sent if it is unclear whether the child had sufficient protein intake when the first test was collected.

1.2. Differential diagnosis

About 2% of all Phe level elevations detected by the newborn screening are due to disorders in BH4 metabolism, highlighting the importance of always considering the differential diagnosis for every even slightly elevated blood Phe level [7]. Frequency of BH4 deficiency is higher in some countries where the rate of consanguineous marriages tends to maintain the presence of genetic disorders within families, e.g. Turkey or Saudi Arabia [8]. BH4 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 BH4 (sapropterin dihydrochloride) is crucial for a good outcome. Analysis of DBS or urine for neopterin and biopterin and measurement of dihydropteridine reductase (DHPR) activity in the DBS is essential for the exact diagnosis and should be performed as early as possible. A BH4 loading test and measurement of neurotransmitter metabolites, pterins, and folates in cerebrospinal fluid add further important information about the severity of the disease [9].

In patients with BH4 deficiency, the pattern of pterins is identical in blood, urine, and CSF. The use of DBS on filter paper (Guthrie card) is, however, more practical and allows measurement of pterins, DHPR activity, and amino acids from a single specimen [10]. It is important to know that patients with classic PKU excrete more pterins in urine compared with healthy controls and the amount of excreted metabolites is directly proportional to blood Phe levels. Diseases that cause activation of the immune system (elevated neopterin), and anticancer therapy or rheumatic disease therapy with methotrexate (inhibition of DHPR), may interfere with the analytic procedures. 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. Fig. 1 shows the algorithm for the diagnostic work-up of elevated blood Phe levels. Table 1 summarizes the most important biochemical parameters used in the differential diagnosis of HPA.

1.3. BH4 loading test

The BH4 loading test was initially used to discriminate between patients with elevated phenylalanine levels due to PAH deficiency and patients with elevated Phe levels due to BH4 deficiency (enzyme defects in the biosynthesis or regeneration of the cofactor BH4) [11,12]. Thus, the loading test is an additional useful tool for the early detection of BH4 deficiencies and it was used in Europe for almost 30 years. In addition, this test detects PKU patients responsive to BH4 administration.

Detection of BH4-responsive PKU patients is important because some PKU patients benefit from oral administration of BH4 (sapropterin dihydrochloride) in that their blood Phe level decreases or even normalizes under pharmacological therapy with BH4 [13]. The phenomenon of BH4-sensitive PKU was initially described in Japanese patients, and confirmed in retrospective and prospective studies with large cohorts of patients [14–16]. Modalities of the BH4 challenge vary in the literature from a 24-hour test with a single administration of BH4 (10–20 mg/kg) to several weeks of administration with daily or weekly monitoring of blood Phe levels [17,18].

There is general agreement that a reduction on blood Phe of at least 30% in response to BH4 loading indicates a clinically significant effect, although in some centers a lower cut-off value may be defined for individual patients, or no specific cut-off value may be used [19]. The frequency of BH4-responsiveness is highest in patients with mild (non-PKU) HPA, or mild PKU resulting from *PAH* mutations that allow for residual enzyme activity [15,20]. Conversely, the response rate among patients with classic PKU (little or no residual

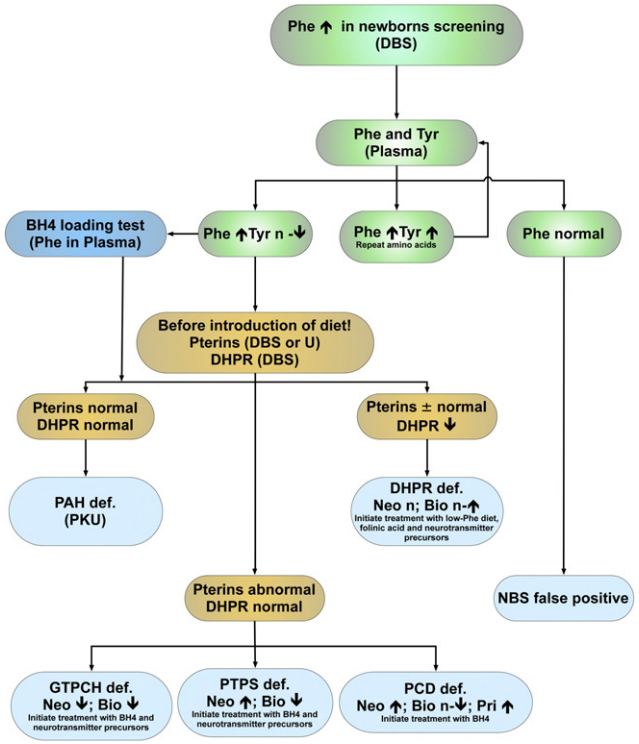

Fig. 1. Diagnostic flow-chart for the laboratory diagnosis of PKU and BH4 deficiencies. Modified according to Opladen et al. [10]. Dried blood spots (DBS) or random urine (U) can be used for the differential diagnosis and depending on the profile of neopterin (Neo), biopterin (Bio), and primapterin (Pri) and dihydropteridine reductase (DHPR) activity in DBS, diagnosis of following BH4 deficiencies can be established: GTP cyclohydrolase I (GTPCH) deficiency (low or no detectable neopterin and biopterin), 6-pyruvoyl-tetrahydropterin synthase (PTPS) deficiency (high neopterin and low or no detectable biopterin), dihydropteridine reductase (DHPR) deficiency (normal neopterin and normal or elevated biopterin and no DHPR activity), and pterins-4a-carbinolamine dehydratase (PCD) deficiency (elevated neopterin, low-normal biopterin, and elevated primapterin). n: normal.

PAH activity) is very low. A number of *PAH* mutations associated with BH4 responsiveness have been identified and genotyping is a useful additional tool for predicting responsiveness [21,22] (see below). Fig. 2 summarizes the proposed procedure for the BH4 loading test in Europe. In newborns the test should be performed before introducing the low-Phe diet and at elevated blood Phe levels (>400 μmol/L). In infant or adult PKU patients on a Phe-restricted diet, the diet needs to be modified by increasing the protein intake (egg or milk powder before and during the test). Table 2 summarizes factors that may potentially influence the outcome of the test.

An international online survey [23] with 92 participants from 30 different countries documented that in 62% of the metabolic centers the BH4 loading test is an integral part of the diagnostic work-up for PKU. The main reason for not using the BH4 test is either relatively high costs or no availability of BH4 in some countries (78%). Most centers use the BH4 test in all age groups (79%) and only in about 11% of the metabolic centers pregnant PKU women are tested. A

Table 1
Biochemical parameters in dried blood spots (DBS) used in the differential diagnosis of PKU and BH4 deficiencies.

Deficiency	Neopterin	Biopterin	Primapterin	DHPR activity	Phe
PKU	n-↑	n-↑	n	n	↑
GTPCH	↓	↓	n	n	↑[a]
PTPS	↑	↓	n	n	↑
PCD	↑	↓-n	↑	n	↑
DHPR	n	n-↑[a]	n	↓	↑

[a] Can be normal in the early neonatal period.

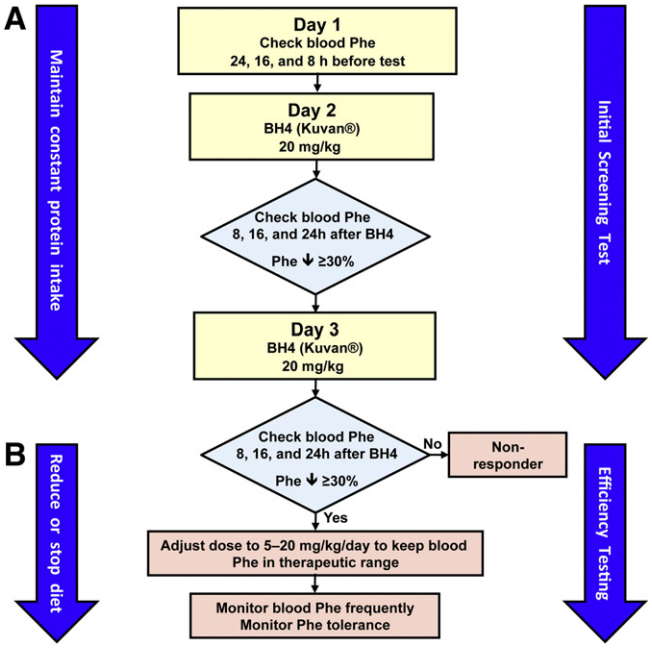

Fig. 2. Proposed algorithms for the BH4 (sapropterin dihydrochloride) challenge, screening, and initiating treatment in BH4-responsive PKU patients. A) Initial screening test with blood Phe monitoring on the first day and BH4 (sapropterin dihydrochloride) administration (20 mg/kg) on two following days; B) Efficiency testing in BH4-reponsive patients over several weeks with BH4 doses adjusted individually according to Phe tolerance and therapeutic blood Phe levels.

dosage of 20 mg/kg is used in 92% of the centers and duration of the test is quite variable: 24 h (33%), 48 h (24%), 72 h (16%), and about 26% run the test over 1–4 weeks, most of them from the U.S. with test duration over at least 4 weeks. About half of the survey participants defined BH4-responsiveness as both increase in tolerance for dietary Phe (by a factor of two) and reduction in blood Phe levels (by at least 30%), while the rest use only blood Phe reduction as criterion.

1.4. Cerebrospinal fluid investigation

BH4 deficiency influences the synthesis of catecholamines, serotonin [24] and nitric oxide [25] in the central nervous system (CNS), and measurement of their metabolites in cerebrospinal fluid (CSF) is important for the diagnosis of different forms (severe v. mild) of BH4 deficiencies. Not only the absolute levels of 5-hydroxyindoleacetic acid and homovanillic acid in CSF, but also differences in the ratios of neurotransmitter levels provide important diagnostic information relating to the severity and outcome of BH4 deficiency [26].

2. Classification

2.1. Phenylalanine loading test

Phenylalanine loading tests were applied since 1956 for the detection of heterozygotes in PKU families [27], until, in the late 1980s, this

Table 2
Factors potentially affecting the BH4 loading test.

- BH4 dosage (higher sensitivity with 20 mg/kg v. 10 mg/kg)
- Duration of the test (24–48 h for initial screening v. 4–8 weeks for efficiency)
- Food intake (better GI absorption of BH4 with high calories food)
- Age (outcome may be different in newborns v. adolescents v. adults)
- Diet (better response to BH4 when out of diet and on higher blood Phe levels)
- GI absorption (may be individually different; monitor blood BH_4 levels)
- Genotype

approach was replaced by molecular analysis of *PAH* gene haplotypes and mutations [28]. Phenylalanine loading tests gained further interest when Guthrie card mass screening uncovered not only the expected cases of classic PKU but also variants of PKU [29]. Because these variants were initially thought not to require dietary treatment, a reliable discrimination of these phenotypes was needed. For practical and ethical reasons, *in vivo* ^{3}H or ^{14}C isotope studies [30] or invasive testing such as enzyme assays from liver biopsies [31] were not appropriate. Therefore, Blaskovics [32] developed in the mid-1960s the standardized three day natural protein loading test with evaporated milk that was applied at 6 months of age in the U.S. [33] and German [34] PKU Collaborative Studies for classification of PKU and for genotype-phenotype analysis. With this test three principal types of Phe blood level response, types 1, 2 and 3, were delineated in both studies. Type 1 is characterized by a 72 h Phe beyond 1200 μmol/L and corresponds to classic PKU. Type 2 is defined by a spontaneous decline of Phe levels, despite continuation of loading, from above 1200 μmol/L after day 2 to a 72 h Phe levels of 1200–600 μmol/L. In both studies, about 10% of the patients belong to this type. In type 3 the plasma Phe fluctuates around 600 μmol/L and the 72 h Phe levels are <600 μmol/L. Clinically, it corresponds to mild HPA. On a free diet, these patients (22% in the U.S., and 13% in the German study) do not need a low-Phe treatment for normal mental outcome [35].

Major successes of the Blaskovics loading test were (i) the discovery of the type 2 response as an indicator of mutant enzyme activation by Phe [36], (ii) the delineation of the type 3 response as the base for the decision to discontinue dietetic treatment, and (iii) its use as a measure of *in vivo* phenotype for genotype–phenotype analysis. In contrast to its proven scientific value, the test has only limited value for the dietetic treatment of patients with classic and mild PKU because the Phe 72 value will not predict the current and future individual dietary requirements (see below) and the patients may manifest during the test signs of intoxication such as nausea, vomiting, irritability, insomnia and EEG changes. The test is no longer necessary [37] and has been replaced in practice by predictive molecular and enzymatic classifications [22,38]. A recent online survey accordingly revealed that only 4% of centers still use it for estimating the phenotype of their patients [23].

2.2. Phenotypes

Depending on the enzyme defect, the genotype and the severity of the disease, different forms of PKU with different clinical phenotypes have been described. Thus, different classifications for PKU phenotypes have been established. PKU may be classified as classic PKU and as variant PKU which includes all milder forms of PKU, (i.e. moderate PKU and mild PKU), as mild HPA or non-PKU HPA, and, additionally, as BH4-responsive PKU [29,39,40]. Definition of PKU phenotypes may be essential in establishing treatment options, e.g. new therapeutic strategies, in counseling and in prediction of the outcome, and in pregnancy. Pretreatment blood Phe levels, the individual Phe tolerance, and the clinical course of the disease may help to discriminate the different phenotypes of PKU, but are not precise parameters and the cut-offs for pretreatment levels collected during the first 24–48 h, as they relate to the different types of PKU, have to be newly defined.

2.3. Blood phenylalanine

In 1980, for the first time, blood Phe levels were used to discriminate between three different phenotypes of PKU [29]. Still, this classification of the various types of PAH deficiency is used for phenotyping PKU: Classic PKU is defined by presenting with Phe pretreatment levels >1200 μmol/L, variant PKU with Phe pretreatment levels of 600–1200 μmol/L, and mild HPA with Phe pretreatment levels<600 μmol/L. More precisely, PAH deficiency may be classified into four different phenotypes: classic PKU presenting with Phe pretreatment levels >1200 μmol/L, moderate PKU with Phe pretreatment levels of 900–1200 μmol/L, mild PKU with Phe pretreatment levels of 600–900 μmol/L, and mild HPA with Phe pretreatment level <600 μmol/L [38,39].

Although pretreatment Phe levels are indispensable for phenotyping PKU, they are dependent on some variables, e. g. on the timing of blood Phe measurement, on the neonatal catabolism, and on the diet received at the time of blood sampling. Due to improvement in newborn screening with an early blood sampling at day three of life, patients with PKU are diagnosed much earlier, thus resulting in significantly lower pretreatment Phe levels. If pretreatment Phe levels will be used for determining PKU phenotypes in the future, this classification needs to be adjusted. In day-to-day-practice, pretreatment blood Phe levels have been shown to be used for phenotyping patients with PKU in about 80% of the treatment centers [23].

2.4. Phenylalanine tolerance

Daily Phe tolerance has been established as a stable parameter for phenotyping the various types of PAH deficiency [29]. Phe tolerance is usually determined at the age of 5 years and indicates the amount of daily Phe intake that a patient can tolerate without an increase of the blood Phe level above the upper target range. Three different phenotypes may be classified by using Phe tolerance: classic PKU with a Phe tolerance <20 mg/kg/day, variant PKU with a Phe tolerance of 20–50 mg/kg/day, and mild HPA with a Phe tolerance >50 mg/kg/day [29]. More detailed is the classification into four different phenotypes, who defines classic PKU with a Phe tolerance <20 mg/kg/day (250–300 mg/day), moderate PKU with a Phe tolerance of 20–25 mg/kg/day (350–400 mg/day), mild PKU with a Phe tolerance of 25–50 mg/kg/day (400–600 mg/day), and mild HPA with patients off diet [38,39].

Recently it has been shown that Phe tolerance may be predictable already at the age of 2 years, and that Phe tolerance at age 2, 3, and 5 years correlates with that at age 10 years [41]. In contrast, reassessment of Phe tolerance may be necessary in adults [42]. Although Phe tolerance is a good indicator for the PKU phenotype, its determination may be unreliable if not determined under standardized conditions. In day-to-day-practice, prescribed Phe intake often is much lower than the effective Phe intake at home. We therefore recommend determining Phe tolerance under standardized in-patient conditions with precise dietary protocols. In clinical follow-up, Phe tolerance is used for phenotyping patients with PKU in 70% of metabolic centers queried [21].

2.5. Clinical course

Furthermore, various types of PAH deficiency may be distinguished by the clinical course of the disease. This includes data on the outcome (e. g. education, IQ), the maximum blood Phe concentrations (e. g. during febrile infection, dietary non-compliance), the fluctuation of blood Phe levels, and the Phe/Tyr ratios [43,44]. Though, in day-to-day-practice, clinical course of the disease is only used in 31% of metabolic centers to distinguish different PKU forms [23].

In day-to-day practice, classification of PKU is essential for choosing the optimal treatment. This may suggest a simplified classification scheme based on treatment requirements: a) patients who do need strict dietary treatment (PKU), b) patients who do not need any treatment (non-PKU HPA), c) patients who may be treated with BH4 (BH4-responsive PKU).

2.6. Software and phenylalanine home monitoring device

The Blaskovics loading tests at 6 months with their resulting 72 h Phe values [32] yield indicators of metabolic phenotypes which belong

to a bimodal distribution with nadir at about 1,200 and 1,600 μmol/L, respectively [33,34], thus establishing a classification into classic and variant PKU. A more detailed separation of the metabolic phenotypes was established by Güttler and Guldberg [45] through estimating Phe tolerance at 5 years of age (see also above). Neither of these systems can, at any age, predict the individual Phe tolerance. This is made possible, however, by mathematical analysis of the data with a kinetic model using per cent body water (PBW), net protein synthesis (PROT), and 1st order rate of metabolic disposal (KOUT) of Phe as variable parameters [46]. Both PBW and PROT are age-dependent parameters, whereas KOUT is expected not to change with age. With age-specific PBW and PROT data from the literature [47], an age-independent KOUT would enable the reliable prediction of evolution of Phe tolerance at all ages. Even in adults, from their Blaskovics test data, provided the patients, or their data, can be 'retrieved' again [48].

By kinetic analysis of the protein loading data of the German PKU Study it has been possible to explain the type 2 response as a consequence of mutant PAH activation by high Phe levels [36]. Also some mild cases, originally classified as type 1, were found to be activated by Phe levels to some extent. The distribution of the KOUT estimates, including the maximal KOUT estimates of the cases with activation, is depicted in Fig. 3. This distribution is apparently multimodal and the peaks appear to represent classic (a), moderate (b), and mild PKU (c), and mild HPA (d), respectively. These findings can be compared to the classification of Güttler and Guldberg [45] by treating the dietary tolerance at the target Phe level as the equilibrium state of Phe metabolism. In the kinetic model [46] this corresponds to:

$$\text{Target Phe} = ((\text{intake}-\text{PROT})\text{mg/day})/\text{PBW} * \text{KOUT/day} \quad (1)$$

With Eq. (1) and taking PBW = 0.65 and PROT = 2.7 mg Phe per kg body weight and day, the classification of Güttler and Guldberg translates into KOUT values of 0.62 and 1.08 per day for moderate and mild PKU, respectively. The data at KOUT = 1.7 (d) corresponds within this system to an equilibrium Phe level of 110 μmol/L at an intake of 120 mg Phe per kg body weight and day (here taken as 'normal diet').

These calculations implicate that the constant of metabolic disposal of Phe is about identical between 6 months and 5 years of age and may therefore be considered as a patient-specific parameter. This is relevant for the day-to-day management of PKU children, because it has been shown (in an observational study) that increased Phe values, in a considerable part of cases, are the consequence of hidden catabolism (as indicated in the model by diminished PROT values) and not of non-compliance [49].

Application of this kinetic model in a user-friendly format (e.g. a so-called App) would support self-control with home-monitoring devices [50] and could be trained e.g. in PKU summer camps. Such a device is presently in development and according to a recent survey [23], 85% would consider home monitoring of Phe useful for both children and pregnant women.

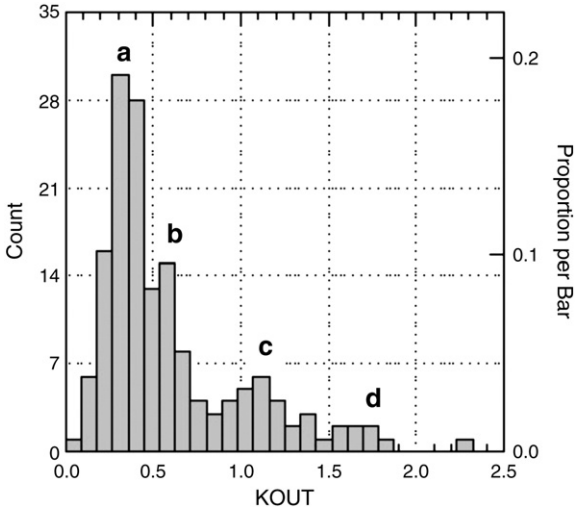

Fig. 3. Distribution of KOUT [per day], the 1st order rate constant of metabolic disposal of Phe, in the German PKU Study [36], N = 157. The data suggest the peaks a, b, c, and d as representing classic, moderate and mild PKU, and mild HPA, respectively. The apparently multimodal distribution implies a frequency in the German population of 15% and 8% for mild PKU and mild HPA, respectively. Normal KOUT values, determined by i.v. loads, range from 10.6 to 24.9 per day, corresponding to a half-life of 0.7 to 1.6 h.

3. Genetics

3.1. PAH mutations and PKU genotypes (incl. databases)

As already stated, knowing whether a patient has residual PAH enzyme activity can be relevant for the therapeutic approach, the likely Phe tolerance, and the expected response to BH4. Delineation of the mutations of the *PAH* gene was initiated immediately after the cloning of the gene in 1983 [28]. Initially the most prevalent mutations in the Western European population were identified and characterized with regard to the *in vitro* residual enzyme activity associated with the respective mutation [51,52]. Subsequently a 'Phenylalanine Hydroxylase Locus Knowledgebase' *PAH*db was created and curated at McGill University [53] (http://www.pahdb.mcgill.ca/) that cataloged knowledge about *PAH* alleles and mutations and their characteristics as reported by clinicians and laboratories from around the world. This database now lists a total of 564 *PAH* mutations discovered world wide as well as the knowledge available about the respective mutation including the residual enzyme activities of ~200 mutations. Of the 564 mutations 60.5% are missense mutations, 13.5% deletions, 11% splice site mutations, (5.7% silent mutations), 5% nonsense mutations, and 1.8% insertions. It was evident early on that the majority of the *PAH* mutations were missense mutations not preventing transcription or translation and that the majority of the patients are compound heterozygotes, meaning they carry a different mutation in each of their alleles. PAH deficiency thus most often results from complex interactions of mutant alleles or rapid intracellular destruction of mutant enzyme subunits making genotype/phenotype correlations based on the knowledge about individual mutations challenging.

3.2. Phenotype–genotype correlation

The first publication with extensive data on genotype/phenotype correlation in PKU was able to establish that the genotype of the patient correlates with the biochemical phenotype [54]. PKU patients had been tested for 8 mutations of the *PAH* gene for which the in vitro residual enzyme activity had been determined. This mutation analysis had allowed for complete genotype determination (identifying both mutations) in 104 patients with PAH deficiency. Stringent classification criteria for the biochemical phenotype were applied to determine genotype/phenotype correlation in these 104 patients. There was a highly significant correlation between the genotype of the patients and the biochemical phenotype ($r = 0.74–0.84$, $p < 0.001$ depending on the parameter) although the genotype was expressed as the predicted residual enzyme activity of the patient and was calculated as the mean of the combined *in vitro* residual enzyme activities of both mutant alleles of the patient, which was quite a simplification compared to the real *in vivo* situation. The goal of the research had been to determine whether genotype analysis after exclusion of BH4 cofactor deficiency could replace more involved clinical testing such as response to a standardized oral protein load at 6 months of age and Phe tolerance assessment in an inpatient setting over the course of 1–2 weeks at 5 years of age. These clinical tests had proven to be the most reliable clinical classification criteria in large

clinical studies [54] but were cumbersome and in the case of the loading test exposed the patient to high Phe concentrations.

In 1998 Guldberg at al. [38] reported the identification of the complete genotype in 686 patients from 7 European centers. Based on the Phe tolerance (or in the case of mild HPA the pretreatment level) of 297 functionally hemizygous patients (patients carrying a null allele and the uncharacterized mutant allele), an arbitrary phenotype category was assigned to each of 105 mutations for which the residual enzyme activity was not known. Using these arbitrary categories phenotype predictions for 650 of the patients were made. In 79% of the patients the predicted phenotype matched the observed phenotype and the authors concluded that differences in the phenotype classification across centers might have accounted for the genotype–phenotype inconsistencies that were observed. Overall, despite the potential shortcomings, this work also led to the conclusion that the genotype is the main determinant of the biochemical phenotype in most patients with PAH deficiency.

Many more studies were published reporting genotype/phenotype correlations in different populations and inconsistencies, i.e. genotypes that were associated with several different phenotypes. It was also pointed out in numerous publications (first by S. Kaufman [55]) and investigated *in vitro* [56] that the combination of the two mutant alleles is important for the residual PAH enzyme activity and that the individual mutations of a patient should not be viewed by themselves. It is clear that the residual activity of an enzyme that is a homotetramer that consists of a combination of different mutant subunits may not simply be the mean of the activity that each subunit produces by itself *in vitro* because there may be negative intra-allelic complementation between different mutant enzyme subunits [55]. However, a correlation between genotype and biochemical phenotype prevailed in the majority of the patients reported. Limitations regarding genotype-based prediction of the phenotype, however, are that the *in vitro* residual enzyme activity is not known for all mutations, that negative intra-allelic complementation may occur, and some exceptions described below may occur.

3.3. BH4-responsive mutations and genotypes

As mentioned above a 'Phenylalanine Hydroxylase Locus Knowledgebase' *PAH*db exists at McGill University [53] (http://www.pahdb.mcgill.ca/) that has catalogued knowledge about *PAH* alleles and mutations including the associated phenotype. It lists the phenotype associated with about 600 allele combinations; however this data is 'self-reported' by clinicians and laboratories using variable criteria for the determination of the phenotype. Another database, the BIOPKU database (www.biopku.org) was created at the University of Zürich. It has catalogued the complete genotypes (complete mutation combinations) of 730 PKU patients, their phenotypes (based on the highest blood Phe levels before starting treatment) and their response to BH4. These 730 patients represent a total of 430 different mutation combinations.

Some of the mutation combinations of the 730 patients listed are not always associated with the same phenotype. 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. However, although different phenotypes are associated with these mutation combinations their BH4 responsiveness is always the same except in a smaller percentage of L48S homozygotes (19%) that may not respond while 81% respond. A similar phenomenon was observed in 40% of Turkish patients that were homozygous for the E390G mutation and did not respond to BH4 [22] while other homozygous patients and all patients compound heterozygous for this very mild mutation listed in the BIOPKU database did respond to BH4. The observations regarding variability for two mild mutations in the homozygous state indicate that certain mutant homotetramers may not allow for stable active enzyme even if the mutations themselves appeared to be mild *in vitro*. Patients homozygous for these particular mutations may occasionally not respond to BH4 or not within 48 h. Finally, for the c.1066-11G>A mutation in the homozygous state different phenotypes and the whole spectrum of BH4 responsiveness (negative, positive, and slow) is cataloged. Given that this is a mutation in an intron that creates a cryptic splice acceptor site it is conceivable that the cryptic splice site does not always come into play and functional enzyme is being translated under certain circumstances creating this variability.

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

1. Mutation combinations that allow for <15% *in vitro* enzyme activity cause classic PKU and do not respond to BH4. Mutation combinations that allow for >20% residual activity responds to BH4. 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 data bases.
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 BH4 responsiveness of many mutation combinations (complete genotypes) has been well established multiple times. Patients that have those genotypes may not need to undergo BH4 responsiveness testing.

After assessment of the genotype (both mutations) of a patient one can now in many cases simply look up the associated phenotype and BH4 responsiveness that is cataloged for this mutation combination in the databases rather than trying to predict it using a simplifying formula and the *in vitro* residual enzyme activity of the mutant alleles as was done in the past and had its limitations (see above). DNA diagnostic laboratories should include the information cataloged in the data bases for a specific mutation combination in their reports about *PAH* mutations to clinicians to allow physicians to make the best informed decisions based on available information.

In an anonymous survey among 65 US metabolic centers 72% of the centers reported that they classify PKU patients into classic PKU, mild PKU, and mild HPA; 40% were ordering mutation testing on all of their patients 53% on some of their patients; 53% of the centers order genotype analysis, when they order it, to understand the phenotype of the patient; 72% order to provide the family with genetic information (multiple answers could be chosen); of those who do not order mutation analysis 40% said they had too little information about the meaning of the mutations (what phenotype to expect), 54% said they do not need mutation analysis to understand the phenotype of their patients, and 36% said the health insurance of their patients does not cover the testing; 20% of the centers said they order mutation analysis on each PKU patient that wants to try BH4 supplementation to see whether the patient should have residual enzyme activity (which would make it more likely that patient responds to BH4).

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References

[1] J.L. Dhondt, Laboratory diagnosis of phenylketonuria, in: N. Blau (Ed.), PKU and BH4: Advances in Phenylketonuria and Tetrahydrobiopterin, SPS Verlagsgesellschaft, Heilbronn, 2006, pp. 161–179.

[2] R. Guthrie, A. Susi, A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants, Pediatrics 32 (1963) 338–343.

[3] D.H. Chace, J.E. Sherwin, S.L. Hillman, F. Lorey, G.C. Cunningham, Use of phenylalanine-to-tyrosine ratio determined by tandem mass spectrometry to improve newborn screening for phenylketonuria of early discharge specimens collected in the first 24 hours, Clin. Chem. 44 (1998) 2405–2409.

[4] M. Zaffanello, G. Zamboni, C. Maffeis, L. Tato, Neonatal birth parameters of positive newborns at PKU screening as predictors of false-positive and positive results at recall-testing, J. Med. Screen 10 (2003) 181–183.

[5] Phenylketonuria (PKU): screening and management, NIH Consens. Statement 17 (2000) 1–33.

[6] J.W. Eastman, J.E. Sherwin, R. Wong, C.L. Liao, R.J. Currier, F. Lorey, G. Cunningham, Use of the phenylalanine:tyrosine ratio to test newborns for phenylketonuria in a large public health screening programme, J. Med. Screen 7 (2000) 131–135.

[7] N. Blau, B. Thöny, R.G.H. Cotton, K. Hyland, Disorders of tetrahydrobiopterin and related biogenic amines, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle, B. Childs, B. Vogelstein (Eds.), The Metabolic and Molecular Bases of Inherited Disease, McGraw-Hill, New York, 2001, pp. 1725–1776.

[8] N. Blau, F.J. Van Spronsen, H.L. Levy, Phenylketonuria, Lancet 376 (2010) 1417–1427.

[9] N. Longo, Disorders of biopterin metabolism, J. Inherit. Metab. Dis. 32 (2009) 333–342.

[10] T. Opladen, B. Abu Seda, A. Rassi, B. Thöny, G.F. Hoffmann, N. Blau, Diagnosis of tetrahydrobiopterin deficiency using filter paper blood spots: further development of the method and 5 years experience, J. Inherit. Metab. Dis. 34 (2011) 819–826.

[11] H.C. Curtius, A. Niederwieser, M. Viscontini, A. Otten, J. Schaub, S. Scheibenreiter, H. Schmidt, Atypical phenylketonuria due to tetrahydrobiopterin deficiency. Diagnosis and treatment with tetrahydrobiopterin, dihydrobiopterin and sepiapterin, Clin. Chim. Acta 93 (1979) 251–262.

[12] A. Ponzone, O. Guardamagna, I. Dianzani, R. Ponzone, G.B. Ferrero, M. Spada, R.G.H. Cotton, Catalytic activity of tetrahydrobiopterin in dihydropteridine reductase deficiency and indications for treatment, Pediatr. Res. 33 (1993) 125–128.

[13] A.C. Muntau, W. Röschinger, M. Habich, H. Demmelmair, B. Hoffmann, C.P. Sommerhoff, A.A. Roscher, Tetrahydrobiopterin as an alternative treatment for mild phenylketonuria, N. Engl. J. Med. 347 (2002) 2122–2132.

[14] C. Bernegger, N. Blau, High frequency of tetrahydrobiopterin-responsiveness among hyperphenylalaninemias: a study of 1919 patients observed from 1988 to 2002, Mol. Genet. Metab. 77 (2002) 304–313.

[15] B. Fiege, N. Blau, Assessment of tetrahydrobiopterin (BH4)-responsiveness in phenylketonuria, J. Pediatr. 150 (2007) 627–630.

[16] H. Levy, A. Milanowski, A. Chakrapani, M. Cleary, P. Lee, F.K. Trefz, C.B. Whitley, F. Feillet, A.S. Feigenbaum, J.D. Bebchuk, H. Christ-Schmidt, A. Dorenbaum, Efficacy of sapropterin dihydrochloride (tetrahydrobiopterin, 6R-BH4) for reduction of phenylalanine concentration in patients with phenylketonuria: a phase III randomized placebo-controlled study, Lancet 370 (2007) 504–510.

[17] N. Blau, Sapropterin dihydrochloride for phenylketonuria and tetrahydrobiopterin deficiency, Expert. Rev. Endocrinol. Metab. 5 (2010) 483–494.

[18] H. Levy, B. Burton, S. Cederbaum, C. Scriver, Recommendations for evaluation of responsiveness to tetrahydrobiopterin (BH(4)) in phenylketonuria and its use in treatment, Mol. Genet. Metab. 92 (2007) 287–291.

[19] N. Blau, Defining tetrahydrobiopterin (BH4)-responsiveness in PKU, J. Inherit. Metab. Dis. 31 (2008) 2–3.

[20] B.K. Burton, D.K. Grange, A. Milanowski, G. Vockley, F. Feillet, E.A. Crombez, V. Abadie, C.O. Harding, S. Cederbaum, D. Dobbelaere, A. Smith, A. Dorenbaum, The response of patients with phenylketonuria and elevated serum phenylalanine to treatment with oral sapropterin dihydrochloride (6R-tetrahydrobiopterin): a phase II, multicentre, open-label, screening study, J. Inherit. Metab. Dis. 30 (2007) 700–707.

[21] M.R. Zurflüh, J. Zschocke, M. Lindner, F. Feillet, C. Chery, A. Burlina, R. Stevens, B. Thöny, N. Blau, Molecular genetics of tetrahydrobiopterin responsive phenylalanine hydroxylase deficiency, Hum. Mutat. 29 (2008) 167–175.

[22] S.F. Dobrowolski, C. Heintz, T. Miller, C.R. Ellingson, C.C. Ellingson, I. Özer, G. Gökcay, T. Baykal, B. Thöny, M. Demirkol, N. Blau, Molecular genetics and impact of residual in vitro phenylalanine hydroxylase activity on tetrahydrobiopterin-responsiveness in Turkish PKU population, Mol. Genet. Metab. 10 (2011) 116–121.

[23] N. Blau, U. Langenbeck, J.B. Hennermann, U. Lichter Konecki, Diagnosis and management of PKU: an international survey J Inherit Metab Dis (abstract), 34 (Suppl 3) (2011) S97.

[24] K. Hyland, R.A.H. Surtees, S.J.R. Heales, A. Bowron, D.W. Howells, I. Smith, Cerebrospinal fluid concentrations of pterins and metabolites of serotonin and dopamine in a pediatric reference population, Pediatr. Res. 34 (1993) 10–14.

[25] G. Zorzi, B. Thöny, N. Blau, Reduced nitric oxide metabolites in CSF of patients with tetrahydrobiopterin deficiency, J. Neurochem. 80 (2002) 362–364.

[26] L. Jäggi, M.R. Zurflüh, A. Schuler, A. Ponzone, F. Porta, L. Fiori, M. Giovannini, R. Santer, G.F. Hoffmann, H. Ibel, U. Wendel, D. Ballhausen, M.R. Baumgartner, N. Blau, Outcome and long-term follow-up of 36 patients with tetrahydrobiopterin deficiency, Mol. Genet. Metab. 93 (2008) 295–305.

[27] K.W. Driscoll, D.Y. Hsia, W.E. Knox, W. Troll, Detection by phenylalanine tolerance tests of heterozygous carriers of phenylketonuria, Nature 178 (1956) 1239–1240.

[28] S.L. Woo, A.S. Lidsky, F. Güttler, T. Chandra, K.J. Robson, Cloned human phenylalanine hydroxylase gene allows prenatal diagnosis and carrier detection of classical phenylketonuria, Nature 306 (1983) 151–155.

[29] F. Güttler, Hyperphenylalaninemia: diagnosis and classification of the various types of phenylalanine hydroxylase deficiency in childhood, Acta Paediatr. Scand. Suppl. 280 (1980) 1–80.

[30] W.D. Lehmann, R. Fischer, H.C. Heinrich, P. Clemens, R. Grüttner, Metabolic conversion of L-[U-14 C]phenylalanine to respiratory 14CO2 in healthy subjects, phenylketonuria heterozygotes and classic phenylketonurics, Clin. Chim. Acta 157 (1986) 253–266.

[31] M.C. Hsieh, H.K. Berry, M.K. Bofinger, P.J. Phillips, M.B. Guilfoile, M.M. Hunt, Comparative diagnostic value of phenylalanine challenge and phenylalanine hydroxylase activity in phenylketonuria, Clin. Genet. 23 (1983) 415–421.

[32] M.E. Blaskovics, Phenylketonuria: loading studies revisited, in: N. Blau (Ed.), PKU and BH4: Advances in Phenylketonuria and Tetrahydrobiopterin, SPS Verlagsgesellschaft, Heilbronn, 2006, pp. 104–119.

[33] M.E. O'Flynn, N.A. Holtzman, M. Blaskovics, C. Azen, M.L. Williamson, The diagnosis of phenylketonuria: a report from the Collaborative Study of Children Treated for Phenylketonuria, Am. J. Dis. Child. 134 (1980) 769–774.

[34] P. Lutz, H. Schmidt, U. Batzler, Study design and description of patients, Eur. J. Pediatr. 149 (Suppl 1) (1990) S5–S12.

[35] J. Weglage, M. Pietsch, R. Feldmann, H.G. Koch, J. Zschocke, G. Hoffmann, A. Muntau-Heger, J. Denecke, P. Guldberg, F. Güttler, H. Möller, U. Wendel, K. Ullrich, E. Harms, Normal clinical outcome in untreated subjects with mild hyperphenylalaninemia, Pediatr. Res. 49 (2001) 532–536.

[36] U. Langenbeck, P. Burgard, U. Wendel, M. Lindner, J. Zschocke, Metabolic phenotypes of phenylketonuria. Kinetic and molecular evaluation of the Blaskovics protein loading test, J. Inherit. Metab. Dis. 32 (2009) 506–513.

[37] J. Donlon, H. Levy, C.R. Scriver, Hyperphenylalaninemia: phenylalanine hydroxylase deficiency, in: D. Valle, A.L. Beaudet, B. Vogelstein, K.W. Kinzler, S.E. Antonarakis, A. Ballabio (Eds.), The Online Metabolic & Molecular Bases of Inherited Disease, McGraw-Hill, Montreal, 2008, Ch. 77.

[38] P. Guldberg, F. Rey, J. Zschocke, V. Romano, B. Francois, L. Michiels, K. Ullrich, G.F. Hoffmann, P. Burgard, H. Schmidt, C. Meli, E. Riva, I. Dianzani, A. Ponzone, J. Rey, F. Güttler, A European multicenter study of phenylalanine hydroxylase deficiency: classification of 105 mutations and a general system for genotype-based prediction of metabolic phenotype, Am. J. Hum. Genet. 63 (1998) 71–79.

[39] P. Guldberg, F. Güttler, Mutations in the phenylalanine hydroxylase gene: methods for their characterization, Acta Paediatr. Suppl. 407 (1994) 27–33.

[40] S. Kure, D.C. Hou, T. Ohura, H. Iwamoto, S. Suzuki, N. Sugiyama, O. Sakamoto, K. Fujii, Y. Matsubara, K. Narisawa, Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency, J. Pediatr. 135 (1999) 375–378.

[41] F.J. van Spronsen, M. van Rijn, B. Dorgelo, M. Hoeksma, A.M. Bosch, M.F. Mulder, J.B. de Klerk, T. de Koning, M.E. Rubio-Gozalbo, M. de Vries, P.H. Verkerk, Phenylalanine tolerance can already reliably be assessed at the age of 2 years in patients with PKU, J. Inherit. Metab. Dis. 32 (2009) 27–31.

[42] E.L. MacLeod, S.T. Gleason, S.C. van Calcar, D.M. Ney, Reassessment of phenylalanine tolerance in adults with phenylketonuria is needed as body mass changes, Mol. Genet. Metab. 98 (2009) 331–337.

[43] V. Anastasoaie, L. Kurzius, P. Forbes, S. Waisbren, Stability of blood phenylalanine levels and IQ in children with phenylketonuria, Mol. Genet. Metab. 95 (2008) 17–20.

[44] M. Humphrey, J. Nation, I. Francis, A. Boneh, Effect of tetrahydrobiopterin on Phe/Tyr ratios and variation in Phe levels in tetrahydrobiopterin responsive PKU patients, Mol. Genet. Metab. (2011), doi:10.1016/j.ymgme.2011.1005.1011.

[45] F. Güttler, P. Guldberg, The influence of mutations on enzyme activity and phenylalanine tolerance in phenylalanine hydroxylase deficiency, Eur. J. Pediatr. 155 (1996) S 6–S 10.

[46] U. Langenbeck, J. Zschocke, U. Wendel, V. Hönig, Modelling the phenylalanine blood level response during treatment of phenylketonuria, J. Inherit. Metab. Dis. 24 (2001) 805–814.

[47] S.J. Fomon, F. Haschke, E.E. Ziegler, S.E. Nelson, Body composition of reference children from birth to age 10 years, Am. J. Clin. Nutr. 35 (1982) 1169–1175.

[48] B.K. Burton, L. Leviton, Reaching out to the lost generation of adults with early-treated phenylketonuria (PKU), Mol. Genet. Metab. 101 (2010) 146–148.

[49] U. Langenbeck, M. Ammar, J. Zschocke, E. Solem, I.M. Knerr, J. Herwig, H. Boehles, Recognizing catabolic states during dietary treatment of phenylketonuria. An application of metabolic modelling J Inherit Metab Dis 34 (Suppl 3) (2011) S102.

[50] U. Wendel, U. Langenbeck, Towards self-monitoring and self-treatment in phenylketonuria—a way to better diet compliance, Eur. J. Pediatr. 155 (Suppl 1) (1996) S105–S107.

[51] D.S. Konecki, U. Lichter Konecki, The phenylketonuria locus: current knowledge about alleles and mutations of the phenylalanine hydroxylase gene in various populations, Hum. Genet. 87 (1991) 377–388.

[52] R.C. Eisensmith, Y. Okano, M. Dasovich, T. Wang, F. Güttler, H. Lou, P. Guldberg, U. Lichter-Konecki, D.S. Konecki, E. Svensson, et al., Multiple origins for phenylketonuria in Europe, Am. J. Hum. Genet. 51 (1992) 1355–1365.
[53] L. Hoang, S. Byck, L. Prevost, C.R. Scriver, PAH mutation analysis consortium database — a database for disease-producing and other allelic variation at the human PAH locus, Nucleic Acids Res. 24 (1996) 127–131.
[54] Y. Okano, R.C. Eisensmith, F. Güttler, U. Lichter-Konecki, D.S. Konecki, F.K. Trefz, M. Dasovich, T. Wang, K. Henriksen, H. Lou, et al., Molecular basis of phenotypic heterogeneity in phenylketonuria, N. Engl. J. Med. 324 (1991) 1232–1238.
[55] S. Kaufman, Phenylketonuria: Biochemical Mechanisms, in: B.W. Agranoff, M.H. Aprison (Eds.), Advances in Neurochemistry, Plenum Press, New York, 1976, pp. 1–132.
[56] P.J. Waters, How PAH gene mutations cause hyper-phenylalaninemia and why mechanism matters: insights from in vitro expression, Hum. Mutat. 21 (2003) 357–369.