MOLECULAR CHARACTERISTICS, PHENOTYPIC DIVERSITY AND GENOTYPE-ESTIMATED THERAPEUTIC RESPONSIVENESS OF SERBIAN PATIENTS WITH PHENYLKETONURIA

MOLEKULARNE KARAKTERISTIKE, FENOTIPSKA RAZNOLIKOST I PROCENA ODGOVORA NA TERAPIJU ZASNOVANA NA GENOTIPIU KOD SRPSKIH PACIJENATA SA FENILKETONURIJOM

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Summary
Phenylketonuria (PKU) is a rare, inherited metabolic disease which is transmitted in an autosomal recessive pattern. PKU is caused by mutations in the gene encoding the phenylalanine hydroxylase (PAH) enzyme. This review cites the most prominent methods for the detection of mutations in the PAH gene. Since the image of PKU transcends »simple« monogenic disease, the known data on non-coding PAH gene variants and their role and PKU modifier genes have been further reviewed. It has been shown that there is a significant correlation between mutant PAH genotypes and PKU phenotypes. However, genotype–phenotype correlation inconsistencies have also been found. This review discusses the possible causes of phenotypic inconsistencies, such as oversight of more than two mutations present in the patient’s PAH genotype, pitfalls of patient phenotypic classification (plasma phenylalanine concentration and phenylalanine tolerance), the inter-allelic complementation (positive and negative) phenomenon. A new therapeutic approach, tetrahydrobiopterin (BH4) supplementation therapy, is an important innovation in the course of PKU patients’ treatment. However, in countries where the BH4-loading test and BH4-supplementation therapy are not available, a genotype-based estimation of responsiveness to the therapy is a valuable approach. It enables BH4-potential benefit estimation, which provides vital information both for the patient and for the population. An optimal molecular diagnostics algorithm, Kratak sadržaj
Fenilketonurija (PKU) jeste retko metaboličko oboljenje koje se nasleđuje autozomalno recessivno. Uzrok PKU su mutacije u genu koji kodira za enzim fenilalaninan-hidroksilazu (PAH). U ovom revijskom radu su opisane najznačajnije metode za detekciju mutacija u PAH genu. Budući da slika o PKU prenosi monogensko oboljenje, prikazani su i podaci o varijantama u nekodirajućim regionima PAH gena, kao i genima modifikatorima za PKU. Iako postoji značajna korelacija između PAH genotipa i PKU fenotipa, uočene su i neusaglašenosti. U ovom revijskom radu su izneti mogući uzroci za neusaglašenost između genotipa i fenotipa, kao što su: propust u detekciji više od dve mutacije prisutne u PAH genotipu pacijenta, neusaglašenost u fenotipskoj klasifikaciji pacijenata (koncentracija fenilalanina u serumu pre terapije i tolerancija fenilalanina), fenomen interalelske komplementacije (pozitivne i negativne). Novi terapijski pristup, terapija tetrahydrobiopterinom (BH4), predstavlja važnu inovaciju u lečenju pacijenata sa PKU. Međutim, u zemljama u kojima test opterećenja sa BH4 i terapija sa BH4 nisu dostupni, procena odgovora na terapiju koja je zasnovana na genotipu predstavlja koristan pristup. Ovakav pristup omogućava procenu potencijalne koristi od terapije sa BH4 što je bitna informacija, kako za pacijenta, tako i na nivou populacije. Predložen je i optimalni algoritam za molekularnu dijagnostiku, uspostavljen prema objavljenim učestalostima mutacija kod pacijenata sa PKU u Srbiji. U budućnosti, molekularno-

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List of abbreviations: PKU, phenylketonuria; PAH, phenylalanine hydroxylase gene; BH4, (6R)-L-erythro-5,6,7,8-tetrahydrobiopterin; Phe, phenylalanine; Tyr, tyrosine.
established according to the published mutation frequencies in Serbian PKU patients, has been suggested. In the future, the molecular-genetic algorithm for PKU could be expanded to include a variety of transcriptional regulatory elements located in noncoding PAH gene regions and yet to be discovered modifier genes.

**Keywords:** genotype-based prediction, mutation detection algorithm, phenylalanine hydroxylase, phenotype-genotype correlation, phenylketonuria, tetrahydrobiopterin

**Introduction**

Phenylketonuria (PKU, MIM# 261600) is a form of hyperphenylalaninemia (HPA), the most frequent inborn error of metabolism (with 1:10 000 average incidence in Caucasians) (1). PKU is a monogenic disease transmitted in an autosomal recessive pattern. Mutations found in the phenylalanine hydroxylase gene (PAH, GenBank accession no. AF404777) are the main determinants of PKU phenotype. A PAH mutation affects the structure and/or function of a hepatic enzyme, phenylalanine hydroxylase (PAH, EC 1.14.16.1), which consequently fails to catalyze the conversion of L-phenylalanine (Phe) to tyrosine (Tyr). Therefore, the main characteristic of the PKU patients’ phenotype, which is to be noted immediately after birth, is the elevated level of Phe in serum. Since normal nutrition introduces Phe that cannot be metabolized, newborn screenings have been established worldwide to identify affected children and start therapy – a low-Phe diet. As a result of good adherence to the effective but difficult treatment, mental retardation and epilepsy are avoided (2).

On the verge of the 21st century, innovation in the way that patients are treated finally happened. It was discovered that PAH alleles, which cause misfolding of proteins, may respond to pharmacological doses of tetrahydrobiopterin (BH4) (3–4). Furthermore, as our knowledge about the variety of genetic factors that contribute to the complexity of PKU metabolic and cognitive phenotype grows, the PKU image is shifting from a «simple» monogenic disease to a more complex disorder (5).

**Molecular Genetic Characteristics of Phenylketonuria**

**Human phenylalanine hydroxylase gene**

The PAH genomic sequence and its flanking regions are located at the 12q23.2 chromosome locus and span around 170 kb (5’UTR upstream covers app. 27 kb, 3’ sequence downstream from the poly(A) site in the last exon covers app. 64.5 kbp (6–8, www.pahdb.mcgill.ca)). Coding region of the PAH gene consists of 13 exons and represents less than 3% of the genomic sequence lying between the start codon and the 3’poly(A) tract. Noncoding regions, the 5’UTR and introns are quite large. The human PAH gene promoter contains several transcription regulatory elements (7). Also, numerous SNPs, multiallelic tandem repeat sequences and RFLPs without known function are embedded in the PAH introns.

More than 770 genetic variants have been identified in the PAH gene and recorded in the literature and PAH mutation databases: PAHdb, http://www.pahdb.mcgill.ca and PAHvdb, http://www.biopku.org (8). Disease-causing PAH gene mutations vary in their type and relative frequency. In general, missense mutations account for two-thirds of all detected mutations worldwide, while other mutation types are less frequent: small deletions (13%), splice (11%), nonsense (5%), small insertions (1%). Large deletions, once thought to be rare, probably account for 3% of the PKU-causing mutations (9). Up to date, no disease-causing mutations have been found in the promoter region (10, PAHdb).

Differences in the mutation spectrum between Caucasian and Asian populations have been recorded. Furthermore, population-specific differences exist among European populations in concordance with historically and geographically recorded population migrations across Europe (11). Therefore, molecular characterization of the PAH gene locus and documentation of the spectrum and frequency of disease-causing mutations found in a particular population is the first step in the development of an optimal molecular diagnostic algorithm. The main goal of a population specific algorithm is the establishment of a rapid genetic analysis at the lowest cost.

**Methods for detection of disease-causing PAH gene mutations**

The main methods used for the screening and identification of PAH gene mutations are based on individual exon amplification, heteroduplex formation and sequencing: «broad range» denaturing-gradient gel electrophoresis (DGGE), denaturing high-performance liquid chromatography (dHPLC), direct DNA sequencing and different variants of PCR-RFLP (12, 13). Since PCR-RFLP methods aim to identify only one mutation at a time, the method is shown to be time-consuming and inefficient in the majority of populations (14). Thus, PCR-RFLP is generally used only as an additional method for mutation detection.
Rarely, in populations where one or a few mutations have enormous prevalence, the PCR-RFLP method could be routinely used for diagnostic purposes (15).

DGGE is a very useful method because it enables screening of the whole PAH gene and enables detection of mutations and polymorphisms in the amplified regions. Multiplex DGGE is particularly practical, because it is possible to screen all exons of a patient and both their parents on the same gel (12).

DNA sequencing of all 13 exons and their flanking intron regions (not necessarily proceeding with DGGE or dHPLC) is used to ensure detection of missense, nonsense, splice site, small deletion or small insertion mutations. Recently, the amazing technology advances have made DNA sequencing methodology the one that is used the most.

However, mutation analysis using exon by exon screening and sequencing may fail to detect the mutant allele in case of large gene deletions as a consequence of primer annealing in the deleted area (9). These deletions have been difficult to detect in compound heterozygotes due to a masking effect of the nondeleted allele. Thus, the method of choice for large deletions identification and genomic quantification is MLPA – multiplex ligation-dependent probe amplification (16).

**Noncoding PAH Gene Variants and Their Role**

Noncoding regions of the PAH gene comprise more than 95% of its genetic sequence and contain a wide variety of genetic elements and variants. However, only a small proportion of genetic entities have been analyzed and characterized.

Analysis of the human PAH gene promoter sequence revealed that PAH gene expression is regulated through synchronized action of different transcription factors (TFs) (7). The most upstream CAP site for the human PAH gene transcript is located 154 bp upstream of the ATG codon. The 3'19 bp long region immediately upstream of the CAP site is characterized by the lack of a proximal TATA box and the presence of sequences similar to GC boxes, CACCC boxes, CCAAT boxes, activator protein 2 (Ap2) sites, partial glucocorticoid response elements (GREs), and partial cyclic AMP response elements (CREs).

Wang et al. analyzed a 9 kb genomic DNA fragment at the 5' end of the 90-kb-long human PAH gene and concluded that this fragment is responsible for the PAH gene tissue- and developmental-specific expression (17). Interestingly, they also noticed that the analyzed fragment may lack some elements required for the precise regulation of PAH gene expression. Furthermore, the minimal promoter was defined, and two binding sites for hepatic nuclear factor 1 (HNF1) were identified (18). Thus, only the 9 kb genomic fragment was analyzed in detail (7, 17–19), leaving approximately 90% of the PAH gene containing mainly noncoding sequences unanalyzed.

The first study that considered other noncoding PAH gene regions was focused on the well-known haplotype-defining RFLP polymorphisms (20). After in silico selection of a genetic sequence with the potential to be a transcriptional regulatory element, Stojilkovic et al. (20) conducted a functional study in the HepG2 cell line as well as DNA-protein interaction studies on the part of intron 8 containing XmnI polymorphism. The study identified the first enhancer element in the PAH gene intron. This demonstrated the importance of analyzing genetic variants embedded into noncoding sequences which could potentially be PAH gene expression modulators. Thus, the understanding of expression complexity could be an important step toward better understanding of phenotype complexity. In the future, the molecular-genetic algorithm for the phenylketonuria phenotype could be expanded to include a variety of transcriptional regulatory elements located in noncoding PAH gene regions.

**Modifier Genes**

Modern understanding of human diseases, even monogenic ones, tends to be comprehensive and systemic. In order to fully understand the genetic base of a disease, it is not enough to look only at one locus. A complex organism is comprised of molecular and cellular modules and networks (21). Therefore, it is necessary to look at the whole picture and identify all the genes that could contribute to the outcome of a disease through various pathways. These modifier genes could encode transporters, receptors, chaperones or other proteins involved in the life of phenylalanine hydroxylase in the hepatocytes, or flux of L-Phe from the moment it enters into an organism.

**Inside the cell.** Phenylketonuria is an example for the concept of conformational diseases (22). From the in vitro assays, it is known that the architecture of the PAH protein, unlike many other enzymes, seems exquisitely sensitive to misfolding and/or misassembly caused by missense mutations (25–28). In general, components of the intracellular quality control system (chaperones and proteases) are the main players in the survival mechanism of unfolded, partially folded and misfolded polypeptides. They determine the fine balance between the proper biogenesis of «normal» proteins and the aggregation or rapid degradation of improperly folded, damaged or mutant proteins. However, what happens with the human PAH protein in vivo is not known. Does it in fact interact with the chaperones and components of the proteolytic pathways, and if so, with which ones (29–30). Moreover, it is not clear if these interactions are indeed important for PAH stability in vivo and to which extent they contribute to the PKU phenotype.
In the body. Concentration of L-Phe in the body is determined by: intestinal absorption of dietary L-Phe on a dedicated membrane transporter; distribution into metabolic amino acid pools in the body; carrier-mediated uptake of L-Phe by the liver (31); controlled transport into the kidney (the other organ where L-Phe is converted to Tyr) (32–33); its incorporation into proteins; its degradation by hydroxylation, transamination (34) and decarboxylation; its excretion from the organism, as well as carrier-mediated transport of L-Phe through the blood–brain barrier (5, 31).

The transport through the blood–brain barrier attracted attention since it bore the promise of a »protector« modifier gene. Different metabolic and cognitive presentations of late-detected phenylketonuria and different dysmyelination changes (found by magnetic resonance imaging) in two brothers with the same PAH genotype were reported (35). In particular, LAT1 and 4F2hc genes, encoding parts of the LNAA transport system (neutral amino acid transporter) were targeted to be significant for the L-Phe blood–brain barrier transport (36). Weglage et al. conducted a study with a group of PKU patients who had a low ratio of brain to blood L-Phe concentrations (37). They thoroughly analyzed the gene sequence responsible for the 4F2hc/LAT1 complex, but did not find any pathogenic genetic variants. In a similar study, three polymorphisms and one potentially pathogenic new mutation (p.G41D) were reported within the PAH gene (38). However, there is still not enough evidence to confirm the role of genes involved in the transport of L-Phe across the blood–brain barrier.

On the other hand, diploid organisms developed a variety of buffering mechanisms during the course of evolution. These mechanisms are depicted by redundancy in genome, reduced specificity in function through flexibility and versatility in the proteins involved, and negative feedback regulation (39). One piece of evidence for the presence of a complex network of factors that buffers potential disturbances of L-Phe metabolic homeostasis is the fact that there is no significant difference in the serum L-Phe level between PKU heterozygous and homozygous normal phenotypes (40).

At this point, we can only hypothesize that the control of metabolic homeostasis of L-Phe in the body is represented by the sum of all mentioned processes. Currently, there is not enough evidence to determine the individual influence of different modifier genes or the integral algorithm for a genotype-based understanding of phenylketonuria.

Phenotypic Diversity

Having in mind the number and variety of genetic components involved, it is not a surprise that the hyperphenylalaninemia phenotype is highly variable. HPA includes PAH deficiency (∼98%) which shows a broad continuum of phenotypes and BH4 deficiency (∼2%) which will not be discussed in this review.

The continuous spectrum of high Phe concentrations detected in patients with a deficient PAH enzyme is, for the diagnostic and therapeutic process, divided into three categories. Thus, complete or near complete deficiency of PAH activity is designated as classical PKU (cPKU) and without treatment it would result in profound and irreversible mental retardation. Less severe forms are designated as mild PKU (mPKU) and mild hyperphenylalaninemia (MHP). Thus, the main difference between these arbitrary categories is in the extent of necessary reduction of phenylalanine consumed through the diet. In the case of cPKU, early started, well-adjusted, well-controlled and continuous restriction of phenylalanine intake is required to ensure normal physical, neurological, and cognitive development. In patients with the milder forms, especially in the case of MHP, dietary correction which prevents neurological symptoms is more flexible or not required at all (2, 41).

In the course of clinical practice, phenotypic categories are determined at neonatal screening according to the pretreatment phenylalanine serum level: classical PKU (Phe >1200 mmol/L), mild PKU (Phe 600–1200 mmol/L) and MHP (Phe <600 mmol/L) (42). Additional phenotypic assessment is performed in some patients after 2 years of age according to phenylalanine tolerance: classical PKU (Phe tolerance <20 mg/kg/day – equivalent 250–350 mg/day), mild PKU (Phe tolerance 20–25 mg/kg/day – equivalent 350–600 mg/day), and MHP (Phe tolerance >25 mg/kg/day – equivalent >600 mg/day). Also, patients with phenylalanine levels <600 mmol/liter on a normal diet are usually classified as having MHP (43).

Generally, phenylalanine tolerance was shown to depict more realistically the ability of a patient to metabolize Phe from the food, thus being a more adequate criterion for phenotypic classification.

Inconsistencies in the genotype-phenotype correlation

Due to neonatal screening programs, PKU patients are regularly diagnosed, treated and monitored around the globe. On the other hand, numerous population-specific studies as well as the Human Genome Project were thoroughly revealing and multiplying the knowledge about the molecular characteristics of PKU patients’ genomes. This set an amazing base for exploring the correlation between genotype (two disease-causing mutations in the PAH gene) and in vivo metabolic phenotype (plasma L-Phe and L-Phe tolerance).
Genotype–phenotype correlation should be performed only in patients in whom one disease-causing mutation acts on its own. Homozygous patients carrying two identical mutations are the obvious choice (43). However, it should be carefully determined that a patient is not carrying a mutation at one, and a large deletion at the other chromosome (parents’ PAH gene analyses and/or MLPA analyses are needed). Since PKU patients usually carry two different disease-causing mutations in the PAH gene, functionally hemizygous patients are very often included in the genotype–phenotype studies. One of the mutations in a functionally hemizygous patient is designated as the null one. Null mutations are known or predicted to completely abolish PAH activity. The majority of these mutations are frame shift mutations, splice-site mutations, and base substitutions that introduce a premature stop codon. Also, missense mutations that result in zero enzyme activity in vitro (less than 10% in comparison to wild-type PAH) are considered as null mutations.

By using this approach, large multicentre studies conducted by Guldberg (43) and Kayalaap (44) greatly contributed to the knowledge of PAH mutation effect and showed that there is a significant correlation between mutant PAH genotypes and PKU phenotypes in most patients.

However, the PAH genotype is not a consistent predictor of the PKU phenotype, and many phenotypic inconsistencies have been found. In some inconsistency cases, we could argue that detection of a mutation was not thorough and complete and can be explained by the presence of a third mutation (45, 46). This problem is easily resolved by complete gene screening which involves the combination of different molecular-genetic methods (47). On the other hand, there is no official guideline for the phenotype classification. Therefore, due to differences in the phenotype classification guidelines used in different clinical centres, a proportion of patients may be misclassified (43). This problem should not exist in one center studies where all patients are classified and diagnosed uniformly. Also, in order to overcome the possible pitfalls of patients’ phenotypic classification (plasma L-Phe and L-Phe tolerance), studies tend to take into consideration both parameters (48, 49).

Still, even when both genotype and phenotype are consistently determined, inconsistencies are recorded (48). These particular exceptions in the genotype–phenotype correlation should attract special attention because they may reveal things of interest (5).

One of the phenomena that was revealed by studying genotype–phenotype inconsistencies is the interallelic complementation (positive and negative). Since the majority of PKU patients are compound heterozygotes, the PAH enzyme – a tetramer, is composed of different subunits bearing different defects. Interallelic complementation is the term which illustrates the protein–protein interactions occurring between these different subunits of the heterotetrameric human PAH enzyme (50–52). It was even suggested that interallelic interaction leads to correct folding of one of the monomers (53). Having this in mind, one should be careful when including missense mutations that resulted in zero enzyme activity in vitro (such as p.R408W and p.P281L mutations) into genotype–phenotype correlation studies. Functionally, null mutations may not contribute to enzyme activity, but may cause interallelic complementation in the heterotetrameric PAH enzyme.

In homozygotes, interallelic complementation could not explain the different levels of serum Phe and/or Phe tolerance observed in some patients (48). This phenomenon will probably be explained in the future by new transcriptional regulators located in the non-coding region of the PAH gene and/or variety of modifier genes.

Nowadays, it is a rule to treat the actual phenotype observed in a patient, not the one predicted from the genotype at the PAH locus. However, as the PKU phenotype becomes better explained by coding and noncoding genetic variations of the PAH gene, as well as genomic variations in yet to be identified modifier genes, the correlation of genotype with phenotype will be better understood. Thus, the algorithm for genome-based prediction of patient’s phenotype and the patient-specific therapy will one day become a reality.

BH4 Responsiveness

Tetrahydrobiopterin (BH4) binds to the catalytic region of the PAH enzyme and acts as an obligatory cofactor. This natural cofactor also acts as a negative regulator of PAH activity through binding to a site distinct from the BH4 catalytic site, and forming an inactive BH4–PAH complex (54, 55). However, if the PAH enzyme has already went through large conformational changes initiated by the high Phe concentration and has reached its Phe-activated form, then it cannot be inhibited by BH4 (54).

As a drug, BH4 was initially used in patients with BH4 deficiency (56). Recently, it was observed that pharmacological doses of BH4 can lower the blood phenylalanine concentration in PKU patients as well (3). After this discovery, BH4 – as a new therapeutic option, came to the center of PKU research. Molecular mechanism of the BH4 effect on the increase in PAH activity is multifactorial and it is still under investigation. It is known, though, that this mechanism is mainly based on the chaperon-like effect which BH4 generates by binding to catalytic and/or regulatory PAH domains. Therefore, while BH4 is a natural cofactor and regulator of PAH activity, if it is present in a high concentration inside the cell, it acts as a pharmacological chaperone. By binding to an unsta-
ble enzyme, BH4 promotes folding, stabilizes altered conformations of the PAH protein structure, protects it from degradation, prolongs its half-life and enables it to perform the conversion of phenylalanine to tyrosine (4, 25, 57, 58).

Clinical studies assessed the effectiveness of BH4-supplementation therapy (Kuvan). It was found that BH4 can be used to loosen or even replace the burdensome dietary treatment of PKU patients (59, 60). However, not all PKU patients are BH4-responsive, because not all mutated proteins gain function through BH4 chaperone-like activity. In order to understand which mutations are good candidates, many studies analyzed the enzyme activity of individual aberrant PAH subunits in vitro in the presence of BH4 precursors (61–63). The fact that the concentration of BH4 (a negative regulator of PAH enzyme activity) is increased during the therapy, while the Phe (a positive regulator of PAH enzyme activity) concentration is decreased because of low-Phe therapy, was also taken into account. It was found that mutant PAH function in response to BH4 administration will be a consequence of the interplay between genotype, metabolic state, and cofactor concentration (64).

Furthermore, it was acknowledged that the interaction of two mutated subunits may exhibit a different response to BH4 and different molecular behavior in comparison to mutant homozygous PAH (65). Due to interallelic complementation (discussed above), a proper investigation of the effect of BH4 supplementation on a particular genotype should be performed in a dual eukaryotic vector system with two variant PAH proteins N-terminally fused to different epitope tags for discernable allele expression. In the future, the study of many different PAH subunit combinations and the knowledge about their interaction will reveal dominance effects for each mutation and lead to algorithms for the prediction of BH4-responsiveness.

The first study which suggested that BH4-responsiveness could be deduced from the PAH genotype stated that at least one BH4-responsive mutation would be enough to lead to the physiological BH4 responsiveness (66). The following studies attempted to predict BH4-responsiveness on the basis of single allele information or a patient’s genotype (48, 49, 67–73). For a number of patients’ genotypes, the genotype-based prediction was found to be a useful complementary tool to the obligatory BH4 loading test.

The awareness of the BH4 effect opened the quest for other, more effective pharmacological chaperones. The rationale for the continuation of research efforts lies in the fact that BH4 is not effective in all PKU patients. Some small chemical chaperones have already been identified as new potential therapeutic agents (74, 75). Further analysis will assess their effect and determine whether they could potentially be independent from the PAH genotype.

As a result of the discovery of genotype-based therapeutics, the process of identification of disease-causing mutations in the PAH gene of PKU patients becomes not only a diagnostic, but also a predictive genetic test with an application in the personalized medical treatment.

Molecular Diagnostic Algorithm for PKU Patients in Serbia

Neonatal PKU screening has been established since 1982 in Central Serbia and the incidence of PAH deficiency is 1:12300 newborn infants. Each year, approximately 3 to 4 new patients are diagnosed through neonatal screening and genetic counseling programs (76). Molecular characterization of the whole PAH gene of 34 Serbian PKU patients identified the spectrum and frequency of mutations, and enabled the genotype–phenotype correlation analysis (47). Recently, an update study with 61 patients reported that an expanded spectrum (26 different disease-causing mutations) was found in the Serbian population (48). However, all the mutations were previously reported among different European populations, in particular Mediterranean and Balkan populations (11). Thus, the evident heterogeneity of the PAH gene locus of the Serbian population finds its explanation in the historically documented migrations across the Balkans.

With the frequency of 31%, p.L48S mutation reassured its status as the most frequent in Serbia. Furthermore, the frequency of p.L48S mutation in the Serbian population is the highest ever reported in a single population. The effect of p.L48S was generally described as inconsistent (43, 49). Since the relative frequency of unrelated genotypes that included p.L48S mutation was 48%, the Serbian study focused on the phenotypic implications of this mutation (48). It was shown that the effect of p.L48S was altered in functional hemizygotes in comparison to homozygotes, implying that one should be careful when including functionally null mutations in the genotype–phenotype correlation studies. Moreover, the phenotypic inconsistency found among homozygotes suggested that interallelic complementation and/or additional factors play a role in the genotype–phenotype correlation.

Both studies conducted in the Serbian population (47, 48) used a combination of mutation-screening (DGGE) and mutation-detection (PCR-RFLP, PCR-ACRS, DNA sequencing) methods, and thus reached a detection rate of 97% and 99% respectively. Furthermore, the screening of the whole PAH gene enabled detection of three mutated alleles in patients, which is one of the critical prerequisites for a meaningful genotype–phenotype correlation study. However, this review also suggests an optimal molecular diagnostic algorithm, based on the known frequencies in the Serbian population, which is intended for faster and cheaper mutation detection (Figure 1).
MOLECULAR DIAGNOSIS OF A PATIENT

Figure 1 An optimal molecular diagnostic algorithm, established according to published PAH mutation frequencies in Serbian PKU patients (47, 48).
In conclusion, the characterization of PAH mutations created the basis for the molecular diagnostics and genetic counseling of patients with phenylketonuria in Serbia.

Genotype-estimated therapeutic responsiveness for PKU patients in Serbia

BH4-loading test is used as a definitive diagnostic test for the assessment of PKU patient response to BH4-supplementation therapy (77). Most PKU centers in Europe use the standard 48 hours test with two consecutive BH4 administrations of 20 mg/kg. However, the BH4-loading test is not available in Serbia.

A recent study in the Serbian population determined that eight BH4-responsive mutations are abundant in the Serbian population (48). Their overall relative frequency is 52.6%. In this study, BH4-responsive mutations were further classified as consistently and inconsistently BH4 responsive. According to this, Serbian PKU patients’ genotypes were categorized into BH4 responsive (one consistently responsive and an inconsistently responsive/nonresponsive mutation), probably BH4 responsive (one inconsistently responsive and an inconsistently responsive/nonresponsive mutation), and non-BH4 responsive (two nonresponsive mutations) (Figure 2).

In countries like Serbia, where BH4 is not an approved drug, genotyping-based prediction of BH4 response is important for two reasons. First, further tests could be advised to patients in order to determine if they would benefit from the BH4-supplementation therapy. Second, at state level, it would provide an estimation of the overall BH4-responsive mutations frequency and could encourage registration of needed drugs.

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Conflict of interest statement

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