

## Molecular genetics of PKU in Poland and potential impact of mutations on BH4 responsiveness

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**Tetrahydrobiopterin (BH4) has been recently approved as a treatment of patients with phenylketonuria. However, as a confirmation of BH4-responsiveness, it might require a very expensive trial treatment with BH4 or prolonged BH4-loading procedures. The selection of patients eligible for BH4-therapy by means of genotyping of the PAH gene mutations may be recommended as a complementary approach. A population-wide genotyping study was carried out in 1286 Polish phenylketonuria-patients. The aim was to estimate the BH4 demand and to cover prospectively the treatment by a National Health Fund. A total of 95 types of mutations were identified. Genetic variants corresponding with probable BH4-responsiveness were found in 28.2% of cases. However, patients with mild or classical phenylketonuria who require continuous treatment accounted for 11.4% of the studied population only. Analysis of the published data shows similar percentage of the "BH4-responsive" variants of a PAH gene in patients from other countries of Eastern Europe. Therefore, it can be concluded, that the proportion of phenylketonuria-patients who could benefit from the use of BH4 reaches approximately 10% in the entire region.**

**Key words:** PKU, hyperphenylalaninemia, inborn error of metabolism, dietary treatment, genetic epidemiology

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### INTRODUCTION

Phenylketonuria (PKU; MIM# 261600) is the most common inborn error of amino acid metabolism caused by the mutations of the phenylalanine hydroxylase (PAH) gene (Bickel *et al.*, 1981). Currently, more than 800 mutations have been described ([www.biopku.org](http://www.biopku.org)) as resulting in PKU or its milder form — a mild hyperphenylalaninemia. PKU treatment consists in low-phenylalanine diet, which, unfortunately, is not maintained properly by the majority of teenagers and adults who typically manifest serious compliance problems (Blau *et al.*, 2010; Walter *et al.*, 2002). In non-compliant persons severe brain dysfunction may develop (Smith *et al.*, 1990; Christ *et al.*, 2010; Bik-Multanowski *et al.*, 2011).

Tetrahydrobiopterin (BH4) has been proposed as a treatment to PKU patients (Kure *et al.*, 1999; Muntau *et al.*, 2002). It has been accepted by FDA and EMA in 2007 and 2008, respectively. Although very expensive, with yearly costs reaching 100 000 Euro per an average adult, this therapy significantly increases treatment efficiency and improves the quality of life. Therefore, the PKU community has enthusiastically greeted it. Unfortunately, only selected patients respond to BH4 and provide clinical confirmation of the treatment effectiveness (demonstrable increase in the permissible protein intake). Several weeks of an expensive trial-treatment is also often required. Therefore, preselection of BH4-non-responders by means of genotyping of the PAH gene mutations (Blau & Erlandsen, 2004) should be recommended to avoid unnecessary (and expensive) BH4-challenge in persons with definitively "BH4-resistant" genotypes.

In the present study, a population-wide genotyping was carried out in Polish PKU-patients aiming at estimating the number of potential BH4-responders and the BH4 demand, as well as financing it prospectively by a National Health Fund.

### PATIENTS AND METHODS

Starting from 2007, all Polish patients with PKU were offered genotyping for mutations of the PAH gene. Patients were informed about possibility of genetic testing in their regional PKU treatment centers as well as during meetings for PKU families and using web pages of patient organizations. Prior to genotyping, all patients interested in this procedure were asked to sign appropriate informed consent form approved by the Ethics Committee.

Blood samples obtained from the patients were sent to the Chair of Pediatrics in Krakow, where genetic testing was performed. DNA was extracted from the leukocytes with the use of standard methods. Screening of all exons and flanking intronic regions of the PAH gene with the use of DHPLC method (WAVE system,

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**Abbreviations:** PKU, phenylketonuria; BH4, tetrahydrobiopterin; PAH, phenylalanine hydroxylase; DHPLC, denaturing high-performance liquid chromatography; MLPA, multiplex ligation-dependent probe amplification

**Table 1. Common genotypes with regard to the BH4-responsiveness**

Genotype	Number of cases
BH4-responsiveness absent or unlikely (825 cases)	
p.R408W/p.R408W	475
p.R408W/IVS10-11g>a	62
p.R408W/p.R158Q	56
p.R408W/p.P281L	34
p.R408W/IVS12+1g>a	27
p.R408W/p.R252W	18
p.R408W/g.47563_51794del4232g.56161_56430ins268	13
p.R408W/p.I283F	12
p.R408W/p.F55fs	11
Other genotypes	117
BH4-responsiveness probable (363 cases)	
Mild hyperphenylalaninemia (216 cases):	
p.A403V/p.R408W	38
p.A300S/p.R408W	34
p.I306V/p.R408W	23
p.R297H/p.R408W	19
p.V245A/p.R408W	10
Other genotypes	92
PKU (147 cases):	
p.R261Q/p.R408W	26
p.Y414C/p.R408W	26
p.V388M/p.R408W	12
p.L348V/p.R408W	11
p.R241H/p.R408W	11
p.R243Q/p.R408W	6
Other genotypes	55

Transgenomics) was used to select the samples containing potential gene variants. Subsequent sequencing (3130 Genetic Analyzer, Applied Biosystems) allowed to diagnose the specific point mutations. To detect the exon deletions MLPA technique (SALSA MLPA kit P055 *PAH*, MRC-Holland) was used followed by PCR-based detection methods as described earlier (Bik-Multanowski & Pietrzyk, 2008). In case of the new deletions microarray-based mapping (Cytogenetics 2.7M Array, Affymetrix) and final sequencing of the regions flanking the deletion (technical details on request) were used. When the above procedure didn't allow to detect both mutations, sequencing of all exons of the *PAH* gene was performed in a given sample.

Assessment of BH4-responsiveness-status of the detected mutations was performed. It was based on previously published observational data — the results of BH4 loading test in patients selected with regard to their genotype (Blau & Erlandsen, 2004; Zurflüh *et al.*, 2008; BIOPKU). The status of the mutations was defined as: — “Absent/Unlikely” (including common variants p.R158Q (c.473G>A) and IVS10-11G>C (c.1066-11G>A) for which only single cases of BH4-responsiveness were reported), — “Probable” (convincing data reported on BH4-responsiveness in carriers of the given mutation) and

— “Undefined” (no data available relating to the given genetic variant).

New, previously not described nonsense mutations and large deletions were assumed as “non-responsive” as well as frameshift variants and splice site mutations (with the exception of variants previously reported and corresponding with the milder types of hyperphenylalaninemia). The clinical severity of hyperphenylalaninemia was assessed on the base of the medical records analysis and the genotyped patients.

## RESULTS

A total of 1286 PKU-patients (approx. 70% of the entire population of registered Polish patients) were genotyped. Genotyping allowed to identify 95 types of mutations including nine genetic variants not described previously. Mutations were identified in 96.9% of the alleles. The most prevalent mutation p.R408W (c.1222C>T) was detected in 1590 alleles (61.8%). In next 534 alleles (20.8%) one of ten genetic variants was found, revealing allelic frequency above 1%: IVS10-11G>A (c.1066-11G>A), p.R158Q (c.473G>A), p.P281L (c.842C>T), IVS12+1G>A (c.1315+1G>A), p.A300S (c.898G>T), p.A403V (c.1208C>T), p.R261Q (c.782G>A), p.Y414C (c.1241A>G), p.I306V (c.916A>G), p.R252W (c.754C>T). The remaining 84 mutation types were rare variants with allelic frequency below 1% or some private mutations.

In 2068 alleles, 44 types of mutations determining absent or unlikely BH4-responsiveness were found, whereas in 384 alleles 31 types of “BH4-responsive” were present and in 39 alleles 20 types of mutations with undefined BH4-status were detected. In 81 patients only one mutation was recognized (3.1% of alleles).

To select a group of potential BH4-responders, analysis of 1223 cases was performed. It included 1205 persons with both mutations and 18 persons with only one identified mutation which was not a non-responsive one. The remaining 63 patients were not analyzed.

BH4-responsiveness was excluded in 64.2% of the studied population (825 patients), in whom two mutations, definitely or most probably non-responsive were identified. Positive response to BH4 was expected in 28.2% of cases (363 patients) in whom at least one “BH4-responsive” mutation was found.

The majority of probable BH4-responders (216 patients; 16.8% of cases) carried *PAH* gene mutations which were theoretically corresponding with the presence of a mild hyperphenylalaninemia (such as p.A403V, p.I306V, p.A300S and p.R297H). As expected, in most patients from this group, medical records analysis revealed blood phenylalanine concentrations constantly below 0.6 mmol/l (typical for mild hyperphenylalaninemia). In some cases, higher concentrations (typical for mild PKU) were recorded in the neonatal screening and incidentally in case of high dietary protein intake or a fever caused by an acute infection. However, as the majority of blood phenyla-

lanine concentrations remained below of 0.6 mmol/l, such borderline-phenotype patients were regarded as having mild hyperphenylalaninemia.

The remaining potential BH4-responders (147 patients; approximately 11.4% of all cases genotyped in our survey) were patients with mild or classical PKU. The mutations: p.R261Q, p.Y414C, p.L348V and p.V388M were the most prevalent genetic variants detected in nearly 3/4 of the alleles in this group.

In 2.7% of patients (35 cases) at least one mutation was found with an undefined BH4-status. Based on the genotyping of the *PAH* gene mutations, the BH4-response could not be predicted.

The most common genotypes identified in the studied population are listed in Table 1. Details on the detected genetic variants can be found in the Supplementary Table 1 and Table 2 at [www.actabp.pl](http://www.actabp.pl).

## DISCUSSION

To our knowledge, the present study has been the largest national *PAH* gene mutation analysis published until now. We intended to test the largest possible group of patients with classical and milder forms of PKU. Adults with this disease often relax or sometimes even abandon their diet. They also miss scheduled control visits in their outpatient clinics. These are especially patients with mild disease course. Therefore, the group of adult patients with mild phenylketonuria and mild hyperphenylalaninemia caused by potentially BH4-responsive mutations of the *PAH* gene might be slightly unrepresentative in our material. On the other hand, vast majority of the genotyped patients were diagnosed after introducing a neonatal screening in all regions of the country (PKU screening did not cover the entire population before 1987). Virtually, all of these persons were registered in PKU treatment clinics and were offered genotyping regardless of their disease severity. Therefore, our data seems to be representative for the whole Polish PKU population.

It seems interesting that previous reports stressed low percentage of “BH4-responsive” mutations in Polish population (Zurflüh *et al.*, 2008; Zschocke, 2003). This appears to be not appropriate in relation to our data. The reason for this discrepancy was probably a low number of patients with PKU who were genotyped and, on the other hand, a selective genotyping which was performed to detect the most common mutations only. In fact, when analyzing the published data on prevalence of “BH4-responsive” variants (Zschocke, 2003), their actual percentage in Poland resembles the one in other countries of Eastern Europe, including Czech Republic, Slovakia, Bulgaria, Lithuania as well as, probably, Ukraine and the European part of Russia, where only screening for specific mutations was performed (Zschocke, 2003; Kozak *et al.*, 1997; Kadasi *et al.*, 1995; Kalaydjieva, 1993; Kasnauskienė, 2003; Nechiporenko & Lalivshits, 2000; Baranovskaya *et al.*, 1996; Charikova, 1993). All these countries represent together a population of approx. 200,000,000 of Europeans. Some conclusions on the prevalence of BH4-responsiveness in Poland may probably be extrapolated into the population of the entire region of Eastern Europe.

Although BH4-responsiveness is probable in nearly 30% of Polish patients with hyperphenylalaninemia, no dietary treatment is needed to the part of them. In particular, in persons with mild hyperphenylalanine-

mia, in whom blood phenylalanine concentration does not exceed 0.6 mmol/l, no treatment is recommended in the majority of European countries except pregnant women and young children. On the other hand, the blood phenylalanine concentration even in patients with mild hyperphenylalaninemia can exceed the level of 0.6 mmol/l in case of incidental excessive protein intake. Such situation may occur in off-diet patients due to their irregular eating habits (as typically observed among adolescents and young adults). Therefore, it seems to be important that these patients remain under clinical control, to increase their safety level. However, the need for continuous BH4-therapy as preventive treatment to avoid episodes of significant hyperphenylalaninemia (and the effectiveness of such therapy in the light of high residual *PAH* activity as well as typically poor treatment adherence) seems to be questionable. Consequently, high costs of BH4-treatment should be individually balanced against its beneficial effects in each patient with mild hyperphenylalaninemia.

When we exclude patients with mild hyperphenylalaninemia the usage of BH4 seems to be useful only in approximately 10% of the studied population – mostly in patients with mild PKU. However, patients with theoretically BH4-responsive genotype do not always respond to the BH4-treatment (Zurflüh *et al.*, 2008). This situation may be caused by several reasons such as modifying effect of neighborhood-genes, presence of additional genetic variants enhancing or inhibiting exon splicing or direct interaction of two given types of mutated *PAH* proteins in compound heterozygotes. Bearing in mind the fact, that around 50% of carriers of the p.R261Q mutation (the most common BH4-response-related genetic variant in the group of our classical/mild-PKU patients) as well as 10–20% of mild-PKU patients carrying other BH4-responsive mutations do not respond to BH4 (BIOPKUdb), it may be assumed that the rate of BH4-responders who require a BH4-treatment, reaches approx. 8–10% in the Polish population of PKU patients. To ultimately select the BH4-responders, BH4-loading procedure and/or trial treatment should be proposed to these patients. It allows a precise assessment of the expected increase in their dietary phenylalanine tolerance in relation to the BH4 therapy.

Several previous studies reported problems with detection of the *PAH* gene mutation in 2–3% of alleles while genotyping patients with PKU (Aulehla-Scholz & Heilbronner, 2003; Bercovich *et al.*, 2008; Cali *et al.*, 2010). In those patients different factors, such as presence of functional genetic variants located in a deep intronic sequences or epigenetic influences could affect the *PAH* gene function. Our results are cohesive with these findings. In 81 patients only one mutation was identified. In this group, an assessment of the potential BH4-responsiveness was not possible due to 63 cases in whom only one, BH4-unresponsive mutation was found. Despite sequencing of all exons of the *PAH* gene knowledge on the second mutation remained unknown in these persons. Therefore BH4-loading test/trial treatment would be necessary to select the BH4-responders.

In conclusion, approximately 8–10% of Polish patients with hyperphenylalaninemia may possibly benefit from the BH4 treatment. This percentage seems to be similar with other countries of Eastern Europe, inhabited by a population of approx. 200,000,000 citizens.

## Contributions

Mirosław Bik-Multanowski designed the study, performed the genotyping, analyzed data and wrote the manuscript. The remaining authors participated in the assessment of a clinical data and in writing of the manuscript.

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