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RĪGAS STRADIŅA UNIVERSITĀTE

Natalja Pronina

The Molecular Basis of Phenylketonuria and Hyperphenylalaninemia in Latvia

Synopsis of the Doctoral Thesis
Speciality – Medical Genetics

Riga, 2012

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Speciality: Medical Genetics

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ABBREVIATIONS

ANOVA	- ANalysis Of VAriance between groups
AVs	- Assigned values
BH4	- tetrahydrobiopterin
CBR1	- Cofactor binding region
CD	- Catalytic domain
cDNA	- Cloned DNA
DGGE	- Denaturation gradient gel electrophoresis
DNA	- Deoxyribonucleic acid
HPA	- Hyperphenylalaninemia
IEMs	- Inborn error of metabolisms
MHP	- Mild hyperphenylalaninemia
PAH	- Enzyme Phenylalaninehydroxylase
PheOH	- Enzyme Phenylalaninehydroxylase
PAH	- Phenylalaninehydroxylase gene
PCR	- Polymerase chain reaction
Phe	- Phenylalanine
PKU	- Phenylketonuria
PolyPhen	- Polymorphism Phenotyping
PSIC	- Position-specific-independent-counts
RFLP	- Restriction length fragment polymorphism
STR	- Short tandem repeats
VNTR	- Variable number of tandem repeats

SUMMARY

Phenylketonuria (PKU) is the most common inborn error of amino acid metabolism in Europeans. It is caused by an autosomal recessive deficiency of the hepatic enzyme phenylalanine hydroxylase (*PAH*) that catalyses the irreversible hydroxylation of phenylalanine to tyrosine. The elevated level of phenylalanine effects the energy production, protein synthesis, and neurotransmitter homeostasis in the developing brain and results in the most important manifestation of PKU - mental retardation.

More than 560 different disease-causing mutations in the *PAH* gene have been identified and reported since the gene was discovered in 1986. Mutations differ in residual enzyme activity, and the genotype could be a good predictor of biochemical phenotype in the majority of patients.

The aim of the study was to investigate the molecular basis of phenylketonuria and hyperphenylalaninemia in Latvian patients and evaluate a PAH gene mutation diagnostic strategy in Latvian population.

Analysis of the molecular basis of PKU in Latvia has revealed 20 different mutations in the *PAH* gene. The most common mutation was R408W that accounted for 73% of all PKU chromosomes that gives the high level of homogeneity ("homozygosity") at the *PAH* locus in Latvia (j=0.514). Frequencies of remained 19 mutations ranged from 0.7 to 5.7% of all mutant alleles. The majority of mutations (12/20) were severe and responsible for the classic PKU phenotype that was observed in 91% of PKU patients.

The evaluation of patients' genotypes can provide the additional information for BH_4-responsiveness. According to the study results 13 of 70 (18%) Latvian PKU patients could potentially benefit from chaperon therapy by sapropterin dihydrochloride while remaining 57 (81%) patients with homozygous R408W mutation should keep the low phenylalanine diet as the only effective form of therapy.

Minihaplotype studies have revealed 16 different minihaplotypes associated to *PAH* gene mutations and 20 different minihaplotypes for normal

PAH alleles. The average probability of heterozygosity for minihaplotypes was about 76% for mutant and 92% for normal chromosomes indicating a greater diversity of normal alleles. Statistical analysis has revealed the significant difference in the distribution of normal and mutant alleles for only two minihaplotypes 3/238 ($p=0.0000572$) and 8/230 ($p=0.0133$), and the tendency to statistically significant difference ($p<0.10$) between normal and mutant alleles in the distribution of minihaplotypes 3/242, 7/246 and 8/234.

Analysis of the distribution of the *PAH* gene mutation R408W together with it strong association with a typical East-European VNTR3/STR238 minihaplotype have confirmed the Balto-Slavic origin of mutation R408W and introduction of this mutation to other European populations by people migrations.

The three-step *PAH* gene mutation detection strategy used in the study is the most effective for routine diagnostics in Latvian population with the sensitivity of the method 99%.

I INTRODUCTION

Metabolic diseases are life threatening inheritable, genetic disorders in which errors of metabolism occur, involving a block where a catalyst or enzyme is absent or malfunctioning. The most part of inherited metabolic disorders are identifies as inborn errors of metabolism or IEMs. IEMs are normally defined as diseases of amino acids, organic acids, the urea cycle, galactosemia, primary lactic acidoses, glycogen storage diseases, lysosomal storage diseases, and diseases involving peroxisomal and mitochondrial respiratory chain dysfunction (Villas-Boas, 2007).

The incidence of inherited metabolic diseases varies from 1 in 10 000 people to diseases that are very rare and affect around 1 in 100 000, and lead to a wide range of special needs in care and education (Hannigan, 2007).

Phenylketonuria (PKU) is the most common inborn error of amino acid metabolism in Europeans with the incidence of about one case per 10 000 live births. It has been frequently described as a paradigm of a Mendelian disorder. PKU was the first metabolic cause of mental retardation to be identified, the first genetic disorder of the central nervous system that could be fully treated by modification of external factors (i.e., the diet), and the first disorder that was successfully diagnosed by universal neonatal screening.

The understanding of the biochemical and molecular basis of PKU was very important for the treatment strategies introduced for these patients and led to significant reduction in morbidity and to an improvement in quality of life.

Untreated PKU is associated with an abnormal phenotype which includes growth failure, poor skin pigmentation, microcephaly, seizures, global developmental delay and severe intellectual impairment. However, since the introduction of newborn screening programs and with early dietary intervention, children born with PKU can now expect to lead relatively normal lives.

The phenylalanine hydroxylase (*PAH)* gene (OMIM 261600) (http://www.pahdb.mcgill.ca) was first cloned in 1983 (Woo, 1983). The most studies of *PAH* gene mutations in different populations were carried out in the 1990s.

Most forms of PKU and hyperphenylalaninaemia (HPA) are caused by mutations in the *PAH* gene on chromosome 12q23.2. Over 560 disease causing mutations have been reported, most of them corresponding to point mutations causing missense changes. Mutations differ in residual enzyme activity, and the genotype could be a good predictor of biochemical phenotype in the majority of patients.

Despite the fact that dietary treatment remains the mainstay of PKU management, it is multifaceted, challenging, and life long. Key dietary behaviours associated with optimal control of blood phenylalanine (Phe) concentrations include avoidance of high-protein foods and supplementation with special medical foods that often are unpalatable. Dietary compliance is influenced by cognitive, emotional, psychological, and cultural factors. Non-compliance with the dietary prescription is commonplace, particularly during adolescence and adulthood. For all these reasons, the contemporary interest in PKU more often is focused on the development of new therapeutic approaches, but it is still closely related to the biochemical and molecular basis of PKU.

Chaperon therapy by sapropterin dihydrochloride is a novel therapeutic approach that is effective in a subset of individuals with PKU. It is specifically indicated to reduce blood Phe levels in patients with HPA due to tetrahydrobiopterin- (BH4-) responsive PKU and could be used alone or in conjunction with a Phe-restricted diet. The effectiveness of the chaperon therapy depends on mutation residual activity in the *PAH* gene. So, the genotypes can provide the additional information for BH_4 responsiveness and are taken into account in selecting the type of PKU treatment (Blau, 2010).

Population studies have revealed at least 87 different haplotypes for *PAH* gene mutations but only a few are prevalent, and most are uncommon. Haplotypes 1 through 4 account for more than 80% of PKU-bearing chromosomes and are mainly used to determine the origin of mutations.

The high degree of polymorphism and strong Mendelian segregation of minihaplotypes (combination of VNTR and STR systems) considerably increases the number of cases that are informative and makes it useful for prenatal diagnosis, detection of rare mutations, and carrier screening determination in PKU families.

Among the most common *PAH* mutations is R408W. In eastern European populations, the R408W mutation is strongly associated with RFLP haplotype 2, the three-copy VNTR allele (VNTR 3), and the 238-bp STR allele. Therefore, we expect a high incidence of this mutation in our study.

Large number of Latvian PKU patients has classic (severe) clinical PKU phenotype that could be possible because of the prevalence of severe mutations in Latvian PKU chromosomes.

THE AIM OF THE STUDY:

The aim of the study was to investigate the molecular basis of phenylketonuria and hyperphenylalaninemia in Latvian patients and evaluate a *PAH* gene mutation diagnostic strategy in Latvian population.

THE TASKS OF THE STUDY:

1. To investigate the *PAH* gene mutation spectrum for Latvian PKU patients and their parents;
2. To detect the association between minihaplotypes and mutations at the *PAH* locus in Latvian PKU patients and their parents;

3. To compare distribution of minihaplotypes in mutant and normal *PAH* chromosomes;
4. To estimate the genotype-phenotype correlation in patients with PKU;
5. To compare the frequency of the mutation R408W in Latvian PKU chromosomes with the frequencies of this mutation in other PKU populations in Europe;
6. To evaluate the diagnostic techniques for the introduction of a *PAH* gene mutation detection strategy in the routine diagnostics in Latvia.

Scientific Novelty of the Study

This study is the first study in the Baltic States region investigating minihaplotype associations for full mutation spectrum including rare and novel mutations observed in the *PAH* gene.

Three novel nucleotide changes were identified and two of them assumed to be disease causing while the third one is going to be mild mutation causing MHP.

R408W was found on high relative frequencies and typical Eastern-European minihaplotype 3/238 confirming the Balto-Slavic origin of the mutation.

Practical Novelty of the Study

The evaluation of patients' genotypes provides the additional information for detection of clinical phenotype for PKU patients and selecting of appropriate therapy. Chaperon therapy was introduced for four Latvian PKU patients.

Results of minihaplotypes studies are useful for prenatal diagnosis, carrier screening and detection of rare mutations in Latvian PKU chromosomes.

Theses to be defended

- Common phenylketonuria mutation for Latvian patients is R408w tah is one of predominant in the *PAH* gene in European populations and the most predominant in Eastern European populations.
- The three-step *PAH* gene mutation detection strategy used in the study is the most effective for routine diagnostics in Latvian population.

II MATERIALS AND METHODS

II.1. SUBJECTS

PKU patients in this study were initially diagnosed as having PAH deficiency through the National Newborn Screening Program. Patients were being treated and monitored in Medical Genetics Clinic in Riga.

Blood samples for DNA analysis were collected from patients and their first degree relatives. Patients with transitory HPA who did not required treatment were not included in this study. However, patients with unclear or borderline parameters were investigated to clarify patients' status.

DNA samples for control group were collected from volunteers from the mixed population and were investigated for the absence of the *PAH* gene sequence changes to check the population specific polymorphism.

Samples from PKU patients were taken with consent of their parents; the study was approved by the Central Medical Ethics Committee.

II.2 GENOMIC DNA EXTRACTION

Genomic DNA was purified from whole blood leucocytes using Genomic DNA Purification Kit („Fermentas", Lithuania).

II.3 IDENTIFICATION OF *PAH* GENE MUTATION R408W

PAH gene mutation R408W is a transition C→T at the position 1222 of cDNA causing amino acid substitution (codon CGG→TGG). Diagnostic identification of mutation R408W is based on the fact that it creates new restriction enzyme *Sty*I site in the exon 12 (MOLGENT, 1999-2002).

II.4 DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE) AND AUTOMATED DIRECT SEQUENCING OF *PAH* GENE EXONS

Non-R408W chromosomes were screened for mutations through denaturing-gradient gel electrophoresis (DGGE) of the 13 exons of the *PAH* gene (Guldberg and Güttler, 1994). Exons showing variant electrophoretic patterns were sequenced by fluorescent automated sequencing of *PAH* gene fragments by automated gene analyser ABI PRISM™ 310 and Big Dye Terminator Sequencing protocol (Applied Biosystems).

II.5 *PAH* GENE MINIHAPLOTYPE ANALYSIS

Minihaplotype analysis (combination of the VNTR and STR alleles) was performed on PCR-based approach with the further electrophoretic separation on 6% PAA gel for VNTR and capillary electrophoresis for STR system.

Electrophoretic resolution of the amplified products for VNTR alleles demonstrated DNA fragments of six discrete sizes -380, 470, 500, 530, 560, and 650 bp that correspond to the 3, 6, 7, 8, 9, or 12 copies of the repeated unit, respectively.

The corresponding STR fragments' length (226 bp; 230 bp; 234 bp; 238bp; 242 bp; 246 bp; 250 bp; 254bp and 258bp) was calculated according to the calibration curve of the GeneScan™ ROX 2500™ size standard.

II.6 PROGRAMMES USED FOR THE RESULTS PROCESSING

Polymorphism Phenotyping. Novel amino acid changes found in Latvian PKU chromosomes that were not previously reported were analysed by *PolyPhen* software to gather information about it possible effect on PAH (http://genetics.bwh.harvard.edu/pph/).

PolyPhen (=*Poly*morphism *Phen*otyping) is an automatic tool for prediction of possible impact of an amino acid substitution on the structure and function of a human protein. This prediction is based on straightforward empirical rules which are applied to the sequence, phylogenetic and structural information characterizing the substitution.

The resulting multiple alignment is used by the new version of the PSIC software (**P**osition-**S**pecific **I**ndependent **C**ounts) to calculate the so-called *profile matrix*. Elements of the matrix (profile scores) are logarithmic ratios of the likelihood of given amino acid occurring at a particular position to the likelihood of this amino acid occurring at any position (background frequency).

PolyPhen computes the absolute value of the difference between profile scores of both allelic variants in the polymorphic position. Big values of this difference may indicate that the studied substitution is rarely or never observed in the protein family. *PolyPhen* also shows the number of aligned sequences at the query position. This number may be used to assess the reliability of profile score calculations.

PolyPhen uses empirically derived rules to predict that an nsSNP is

- **probably damaging**, i.e., it is with high confidence supposed to affect protein function or structure
- **possibly damaging**, i.e., it is supposed to affect protein function or structure
- **benign**, most likely lacking any phenotypic effect
- **unknown**, when in some rare cases, the lack of data do not allow *PolyPhen* to make a prediction

Calculation of Homozygosity.Homozygosity (*j*) at the PAH locus in the population is determined using the equation $j = \Sigma x_i^2$, where x_i is the frequency of the *i*th allele. In populations where ascertainment of mutations is

not 100%, each of the uncharacterised alleles is defined as having a frequency of 1/N, where N is the total number of mutant chromosomes investigated.

This value is the theoretical frequency of patients carrying two identical mutations. Homozygosity values of different populations reflect their mutational heterogeneity for the particular locus (Guldberg, 1996).

Calculation of Expected Heterozygosity for PKU minihaplotypes. The average level of heterozygosity of the VNTR/STR system was calculated according to the formula provided by Daiger *et al.*: $1 - \sum p_i^2$. (Daiger, 1989).

Statistical methods. Statistical calculations were performed with STATISTICA 7 software using sub-module General linear/Non-linear model with one-way ANOVA, distribution binomial and logit function. The result for comparison of R408W mutation frequencies between two geographic populations was expressed as mean frequency ± 95% confidence interval.

Comparisons between minihaplotypes' variants in normal and mutant *PAH* chromosomes with "0" and "1" parameters were done using Chi-square (χ^2) 2x2 contingency table analysis.

Absolute allele frequencies in a population were estimated using incidence data and the Hardy-Weinberg formula: $p^2 + 2pq + q^2 = 1$ (http://www.changbioscience. com/genetics/hardy.html).

Chi-square test was used for comparison of absolute and relative *PAH* allele frequencies, as well as for comparison of two different approaches for classifying the PKU clinical phenotype (http://www.graphpad.com/quickcalcs/index.cfm).

Sensitivity measurements for methods used in our study were performed according to Altman and Bland, 1994. Sensitivity is the proportion of true positives that are correctly identified by the test.

III RESULTS

A total of 74 Latvian patients with PKU, corresponding to 70 unrelated families and their 110 first degree relatives, were investigated. Most (57/70) of the patients, accounting for 81.5%, were identified in neonatal screening, the remaining (13/70) accounting for 18.5%, when they showed mental retardation in period between 1 and 5 years old. The oldest patient was born in 1967 but the youngest one in 2011. Six patients from five unrelated families were born before the National Newborn Screening Program was launched in the whole country.

Preliminary patients' phenotypes were classified according to the pre-treatment level of Phe: 64.3% (45/70) had severe PKU (Phe > 1200 μmol/L), 22.9% (16/70) had mild PKU (Phe 360–1200 μmol/L), 5.7% (4/70) had MHP (Phe < 360 μmol/L) and in 7.1% (5/70) cases Phe level was unavailable. Using the Phe tolerance (amount of dietary phenylalanine per day to keep plasma concentration of Phe at a safe level) as the additional classification parameter, 91.4% (64/70) patients were classified as having severe PKU, 5.7% (4/70) as having mild PKU and 2.9% (2/70) as having MHP (Table 3.1). Phe measurement units used in Latvia are mg% (1 mg% corresponds to 60 μmol/L).

Table 3.1. Classification of PKU clinical forms in Latvian patients.

PKU clinical form	By Phe pre-treatment level	N	%	By Phe tolerance	N	%
Severe	> 1200 μmol/L (>20 mg%)	45	64.3	250-350 mg	64	91.4
Mild PKU	360–1200 μmol/L (6 - 20 mg%)	16	22.9	350-600 mg	4	5.7
MHP	< 360 μmol/L (< 6 mg%)	4	5.7	< 600 mg	2	2.9
Unclassified	Unknown	5	7.1	—	—	—

III.1 MUTATION SPECTRUM IDENTIFIED FOR LATVIAN PKU PATIENTS

Mutation analysis of 140 independent Latvian PKU chromosomes has revealed 20 different *PAH* gene mutations, representing a mutation detection rate 99%, one allele remained unknown. Thirteen were predicted missense mutations, 3 nonsense mutations, 3 splice site mutations, and one small deletion. PAH molecular lesions were identified in 8 different *PAH* gene exons (2, 3, 5, 6, 7, 8, 9, 11 and 12), while none was found in exons 1, 4, 10, and 13. Two mutations were identified in intron 12 and one mutation in intron 10.

The most common mutation was R408W, accounted for 73% of mutant alleles (Fig.1). Next most common mutation E280K presented on 5.7% of all PKU chromosomes. The frequency of the other five mutations (R261Q, R158Q, P281L, IVS10-11G>A and A104D) ranged from 1.4% to 3%.

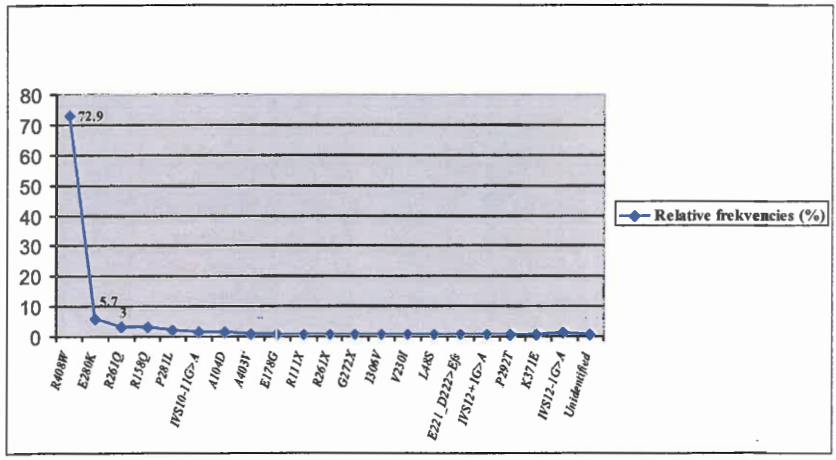

Fig. 1. *PAH* gene mutation spectrum for Latvian PKU chromosomes.

Twelve mutations were identified on single chromosome only, corresponding to a frequency of 0.7%. Three mutations (P292T, K371E and IVS12-1G>A) had not been previously identified; two of them were found only

once, but the third one was identified on two unrelated PKU chromosomes. None of these mutations have been examined by in vitro expression analysis. A novel mutation was assumed to be disease-causing when (1) it was either non-silent or a potential splicing mutation, (2) no other mutation was identified in the coding region of the *PAH* gene, (3) the allele was inherited from the parent who did not carry the other PKU mutation, and (4) the mutation had not been previously identified on normal or mutant chromosomes (Zschocke, 1999). To check the population specific polymorphisms for these novel single nucleotide changes control samples of 100 volunteered individuals without PKU were tested.

PAH gene mutations, their location and frequencies are summarised in table 2.

Table 2. Frequencies of *PAH* gene mutations in Latvian PKU patients.

No.	Mutation Name	Syst. name	Location	Characters of mutation	No.	RF %
1	R408W	c.1222C>T	Ex 12	Missense	102	72.9
2	E280K	c.838G>A	Ex 7	Missense	8	5.7
3	R261Q	c.782G>A	Ex 7	Missense	4	3.0
4	R158Q	c.473G>A	Ex 5	Missense	4	3.0
5	P281L	c.842C>T	Ex 7	Missense	3	2.1
6	IVS10-11G>A	c.1066-11G>A	I10	Splice site	2	1.4
7	A104D	c.311C>A	Ex 3	Missense	2	1.4
8	A403V	c.1208C>T	Ex 12	Missense	1	0.7
9	E178G	c.533A>G	Ex 6	Missense	1	0.7
10	R111X	c.331C>T	Ex 3	Nonsense	1	0.7
11	R261X	c.781C>T	Ex 7	Nonsense	1	0.7
12	G272X	c.814G>T	Ex 7	Nonsense	1	0.7
13	I306V	c.916A>G	Ex 9	Missense	1	0.7
14	V230I	c.688G>A	Ex 6	Missense	1	0.7
15	L48S	c.143T>C	Ex 2	Missense	1	0.7
16	E221_D222>Efs	c.663_664delAG	Ex 6	Deletion	1	0.7
17	IVS12+1G>A	c.1315+1G>A	I12	Splice site	1	0.7
18	**P292T***	**c.874C>A**	Ex 8	Missense	1	0.7
19	**K371E***	**c.1111A>G**	Ex 11	Missense	1	0.7
20	**IVS12-1G>A***	**c.1316-1G>A**	I12	Splice site	2	1.4
21	Unidentified	-	-	-	1	0.7
Total					**140**	**100**

*Novel mutations are marked in bold

The sensitivity of tests used for mutation detection was calculated as the proportion of alleles with identified *PAH* mutation that test positive for it. This can also be written as:

$$\text{sensitivity} = \frac{\text{number of true positives}}{\text{number of true positives} + \text{number of false negatives}}$$

Using these calculations the sensitivity of approach used for mutation detection in *PAH* gene was detected as 99.28%.

III.2 GENOTYPES IDENTIFIED IN LATVIAN PKU PATIENTS

The most prevalent genotype among Latvian PKU patients was R408W/R408W. Thirty-six (51.4%) of 70 unrelated characterised PKU patients were homozygous for R408W, the remaining 34 patients were compound heterozygous. The homozygosity value (*j*) for the PKU population of Latvia is 0.514.

The majority on compound heterozygote PKU patients had R408W mutation in one allele (42.9%); only four patients (5.7%) had no R408W mutations in their chromosomes (Fig. 2).

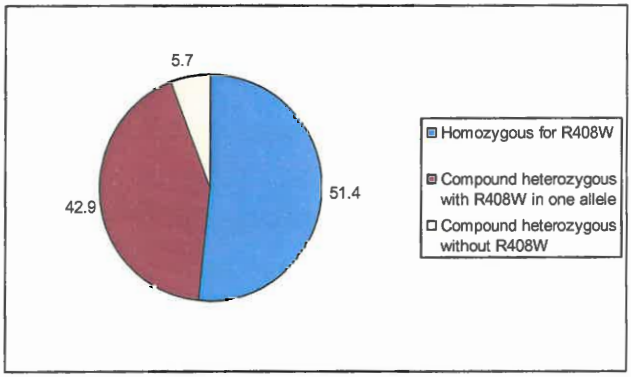

Fig. 2. The distribution of genotypes for Latvian PKU patients (%).

The full list of indentified genotypes for Latvian PKU patients and frequency for each genotype are shown on Fig. 3.

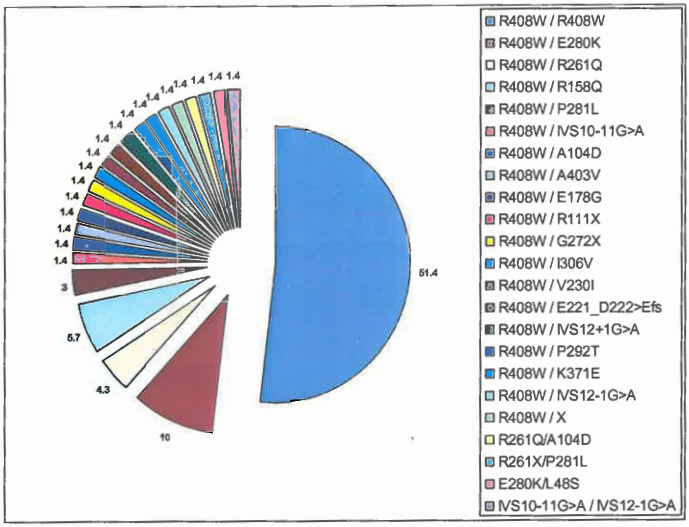

Fig. 3. Relative frequencies of genotypes identified in Latvian PKU patients.

Hardy-Weinberg equilibrium was used to determine whether the population is balanced. We used the R408W mutation number (n=102) obtained from our study to calculate the absolute frequencies for PKU genotypes.

According to Hardy-Weinberg equilibrium the absolute frequency for R408W homozygote patients has to be 53.1% and 39.6% for compound heterozygous patients with R408W mutation. Our study did not reveal the presence of other homozygous patients in PKU population that according to Hardy-Weinberg equilibrium has to be up to 7%.

To estimate the difference between the PKU genotypes' absolute frequencies and current relative frequencies in Latvian PKU population we regarded PKU patients without R408W mutation as *aa* homozygous to make calculations. Statistical analysis showed that this difference is considered to be

not statistically significant ($p = 0.8312$, χ^2 equals 0.370 with 2 d.f.), as we expected. These data means, that the Latvian PKU patients pool is balanced.

In that way our results suggest that for the Latvian population, the allele and genotype frequencies at the *PAH* locus are in Hardy-Weinberg equilibrium. In other words, we can expect these allele frequencies to remain constant over time (barring any specific evolutionary forces acting upon this locus), thus ensuring genetic variation in the population at the *PAH* locus.

III.2.1 Genotype-phenotype correlations in Latvian PKU patients

On the basis of individual data on Phe tolerance and pre-treatment serum Phe levels, the patients were assigned to one of four phenotype categories: 64 (91.4%) had severe PKU, four (5.7%) had mild PKU and two (2.9%) had MHP. No one patient was classified as having moderate PKU clinical form. Genotype and phenotype correlation in PKU patients is shown in Table 3.

Table 3. Observed correlation between *PAH* genotype and phenotype in Latvian PKU patients.

No.	PAH locus genotype	No. of cases	RF%	Clinical phenotype
1	R408W / R408W	36	51,4	Severe PKU
2	R408W / E280K	7	10,0	Severe PKU
3	R408W / R261Q	3	4,3	Severe PKU
4	R408W / R158Q	4	5,7	Severe PKU
5	R408W / P281L	2	3,0	Severe PKU
6	R408W / IVS10-11G>A	1	1,4	Severe PKU
7	R408W / A104D	1	1,4	Severe PKU
8	R408W / R111X	1	1,4	Severe PKU
9	R408W / E221_D222>Efs	1	1,4	Severe PKU
10	R408W / IVS12+1G>A	1	1,4	Severe PKU
11	R408W / E178G	1	1,4	Mild PKU
12	R408W / A403V	1	1,4	Mild PKU
13	R408W / G272X	1	1,4	Mild PKU
14	R408W / I306V	1	1,4	Mild PKU
15	R408W / V230I	1	1,4	MHP

Table 3 (continued)

16	R408W / **K371E**	1	1,4	MHP
17	R408W / **P292T**	1	1,4	Severe PKU
18	R408W / **IVS12-1G>A**	1	1,4	Severe PKU
19	R408W / X	1	1,4	Severe PKU
20	R261Q/A104D	1	1,4	Severe PKU
21	R261X/P281L	1	1,4	Severe PKU
22	E280K/L48S	1	1,4	Severe PKU
23	IVS10-11G>A / **IVS12-1G>A**	1	1,4	Severe PKU
	Total	**70**	**100**	

According to multicenter study (Guldberg, 1998) 10 of 17 known *PAH* gene mutations observed in Latvian PKU patients were classified as severe or classic-PKU mutations, one as moderate-PKU mutation, two mild-PKU and 4 as mutations causing MHP. In compliance with Guldberg *et al.,* 1998 proposed model for phenotypic effect of two mutant *PAH* alleles, expressed as the sum of their assigned values (AVs), we calculated the phenotype for Latvian PKU patients using the each mutation assigned value (Table 4).

Table 4. Genotype and phenotype correlation in Latvian PKU patients according to Guldberg model.

No.	PAH locus genotype	AV_1+AV_2	AVs sum	Clinical phenotype
1	R408W / R408W	1+1	2	Severe PKU
2	R408W / E280K	1+1	2	Severe PKU
3	R408W / R261Q	1+2	3	Moderate PKU
4	R408W / R158Q	1+1	2	Severe PKU
5	R408W / P281L	1+1	2	Severe PKU
6	R408W / IVS10-11G>A	1+1	2	Severe PKU
7	R261X/P281L	1+1	2	Severe PKU
8	R408W / R111X	1+1	2	Severe PKU
9	R408W / E221_D222>Efs	1+1	2	Severe PKU
10	R408W / IVS12+1G>A	1+1	2	Severe PKU
11	R408W / **IVS12-1G>A***	1+1	2	Severe PKU
12	IVS10-11G>A / **IVS12-1G>A**	1+1	2	Severe PKU
13	R408W / G272X	1+1	2	Severe PKU
14	R408W / A104D	1+4	5	Mild PKU
15	R261Q/A104D	2+4	6	Mild PKU

Table 4 (continued)

16	E280K/L48S	1+4	5	Mild PKU
17	R408W / I306V	1+8	9	MHP
18	R408W / V230I	1+8	9	MHP
19	R408W / E178G	1+8	9	MHP
20	R408W / A403V	1+8	9	MHP

* we assume a novel splice site mutation as severe (classic)

In comparison with observed correlation between *PAH* genotype and phenotype in our study, some deviations took place using the Guldberg model. Mostly these deviations occurred in classification of severe PKU and MHP (Fig. 4). It shows that determining the severity of the disease not only mutation characteristics but also the individual characteristics of the organism have to be taken into account.

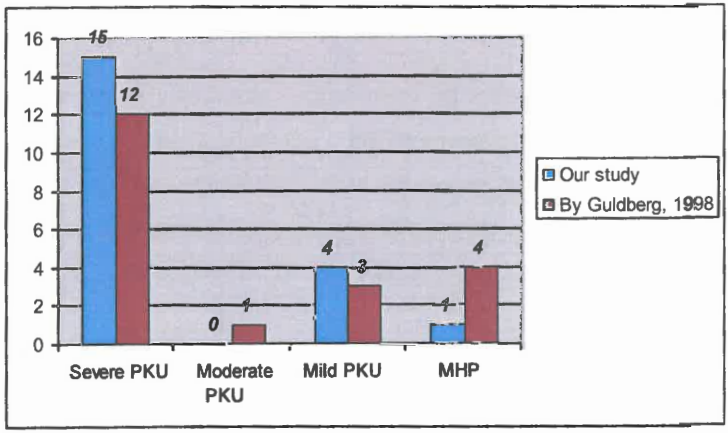

Fig. 4. Comparison of genotype and phenotype correlation in Latvian PKU patients using different classification models.

Chi-squared test was used to estimate the difference between two variants of the PKU classification showed that the difference is considered to be not statistically significant ($p=0.2276$, χ^2 equals 4.333 with 3 d.f.) It could be explained by the prevalence of severe mutations in Latvian PKU chromosomes and relatively small number of mutations causing variant PKU phenotypes.

III.2.2 Silent mutations in the *PAH* gene

Nine different silent mutations were found in Latvian PKU patients' *PAH* chromosomes in different combinations (Table 5). The most frequent polymorphisms were IVS2+19T->C (found in 11 patients) and IVS4+47C->T (found in 8 patients). Silent mutations were determined in 18 compound heterozygous patients by direct sequencing analysis of *PAH* gene exons that formed heteroduplexes during DGGE. All silent mutations were found in heterozygote condition.

Table 5. Silent mutations found in Latvian PKU patients.

No.	Genotype	SNPs	Location
1	R408W/E221_D222>Efs	IVS3-22C->T IVS4+47C->T V245V	Ex 4 Ex 4 Ex 7
2	R408W/E280K	IVS2+19T->C	I2/Ex 2
3	R408W/E280K	IVS2+19T->C	Ex 2
4	R408W/IVS12+1G>A	Q232Q	Ex 6
5	R111X/R408W	IVS2+19T->C	Ex 2
6	R408W/E280K	IVS2+19T->C IVS4+47C->T	Ex 2 Ex 4
7	R408W/E280K	IVS2+19T->C IVS4+47C->T	Ex 2 Ex 4
8	R408W/IVS10-11G>A	IVS2+19T->C	Ex 2
9	R408W/E280K	IVS2+19T->C IVS4+47C->T	Ex 2 Ex 4
10	R408W/E280K	IVS2+19T->C	Ex 2
11	IVS10-11G>A/IVS12-1G>A	IVS2+19T->C IVS3-22C->T IVS5-54A->G	Ex 2 Ex 4 Ex 6
12	R408W/I306V	IVS3-22C->T IVS4+47C->T Q232Q V245V IVS9+43G->T	Ex 4 Ex 4 Ex 6 Ex 7 Ex 9
13	R408W/V230I	IVS3-22C->T Q232Q V245V	Ex 4 Ex 6 Ex 7
14	R408W/K371E	IVS4+47C->T Q232Q V245V IVS9+43G->T	Ex 4 Ex 6 Ex 7 Ex 9

Table 5 (continued)

15	E280K/L48S	IVS2+19T->C IVS3-22C->T Q232Q V245V	Ex 2 Ex 4 Ex 6 Ex 7
16	R408W/P292T	-71A->C IVS4+47C->T Q232Q IVS9+43G->T L385L	5'UTR Ex 4 Ex 6 Ex 9 Ex 11
17	R408W/E280K	IVS2+19T->C IVS4+47C->T	Ex 2 Ex 4
18	X/R408W	IVS3-22C->T Q232Q V245V IVS9+ 43G>T	Ex 4 Ex 6 Ex 7 Ex 9

III.3 ANALYSIS OF *PAH* GENE MUTATIONS' MINIHAPLOTYPES

Results of VNTR and STR systems analysis were used to form *PAH* gene mutations' minihaplotypes. *PAH* minihaplotypes for the mutant chromosomes have been identified in 34 compound heterozygote patients when parents were available and two homozygote patients for mutation R408W.

Sixteen different *PAH* gene minihaplotypes have been identified associated with Latvian PKU chromosomes (Table 6). The most frequent minihaplotype was 3/238 that associated with the mutation R408W that is typical for Eastern-European populations and is spread across Europe from the northeast to the southwest.

Among the sixteen minihaplotypes associated to specific *PAH* mutations five were associated to more than one mutation (Table 7). On the other side, more common PKU mutations, including the most common mutation R408W and mutations E280K, R261Q, R158Q and P281L, were associated to more than one minihaplotype that could be result of different origin of the mutations (Table 6).

Table 6. Associations with PAH haplotypes and minihaplotype observed for mutations in the Latvian population.

No.	Mutation	Total alleles investigated	Minihaplotype	Alleles
1	R408W	34	3/238	28
			3/242	3
			3/234	2
			8/238	1
2	E280K	8	9/250	7
			9/246	1
3	R261Q	4	3/238	2
			8/238	2
4	R158Q	4	3/238	2
			3/234	1
			7/234	1
5	P281L	3	7/242	2
			8/242	1
6	IVS10-11G>A	2	7/250	2
7	A104D	2	8/242	2
8	A403V	1	8/246	1
9	E178G	1	7/242	1
10	R111X	1	8/250	1
11	R261X	1	7/238	1
12	G272X	1	9/234	1
13	I306V	1	3/234	1
14	V230I	1	3/246	1
15	L48S	1	3/234	1
16	E221_D222>Efs	1	3/242	1
17	IVS12+1G>A	1	8/242	1
18	**P292T**	1	8/226	1
19	**K371E**	1	3/238	1
20	**IVS12-1G>A**	2	7/242	2
21	Unidentified	1	3/234	1
	Total	72		72

Table 7. *PAH* minihaplotypes associated to more than one mutation

No.	Minihaplotype	Mutation	Alleles
1	3/238	R408W	28
		R261Q	2
		R158Q	2
		K371E	1
2	3/242	R408W	3
		E221_D222>Efs	1
3	3/234	R408W	2
		R158Q	1
		I306V	1
		L48S	1
		Unidentified	1
4	7/242	P281L	2
		E178G	1
		IVS12-1G>A	2
5	8/238	R408W	1
		R261Q	2
6	8/242	A104D	2
		P281L	1
		IVS12+1G>A	1

Minihaplotypes for 61 normal *PAH* alleles were obtained from the patients' parents. Most of the minihaplotypes were present on both normal and mutant *PAH* chromosomes. A continuous distribution from the smallest STR allele (226bp) to the largest (250bp) was observed on both allele types. Alleles 254bp and 258bp were absent in our study. The largest VNTR allele 12 was found only on normal *PAH* chromosome. The most common minihaplotype among mutant alleles was 3/238 due to the high prevalence of R408W mutation. Minihaplotype 3/242 was the most prevalent among normal chromosomes (Table 8).

The average probability of heterozygosity for minihaplotypes was about 76% for mutant and 92% for normal chromosomes.

Table 8. Frequencies of minihaplotypes on normal and mutant *PAH* chromosomes

No.	Minihaplotype	Frequency		p_i^2	
		Mutant	Normal	Mutant	Normal
1	3/234	0.0833	0.0492	0.00693	0.00242
2	3/238	0.4583	0.1147	0.21	0.013156
3	3/242	0.0556	0.1475	0.0031	0.02176
4	3/246	0.0139	0.0164	0.00019	0.00027
5	7/230	—	0.0164	—	0.00027
6	7/234	0.0139	0.0327	0.00019	0.00107
7	7/238	0.0139	0.0164	0.00019	0.00027
8	7/242	0.0694	0.0492	0.0048	0.00242
9	7/246	—	0.0492	—	0.00242
10	7/250	0.0278	—	0.00077	—
11	8/226	0.0139	0.0164	0.00019	0.00027
12	8/230	—	0.082	—	0.0067
13	8/234	—	0.0492	—	0.00242
14	8/238	0.0416	0.0984	0.00173	0.00968
15	8/242	0.0556	0.082	0.0031	0.0067
16	8/246	0.0139	0.0655	0.00019	0.00429
17	8/250	0.0139	0.0164	0.00019	0.00027
18	9/234	0.0139	0.0164	0.00019	0.00027
19	9/246	0.0139	0.0164	0.00019	0.00027
20	9/250	0.0972	0.0492	0.00945	0.00242
21	12/230	—	0.0164	—	0.00027
	Total	**1.0000**	**1.0000**	$\Sigma_m = 0.2414$	$\Sigma_n = 0.077616$
	1 - Σ			**0.7586**	**0.9224**

There was a statistically significant difference observed between normal and mutant alleles in the distribution of minihaplotype 3/238 and 8/230 (Fig. 5 (a) and (b)).

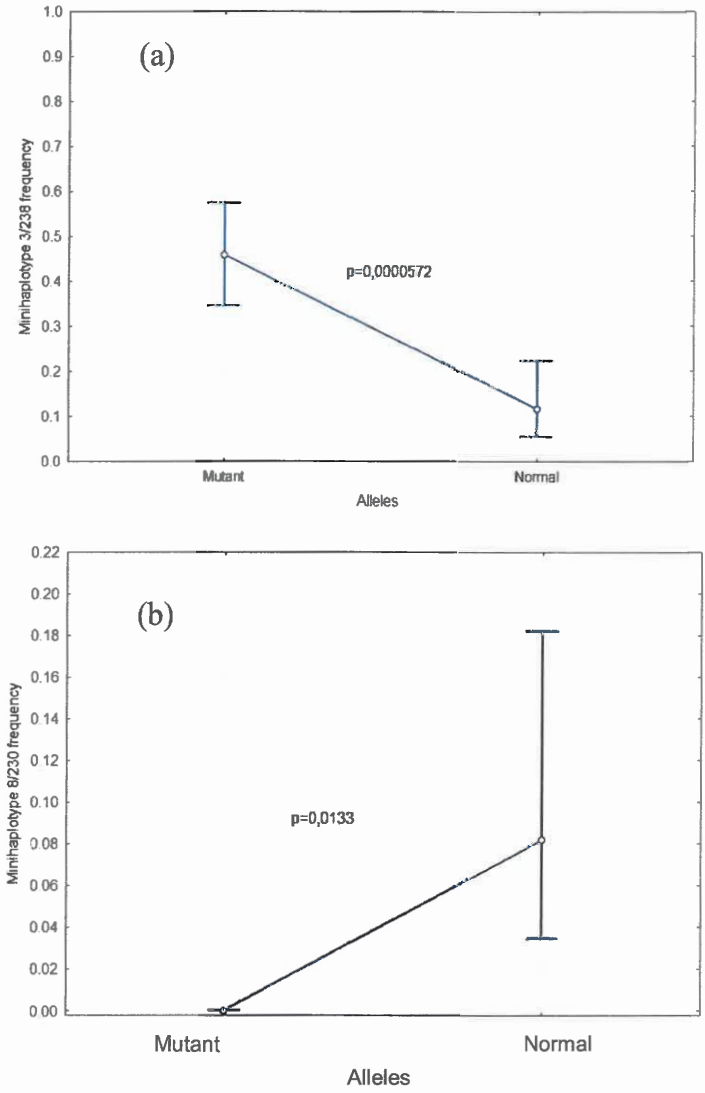

Fig. 5. Results of statistical analysis for distribution of minihaplotypes 3/238 (a) and 8/230 (b) between normal and mutant alleles.

Statistical analysis showed the tendency to statistically significant difference ($p<0.10$) between normal and mutant alleles in the distribution of minihaplotypes 3/242, 7/246 and 8/234. There was no statistically significant difference overall in the relative frequencies of other minihaplotypes.

III.4 ANALYSIS OF THE DISTRIBUTION OF *PAH* GENE MUTATION R408W

Distribution of *PAH* gene mutation R408W was similar in Latvia, Lithuania and St. Petersburg region. Estonia keeps the highest number of *PAH* chromosomes harbouring mutation R408W (Fig. 6). Statistical analysis comparing the relative frequencies of mutation R408W overall among the different geographical regions showed this to be highly significant for Latvia and other European countries (Table 9). Results of statistical analysis confirm the Balto-Slavic origin of mutation R408W and introduction of this mutation to other European populations by people migrations (Fig. 7).

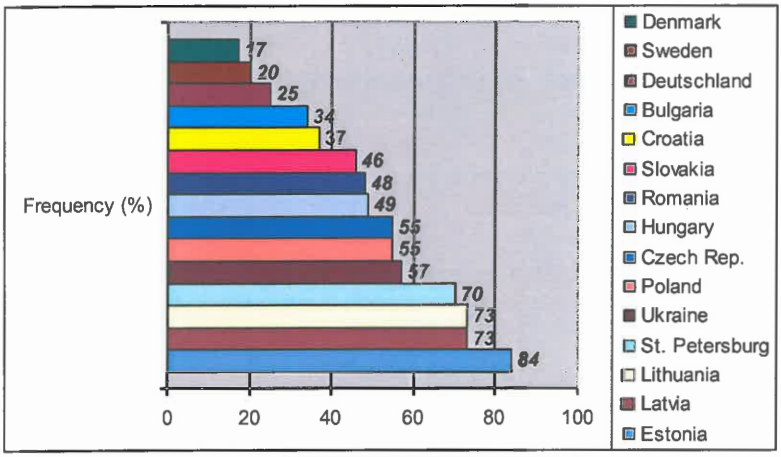

Fig. 6. Geographic distribution of the mutation R408W within different European populations.

Table 9. Comparison between the frequency of the mutation R408W in Latvian PKU population and other European populations.

Country	Frequency (%)	Alleles investigated	References	$p^{\#}$
Estonia	84	68	Lilleväli, 1996	0.0836
Latvia	73	140	Present study	*
Lithuania	73	184	Kasnauskiene, 2003	—
St. Petersburg	70	140	Baranovskaya, 1996	—
Ukraine	57	202	Nechyporenko and Livshits, 2002	0.00283
Poland	55	182	Zschocke, 2003	0.00109
Czech Rep.	55	266	Kozak, 1997	< 0.001
Hungary	49	70	Zschocke, 2003	< 0.001
Romania	48	44	Zschocke, 2003	< 0.001
Slovakia	46	98	Kadasi, 1995	< 0.001
Croatia	37	79	Zschocke, 2003b	< 0.001
Bulgaria	34	60	Zschocke, 2003	< 0.001
Deutschland	25	438	Zschocke, 1999	< 0.001
Sweden	20	176	Svensson, 1993	< 0.001
Denmark	17	308	Guldberg, 1993a	< 0.001

[#] the p value is for the comparison of the relative frequencies between two geographical groups.

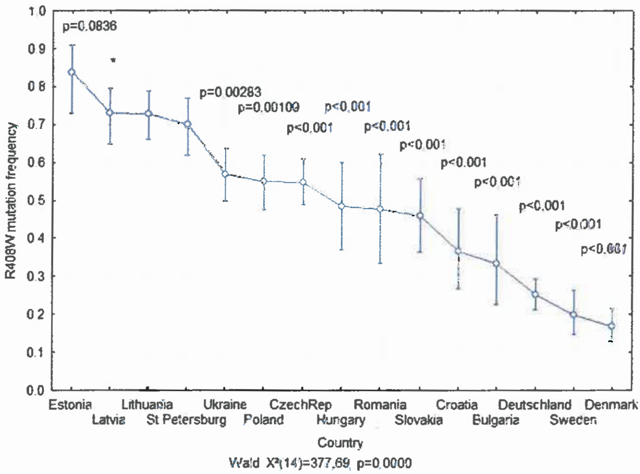

Fig.7. Results of statistical analysis between R408W mutation frequencies for different European countries. Significance level set at 0.05 was used to compare Latvia (*) to the other geographic populations.

IV DISCUSSION

Phenylketonuria is the most common inborn error of amino acid metabolism in Europeans. It has been frequently described as a paradigm of a Mendelian disorder. PKU was the first metabolic cause of mental retardation to be identified, the first genetic disorder of the central nervous system that could be fully treated by modification of external factors (i.e., the diet), and the first disorder that was successfully diagnosed by universal neonatal screening.

The understanding of the biochemical and molecular basis of phenylketonuria and the innovative treatment strategies introduced for these patients during the last 60 years, were transferred to other inborn errors of metabolism and led to significant reduction in morbidity and to an improvement in quality of life.

The *PAH* gene was first cloned in 1983 (Woo, 1983). Almost 30 years have now passed since the first molecular tests were carried out in PKU families. The most studies of *PAH* gene mutations in different populations were carried out in the 1990s and a *PAH* Mutation Analysis Consortium was formed, as a result PAHdb, an online relational database, was created.

Contemporary interest in PKU more often is focused on the development of new therapeutic approaches, but it is still closely related to the biochemical and molecular basis of PKU.

IV.1 MUTATIONS IDENTIFIED IN LATVIAN PKU CHROMOSOMES

In our study the most common mutation in Eastern Europe R408W was found in relative frequency of 73% in Latvian PKU patients. Similar frequencies of R408W are found in Lithuania and St. Petersburg region. The highest relative frequency of this mutation is found in Estonia (84%). Statistical

analysis did not show a significant difference in the distribution of the R408W allele between Latvia, Lithuania, Estonia and St. Petersburg region. So the high frequency of this mutation is explained by Balto-Slavic origin of R408W mutation that also is confirmed by our results. The relative frequency of R408W is progressively lower the greater the geographic distance from regions with the highest relative frequency ($p<0.001$) suggesting that this mutant allele may have been introduced into these populations by the relatively recent spread of European peoples across the Eurasian landmass.

We did not investigate the haplotype of mutation R408W because the significant differences in comparison with other Eastern European populations were not expected. Thus we assume that mutation R408W in Latvian population is associated with haplotype 2. Instead of this we provided minihaplotype studies for this mutation and found that 97% of R408W alleles are associated with three-copy VNTR allele but 82% with VNTR3/STR238 minihaplotype. This founding also suggests that our assumption about haplotype association could be truthful because for eastern European populations the R408W mutation is strongly associated with RFLP haplotype 2, the three-copy VNTR allele (VNTR 3), and the 238-bp STR allele. Three R408W alleles (9%) were found in association with minihaplotype 3/242, two alleles (6%) in association with minihaplotype 3/234 and only one R408W allele (3%) was found on minihaplotype 8/238. In comparison with Estonian and Lithuanian PKU populations, R408W was found on Eastern-European minihaplotype 3/238 on 98% and 68% of R408W alleles, respectively. Minihaplotype 3/242 for R408W was also found in Polish population on 6 mutant chromosomes (6.5%) and on single chromosome in Estonia. No "Celtic" allele VNTR8/STR242 that is the most common in north-western European populations was found in Latvian PKU chromosomes.

Mutation R408W was also found in association with minihaplotypes 8/230, 8/246, 9/242 and 9/246 in other European populations but on small

number of chromosomes. The association of a mutation with different VNTR and STR background is common in different populations and reflects a hypervariability of tandem-repeat DNA sequences. The other mechanisms leading to minihaplotype heterogeneity include a founder effect and a recurrence mechanism of the mutation. R408W allele's codon contains a methylated cytosine, which can experience methylation mediated deamination of the 5mC nucleotide to create thymine (c.1222C>T). In CpG dinucleotides mutation has been estimated to occur much greater than mutations at non-CpG dinucleotides. This process of recurrent mutation provides one explanation for the high relative frequency (<10%) of the R408W allele in European PKU genomes.

The high degree of minihaplotype variability associated with one mutation could also indicate an ancient origin.

PKU in Latvia is homogeneous. Calculation of the homogeneity ("homozygosity") at the *PAH* locus in Latvia gives a value of 0.514.

In comparison, the most homogeneous population described so far are Yemenite Jews, in whom a single molecular defect (large deletion covering the exon 3 of the *PAH* gene) is responsible for all the PKU cases in the population. Accordingly, the homozygosity value for this population is 1 (Avigad, 1990). An analysis of family histories of the Yemenite Jewish community has traced the origin of this defect to a common ancestor from Sana, Yemen, before 18th century (Killeen, 2008).

Other populations of Eastern European countries are also quite homogeneous. Homozygosity value for Estonia is 0.62 (Lilleväli, 1996), for Lithuania is 0.54 (Kasnauskiene, 2003), for Poland is 0.35 (Jaruzelska, 1993), for Southern Poland is 0.44 (Zygulska, 1991). Homozygosity value is rather low in ethnically mixed populations, for example, in Germany – j = 0.08 (Zschocke and Hoffmann, 1999). Germany has been at the crossroads of migration throughout the history of Europe. At present, almost 20% of people

living in Germany are relatively recent immigrants and the largest ethnic group of non-German origin is the Turkish.

The homozygosity value has to be considered when a *PAH* gene mutation detection strategy for a particular population is being created. The high level of homozygosity may facilitate genotyping and to raise the mutation detection rate.

Relatively high homozygosity level also has an impact on clinical picture of the disease for a significant number of patients. Mutation R408W is a severe mutation that is associated with < 0.3% of normal activity and a severe PKU phenotype. Taking into account that thirty-six (51.4%) of 70 unrelated characterised PKU patients are homozygous for R408W, the prevalent clinical form of the PKU supposed to be severe. Remaining 34 (48.6%) patients are compound heterozygous. The majority of compound heterozygote patients (43%) had R408W mutation in one allele that means that their clinical form of PKU depends on the mutation in other allele of the *PAH* gene. Only four Latvian PKU patients (5.7%) had no R408W mutations in their chromosomes.

Next most common mutation found in Latvian PKU patients was E280K presented on 5.7% of all PKU chromosomes. Mutation E280K is a severe mutation with enzyme residual activity ~0.9% of wild type. Glu280 is located in a stretch of 27 PAH amino acids (His263 to His289), which is highly conserved and is closed to the active site. It forms a salt bridge to Arg158 plus hydrogen-bond to His146. The mutation E280K would be expected to affect severely the electrostatic potential in the active site (Jennings, 2000).

Seven of eight (7/8) E280K alleles were found with R408W in homologous allele, these patients had severe form of PKU due to very low enzyme activity determined by both severe *PAH* gene mutations. One patient had genotype E280K/ L48S.

E280K allele, involving a CpG dinucleotide, was found in 3 different haplotypes that suggests the recurrent mechanism for this mutation. In Latvian

PKU chromosomes mutation E280K was found in strong association with minihaplotype 9/250, one allele was found on minihaplotype 9/246. In comparison with other populations it was found on different minihaplotypes 7/246 (Germany), 8/238 and 8/246 (Northern Ireland), 9/234 (Spain). Mutation E280K is not very common in European populations and usually was found only in few PKU chromosomes.

Two mutations were detected with frequency 3% each - R261Q and R158Q. The R158Q mutation is a frequent mutation in patients with PKU in European countries but usually represents less than 10% of PKU chromosomes. As mentioned above, Arg158 forms a salt bridge to Glu280, but also forms a hydrogen bond to Tyr268. Both of these interactions are important for conserving the shape of the active site, and substitution into a glutamine residue will alter the active site architecture and lower enzymatic activity. Mutation R158Q is classified as severe with the residual activity of 10% for PAH enzyme (Jennings, 2000). However, sometimes it was classified as moderate. So R158Q is one of mutations that are frequently represented in discordant genotype-phenotype associations. A potential cause of these inconsistencies may relate to the biological properties and functions of the mutant protein. It was noticed that some patients with mild PKU have a relatively low phenylalanine tolerance when treated, although plasma phenylalanine levels were only a little above the therapeutic threshold when these patients were untreated. Mutation R158Q is considered to be BH4-responsive. In our study mutation R158Q was found only with R408W mutation in homologous allele and all four patients were characterised as having severe PKU.

Minihaplotypes studies have revealed that mutation R158Q was associated with three different minihaplotypes: 3/238 (two alleles), 3/234 (one allele) and 7/234 (one allele). In other populations this mutations was found on minihaplotype 3/234 (Germany, Italy and Northern Ireland). This mutation also involves a CpG dinucleotide and different minihaplotypes could be the result of

the recurrent mechanism of this mutation but available data are not sufficient to make conclusions.

Mutation R261Q is a moderate PKU mutation with residual activity >30% of wild type of *PAH* enzyme. R261Q was found throughout the Europe but the highest frequency was discovered in Switzerland (>30%). Arg261 is located in the cofactor binding domain (CBR1), where it hydrogen bonds to Gln304 and Thr238. This helps to stabilise the structure of the active site, and substituting the arginine would destabilise the active site. Mutation R261Q is considered to be BH4-responsive. It was associated with responsiveness both in combination with null mutations and not. However, in another studies it was found not to be responsive.

Genotyping of Latvian PKU patient has revealed that three patients are compound heterozygotes for R261Q and R408W mutations and one patient for mutations R261Q and A104D. As a confirmation of mentioned above all patient were classified as having severe form of PKU in despite of moderate phenotype of R261Q.

Four R261Q alleles were associated with two minihaplotypes – 3/238 and 8/238 equally. In other European populations the most common minihaplotype for R261Q was found 8/238 (Germany and Italy) but it was also found on minihaplotypes 3/246, 7/242, 8/234, 8/242 and 8/246. Association with several minihaplotypes and distribution among different European populations could be result of the presence of hypermutable CpG dinucleotide and also indicate an ancient origin of this mutation.

P281L mutation is not very common in Europe, the highest frequency 19% was found in Iceland, Croatia and Greece showed similar frequencies 11% and 10%, respectively. In other European populations the most common frequency of P281L was 1-3% and did not exceed 6%. Three Latvian PKU chromosomes (2.1%) were found to harbour P281L allele. Pro281 is located in the active site and helps to define the shape of the active site very close to the

iron in the PAH structure. A substitution to a less rigid leucine will change the conformation of the active site by removing the conformational constraints imposed by the proline and resulting in <1% of enzyme activity.

In Latvian PKU patients P281L mutation was found in two different genotypes: R408W/P281L for two patients and R261X/P281L for one patient. All patients had severe PKU that is compatible with metabolic phenotype of mutations.

P281L mutation's the most common minihaplotypes 7/242 and 8/242 both were found in Latvian P281L alleles on two and one chromosome, respectively. Additionally, minihaplotypes 8/238, 8/234 and 3/234 were found in Italian population, minihaplotype 3/242 in one Polish PKU patient. Association with several minihaplotypes and distribution among different European populations could be result of hypermutable CpG dinucleotide in proline codon that indicates the recurrent mechanism of this mutation. Variability of minihaplotypes also could indicate an ancient origin of this mutation.

IVS10-11G>A is the most common mutation in the Mediterranean, particularly in Turkey where it accounts for more than 30% of PKU alleles. Mutation is categorised as severe because completely abolishes PAH enzyme activity. This splicing mutation most likely results in truncated protein lacking C-terminal 97 amino acid residues (residues 356-402). As a result an unstable protein is produced.

We found mutation IVS10-11G>A in two PKU chromosomes corresponding to a frequency of 1.4%. Both patients had severe form of PKU but different genotypes - IVS10-11G>A/IVS12-1G>A and R408W/IVS10-11G>A.

Both Latvian IVS10-11G>A alleles were found in association with the most prevalent for it minihaplotype 7/250, although, it was found associated with minihaplotype 7/230 in Spanish Gypsies, Czech, English and German patients. IVS10-11G>A is probably an ancient mutation that originated long

before the end of last ice age and separated into different alleles early in prehistory. The east-west gradient in the Mediterranean basin with the highest focus in Turkey, has suggested a spread from Asia Minor during the Neolithic period (Calì, 1997). Moreover, recent migration has brought the mutation to Northern European countries like Germany.

Mild mutation A104D with residual activity <25% was found on two Latvian chromosomes – 1.4%. A104D is not frequent mutation and presents in small frequencies in Central and Northern Europe. Mutation is located in regulatory domain of PAH enzyme and is classified into BH4-responsive alleles. This mutation is associated with variant PKU, and neutral Ala104 is located in a loop between Rα2 and Rβ4 in the regulatory domain. Substitution into a larger and charged residue may destabilise this loop structure.

Mutation A104D was found in two compound heterozygous genotypes with mutations R408W and R261Q in homologous alleles. Both patients had severe PKU despite the fact that mutation R261Q was considered to be BH4-responsive also confirming the lack of the strong correlation between genotype and BH4-responsiveness.

Information about A104D allele's minihaplotypes was available only from German PKU population where it was associated with 8/242 and 8/246 minihaplotypes. Latvian A104D alleles were found in association with 8/242 minihaplotype both. Alanine codon does not involve hypermutable CpG dinucleotide that could be the reason for mutation rarity.

Three nonsense mutations, R111X, R261X and G272X, were found only once corresponding to a frequency of 0.7% each. All three alleles are classified as severe with residual activity <1% of wild type PAH enzyme activity. Mutation R111X is located in regulatory domain (exon 3), mutations R261X and G272X in catalytic domain (exon 7) of the protein. Mutations result in C-terminally truncated protein.

As in the case of mutation R261Q, substitution of the arginine to a premature stop codon (R261X) will necessarily destabilise the active site structure of the enzyme.

Glu272 is located in a loop just before the active site histidines that bind the catalytic iron and the mutation results in a truncated form of PAH that has none of the residues that are responsible for binding iron; thus, no catalytic activity will be observed.

Mutation R111X causes the loss of approximately two-thirds of the PAH polypeptide. It is more common in Orientals, in European populations frequency of R111X was found about 1-2%. It was also found in Turkey, Italy, Sicily, and Australia. Mutation G272X is common in Norway (16%), while R261X frequency varies within 1-2% in Europe.

Mutations G272X and R111X were found in null/null genotypes with R408W mutation in homologous allele. Mutation R261X was found together with P281L allele. All three patients showed severe PKU phenotype.

In Latvian PKU chromosomes mutations R111X, R261X and G272X were found in association with minihaplotypes 8/250, 7/238 and 9/234, respectively.

Minihaplotypes 3/238 and 3/246 was identified for R261X allele in German PKU patients, but minihaplotypes 7/242 (the most common), 3/238 and 3/242 in Italian PKU patients. Since a CpG dinucleotide is involved the mutation could have arisen independently on different populations and minihaplotype backgrounds.

Mutation G272X was found on minihaplotype 8/226 in Germany. No more minihaplotype data are available for G272X allele from other European populations and there is a total lack of data about R111X mutation's minihaplotype association.

Four mild mutations, A403V, E178G, I306V and V230I, were classified as mutations caused MHP with residual activity of PAH enzyme 32%, 39%,

39% and 63%, respectively. Each mutation was found in single chromosome only. Mutations A403V, E178G and V230I were previously reported as BH4-responsive.

Mutation A403V has been detected in southern Europe, and in Spain it is a relatively common mutation (14%).

Ala403 is located at the end of helix Cα12, close to Ala309 in helix Cα8. Alanine or another smaller residue might be necessary for close packing of helices Cα8 and Cα12. Substitution into a larger valine might result in a less stable protein because it would require the surrounding protein to adjust and create space for the accommodation of the bulkier side chains. Thus, the BH4 binding site in mutant PAH might be only slightly different as compared with the wild-type PAH structure, explaining the BH4-responsiveness of this genotype.

Mutation V230I has the same effect on the enzyme as mutation A403V due to similar substitution of smaller hydrophobic valine residue to larger isoleucine that in the same would require the adjustment of surrounding protein and creation of space for the accommodation of the bulkier side chains.

Mutation E178G is not frequent in Europe. E178G is located on the surface of catalytic domain. Substitution to a small and flexible hydrophobic residue may be very unfavourable, because it can change the fold of the catalytic domain core, which is important for maintaining proper catalytic function. The enzyme is quite susceptible to mutations that destroy the cooperative activation mechanism probably by hindering the transmission of the conformational change.

Mutation I306V is located close to the active site. It results in change of large buried hydrophobic residue to smaller one and destabilises the protein by creating cavity in the hydrophobic core and, the effect depends on the size of the resulting cavity.

All four mild mutations were found together with R408W mutation in functionally hemizygous genotypes. Three patients were classified as having mild PKU phenotype. Patient with genotype R408W/V230I had MHP and did not require treatment.

Mutations A403V, E178G, I306V and V230I were found in associations with minihaplotypes 8/246, 7/242, 3/234 and 3/246, respectively.

Association with the same minihaplotype for mutation A403V was found in Poland, Italy and Spain. Strong association with minihaplotype 8/242 was identified in German PKU patients; it was also prevalent minihaplotype in Italian and Polish PKU chromosomes.

Minihaplotype data for mutation V230I are available only from German PKU population where it was associated with 3/242 minihaplotype. No data were available for mutations E178G and I306V.

Mutation L48S is quite common in the Southern Europe. It was more common in PKU chromosomes in Serbia (21%) and in South of Italy (11%). L48S mutation was classified as mild and BH4-responsive but was frequently represented in discordant genotype-phenotype associations. Mutation is located in regulatory domain and results in change of buried hydrophobic leucine residue to polar serine. Burial of polar side chains results in decrease of protein stability. L48S mutation was found in single Latvian PKU chromosome with null mutation E280K in homologous allele. Patient was functionally hemizygous but was classified as having severe PKU form.

In our study mutation L48S was found on minihaplotype 3/234 that was prevalent minihaplotype for it in German and Italian PKU alleles. Minihaplotypes 3/230, 3/238 and 8/238 were also identified for L48S in single Italian PKU chromosome each.

Only one small deletion was identified in *PAH* gene of Latvian PKU patients - mutation E221_D222>Efs. Mutation results in fusion protein. Such kind of mutations introduces frameshifts and result in mutant proteins

containing a truncated PAH sequence fused to an unrelated sequence at the C-terminus. No data about the protein residual activity was available but mutation was classified as causing severe PKU.

Mutation E221_D222>Efs was identified in genotype with other severe mutation R408W and PKU form was classified as severe.

We found this mutation in association with minihaplotype 3/242. In two German PKU chromosomes it was found on minihaplotypes 3/238 and 3/242.

The mutation IVS12+1G>A results in a truncated protein lacking the C-terminal 52 residues (residues 401–452). *In vitro* expression of the mutant protein comprising residues 1–400 suggests an unstable protein is produced possibly because residues 409–422 participate in the dimer and domain interfaces. Therefore in genotype with other null mutation as R408W it gives severe PKU phenotype.

IVS12+1G>A is quite common in Scandinavia and it frequency reaches 37% in Denmark. In Latvia it was found in a single PKU chromosome (0.7%) and was associated with minihaplotype 8/242 that is only minihaplotype reported for this mutation in Germany, Italy and Northern Ireland. Latvian patient harbouring this mutation had R408W in the other allele, so, patient's severe PKU was compatible with metabolic phenotype of both mutations.

Three mutations (P292T, K371E and IVS12-1G>A) identified in present study had not been previously reported; two of them were found only once, but the third one was identified on two unrelated PKU chromosomes. None of these mutations have been examined by *in vitro* expression analysis but also none of them had been observed in the 100 normal subjects tested (200 chromosomes). These results do not fully exclude, but reduce the possibility of population-specific polymorphism. The all 13 exons of *PAH* gene were sequenced for these four patients but other PKU mutations were not found. Of course, *in vitro* expression analysis remains the most effective way to confirm that a "disease-associated" mutation is truly pathogenic.

In one family a PKU patient presented the P292T mutation that was a c.874C>A substitution in the *PAH* gene in exon 8 at amino acid 292, resulting in a missense mutation – hydrophobic proline is substituted by hydrophilic threonine. Mutation was linked to minihaplotype 8/226. The patient had a neonatal diagnosis, with phenylalanine level indicating severe PKU (~25mg%), and received diet therapy soon after. Mutation P292T was found in heterozygosity with severe R408W mutation. Considering that PKU clinical form depends on the combination of mutant alleles inherited, we suggest that mutation P292T has to be associated with low residual activity of PAH enzyme. This variant was also predicted to be probably damaging by using an automatic tool for prediction of possible impact of an amino acid substitution on the structure and function of a human protein (PolyPhen).

In parents: mutation R408W was inherited from patient's mother but the paternal chromosomes did not carry either of the PKU mutations identified in the child. Since the results of paternity testing for this family were compatible with paternity, we concluded that the second mutation has to be arisen *de novo*. Thereby, the risk for another child with PKU in this family will be low.

These findings demonstrate two important points: the necessity of screening the whole coding region of the *PAH* gene for diagnostic purposes on the one hand, and second the usefulness of confirming inheritance of mutations from both parents when possible. Otherwise the prediction of the expected phenotype or the calculation of risk for another child with PKU may be incorrect.

The 1111A>G substitution in exon 11 of the *PAH* gene at amino acid 371 results in a missense mutation – lysine is substituted by glutamic acid. Both amino acids are classified as polar. This substitution was found in a PKU patient and his father and was associated with minihaplotype 3/238. The patient was diagnosed through the neonatal screening and had slightly elevated phenylalanine level (2.7mg%) that did not require treatment. Mutation K371E

also was found in heterozygosity with R408W mutation. This finding suggests that K371E is a mild mutation with enough PAH residual activity for normal clinical phenotype. These findings match with PolyPhen analysis results that predicted this change as benign. Genotype K371E/R408W could be defined as functionally hemizygous. The normal individuals tested for this mutation did not present it.

The c.1316-1G>A substitution in intron 12 of the *PAH* gene results in a mutation. This substitution was found in two unrelated patients with severe PKU. The mutation IVS12-1G>A is located at the boundary of intron 12 and exon 13 and affects the conserved dinucleotide AG at the 3' splice site. According to its location this mutation results in a truncated protein lacking the C-terminal at least 14 amino acid residues (residues 439-452). This mutation also was not found in 200 chromosomes of 100 healthy individuals.

In one patient this mutation was found in association with mutation R408W but in another one in association with another known splice site mutation IVS10-11G>A. Both patients were diagnosed through the neonatal screening. Patient with genotype IVS12-1G>A/R408W received diet therapy soon after and is currently asymptomatic despite the severe clinical form of PKU. Patient with genotype IVS12-1G>A/IVS10-11G>A has severe mental retardation because of parents' refusal to maintain the adequate dietary treatment. In both cases mutation IVS12-1G>A was linked to minihaplotype 7/242.

Remaining unknown allele may harbour large deletion and thus deserve further investigation by other techniques.

During our work we are faced with some difficulties comparing the frequency of mutations in different populations, especially concerning rare mutations in the *PAH* gene. Some studies were conducted in early 1990s and only the most common mutations were identified using that time available techniques.

Minihaplotype analysis is possible in two ways: in case of homozygous mutation or if patient's parents are available for investigation. Unfortunately, only in few populations minihaplotype analysis was performed for full spectrum of *PAH* gene mutations, more often it was made only for more common mutations. All together, these limitations do not allow tracing the origin of mutations and later distribution to other populations.

Sequencing analysis revealed the presence of 9 different silent mutations in different combinations in Latvian PKU chromosomes. Three of them were located in exons and 6 were located in introns. No specific distribution of *PAH* gene polymorphisms was observed. Polymorphisms detection rate depends on primers used for sequencing analysis. Intron polymorphisms located more distantly from the exon/intron boundaries may remain undetected.

According to Hardy-Weinberg equilibrium Latvian PKU patients' pool is balanced. Statistical analysis confirmed that the observed and expected genotypes' frequencies are not significantly different from one another ($p = 0.8312$). However, our study did not reveal the presence of other homozygous patients in PKU population that according to Hardy-Weinberg equilibrium has to be up to 1%. It could be explained by MHP patients that is part of the PAH deficiency spectrum and is frequently caused by compound heterozygosity for classical PKU mutations and specific mild mutations or, possibly, by two mild mutations. These patients may or may not have been included in the molecular studies. This poses some deviations when absolute allele frequencies in a population are estimated using incidence data and the Hardy-Weinberg formula.

IV.2 GENOTYPE-PHENOTYPE CORRELATIONS IN LATVIAN PKU PATIENTS

Compiling data on mutations observed in Latvian PKU chromosomes, we can conclude that the majority of mutations (12/20) are severe and responsible for the severe PKU phenotype. Two mutations are mild and five are

MHP causing; only one is responsible for moderate PKU. So the high proportion of patients with severe or classic PKU is explained by the mutation severity. We compared two different approaches for classifying the PKU clinical phenotype and results did not shown the significant difference ($p = 0.2276$). The traditional method for PKU phenotype classification is based on Phe pre-treatment serum level and Phe tolerance. The other model is based on an arbitrary value for phenotypic prediction system. By means of this classification, a phenotype resulting from the combination of two mutant *PAH* alleles may be expressed numerically as the sum of the AVs of two mutations. In our study the majority of PKU patients corresponded to that rule but some deviations were observed. Mostly it relates to mutations with residual *in vitro* activities. Many factors can influence phenotypic variation in PKU, such as inter-individual variations in intestinal absorption, hepatic uptake of dietary phenylalanine, rate of incorporation of phenylalanine into proteins, rates of influx of phenylalanine across the blood brain barrier, mutations located close to the cofactor binding site and affecting the activity of the enzyme, as well as interactions of the *PAH* gene with other loci.

The efficiency of the method based on AVs estimates will vary depending on the set of mutations in a specific population. For populations in which the most common mutations are null mutations, the system could be highly useful.

Contemporary therapy for PKU is centered upon tight restriction of dietary Phe intake and requires supplementation with special medical foods that supply sufficient essential amino acids and energy from fat and carbohydrate. Institution and maintenance of the PKU diet are difficult, and the required medical foods are often unpalatable. Dietary therapy is recommended for life (Anonymous, 2001), but non-compliance with the dietary prescription is commonplace, particularly during adolescence and adulthood. Hyperphenylalaninaemia in adults is often associated with attention problems, mood

instability and poor job performance. Chronically elevated Phe may cause a progressive neurodegenerative disorder affecting white matter that leads to seizures and gait disturbance. Finally, untreated maternal hyperphenylalaninaemia during pregnancy is the only teratogen guaranteed to cause birth defects, which include microcephaly, mental retardation and congenital heart disease.

There is no other effective and relatively simple type of treatment that could completely replace the dietary treatment, although, research in this direction is made constantly. However, chaperon therapy by tetrahydrobiopterin supplementation is effective in a subset of individuals with BH4-responsive hyperphenylalaninemia that has been recently described as a variant of PAH deficiency caused by specific mutations in the *PAH* gene.

The evaluation of the data contained in the BIOPKU data base we would expect BH4-responsiveness in about 18% (13/70) of all Latvian patients with PAH deficiency. Seven mutations from 20 observed in Latvian PKU patients have substantial residual activity of PAH (10-39%): mutation R158Q with residual activity 10%, A104D – 26%, A403V – 32%, mutations R261Q, I306V, E178G and L48S with residual activity 39%.

There is still some inconsistency reported of BH4-responsiveness in patients harbouring L48S, R158Q and R261Q mutations. This inconsistency confirms the lack of the strong correlation between genotype and BH4-responsiveness. In this case, in which this residue is implicated in the interaction of two neighbouring subunits, the second PKU allelic variant in the patient could have a high significance in determining the responsiveness to BH4.

A genotype can be considered as associated with BH4-responsiveness if one of alleles harbours BH4-responsive mutation with a substantial residual PAH activity. Among 70 Latvian PKU patients 13 can be considered as potentially BH4-responsive: three patients with genotype R408W /R261Q,

four patients with genotype R408W /R158Q, six patients with genotypes R408W/A104D, R408W/A403V, R408W/E178G, R408W/I306V, R261Q/A104D and E280K/L48S. Twelve patients have null mutation in homologous allele. In comparison, BH4-responsiveness is much higher (<75%) in southern regions of Europe with a high frequency of BH4-responsive alleles. Due to the high prevalence of mutation R408W and other null mutations in Baltic countries, the number of potentially BH4-responsive patients is relatively low.

Despite the fact that there is no strong correlation between genotype and BH4- responsiveness, mutation analysis provides useful information on potential nonresponders in patients harbouring two null alleles and may, to some extent, predict possible BH4-responders.

IV.3 MINIHAPLOTYPE STUDIES FOR MUTANT AND NORMAL *PAH* ALLELES

Minihaplotype studies have revealed 16 different minihaplotypes associated to *PAH* gene mutations and 20 different minihaplotypes for normal *PAH* alleles. The most common minihaplotype for mutant alleles was 3/238 due to the high prevalence of mutation R408W among Latvian PKU chromosomes, while for normal alleles more common minihaplotype was 3/242. Distribution of STR alleles is consistent with the previously reported data about higher frequency of the 238bp allele among mutant chromosomes and the higher frequency of the 242bp and 246bp alleles among normal chromosomes in Caucasians from different European populations. Contrary, distribution of VNTR alleles is different from the accepted opinion that the VNTR allele containing 8 repeats is the most prevalent (about 60%) among both normal and mutant chromosomes. The prevalence of VNTR 3 allele among mutant chromosomes is explained by the most common R408W

mutation, but distribution of VNTR 8 allele in normal chromosomes is consistent with this statement. This is partially confirmed by statistical analysis that has revealed the significant difference in the distribution of normal and mutant alleles for only two minihaplotypes 3/238 ($p=0.0000572$) and 8/230 ($p=0.0133$).

Statistical analysis showed the tendency to statistically significant difference ($p<0.10$) between normal and mutant alleles in the distribution of minihaplotypes 3/242, 7/246 and 8/234. The approval of these trends requires an investigation of greater number of alleles.

The average probability of heterozygosity for minihaplotypes was about 76% for mutant and 92% for normal chromosomes indicating a greater diversity of normal alleles. The association of minihaplotypes with specific mutations results in it limitation in comparison with normal alleles that makes it useful for prenatal diagnosis and carrier screening determination in PKU families.

One of the limitations in performed minihaplotype studies was insufficient number of analysed chromosomes due to relatively small Latvian PKU population.

IV.4 THE MOST EFFECTIVE STRATEGY FOR ROUTINE DIAGNOSTICS OF *PAH* GENE MUTATIONS IN LATVIA

The three-step *PAH* gene mutation detection strategy based on the information of PKU causing mutation spectrum and PKU population homozygosity value can be found to be the best for routine diagnostics for Latvian PKU patients. In the first step, the common mutation R408W detection with restriction enzyme assay that identifies both alleles in >50% of patients and one allele in a further 43% is used. In the second step, denaturant gradient-gel electrophoresis is used to determine the possible location of other *PAH* gene

mutations. In the final third step, depending on DGGE results (number of exons that showing variant electrophoretic patterns) direct sequencing analysis or minihaplotypes combining STR and VNTR data could be used to determine rare mutations. In case of prenatal diagnostic or the need to quickly provide information on *PAH* gene mutations minihaplotype analysis could be used after DGGE and prior to direct sequencing analysis. For example, exon 7 harbours the majority of PKU mutations and several silent mutations. Minihaplotype analysis can provide faster and less time-consuming response for determining the nature of sequence changes.

Taking into account that the diagnostic strategy has to be designed to identify a great number of mutations, the detection rate of 99% achieved in our study confirms that the diagnostic approach used had the best possible design.

V CONCLUSIONS

1. Analysis of the molecular basis of PKU in Latvia has revealed 20 different mutations in the *PAH* gene: the most common mutation R408W accounted for 73% of all PKU chromosomes, the frequencies of remained 19 mutations ranged from 0.7 to 5.7% of all mutant alleles.

2. Minihaplotypes (combinations of *PAH* gene STR and VNTR systems) were determined for all 20 mutations indentified in Latvian PKU chromosomes; a strong association of mutation R408W with VNTR3/STR238 minihaplotype was indicated.

3. The average probability of heterozygosity for minihaplotype system was found lower for mutant chromosomes (0.76) compared to normal *PAH* chromosomes (0.92) indicating a greater diversity of normal alleles.

4. The estimation of genotype-phenotype correlation has revealed that Latvian PKU patients are homogeneous in terms of clinical PKU form due to the high frequency of severe R408W mutation and the high level of homogeneity (j=0.514) at the *PAH* locus.

5. Analysis of the distribution of the *PAH* gene mutation R408W has confirmed the Balto-Slavic origin of mutation R408W and introduction of this mutation to other European populations by people migrations.

6. The three-step *PAH* gene mutation detection strategy used in the study is the most effective for routine diagnostics in Latvian population with the sensitivity of the method 99%.

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